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GIGA-Neurosciences
Laboratoire du Vieillissement Pathologique et Epilepsies

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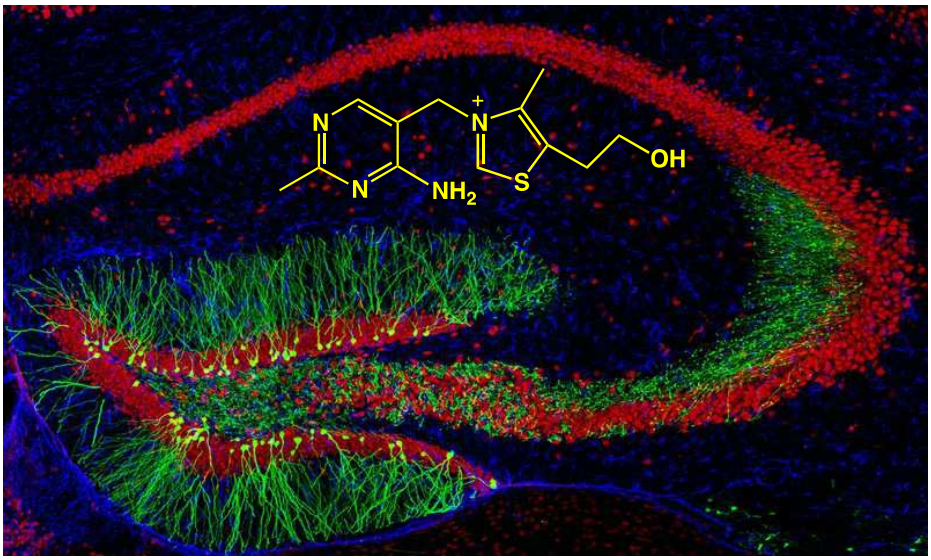
Mécanisme des effets neuroprotecteurs de la thiamine et de précurseurs à plus grande biodisponibilité



Mémoire présenté par Julie VIGNISSE

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"Life is like a box of chocolates.
You never know what you're gonna get"

Forrest Gump, 1994

L'image de couverture a été modifiée et provient du laboratoire de FH Gage at the Salk Institute For Biological Studies, USA.

Thiamine is very important for brain functioning and its deficiency causes specific lesions. This is mainly due to decreased levels of its diphosphorylated derivative thiamine diphosphate (ThDP), an essential cofactor for key enzymes in brain energy metabolism. Brain thiamine deficiency is not only the result of reduced thiamine intake, but also a consequence of chronic alcoholism, gastrointestinal diseases, diabetes, absorption of anti-thiamine factors, aging or reduced transport activity. As thiamine transport across the blood-brain barrier is relatively slow, thiamine precursors with higher bioavailability have been developed. One such compound is benfotiamine (BFT). After oral intake, BFT is dephosphorylated by intestinal alkaline phosphatase to the lipophilic S-benzoylthiamine, which freely diffuses across the intestinal mucosa and is transformed to thiamine. After administration of BFT, much higher blood thiamine levels are reached than after administration of an equivalent amount of thiamine. BFT was first shown to be efficient against diabetes-related complications. More recently, it was shown to have highly beneficial effects in mouse models of neurodegenerative diseases. In particular, it decreases brain amyloid deposits and tau hyperphosphorylation. The aim of our thesis was to investigate the mechanisms involved in central nervous system effects of BFT. In a first part, using the mouse neuroblastoma cell line N2a, we demonstrated that BFT indeed requires prior dephosphorylation to S-benzoylthiamine in order to enter the cells and raise intracellular thiamine concentrations. Surprisingly, when orally administered to mice, BFT strongly increased blood thiamine concentration but did not increase brain ThDP levels, suggesting that potential central nervous system effects are cofactor-independent. It has been suggested that treatment with benfotiamine induces an increase in brain GSK-3ß phosphorylation, thereby decreasing its activity. As GSK-3ß is in part responsible for tau hyperphosphorylation, such a mechanism might explain a reduced formation of neurofibrillary tangles in the above-mentioned models of neurodegeneration. Using N2a cells, we indeed confirm a stimulation of the RTK – PI3K – Akt pro-survival pathway. As it is known that benfotiamine treatment has potent beneficial effects in 2 different mouse models of neurodegeneration and that exposure of WT mice to intense stress is also harmful for the hippocampus, we investigated the effects of predator stress on adult hippocampal neurogenesis. The latter has been shown to be impaired by stress in rodents. We therefore tested the effects of thiamine and BFT treatment on hippocampal neurogenesis in predator-stressed mice. Our results show that both thiamine and BFT prevented the reduction of neurogenesis induced by stress, benfotiamine being most effective. Moreover, we show that thiamine and benfotiamine counteract stress-induced bodyweight loss and increase of anxiety-like behavior. Both treatments elevated brain levels of thiamine, but not of the coenzyme thiamine diphosphate (ThDP), again suggesting that the beneficial effects observed are not linked to the cofactor role of ThDP. Our study demonstrates for the first time that thiamine and benfotiamine prevent stress-induced inhibition of hippocampal neurogenesis and accompanying physiological changes, probably by non-cofactor-dependent mechanisms. The use of thiamine precursors might thus be considered as a complementary therapy in several neuropsychiatric disorders, especially depression caused by chronic stress.

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LISTE DES ABREVIATIONS

AD	Antidépresseur
AGEs	« Advanced Glycation End products »
Akt	Protéine kinase B
AThDP	Adénosine thiamine diphosphate
AThTP	Adénosine thiamine triphosphate
BDNF	« Brain-Derived Neurotrophic Factor »
BFT	Benfotiamine
BHE	Barrière hémato-encéphalique
BPA	Benfotiamine (B) préalablement incubée avec de la phosphatas alcaline (PA)
BrdU	Bromodéoxyuridine
CGL	Couche des cellules granulaires (« Granular Cell Layer »)
DAPI	4',6'-Diamidino-2-phénylindole
DG	Gyrus denté (« Dentate Gyrus »)
DMEM	Milieu Eagle modifié par Dulbecco («Dulbecco's Modified Eagle Medium »)
EDTA	Acide éthylène diamine tétraacétique
FBS	Sérum fœtal bovin (« Fetal Bovin Serum »)
GSK-3β	Glycogène synthase kinase - 3β
HPLC	Chromatographie liquide à haute performance (« High performance Liquid Chromatography »)
HPRT	Hypoxanthine-guanine phosphoryltransférase
IGF-1	« Insulin-Growth Factor-1 »
Ins	Insuline
LCR	Liquide céphalo-rachidien
MA	Maladie d'Alzheimer
MDT	Milieu déficient en thiamine
MPT	Milieu pauvre en thiamine (10 nM de thiamine)
MRT	Milieu riche en thiamine (10 µM de thiamine)
NeuN	« Neuronal Nuclei »
N2a	Cellules de neuroblastomes 2a de souris

NHA	Neurogenèse hippocampique adulte
OGDH	Oxoglutarate déshydrogénase
PA	Phosphatase alcaline
PFA	Paraformaldéhyde
PBS	Tampon phosphate salin (« Phosphate Buffer Saline »)
PDH	Pyruvate déshydrogénase
P_i	Phosphate inorganique
PI3K	Phosphoinositide 3-kinase
RTK	Récepteurs tyrosine kinase
RT-PCR	Amplification en chaîne par polymérase après transcription inverse (« Reverse Transcription Polymerase Chain Reaction »)
SBT	Sulbutiamine
SDS	Sodium dodécyl sulfate
SGZ	Zone sous-granulaire
SNC	Système nerveaux central
TBS	Tampon tris salin (« Tris Buffer Saline »)
TD	Déficience en thiamine (« Thiamine Deficiency »)
TK	Transcétolase
ThDP	Diphosphate de thiamine
ThDPase	Thiamine diphosphatase
ThMP	Monophosphate de thiamine
ThMPase	Thiamine monophosphatase
ThTP	Triphosphate de thiamine
ThTR-1/2	Transporteur de la thiamine (« Thiamine Transporter »)
WB	Western blot

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INTRODUCTION

1. LA THIAMINE ET SES DERIVES PHOSPHORYLES

1.1. Rôle biologique fondamental de la thiamine : du facteur antibéribéri au métabolisme énergétique

La découverte de la thiamine est étroitement liée aux recherches sur l'origine du béribéri, une maladie entraînant polynévrite et paralysie et touchant la population d'Asie de l'Est au 19ème siècle. Il s'est avéré que cette polyneuropathie était liée au régime alimentaire de la population, principalement constituée de riz poli, dont la thiamine était absente. Ces malades souffraient donc d'une déficience nutritionnelle en thiamine. Il a été remarqué qu'un composé, alors inconnu à l'époque et présent dans le son du riz, le lait, le pain, et la viande, avait la capacité de prévenir l'apparition de cette maladie. La thiamine (vitamine B1) a dès lors d'abord été caractérisée comme facteur anti-neuritique (aneurine) et sa découverte est à l'origine du concept de vitamine. Comme toute vitamine, celle-ci ne peut-être synthétisée de novo par les animaux supérieurs et est donc apportée par une source exogène : l'alimentation. En l'occurrence, la thiamine est présente dans la plupart des aliments mais les céréales complètes, la viande et les levures en sont particulièrement riches. La vitamine B1 est principalement connue pour son implication, sous forme diphosphate (ThDP), comme coenzyme essentielle du métabolisme oxydatif. Dès lors, une déficience en thiamine a des effets néfastes chez les mammifères, en particulier sur le système nerveux. En effet, d'une part, les cellules nerveuses dépendent fortement de l'oxydation du glucose pour leur besoin en énergie et d'autre part, l'absorption de thiamine est lente, particulièrement chez les êtres humains (Bettendorff 2014).

1.2. Structure et propriétés chimiques de la thiamine et de ses dérivés phosphorylés.

La thiamine (M = 265.36 g/mol) est une molécule organique constituée d'un hétérocycle pyrimidine et d'un hétérocycle thiazolium, reliés par un pont méthylène (Figure 1). Comme son appellation le sous-entend, elle contient donc un atome de soufre ("thio") et une fonction amine ($-NH_2$, "amine"). Le cycle thiazolium est une structure rare dans les molécules biologiques et la thiamine est la seule coenzyme présentant cet hétérocycle.
La molécule de thiamine comporte 3 parties fonctionnelles (Figure 1):

1) L'hétérocycle aromatique thiazolium, directement impliqué dans la catalyse par les enzymes utilisant le ThDP comme coenzyme,
2) L'hétérocycle aromatique pyrimidine, aidant dans la catalyse et important pour la reconnaissance de la thiamine par les apoenzymes et les transporteurs,
3) Le groupement hydroxyéthyle qui peut être estérifié pour former les dérivés phosphorylés mono-, di- et tri- phosphate de la thiamine (ThMP, ThDP et ThTP) et les dérivés adénylés, adénosine thiamine di- et tri-phosphate (AThDP et AThTP).

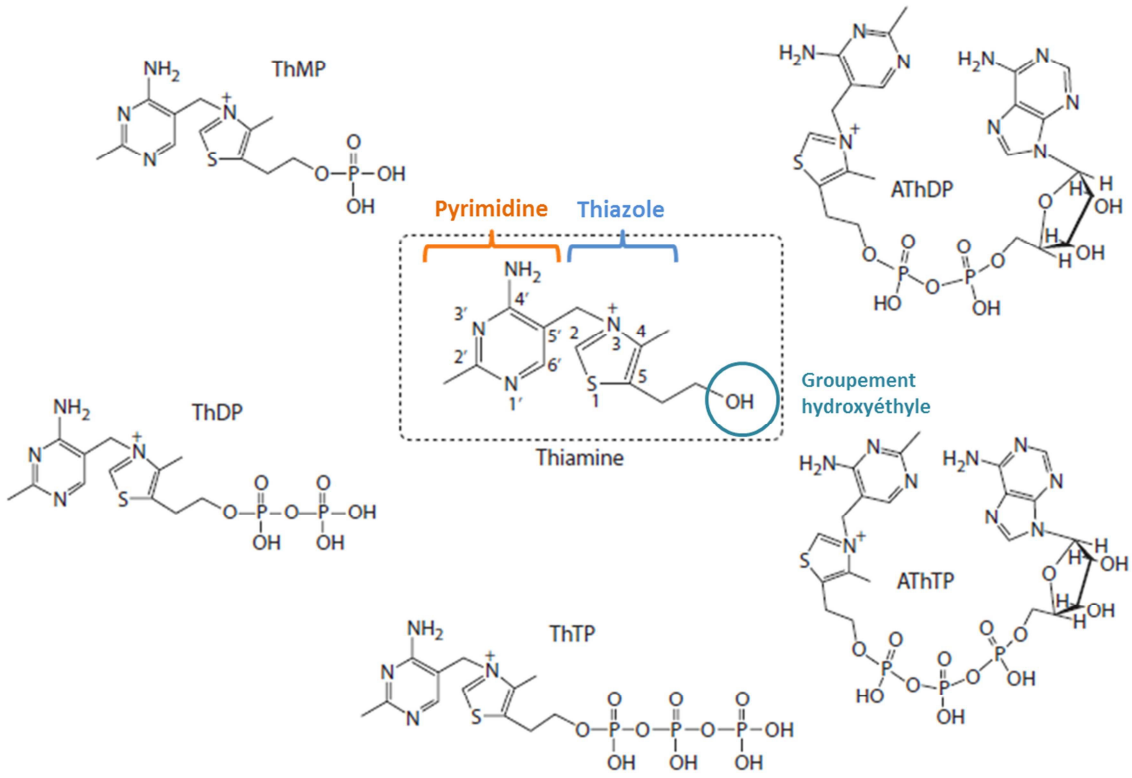


Figure 1 : **Structure moléculaire de la thiamine et de ses différents dérivés phosphorylés et adénylés.** [Tiré et modifié de Bettendorff 2014]**.**

La thiamine est hautement hydrosoluble mais peu soluble dans les solvants organiques. Elle est également hautement réactive et peut subir diverses modifications en présence d'ions hydroxyde (OH^-) et d'autres agents nucléophiles (ouverture du noyau thiazolium) ou d'oxydants (formation de thiochromes à pH alcalin, permettant sa détection en HPLC). La thiamine est stable en solution aqueuse à pH acide (2 – 4) et instable à pH alcalin. Le N1' and N3' ont un caractère basique et peuvent être protonés en milieu très acide (pH<4). L'amine primaire C4'-NH_2 n'est jamais protonée. La thiamine peut être détruite par la chaleur et les

rayons ultraviolets. *In vivo*, la thiamine est généralement administrée sous forme chlorhydrate car c'est la forme qui possède la meilleure biodisponibilité (Loew 1996).

Le ThMP et le ThDP sont relativement stables vis-à-vis de l'hydrolyse en solution aqueuse (sauf à des valeurs extrêmes de pH) ; le ThTP est plus labile.

1.3. Transport de la thiamine

La thiamine est une molécule hydrophile portant une charge positive et ne peut dès lors pas traverser les membranes biologiques par simple diffusion. Le transport de la thiamine à travers les membranes cellulaires nécessite donc des transporteurs spécifiques. A faible concentration (< 1 µM), la thiamine est absorbée via un transporteur saturable de haute affinité, alors que pour les concentrations plus élevées, un transport passif de faible affinité semble prévaloir (Bettendorff 2013; Bettendorff 2014). Ces transporteurs sont décrits plus en détails dans la section 1.3.4.

L'homéostasie de la thiamine est maintenue par un équilibre entre l'absorption intestinale et la perte rénale. L'excrétion de la thiamine et de certains de ses métabolites (2-methyl-4-amino-5-pyrimidine carboxylic acid, 4-methyl-thiazolium-5-acetic acid) se fait dans l'urine via les reins. L'excrétion urinaire de thiamine est réduite pendant le jeûne et est augmentée en cas d'administration de doses élevées de thiamine (Bettendorff 2014).

L'apport journalier recommandé (AJR) est de 1.4 mg/ jour mais dépend de l'absorption de glucose. La thiamine est sensible à des élévations de température et à des procédés tels que la pasteurisation du lait, une cuisson trop importante, ou la conservation. Ces procédés peuvent provoquer des pertes importantes de la vitamine. Pour cette raison, beaucoup d'aliments sont enrichis en thiamine. L'administration orale de doses de thiamine en excès n'engendre généralement pas d'effets secondaires mais son administration par voie veineuse (125 mg/kg) peut conduire à une dépression respiratoire et une paralysie neuromusculaire chez la souris. Cependant, chez l'homme, l'administration par voie veineuse (50-200 mg/jour) entraîne rarement des complications.

1.3.1. Absorption intestinale et transport dans les tissus périphériques (sang, foie, muscles).

Les dérivés phosphorylés de la thiamine provenant de la nourriture sont hydrolysés, probablement par des phosphatases alcalines (PA) intestinales (qui sont des ectoenzymes) en thiamine ou en ThMP. Ensuite, la thiamine libre est transportée de la lumière intestinale à travers la bordure en brosse dans les entérocytes grâce à un transporteur de haute affinité (ThTR-2, K_m=20-40 nM, voir point 1.3.4). Ce transport est un antiport électroneutre thiamine/H^+ et est facilité par un gradient de protons dirigé vers l'extérieur (le pH est plus alcalin dans la lumière de l'intestin). Dans les entérocytes, la thiamine subit un processus de phosphorylation-déphosphorylation. La phosphorylation de la thiamine intracellulaire peut être un facteur additionnel favorisant l'absorption de la thiamine. Cependant, seule la thiamine libre peut traverser la membrane basolatérale. Le transport basolatéral au niveau des entérocytes s'effectue également par un transport de moins haute affinité (ThTR-1, K_m=1-2 µM, voir point 1.3.4) indépendant de Na^+ mais dépendant d'un gradient de pH vers l'extérieur. La thiamine (à proportion de 0.2%) peut également être transphosphorylée en ThMP par la phosphatase alcaline pour entrer dans l'entérocyte. Le ThMP peut aussi être transporté à travers l'entérocyte mais il ne représente que 10% du transport total de la thiamine. La consommation chronique d'alcool diminue le transport intestinal de la thiamine. Le transport intestinal semble également être affecté par l'âge: l'absorption de thiamine diminue avec l'âge d'où une carence en thiamine plus fréquente chez les personnes âgées (Bettendorff et al. 1996; Wilkinson 2000).

Après le transport intestinal, la vitamine est collectée par le système portal et amenée au foie. Elle circule dans le sang où elle est en partie (mineure par rapport au foie) transportée à l'intérieur des érythrocytes et transformée en ThDP. La thiamine libre est présente à basse concentration dans les 2 composantes du sang (érythrocytes et plasma), tandis que le ThDP n'est présent que dans les érythrocytes et le ThMP que dans le plasma. Au niveau du foie, la thiamine est en grande partie capturée par les hépatocytes et convertie en ThDP. Le reste de la thiamine est redistribué vers les tissus (muscles, reins, cerveau…). Il n'existe pas réellement de lieu de stockage de la vitamine dans le corps bien que le foie contienne 10-20% de la quantité totale en thiamine. Lorsque des doses élevées de thiamine sont administrées, une grande partie est capturée par les érythrocytes et les hépatocytes.

Dès lors, ces cellules jouent un rôle important dans l'homéostasie : l'excès de thiamine peut être stocké dans ces cellules et être libéré et transporté via le sang jusqu'aux autres tissus en cas de déficit alimentaire.

1.3.2. Transport à travers la barrière hémato-encéphalique

Le transport de la thiamine à travers la barrière hémato-encéphalique (BHE) est un processus lent "énergie-indépendant" médié par un transporteur comportant une composante de haute affinité et une composante de basse affinité (qui représente moins de 10% du transport total). Le transport de haute affinité est un système de diffusion facilitée de faible capacité, spécifique et saturable, qui permet le transfert de la thiamine à travers la BHE de façon bidirectionnelle (Spector & Johanson 2007). Les niveaux cérébraux de thiamine et de ses dérivés sont fortement régulés, probablement par des mécanismes régulateurs à la fois de l'entrée et de la sortie de la thiamine. Dans des cellules de neurobastome en culture, un système antiport thiamine/thiamine a été mis en évidence (Bettendorff 1995). Il pourrait s'agir d'un mécanisme empêchant une accumulation de thiamine dans le cerveau. Des mécanismes homéostatiques similaires, tendant à maintenir des niveaux plasmatiques et cérébraux constants, existent pour d'autres vitamines (vitamines B3, B6, B5 et B8) (Spector & Johanson 2007).

Le ThMP aussi est transporté à travers la BHE mais à un taux moindre que la thiamine. Les plexus choroïdes semblent jouer un rôle important dans l'homéostasie cérébrale de la thiamine, en accumulant la thiamine par un processus actif et en relarguant de la thiamine et du ThMP dans le liquide céphalo-rachidien (LCR).

Chez le rat, seul 1.5% de la thiamine totale est retrouvée dans le cerveau. L'administration de hautes doses de thiamine engendre une élévation des niveaux de thiamine dans le sang et le foie (surtout sous forme de ThDP dans le foie), mais pas dans le cerveau (Bettendorff 2014).

1.3.3. Transport dans les neurones

Dans des coupes de cerveau, l'entrée de la thiamine est médiée par un transporteur Na^+- et énergie-indépendant et semble être dépendante du potentiel de membrane. Dans les cellules de neuroblastome *in vitro*, l'entrée de la thiamine dépend d'un transporteur

saturable de haute affinité (K_m = 35 nM) et d'un transporteur de faible affinité (K_m = 0.8 mM). Le transport de haute affinité semble être un transport actif secondaire où l'entrée de thiamine est couplée à sa conversion en diphosphate de thiamine par une pyrophosphokinase de la thiamine (TPK, 1, Figure 2) (Bettendorff & Wins 1994). Le transport de la thiamine semble également être influencé par le potentiel de membrane.

1.3.4. Les différents transporteurs caractérisés au niveau moléculaire

Trois gènes, appartenant tous à la famille des transporteurs de soluté SLC19A (famille des transporteurs de folate/thiamine), codant pour des protéines possédant 12 domaines transmembranaires, sont impliqués dans le transport de la thiamine (Oishi et al. 2002; Lindhurst et al. 2006; Subramanian et al. 2006) :

1) Le gène *SLC19A1* code pour un transporteur de folate réduit (RCF-1), capable de transporter le ThMP et le ThDP mais pas la thiamine (Zhao et al. 2001; Zhao et al. 2002). Cette protéine est hautement exprimée à la face apicale du plexus choroïde et est probablement à l'origine de la présence de ThMP dans le LCR. Il est également exprimé dans les neurones et pourrait constituer un mécanisme d'entrée et de sortie de faible affinité pour la thiamine dans les cellules nerveuses.

2) Le gène *SLC19A2* code pour le transporteur-1 de la thiamine (hTHTR-1) et le gène *SLC19A3* code pour le transporteur-2 de la thiamine (hTHTR-2). Ces deux protéines sont des échangeurs spécifiques de protons (H^+) et de thiamine (antiport 1/1) qui sont exprimés de façon ubiquitaire (cœur, foie, rein,…) dans les tissus mammaliens, avec des valeurs de K_m situées entre 10^{-6}–10^{-5} M pour THTR-1 et entre 10^{-8}–10^{-7} M pour THTR-2.

   Ils sont tous les deux fortement exprimés dans les intestins. Cependant, ThTR-1 est localisé au niveau des membranes apicales et baso-latérales alors que ThTR-2 n'est exprimé qu'au niveau de la membrane apicale des entérocytes (Said 2011). ThTR-2 est également présent dans les colonocytes du gros intestin, laissant penser qu'une partie de la thiamine absorbée pourrait provenir de la microflore bactérienne (Said 2011). Au niveau de la BHE et des plexus choroïdes, hThTR-2 est exprimé dans les

péricytes entourant les cellules endothéliales tandis que hThTR-1 est localisé à la face luminale.

3) Le gène *SLC25A19* code pour un transporteur mitochondrial de ThDP. Il semble agir comme un antiport, en échangeant le ThDP extramitochondrial contre des nucléotides intramitochondriaux (principalement de l'ATP) (Lindhurst et al. 2006; Iacopetta et al. 2010).

1.4. Métabolisme des dérivés phosphorylés de la thiamine

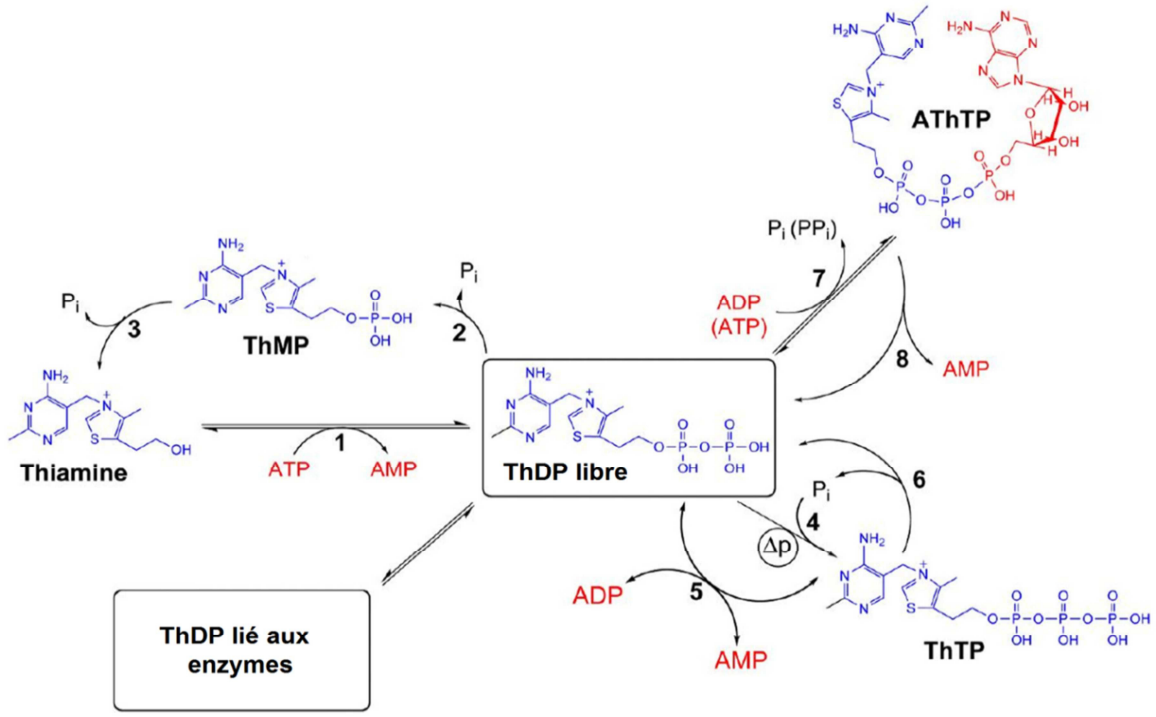


Figure 2 : **Schéma illustrant le métabolisme de la thiamine et de ses dérivés.** L'entrée de la thiamine dans la cellule est couplée à sa transformation en ThDP par la thiamine pyrophosphokinase (1, TPK). Le ThDP peut alors exercer son rôle de cofacteur en se liant à la transcétolase cytoplasmique ou en entrant dans les mitochondries. Le ThDP peut également donner lieu à la formation de ThTP soit grâce à l'adénylate kinase cytosolique (5, principalement dans les muscles), soit par un mécanisme chimi-osmotique impliquant une ThTP synthase (sans doute similaire à F_OF_1) associée à la membrane mitochondriale (4, dans le cerveau). Le ThDP permet la synthèse de l'AThTP grâce à une ThDP adénylyl transférase soluble (7). La thiamine diphosphatase (2) déphosphoryle le ThDP en ThMP en libérant un phosphate inorganique (P_i). La thiamine résulte de la déphosphorylation du ThMP par une thiamine monophosphatase (hypothétique, 3). (8) AThTP hydrolase (hypothétique). Les mécanismes de synthèse et de dégradation de l'AThDP sont inconnus. Δp, gradient de protons transmembranaire. [Tiré et modifié de Gangolf et al. 2010a].

Les dérivés phosphorylés de la thiamine peuvent facilement être interconvertis (Figure 2) mais seulement deux enzymes impliquées dans ces réactions (la TPK et la 25-kDa ThTPase) ont été caractérisées au niveau moléculaire.

1.4.1. Synthèse du diphosphate de thiamine (ThDP)

Le diphosphate ou pyrophosphate de thiamine (ThDP) est la forme la plus abondante de la thiamine et représente généralement 80 à 90% de la thiamine totale. Il est présent dans le cytosol et, en plus grandes quantités, dans les mitochondries. En conditions physiologiques, il est absent du milieu extracellulaire. Chez les mammifères, la thiamine libre absorbée par les cellules est rapidement convertie en ThDP par une enzyme cytosolique ubiquitaire, la ATP : pyrophosphokinase de la thiamine (TPK, Figure 2). Le K_m pour la thiamine est de l'ordre de 10^{-7} - 10^{-6} M alors que le K_m pour l'ATP est très élevé (10^{-2} M). L'équilibre de cette réaction est normalement en faveur des réactifs. Cependant, à l'intérieur de la cellule, l'équilibre est déplacé vers la droite à cause de la liaison du ThDP à la transcétolase (TK) cytsolique et son transport dans les mitochondries. Il existe 2 pools de ThDP intracellulaire (Bettendorff 1994a) : un pool à turnover lent (6-20 h), où le ThDP est principalement (90 à 95% du ThDP total) lié aux enzymes ; et un pool à turnover rapide (1-3 h) dans lequel le ThDP est libre dans le cytosol, où il sert de précurseur pour la synthèse de ThMP, ThTP et AThTP (Figure 2).

1.4.2. Transport mitochondrial du ThDP

Dans les cellules animales, la thiamine est rapidement phosphorylée en ThDP qui peut être transporté dans les mitochondries (Iacopetta et al. 2010). Le transporteur mitochondrial (SLC25A19, issu du gène éponyme) semble agir en échangeant le ThDP extramitochondrial contre des nucléotides intramitochondriaux (principalement de l'ATP) (Iacopetta et al. 2010). Le ThDP est plus concentré dans les mitochondries que dans le cytosol. Il est au moins en partie lié aux complexes des pyruvate et oxoglutarate déshydrogénases très abondants dans la matrice mitochondriale.

1.4.3. Dégradation du ThDP

Le ThDP peut être hydrolysé par des thiamine diphosphatases (ThDPases) plus ou moins spécifiques ainsi que par d'autres phosphohydrolases, ce qui mène à la formation de

ThMP (Figure 2). Le ThMP représente, avec la thiamine, 5 à 15% du pool total de thiamine cellulaire (Bettendorff 1994a). En dehors des cellules, on le retrouve également dans le liquide céphalo-rachidien (LCR) et le plasma (Zhao et al. 2002). Aucun rôle spécifique ne lui a été découvert à l'heure actuelle. Le ThMP peut être hydrolysé en thiamine (Figure 2) par des phosphatases acides ou alcalines ou encore par une ecto-5'-nucléotidase, mais aucune ThMPase spécifique n'a été caractérisée à ce jour.

1.4.4. Synthèse et dégradation des dérivés triphosphorylés

Une fraction variable de ThDP peut servir de précurseur à la synthèse de triphosphate de thiamine (ThTP) et d'adénosine thiamine triphosphate (AThTP) (Figure 2).

Le triphosphate de thiamine (ThTP) est toujours présent dans les tissus nerveux (mitochondries des neurones) et les muscles squelettiques (cytoplasme) mais est peu abondant et représente généralement moins de 1% de la thiamine totale. Deux hypothèses existent quant à son mécanisme de synthèse, qui dépend aussi du tissu. Dans les muscles squelettiques, l'adénylate kinase (AK) cytosolique formerait du ThTP selon la réaction : ThDP + ADP = ThTP + AMP (Shikata et al. 1989a; Shikata et al. 1989b; Miyoshi et al. 1990; Shioda et al. 1991). Le second mécanisme a été récemment décrit (Gangolf, et al. 2010b) dans les mitochondries du cerveau de rat (mais pas dans celles du foie). Le ThTP serait synthétisé par un mécanisme chimiosmotique couplé à la chaîne respiratoire (ThTP synthase) semblable à celui de la synthèse de l'ATP par phosphorylation oxydative selon la réaction ThDP + P_i = ThTP + H_2O. Le ThTP peut ensuite être relargué dans le cytoplasme en échange d'un phosphate. Dans la plupart des cellules de mammifères, sa concentration est maintenue à un niveau faible et sensiblement constant (0.1 à 1 µM) car il est continuellement hydrolysé par une thiamine triphosphatase cytosolique de 25 kDa (ThTPase) (Makarchikov et al. 2003). Cette enzyme hydrolyse le ThTP avec une grande spécificité et une efficacité catalytique élevée (Lakaye et al. 2002; Delvaux et al. 2013). Il existe également des ThTPases membranaires mais seule la 25 kDa ThTPase soluble a été purifiée et caractérisée.

Bien que non confirmés, plusieurs rôles potentiels ont été décrits pour le ThTP. Etant donné que le ThTP est un phosphoanhydride, c'est un composé riche en énergie et donc possédant un potentiel élevé pour le transfert de groupe phosphate. Il a en effet été démontré que le ThTP pouvait phosphoryler des protéines dans des tissus électriques et dans le cerveau de

rat (Nghiêm et al. 2000). Par ailleurs, Bettendorff et al. (1994) ont montré que le ThTP était capable d'activer un canal chlorure à haute conductance dans des patchs excisés de cellules de neuroblastome, peut-être par un mécanisme de phosphorylation.

L'adénosine thiamine triphosphate, récemment découvert chez *E. coli* (Bettendorff et al. 2007; Frédérich et al. 2009), est présent dans plusieurs tissus chez l'homme et les mammifères ainsi que dans les racines de plantes mais en plus faibles quantités que le ThTP. Bien que le rôle de l'AThTP ne soit pas encore déterminé, on lui soupçonne une fonction de signal plutôt que de cofacteur. En effet, l'AThTP s'accumule chez *E. coli* seulement en conditions de stress, en réponse à une absence de source carbonée.

1.5. Rôle du ThDP comme cofacteur indispensable dans le métabolisme énergétique

1.5.1. Réactions de décarboxylation

Dans le cerveau, le ThDP est principalement lié aux apoenzymes dont il est le cofacteur. Le ThDP est essentiel au métabolisme énergétique oxydatif, principalement parce qu'il est le coenzyme des complexes enzymatiques impliqués dans la décarboxylation oxydative. En effet, dans les mitochondries, on le retrouve lié aux complexes de l'α-cétoglutarate ou oxoglutarate déshydrogénase (OGDH) et de la pyruvate déshydrogénase (PDH). L'OGDH est une enzyme limitante du cycle de Krebs et permet la transformation d'oxoglutarate en succinyl-CoA. Rappelons par ailleurs que l'oxoglutarate est le précurseur du glutamate, un neurotransmetteur important. Le complexe PDH est le point d'entrée du cycle de Krebs dans la mitochondrie et permet la décarboxylation oxydative du pyruvate en acétyl-CoA. L'acétyl-CoA est le précurseur d'un autre neurotransmetteur, l'acétylcholine. Le ThDP est également lié à la 2-hydroxylacyl-CoA lyase (HACL1) dans les peroxysomes et donc impliqué dans le catabolisme des acides gras à longue chaîne. Par ailleurs, le ThDP intervient aussi dans le catabolisme des acides aminés branchés (leucine, isoleucine et valine) via l'enzyme mitochondriale BCOAD (branched-chain-2-oxoacid-dehydrogenase).

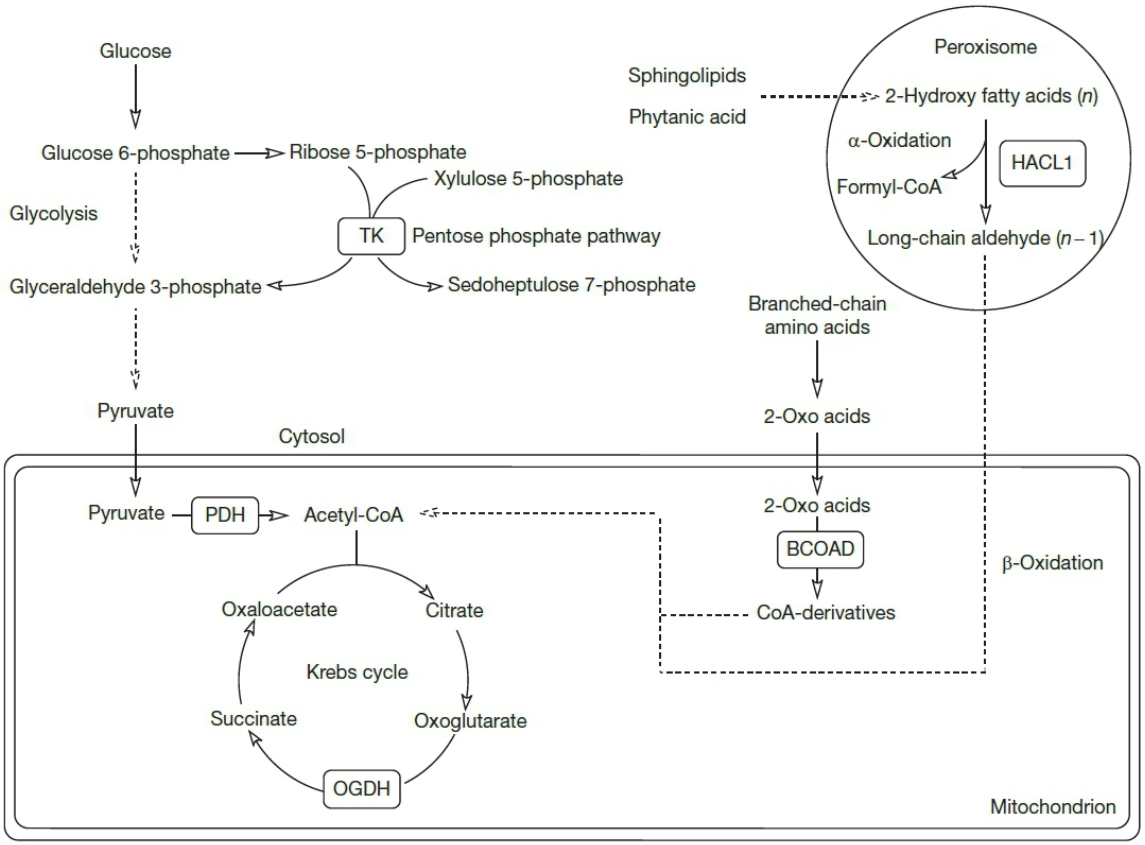


Figure 3 : **Réactions enzymatiques requérant le ThDP comme coenzyme dans le cerveau.** Dans la plupart des cellules, le glucose est dégradé en pyruvate via la glycolyse. Le pyruvate est transporté dans la mitochondrie où il est transformé en acétyl-CoA par le complexe pyruvate déshydrogénase (PDH). La transcétolase (TK) est impliquée dans le shunt des pentoses phosphates conduisant à la formation de NAPDH pour la synthèse des acides gras et à la formation de ribose-5-phosphate nécessaire à la synthèse des acides nucléiques. Les acides aminés branchés (leucine, isoleucine et valine) sont transformés en leur 2-oxo acides correspondants, qui sont transportés dans la mitochondrie où ils sont décarboxylés en dérivés CoA correspondants par la branched-chain-2-oxoacid-dehydrogenase (BCOAD). La 2-hydroxylacyl-CoA lyase (HACL1) est impliquée dans la dégradation des acides gras à longue chaîne. [Tiré de Bettendorff & Wins 2013].

1.5.2. La transcétolase

Dans le cytosol, le ThDP est lié à la transcétolase (TK). La transcétolase permet le transfert d'un groupement cétol (-CO-CH_2OH) d'un donneur cétosique (R-CHOH-CO-CH_2OH) sur un accepteur aldéhydique (R-COH). La réaction est une étape limitante pour la partie non oxydative de la voie des pentoses-phosphates.

1.6. Rôle particulier de la thiamine dans le système nerveux

Comme expliqué précédemment, la thiamine, sous sa forme diphosphate, est principalement connue pour son rôle essentiel de coenzyme dans le métabolisme énergétique cellulaire où elle intervient à de nombreux niveaux : production énergétique, synthèse d'acides nucléiques, biosynthèse des lipides et synthèse des neurotransmetteurs. Cependant, depuis de nombreuses années, l'hypothèse selon laquelle la thiamine et/ou certains dérivés phosphorylés posséderaient un rôle autre que coenzyme, a été soulevée (Bettendorff 1994b). Nous allons développer dans les points ci-dessous les rôles potentiels autres que coenzyme mis en évidence au cours de ces dernières années.

1.6.1. Régulation de la transmission synaptique par la thiamine

Depuis l'observation initiale de Minz (1937), plusieurs études indépendantes ont montré que la stimulation électrique de nerfs isolés engendre une libération de thiamine. Cette libération a été observée conjointement à un shift vers la déphosphorylation des dérivés phosphorylés (ThDP et ThTP). L'explication avancée est que la stimulation électrique des neurones accélère l'hydrolyse de l'ATP car la Na^+-K^+-ATPase est stimulée pour maintenir les gradients de Na^+ et de K^+. Sachant que le K_m de la TPK pour l'ATP est très élevé, le taux de formation de ThDP dépend directement de la concentration en ATP. Une réduction de la charge énergétique pourrait expliquer la formation de thiamine libre à partir de ThDP et la libération de thiamine.

Il a également été montré que la thiamine (et/ou ses dérivés phosphorylés) facilitait la neurotransmission dans différents types de synapses (préparations nerf-muscle, organe électrique de torpille, cerveau de mammifère…), probablement par potentialisation de la libération de neurotransmetteurs tels que l'acétylcholine (ACh) (Dyatlov 1995; Eder et al. 1976; Romanenko et al. 1994), la dopamine (Yamashita et al. 1993) et la noradrénaline (Romanenko et al. 1994). A l'opposé, la déficience en thiamine conduit à une diminution de la libération d'ACh (Jankowska-Kulawy et al. 2010), de glutamate (Lê et al. 1991) ou de dopamine (Mousseau et al. 1996). Au vu de l'existence de protéines liant la thiamine dans les membranes synaptiques, on peut imaginer l'existence d'un récepteur présynaptique à la thiamine. Dès lors, la thiamine pourrait agir comme neuromodulateur au niveau de certaines synapses, régulant ainsi la libération de neurotransmetteurs.

1.6.2. Protéines liant la thiamine (PLT)

Chez les mammifères, des protéines capables de lier les formes libres ou phosphorylées de la thiamine (en dehors des transporteurs, de la TPK ou des apoenzymes) ont été décrites même si leurs rôles restent inconnus. Des protéines capables de lier le ThDP ont été trouvées dans le cerveau de mammifères (Yoshioka et al. 1987; Yoshioka et al. 1990). D'autres études ont montré que des protéines cérébrales, associées aux vésicules synaptiques et aux membranes synaptiques, étaient capables de lier le ThMP et le ThTP (et dans une moindre mesure, le ThDP) et posséderaient même une activité ThTPase (Parkhomenko et al. 2001; Parkhomenko et al. 2010). Dans le cerveau, la thiamine semble être hautement localisée dans les structures membranaires synaptiques et il a été suggéré que celle-ci pourrait être une composante intrinsèque des membranes intervenant dans leur stabilisation (Cooper & Pincus 1979; Matsuda & Cooper 1981; Bâ 2008).
Il a également été montré que la thiamine, le ThMP et le ThDP étaient capables de lier la protéine prion (Perez-Pineiro et al. 2011). Par ailleurs, Tanaka et al. (2011) ont montré que, *in vitro*, l'AThTP peut lier et inhiber l'activité de la poly (ADP-ribose) polymérase 1 (PARP-1), un facteur impliqué dans la pathogenèse et les complications diabétiques (Tanaka et al. 2011).

1.6.3. Le problème de la vulnérabilité sélective des structures diencéphaliques

Le cerveau est un organe dont le fonctionnement dépend essentiellement de l'énergie fournie par le métabolisme oxydatif du glucose. Nous avons vu que la thiamine, via les enzymes ThDP-dépendantes, joue un rôle important dans le métabolisme du glucose, le fonctionnement des mitochondries et la synthèse de neurotransmetteurs. La carence en thiamine engendre donc une détérioration du métabolisme oxydatif conduisant à un déficit énergétique et à du stress oxydatif, mais certaines régions du cerveau sont affectées sélectivement : on observe une perte des neurones dans des régions spécifiques du cerveau. Par exemple, les alcooliques chroniques (souvent en état de carence aigüe en thiamine) peuvent développer une encéphalopathie de Wernicke qui, si elle est n'est pas soignée rapidement par l'administration d'une forte dose de thiamine, peut dégénérer en syndrome de Wernicke-Korsakoff dont les lésions sont irréversibles. Il s'agit de petites lésions hémorragiques, c'est-à-dire des ruptures de l'endothélium des capillaires et perte neuronale

liées à l'excitotoxicité et au stress oxydatif. Ces lésions sont spécifiquement situées dans le thalamus, les corps mamillaires et parfois le cervelet alors que le cortex est épargné. Or, hypothétiquement, la carence en thiamine devrait engendrer une diminution des niveaux de ThDP et de l'activité des enzymes associées (surtout l'OGDH), et donc du métabolisme oxydatif, dans tout le cerveau. A l'heure actuelle, il n'existe aucune explication quant à la vulnérabilité préférentielle de ces structures diencéphaliques à la déficience en thiamine, renforçant l'idée d'un rôle de la thiamine indépendamment de son rôle de coenzyme dans ces régions vulnérables.

1.6.4. Thiamine et maladies neurodégénératives

Plusieurs études (Gibson & Blass 2007) ont été consacrées au rôle de la thiamine et de sa possible déficience dans diverses maladies neurodégénératives liées à l'âge (maladie d'Alzheimer, de Parkinson,…). En effet, ces 2 processus dégénératifs partagent des caractéristiques communes telles qu'une altération chronique du métabolisme oxydatif, une perte régionale sélective de neurones (mais pas d'autres cellules), des modifications vasculaires, des réponses inflammatoires et du stress oxydatif. Dans des cerveaux post-mortem de patients Alzheimer, on a montré une diminution de la teneur en ThDP (souvent concomitante à une augmentation du ThMP) ainsi qu'une diminution de l'activité d'enzymes ThDP-dépendantes, notamment l'OGDH (Héroux et al. 1996; Shikata et al. 1996a; Mastrogiacomo et al. 1996b). L'activité de l'OGDH (Mizuno et al. 1994) et les taux de thiamine dans le LCR sont également diminués chez les patients atteints de la maladie de Parkinson (Gibson & Zhang 2002; Jiménez-Jiménez et al. 1999). Il est nécessaire de préciser ici que les réductions des teneurs en thiamine observées dans ces maladies neurodégénératives sont indépendantes d'une malnutrition.

D'autre part, la déficience en thiamine est responsable d'une aggravation des caractéristiques pathologiques (augmentation des dépôts amyloïdes, des lésions neurofibrillaires et du stress oxydatif) dans le cerveau de modèles murins de la MA (Karuppagounder et al. 2009; Zhao et al. 2011). Enfin, dans des cellules en culture de neuroblastome humain (cellules SH-ZY5Y) surexprimant le β-amyloïde, Zhang et al. (2009) ont montré que la déficience en thiamine augmentait la production du β-amyloïde et celle des radicaux libres dérivés de l'oxygène (ROS).

Des essais cliniques ont été réalisés sur des patients atteints de la maladie d'Alzheimer ou de Parkinson avec des doses élevées de thiamine (100-200 mg). Dans le premier cas, les effets bénéfiques sont mitigés voire inexistants alors que pour la maladie de Parkinson, les premiers résultats semblent prometteurs (Gibson & Blass 2007; Lương & Nguyen 2011).

1.7. Précurseurs synthétiques ayant une meilleure biodisponibilité que la thiamine

Comme mentionné précédemment, la thiamine est transportée à une vitesse relativement faible, et même lorsque les concentrations sanguines sont élevées, il est difficile d'obtenir une augmentation nette des niveaux de thiamine dans le cerveau. Dès lors, des analogues/prodrogues de la thiamine avec une meilleure biodisponibilité ont été développés dans le but de contrecarrer les effets de la déficience en thiamine plus rapidement et plus efficacement qu'avec la thiamine administrée oralement.

1.7.1. Allithiamines et autres analogues lipophiles (fursultiamine, sulbutiamine)

Le concept d'analogues/précurseurs de la thiamine a émané de la découverte dans les années 50 de l'allithiamine (thiamine allyl disulfure), un analogue présent dans l'ail (*Allium sativum*) (Wada et al. 1961). Ce composé, plus liposoluble que la thiamine, a la capacité d'être résorbé par l'organisme sans nécessiter l'intervention de transporteurs puisqu'il diffuse facilement à travers les membranes des muqueuses intestinales. Par la suite, d'autres analogues tels que des disulfures synthétiques de la thiamine, ont été développés, comme la fursultiamine et la sulbutiamine (Figure 4). Ces molécules sont généralement lipophiles, traversent les membranes et engendrent une augmentation rapide et importante des niveaux sanguins et périphériques de thiamine totale. Ces composés sont également responsables d'une augmentation des niveaux de thiamine libre dans le cerveau. Dans la cellule, elles sont transformées en thiamine après réduction du pont disulfure et fermeture de l'anneau thiazolium. Bien que ces composés aient potentiellement les propriétés pour traverser la BHE, aucun de ces composés n'a été détecté dans le parenchyme cérébral jusqu'à ce jour (Bettendorff & Wins 2013). En fait, même dans le sang, on ne mesure qu'une augmentation (souvent importante) de la teneur en thiamine.

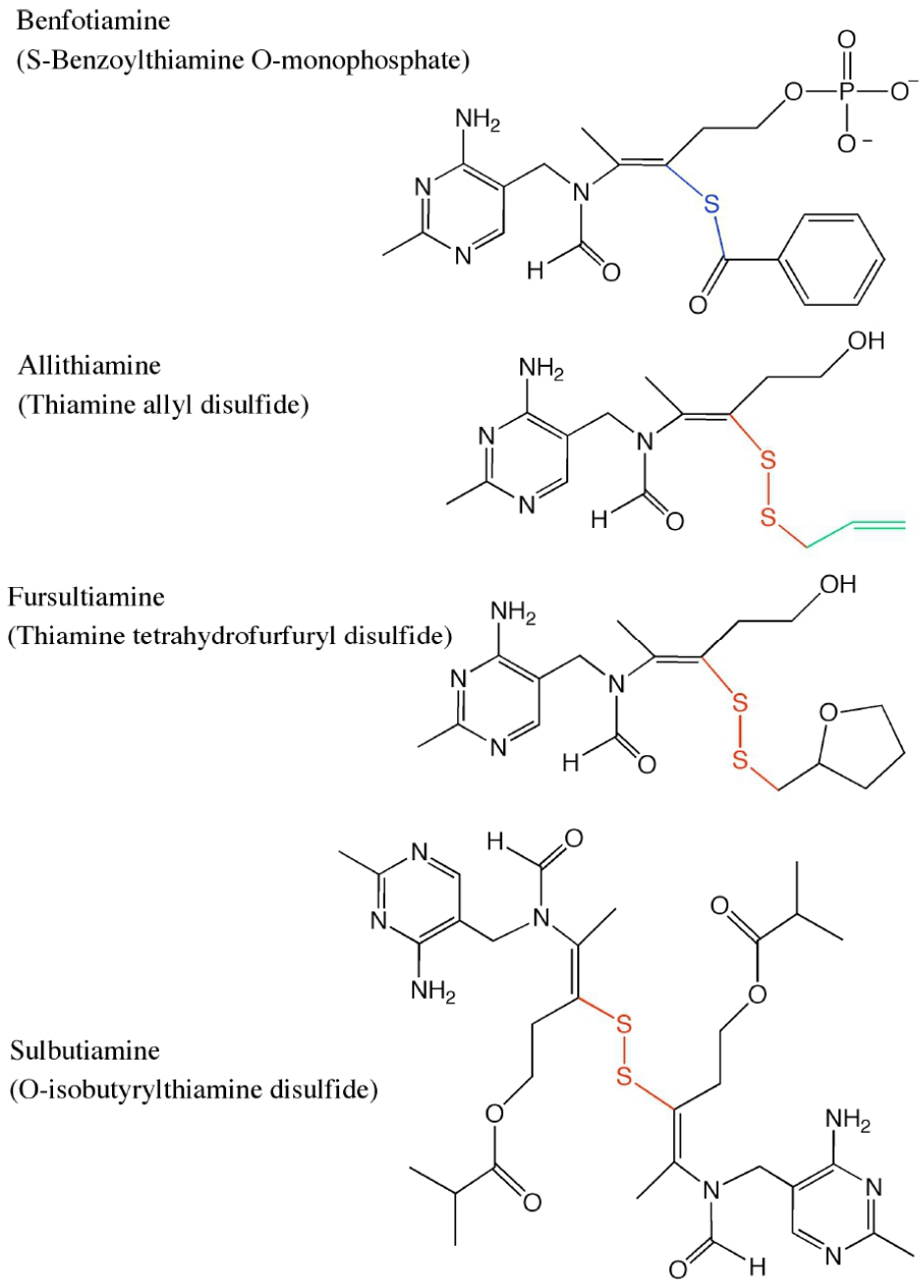


Figure 4 : **Structures de la benfotiamine, de l'allithiamine, de la fursultiamine et de la sulbutiamine.** La liaison thioester de la benfotiamine est indiquée en bleu. Les ponts disulfures de l'allithiamine, de la fursultiamine et de la sulbutiamine sont indiqués en rouge. Le groupement allyle de l'allithiamine est en vert. [Tiré de Volvert et al. 2008].

La fursultiamine ($C_{17}H_{26}N_4O_3S_2$, M = 398.5 g/mol) a démontré un effet bénéfique léger sur les symptômes émotionnels, mentaux et cognitifs des patients légèrement atteints de la maladie d'Alzheimer mais aucune amélioration n'a été notée chez les cas sévères (Mimori et al. 1996). Pourtant, dans un modèle murin de la maladie d'Alzheimer, la fursultiamine n'a pas montré les effets neuroprotecteurs de la benfotiamine (Pan et al. 2010)(voir le point 1.7.2). Au niveau central, la fursultiamine engendre une augmentation de la thiamine libre et du ThTP dans le cerveau de rat (Nozaki et al. 2009) mais de la thiamine seule dans le cerveau de souris (Pan et al. 2010).

La sulbutiamine ($C_{32}H_{46}N_8O_6S_2$, M = 702.8 g/mol) est un médicament utilisé comme psychotrope pour le traitement symptomatique de l'asthénie fonctionnelle (fatigue provenant d'une mauvaise gestion des ressources énergétiques), de la dépression majeure et pour améliorer la mémoire épisodique chez les schizophrènes (Crocq et al. 1978; Lôo et al. 2000). Chez les rongeurs, un traitement à la sulbutiamine engendre une amélioration de la mémoire de travail et de la mémoire à long terme (Kang et al. 2010; Micheau et al. 1985). La sulbutiamine est également capable de moduler les neurotransmissions cholinergiques, glutamatergiques et dopaminergiques (Micheau et al. 1985; Trovero et al. 2000). Des effets neuroprotecteurs *in vitro* (effets antioxydants et anti-apoptiques) et *in vivo* (contrecarre l'amnésie induite par un bloqueur des récepteurs NMDA) ont également été rapportés pour cette molécule (Bizot et al. 2005; Kang et al. 2010; Kwag et al. 2011). Chez le rat, la sulbutiamine a la particularité d'augmenter les niveaux de la thiamine et de ses esters phosphorylés dans le cerveau, particulièrement le ThTP (Bettendorff et al. 1990).

1.7.2. La benfotiamine

1.7.2.1. Propriétés chimiques, absorption et métabolisme

Contrairement à une idée répandue dans la littérature, la benfotiamine est un analogue unique de la thiamine car c'est une prodrogue non lipophile qui n'est ni une allithiamine ni un dérivé disulfure de la thiamine (Figure 4). En effet, la benfotiamine, ou S-benzoylthiamine-O-phosphate ($C_{19}H_{23}N_4O_6PS$; M = 466.45 g/mol), est un dérivé S-acyle (liaison thioester) avec un cycle thiazolium ouvert (Figure 4). Elle a été synthétisée pour la première fois en 1959 (Wada et al. 1961). Elle est peu soluble dans les solvants organiques (benzène, chloroforme, méthanol, octanol ou huile) ou dans l'eau dont le pH est inférieur ou

égal à 7 (0.004 g/mL à pH 4). Elle est par contre facilement dissoute (≥ 1 g/mL) dans de l'eau à pH ≥ 8 car son groupement phosphoryle acquiert 2 charges négatives à pH alcalin (Wada et al. 1961; Shindo et al. 1967; Volvert et al. 2008). Elle est également facilement soluble dans l'acide acétique. La benfotiamine est incapable de diffuser au travers des membranes cellulaires en tant que telle (Shindo et al. 1967; Volvert et al. 2008) vu la charge négative du groupement phosphate.

Pourtant la benfotiamine possède une biodisponibilité orale 5 à 10 fois plus élevée que la thiamine et ce, peu importe le mode d'administration utilisé (Loew 1996; Schreeb et al. 1997; Xie et al. 2014). Des études autoradiographiques comparatives ont étudié la distribution de thiamine et de benfotiamine marquée (aux isotopes S^{35} ou H^{3}) dans différents tissus (cœur, muscle, foie, sang, rein, cerveau) (Nakajima et al. 1968; Karpov et al. 1986; Hilbig & Rahmann 1998). Les deux organes les plus marqués par ces substances sont le foie et le rein. Dans les muscles et le cerveau, la radioactivité a été retrouvée en quantité 5 à 25 fois plus importante après administration de benfotiamine marquée qu'après administration de thiamine marquée (Nakajima et al. 1968; Karpov et al. 1986; Hilbig & Rahmann 1998). Par ailleurs, ces études ont également montré que 1) l'absorption était plus rapide et que 2) la rétention dans les tissus était plus longue pour la benfotiamine que pour la thiamine, en particulier dans le cerveau (matière grise > matière blanche) et les muscles squelettiques et cardiaques (Wada et al. 1961; Nakajima et al. 1968; Karpov et al. 1986; Loew 1996). Par ailleurs, il est intéressant de souligner que la benfotiamine ou ses métabolites ont tendance à se déposer dans les structures lipophiles (Shindo et al. 1967; Hilbig & Rahmann 1998; Ziems et al. 2000).

Dans les études pharmacocinétiques, l'augmentation la plus importante est observée dans le sang, particulièrement pour la thiamine et ensuite pour le ThMP et le ThDP (par exemple, selon Volvert et al., 2008, respectivement d'un facteur de 822, 14 et 4.7 fois). Rappelons que la thiamine est présente dans le plasma et les érythrocytes tandis que le ThMP n'est présent que dans le premier et que le ThDP ne l'est que dans le second. La concentration sanguine maximale en thiamine (Cmax) est généralement atteinte après une à deux heures chez la souris ou chez l'homme (Loew 1996; Greb & Bitsch 1998; Volvert et al. 2008; Xie et al. 2014) avant de diminuer lentement au cours du temps (Greb & Bitsch 1998; Volvert et al. 2008; Xie et al. 2014). Les augmentations les plus importantes observées sont ensuite celles de la

thiamine, du ThMP et du ThDP dans le foie et les reins (Volvert et al. 2008; Hurt et al. 2012). La Cmax dans le foie est atteinte après une heure pour la thiamine et ses dérivés (Volvert et al. 2008). La concentration en thiamine et dérivés dans les tissus est proportionnelle à la dose de benfotiamine administrée (Hurt et al. 2012).

Autant les études pharmacocinétique qu'autoradiographiques montrent que l'administration unique ou chronique de benfotiamine engendre une augmentation rapide de la thiamine et de ses dérivés dans les différents tissus périphériques (Wada et al. 1961; Loew 1996; Greb & Bitsch 1998; Hilbig & Rahmann 1998; Volvert et al. 2008; Pan et al. 2010; Hurt et al. 2012; Xie et al. 2014). Un tel consensus n'est pas présent dans la littérature en ce qui concerne l'impact de l'administration de benfotiamine sur les niveaux cérébraux de thiamine et de ses dérivés. En effet, Volvert et al. (2008) n'ont démontré aucune augmentation significative de la teneur en thiamine dans le cerveau de souris suite à l'administration unique ou répétée de de benfotiamine. Par contre, dans les études de Pan et al. (2010) et de Hurt et al., (2012), une dose unique de benfotiamine (0-300 mg/kg) engendrait une hausse légère et concentration-dépendante de la thiamine libre. La benfotiamine marquée radioactivement se retrouve également en quantité importante dans le cerveau mais dans ce cas, il est impossible de déterminer s'il agit de benfotiamine ou d'un de ses métabolites (Nakajima et al. 1968; Hilbig & Rahmann 1998).

Ce qui nous amène à la métabolisation de la benfotiamine. Comme cité précédemment, la benfotiamine ou S-benzoylthiamine-O-phosphate est une molécule hydrophile, elle est donc incapable de diffuser à travers les membranes biologiques. Lorsque la benfotiamine est administrée par voie orale à un sujet, elle est d'abord déphosphorylée dans la muqueuse intestinale par une ectophosphatase alcaline en S-benzoylthiamine ($C_{19}H_{22}N_4O_6S$; M=385 g/mol) qui diffuse alors à travers la bordure en brosse des cellules épithéliales de l'intestin avant de se retrouver sous cette forme dans le système sanguin mésentérique (Yamazaki 1968; Mizuhira et al. 1968; Shindo et al. 1968). Signalons que la benfotiamine n'a jamais été détectée dans le sang en tant que telle après administration orale (Shindo et al., 1968; communication personnelle de Mark J. Zylka) sauf dans une étude (Ziems et al. 2000). La S-benzoylthiamine quant à elle, se retrouve en quantité certaine (≈ 15% de la dose de benfotiamine administrée) dans le sang carotidien (Shindo et al. 1968; Ziems et al. 2000) bien qu'il n'ait pas été déterminé si elle pouvait traverser la BHE. La S-benzoylthiamine est

ensuite transformée en thiamine soit par 1) hydrolyse via une thioestérase dans le foie (Yamazaki 1968), soit par 2) réduction avec des groupements SH du glutathion dans les globules rouges (Shindo et al. 1967; Shindo et al. 1968). La réduction du groupement thioester de la benfotiamine engendre la fermeture du cycle thiazolium et la libération d'un acide benzoïque conduisant ainsi à la formation de thiamine. Comme expliqué précédemment, la thiamine a tendance à s'accumuler et à rester plus longtemps dans les tissus après administration de benfotiamine qu'après administration de thiamine. D'après Shindo et al. (1967), la transformation intracellulaire rapide (au moins dans les globules rouges) de la S-benzoylthiamine diffusible en thiamine non diffusible engendrerait un gradient de S-benzoylthiamine favorisant son entrée dans les cellules et ainsi l'accumulation de thiamine. La benfotiamine est excrétée par les reins, au moins sous forme de thiamine (Wada et al. 1961; Karpov et al. 1986; Itokawa et al. 1992; Schreeb et al. 1997) et d'acide hippurique (Xie et al. 2014). L'acide hippurique est un produit métabolique résultant de de la conjugaison hépatique d'un acide aminé, la glycine, avec de l'acide benzoïque (une molécule exogène provenant dans ce cas-ci de la benfotiamine) et de glycine.

1.7.2.2. Effets *in vitro*

In vitro, la benfotiamine a démontré des propriétés anti-oxydantes directes et/ou stimule l'activité ou l'expression d'enzymes (catalase, eNOS,…) dans différents types de cellules: cellules sanguines (Marouf et al. 2011; Schupp et al. 2008; Shoeb & Ramana 2012; Yadav et al. 2010), (progéniteurs de) cellules endothéliales (Du et al. 2010; Marchetti et al. 2006), myotubes (Fraser et al. 2012) et cellules rénales (Schmid et al. 2008). Elle possède également des propriétés anti-inflammatoires en inhibant notamment l'activation de NFκB (un facteur pro-inflammatoire) et l'expression de cytokines, de chemokines et de marqueurs inflammatoires (Du et al. 2010; Yadav et al. 2010; Shoeb & Ramana 2012). Par ailleurs, elle a également démontré des caractéristiques protectrices contre la mort cellulaire induite par hyperglycémie, stress oxydatif ou inflammation ; soit en favorisant des facteurs anti-apoptotiques soit en inhibant des facteurs pro-apoptotiques (Beltramo et al. 2008; Du et al. 2010; Gadau et al. 2006; Marchetti et al. 2006; Schmid et al. 2008; Yadav et al. 2010; Shoeb & Ramana 2012). Elle peut également favoriser la prolifération des (progéniteurs de) cellules endothéliales (Gadau et al. 2006; Marchetti et al. 2006; Pomero et al. 2001).

1.7.2.3. Actions neuroprotectrices *in vivo*

Avant de parler du système nerveux, il est intéressant de mentionner que la benfotiamine possède des effets protecteurs dans le diabète. En effet, elle prévient les complications diabétiques vasculaires (rétinopathies, néphropathie, neuropathie) en bloquant les 3 voies principales des dommages hyperglycémiques (hexosamine, PKC-DAG et AGEs) par activation de la transcétolase (Hammes et al. 2003; Balakumar et al. 2010). Ces effets bénéfiques concernent essentiellement les cellules endothéliales. Rappelons que l'endothélium des capillaires est également altéré dans les maladies neurodégénératives et dans les neuropathologies dues à une carence en thiamine.

Au niveau du système nerveux périphérique, la benfotiamine a des effets antinociceptifs et anti-allodyniques dans les modèles animaux de douleur inflammatoire et neuropathique ainsi que des effets analgésiques chez les êtres humains (notamment dans la neuropathie diabétique) (Hurt et al. 2012; Sanchez-Ramirez et al. 2006).

Au niveau du SNC, la benfotiamine confère une protection contre le stress oxydatif dans le cortex cérébral (Sanchez-Ramirez et al. 2006). L'administration chronique de benfotiamine à des souris APP/PS1 (un modèle murin de la maladie d'Alzheimer) confère un effet neuroprotecteur contre les altérations cognitives et histopathologiques observées dans ce modèle. En effet, la benfotiamine induit une nette amélioration de la mémorisation dépendante de l'hippocampe ainsi qu'une diminution spectaculaire des plaques amyloïdes (Pan et al. 2010). Au contraire, la pyrithiamine, un antimétabolite de la thiamine, a des effets opposés (Zhao et al. 2011). Les mêmes travaux (Pan et al. 2010; Zhao et al. 2011) ont montré que le traitement par la benfotiamine augmente le niveau de phosphorylation (et par conséquent, diminue l'activité) de la glycogène synthase kinase 3β (GSK-3β) dans le cerveau des souris transgéniques utilisées, alors que la pyrithiamine a l'effet inverse. Une observation importante est que la fursultiamine, un autre précurseur de la thiamine, ne semble exercer aucun des effets « anti-Alzheimer » de la benfotiamine, ce qui suggère un effet thérapeutique spécifique de ce composé (Pan et al. 2010).

2. LA NEUROGENESE ADULTE DANS L'HIPPOCAMPE

2.1. La neurogenèse

La neurogenèse est un processus impliquant la prolifération cellulaire, la survie, la maturation des neurones nouvellement formés en neurones fonctionnels ainsi que leur intégration dans les circuits synaptiques existants (Christie and Cameron, 2006). Fin des années 90, il a été accepté et démontré que la neurogenèse se produisait également à l'âge adulte et pas seulement durant le développement embryonnaire (Eriksson et al. 1998; Kempermann & Gage 1999). La neurogenèse adulte en conditions normales a lieu dans 2 régions neurogéniques cérébrales : dans la zone sous-ventriculaire des ventricules latéraux et dans la zone sous-granulaire (SGZ) du gyrus denté (DG) de l'hippocampe. L'hippocampe, structure limbique impliquée dans la mémoire et l'apprentissage, comprend 2 grandes structures : la corne d'Ammon (CA1, CA2 et CA3) et le gyrus denté (DG) divisé en 3 parties : le hile, la zone granulaire (CGL) et la zone sous-granulaire (SGZ). Les cellules néoformées dans la SGZ migrent vers la CGL où elles se différencient en neurones matures projetant leurs axones vers CA3 (Figure 5) (Toni et al. 2008). Seuls 50% des neurones néoformés survivent et s'intègrent dans le réseau synaptique du DG. La neurogenèse adulte est régulée par de nombreux facteurs : elle est stimulée par l'enrichissement environnemental, l'exercice physique, le traitement par les antidépresseurs et la thérapie électroconvulsive; à l'opposé, elle est diminuée par le stress et les pathologies qui causent des processus inflammatoires et un stress oxydatif dans l'hippocampe (Ming & Song 2011).

Il est possible d'étudier la neurogenèse adulte à l'aide de la bromodéoxyuridine (BrdU), un analogue de la thymidine. Le BrdU, injecté intrapéritonéalement aux rongeurs, va s'intercaler dans l'ADN des cellules durant la phase S de la réplication des progéniteurs neuronaux. Le BrdU incorporé peut être ensuite détecté grâce à des anticorps spécifiques par immunohistochimie à différents temps après l'injection. Les cellules neoformées expriment différents antigènes en fonction de leurs stades de maturation (par exemple : Ki67 est un marqueur des progéniteurs proliférants tandis que NeuN (Neuronal Nuclei) est un marqueur des neurones postmitotiques) qui peuvent eux aussi être détectés via des anticorps spécifiques. Un comarquage permet donc de réaliser des analyses phénotypiques et des quantifications stéréologiques grâce à la microscopie confocale. L'évolution et le

destin (prolifération, différentiation et survie) des cellules néoformées peuvent ainsi être étudiés au cours du temps (Figure 5).

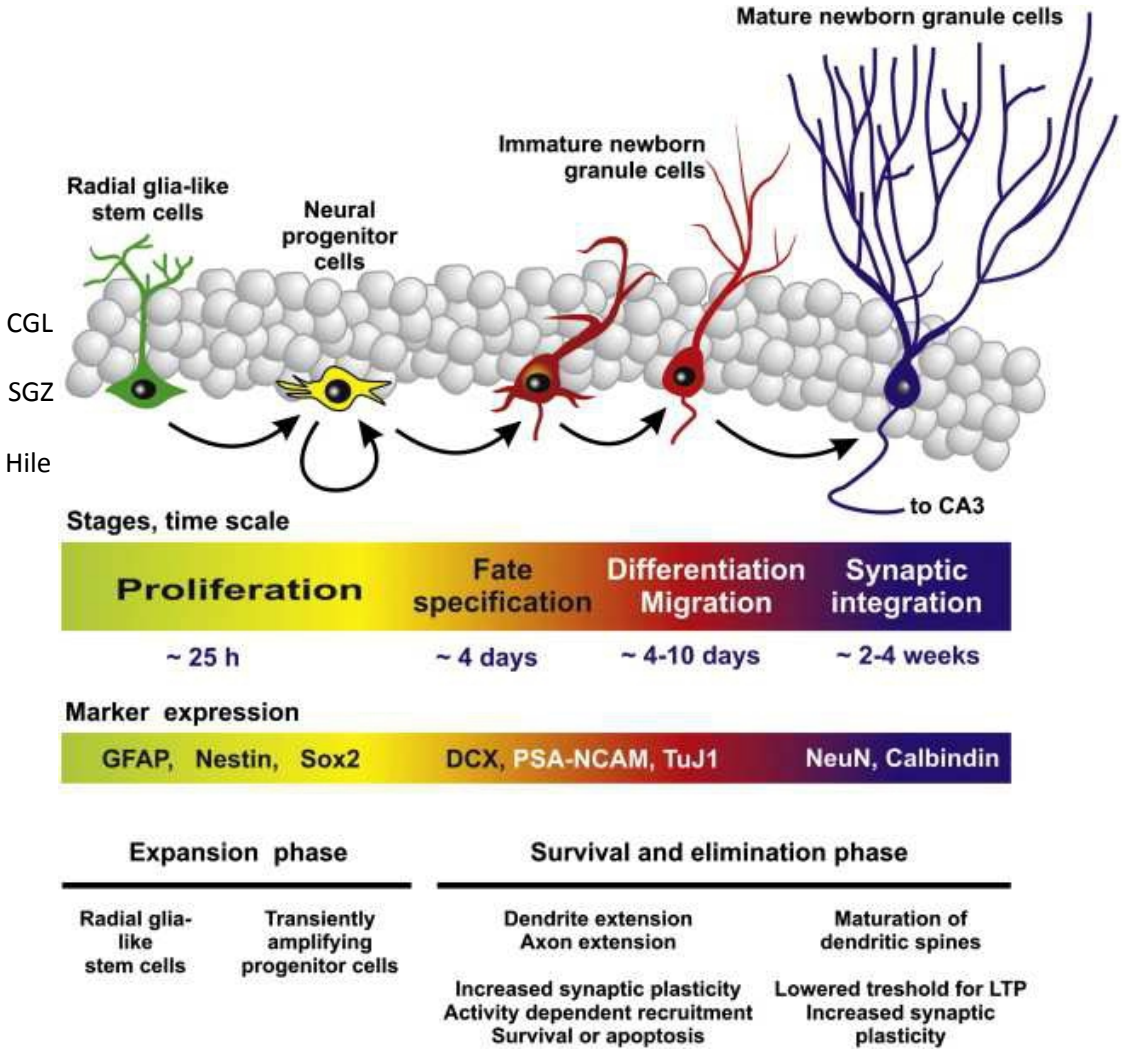


Figure 5 : **(A) Schéma de la structure de l'hippocampe:** la corne d'Ammon (CA1, CA2 et CA3) et le gyrus denté (DG) divisé en 3 parties : le hile, la zone granulaire (CGL) et la zone sous-granulaire (SGZ) **Figure schématique illustrant la cascade neuronale de différenciation des cellules néoformées localisées dans le gyrus denté de l'hippocampe adulte.** [Tiré et modifié de Lucassen et al. 2010].

2.2. Impact du stress sur la neurogenèse

Comme mentionné précédemment, la neurogenèse est influencée par de nombreux facteurs environnementaux, dont le stress. Chez les rongeurs, il a été montré que des paradigmes de stress de différentes natures (physique, psychosocial, prédictible ou non) et de différentes durées (aigu ou chronique) sont capables d'altérer une ou plusieurs étapes de

la neurogenèse hippocampale (Warner-Schmidt & Duman 2006). D'une manière générale, la neurogenèse hippocampique adulte (NHA) est d'autant plus diminuée que le stress est intense et prolongé (Hanson et al. 2011). L'exposition des souris à un prédateur semble être particulièrement efficace (Tanapat et al. 2001; Strekalova et al. 2015).

2.3. Relation entre thiamine et neurogenèse

Dans les modèles murins du syndrome de Wernicke-Korsakoff, la déficience en thiamine engendre des altérations de la neurogenèse hippocampale adulte avant l'apparition de lésions diencéphaliques irréversibles (Zhao et al. 2008; Vetreno et al. 2011a; Hazell et al. 2014). La déficience en thiamine induite par la pyrithiamine (un antimétabolite de la thiamine) provoque d'abord une augmentation de la prolifération cellulaire et de la survie des progéniteurs avant d'engendrer une réduction à long terme de la neurogenèse (Vetreno et al. 2011b). Une déficience induite de façon alimentaire conduit à une altération de la prolifération cellulaire et de la survie des cellules progénitrices ainsi qu'à une diminution de la neurogenèse hippocampale associée à une diminution de la capacité d'apprentissage, toutes deux contrecarrées par une supplémentation en thiamine (Zhao et al. 2008).

3. LA GLYCOGENE SYNTHASE KINASE-3B ET LA CASCADE RTK-PI3K-Akt-GSK-3B

Comme décrit précédemment, Pan et al. (2010) ont montré que la benfotiamine administrée chroniquement à un modèle murin de la maladie d'Alzheimer confère un effet neuroprotecteur contre les altérations cognitives et histopathologiques observées dans ce modèle. En effet, la benfotiamine induit une nette amélioration de la mémorisation dépendante de l'hippocampe ainsi qu'une diminution spectaculaire des plaques amyloïdes et des niveaux de protéine Tau phosphorylée. Dans le même modèle, la fursultiamine, un autre précurseur augmentant les concentrations plasmatiques en thiamine, ne présente pas ces effets bénéfiques. Afin de préciser les mécanismes responsables de ces effets, les auteurs ont étudié les niveaux de phosphorylation et d'activité de la glycogène synthase kinase - 3β (GSK-3β), une enzyme impliquée dans l'hyperphosphorylation de la protéine Tau et donc dans la formation des lésions neurofibrillaires. Ils ont constaté que l'activité de la GSK-3β était réduite par la benfotiamine mais pas par la fursultiamine.

La glycogène synthase kinase 3 (GSK-3) est une enzyme possédant un nombre incroyable de fonctions dans les mécanismes de signalisation intracellulaire. Bien que cette enzyme possède 2 isoformes (α et β), nous nous intéresserons ici uniquement à la GSK-3β, particulièrement abondante dans le cerveau. Elle est composée de 482 acides aminés (43 kDa) et est une enzyme à activité sérine-thréonine kinase. Son activité, bien qu'opérationnelle sans, est régulée par la phosphorylation de deux sites : soit une phosphorylation en sérine 9 qui a pour conséquence d'inhiber son activité ou bien une phosphorylation en tyrosine 216 qui a l'effet inverse. La plupart des substrats de GSK-3β (mais pas tous) nécessitent une phosphorylation préalable par diverses kinases avant que GSK-3β ne puisse procéder elle-même à la phosphorylation. Un site de liaison pour ces substrats phosphorylés, proche de la sérine 9 inhibitrice, est présent chez GSK-3β, permettant de reconnaître le substrat et d'assurer le positionnement optimal de celui-ci au niveau de son site devant être phosphorylé. La sérine située en position 9, une fois phosphorylée, agit en fait comme un pseudo-substrat et empêche les protéines d'atteindre l'enzyme.

Dans le système nerveux central, GSK-3β est impliquée dans la régulation du cycle cellulaire (Manning & Cantley 2007), dans la prolifération cellulaire (van der Heide et al. 2006), dans la survie cellulaire (Lawlor & Alessi 2001; Jope & Johnson 2004; van der Heide et al. 2006), dans la plasticité synaptique (van der Heide et al. 2006; Bradley et al. 2012), dans la neurogenèse (Cole 2013) et dans la neuroprotection (Dal-Cim et al. 2012; Kitagishi et al. 2012). Nous nous intéressons ici plus particulièrement à la voie de signalisation « pro-survie » RTK-PI3K-Akt-GSK-3β (Jope & Johnson 2004). Dans cette voie, l'insuline ou d'autres facteurs de croissance (Insulin Growth Factor-1 ou IGF-1) se lient à des récepteurs tyrosine kinase (RTK) membranaires, lesquels sont fortement exprimés dans les neurones (au niveau des corps cellulaires et des synapses), particulièrement au niveau du bulbe olfactif, du cortex cérébral, de l'hypothalamus et de l'hippocampe. La cascade de signalisation comporte les étapes suivantes (voir Figure 6) :

1) La fixation de l'insuline aux sous-unités du récepteur induit un changement de conformation de celles-ci de sorte que chaque sous-unité catalyse la phosphorylation rapide

de l'autre sous-unité sur des résidus tyrosine. Ce mécanisme est commun à toute la famille des RTK.

2) Une fois phosphorylé, le RTK est capable de recruter diverses protéines cibles, dont la principale est la protéine intracellulaire, IRS-1 ("Insulin receptor substrate 1"). Grâce à son interaction avec les sous-unités phosphorylées du RTK, IRS-1 va à son tour s'autophosphoryler sur des résidus tyrosine.

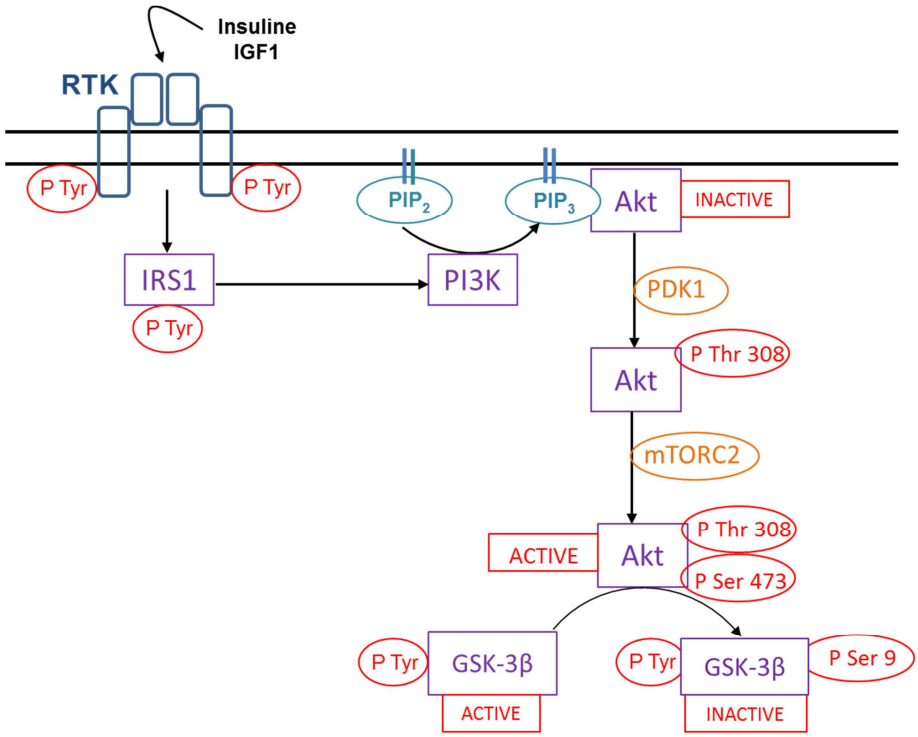


Figure 6 : **Schéma de la voie de signalisation « pro-survie » RTK-PI3K-Akt-GSK-3β.**

3) Une fois phosphorylé, IRS-1 va activer une autre kinase, la phosphoinositide 3-kinase (PI3K).

4) La PI3K activée se fixe alors à la membrane et catalyse la phosphorylation d'un phospholipide membranaire, le phosphoinositol 4,5-biphosphate (PIP2) en phosphoinositol 3,4,5-triphosphate (PIP3). La réaction inverse peut être catalysée par la PIP3 phosphatase PTEN.

5) La protéine kinase B ou Akt qui, sous sa forme non phosphorylée, est cytosolique, va former un complexe avec PIP3 au niveau de la membrane.

6) Au niveau de la membrane, Akt va interagir avec une autre kinase appelé 3-phosphoinositide-dependent kinase-1 (PDK1) qui es également liée au PIP3. PDK1 peut alors

catalyser la phosphorylation d'Akt sur le résidu thréonine 308 de la sous-unité catalytique. Cette phosphorylation induit une activation partielle d'Akt.

7) Pour qu'Akt soit pleinement active, il faut encore que sa sous-unité régulatrice soit phosphorylée sur la sérine 473. La principale kinase responsable de cette dernière réaction semble être le complexe mTORC2 (où TOR signifie "Target of Rapamycin").

8) Une fois activée, Akt se détache de la membrane et va phosphoryler de nombreuses protéines (dans le cytoplasme et aussi dans le noyau). Il s'agit le plus souvent de protéines impliquées dans la survie et la prolifération des cellules dont GSK-3β (Figure 7).

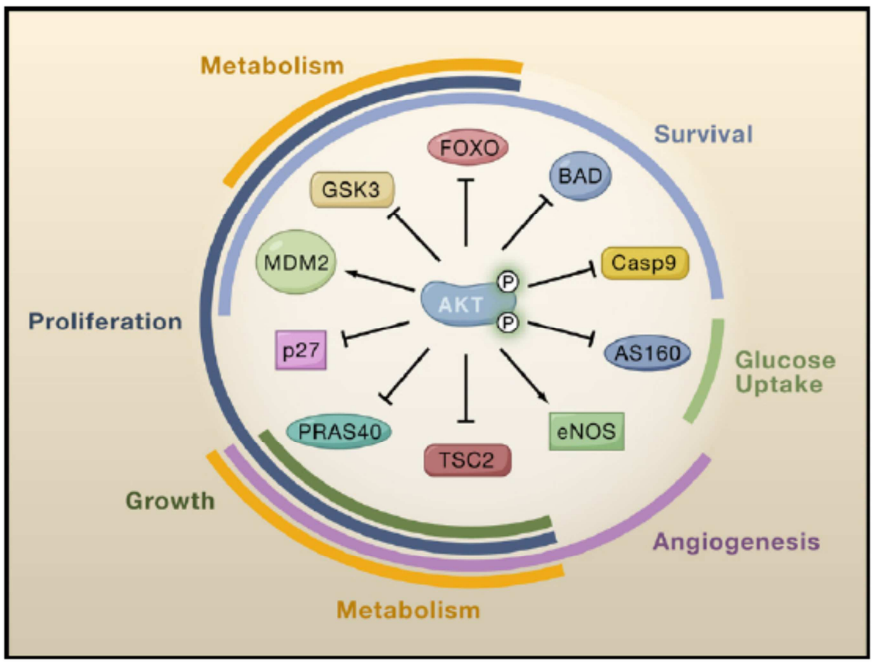


Figure 7 : **Fonctions cellulaires de différents substrats d'Akt dont GSK-3β.** [Tiré de Manning & Cantley 2007].

OBJECTIFS

Notre laboratoire étudie depuis longtemps le métabolisme des dérivés de la thiamine et, dès le début des années 90, un précurseur lipophile, la sulbutiamine, a été utilisé pour augmenter fortement les concentrations cellulaires en thiamine. Dans les années 2000, un autre précurseur, la benfotiamine, a été largement utilisé par les diabétologues en vue de corriger les complications vasculaires du diabète de type 2. Dans notre laboratoire, on a alors étudié de plus près l'action de ce précurseur (Volvert et al. 2008) et, en 2010, des auteurs chinois ont montré, dans un modèle murin de la maladie d'Alzheimer, que le traitement par la benfotiamine avait des effets bénéfiques remarquables sur les caractéristiques histopathologiques et les capacités cognitives des animaux (Pan et al. 2010). Plus récemment, l'équipe américaine du Professeur Beal a montré que la benfotiamine exerçait aussi des effets neuroprotecteurs dans un modèle de tauopathie.

Le mécanisme d'action de la benfotiamine restait toutefois mystérieux car, s'il est démontré que le traitement par ce précurseur augmente fortement les concentrations sanguines en thiamine, il n'y a pas d'augmentation du coenzyme thiamine diphosphate dans le cerveau et donc vraisemblablement pas de stimulation du métabolisme énergétique. Pan et al. (2010) ont proposé que le traitement par la benfotiamine induisait une augmentation de la phosphorylation de la glycogène synthase kinase-3β (GSK-3β) et donc une diminution de l'activité de l'enzyme. Comme celle-ci est en partie responsable de l'hyperphosphorylation des protéines tau, le traitement par la benfotiamine pourrait inhiber la formation de lésions neurofibrillaires caractéristiques de la maladie d'Alzheimer. Toutefois, dans le modèle de tauopathie utilisé par Beal et al., on n'a pas observé d'effets significatifs sur GSK-3β. De toute manière, lorsque nous avons entrepris nos recherches, il n'y avait pas d'explication proposée pour l'effet de la benfotiamine sur la GSK-3β ni, plus généralement, pour ses effets neuroprotecteurs.

Nous avons donc décidé de reprendre d'abord le problème à la base en étudiant les effets de la thiamine et de ses précurseurs non plus sur l'animal entier mais sur des cellules de type neuronal en culture (*in vitro*). Nous avons ainsi pu étudier le métabolisme des dérivés de la thiamine, la phosphorylation de GSK-3β et sa régulation par la voie de signalisation « pro-survie » RTK – PI3K – Akt. En ce qui concerne l'approche *in vivo*, nous avons mesuré le contenu des dérivés phosphorylés de la thiamine dans le cerveau des souris transgéniques utilisées comme modèle de tauopathie. Nous nous sommes ensuite intéressés

aux effets du stress sur la neurogenèse adulte dans l'hippocampe de souris WT. En effet, il a été récemment montré que le stress diminue la neurogenèse dans l'hippocampe (Strekalova et al. 2015). En outre, on sait que la déficience en thiamine diminue également la neurogenèse dans l'hippocampe. Nous avons donc envisagé l'hypothèse que le traitement par la thiamine ou la benfotiamine pourrait contrecarrer les effets du stress sur la neurogenèse.

Notre contribution à la compréhension des effets bénéfiques de la thiamine et de la benfotiamine sur le cerveau aura été de montrer *in vitro* une potentialisation par la thiamine de la voie « pro-survie » RTK – PI3K – Akt et, *in vivo*, une forte stimulation de la prolifération et de la survie des cellules progénitrices dans le gyrus denté des animaux stressés.

MATERIEL ET METHODES

1. MATERIEL

Toutes les solutions ont été préparées à l'aide d'eau Milli-Q (Millipore S.A. / N.V., Brussels, Belgium) à l'exception des solutions destinées aux souris qui ont été préparées avec de l'eau du robinet. Les solvants utilisés pour la chromatographie (qualité HPLC) et l'éther diéthylique proviennent de Biosolve (Valkenswaard, The Nederlands). Les composés utilisés pour préparer les solutions proviennent soit de chez Sigma-Aldrich, soit de chez VWR.

2. METHODES

2.1. EXPERIMENTATION *IN VITRO* : Effet de l'addition de thiamine et de ses précurseurs, avec ou sans insuline, sur des cellules de neuroblastome en culture

2.1.1. Culture cellulaire de cellules de Neuroblastome N2a

Les cellules de neuroblastome de souris (Neuro-2a ou N2a) sont cultivées à 37 °C en atmosphère humide contenant 95% d'air et 5% de CO_2.

Nous avons réalisé en parallèle deux conditions de cultures pour les cellules N2a. Dans la première, dite « riche » en thiamine, les cellules sont cultivées dans des boîtes T75 (430641, Corning) dans 10 mL de milieu classique DMEM avec 4.5 g/L de glucose et de la L-glutamine (Dulbecco's Modified Eagle's Medium, Lonza) supplémenté avec 10% de sérum bovin fétal (FBS, Greiner Bio-one). Dans ce milieu de culture normal, la concentration en thiamine est 10 µM, ce qui est beaucoup plus élevé que les concentrations physiologiques de thiamine dans le plasma sanguin. On parle donc de « milieu riche » en thiamine (MRT). Dans la deuxième condition, dite « pauvre en thiamine », la culture des cellules est réalisée dans des boîtes de Petri de 10 cm de diamètre (Greiner Bio-One) dans un milieu DMEM déficient en thiamine (AS52100 W/O thiamine, Dubelcco's Medium) supplémenté avec 10% de FBS. Le FBS contenant une concentration d'environ 100 nM en thiamine, le milieu « pauvre en thiamine » (MPT) contient dès lors une concentration en thiamine de 10 nM. L'entretien des cellules s'effectue tous les 2-3 jours. La division s'effectue tous les 4-5 jours avec un ratio de 1/10. La croissance n'est que très légèrement ralentie en milieu pauvre en thiamine.

Pour les expériences, les cellules sont préalablement ensemencées à la densité par puits de 100.000 cellules pour la culture normale et 150.000 pour la culture pauvre en thiamine dans des plaques 6 puits afin que la confluence soit de 85-90% le jour de l'expérience.

2.1.2. Design expérimental

Les cellules N2a sont cultivées dans un milieu soit riche (10 µM) soit pauvre (10 nM) en thiamine pendant 1 semaine. La veille de l'expérience, le milieu des cellules est remplacé par du milieu sans FBS contenant 10 nM thiamine pour remplacer la thiamine apportée par le FBS. En effet, les cellules restent une nuit sans sérum afin de minimiser la variation de la phosphorylation au niveau des protéines. Le jour 8, le milieu est remplacé par le milieu pauvre ou riche en thiamine sans sérum et contenant le composé à tester. Les différentes solutions stock (voir Tableau 1) sont stérilisées préalablement à leur usage sur les cellules. Elles sont ensuite diluées dans le milieu pour atteindre la concentration désirée. Le temps 0 h correspond aux cellules récoltées avant l'application des conditions. Les cellules sont ensuite récoltées pour l'analyse par HPLC ou par WB selon le protocole décrit dans les sections correspondantes (Tableau 2). Les mêmes conditions sont appliquées pour les cellules destinées à l'analyse du contenu en thiamine par HPLC.

Tableau 1 : **Concentrations et préparations des solutions-stocks utilisées dans les expériences *in vitro.***

	Concentration	Préparation
Thiamine	1 mM	3.4 mg de chlorhydrate de thiamine (T4625, sigma-Aldrich) dans 10 mL d'eau milliQ
Benfotiamine	1 mM	4.7 mg de benfotiamine (B9636, Sigma-Aldrich) dans 10 mL de TBS pH=8.5 (25 mM Tris-Base, 138 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$)
S-benzoylthiamine	1 mM	Benfotiamine 1 mM + 100 unités DEA de phosphatase alcaline, incubée 1 h à 37 °C dans du TBS pH = 8,5
Sulbutiamine	1 mM	7.03 mg de sulbutiamine (41193, Technologie Servier) dans 10 mL d'eau milliQ
LiCl	200 mM	424 mg de chlorure de lithium (Sigma-Aldrich) dans 50 mL d'eau milliQ
Insuline (I500, Sigma-Aldrich)	344 µM	

Tableau 2: **Récapitulatif des conditions d'expériences menées *in vitro* sur les cellules N2a.** Les cellules destinées à l'analyse à la fois par HPLC et par WB ont été incubées avec un milieu sans sérum pendant 12 h avant l'expérience.

Etudes	Conditions	Milieu	Solution	Concentration	Temps d'incubation	Analyse
Teneur en thiamine et dérivés phosphorylés dans des cellules N2a cultivées en milieu riche ou pauvre en thiamine	Milieu riche en thiamine (MRT)	MRT	/	10 µM thiamine	0, 4, 7, 21, 28, 35, 42 jours	HPLC
	Milieu pauvre en thiamine (MPT)	MPT	/	10 nM thiamine		
Evolution de la teneur en thiamine, ThMP et ThDP lorsque les cellules N2a cultivées en milieu pauvre en thiamine sont incubées en présence de thiamine et de différents précurseurs.	Contrôle (Con)	MPT sans FBS	/	10 nM thiamine	0, 2, 4 et 6 h	HPLC WB (pGSK3 et GSK3)
	Thiamine (Thia)		Thiamine 1 mM	1, 10 et 50 µM		
	Benfotiamine (BFT)		Benfotiamine 1 mM	1, 10 et 50 µM		
	Sulbutiamine (SBT)		Sulbutiamine 1 mM	0,5, 25 et 50 µM		
	LiCl (contrôle positif)		LicL 200 mM	20 mM		
Accumulation de thiamine dans les cellules N2a pauvres en thiamine en présence de benfotiamine traitée par de la phosphatase alcaline	Contrôle (Con)	MPT sans FBS	/	10 nM thiamine	0, 5, 15, 20, 30, 40, 60 min	HPLC
	Thiamine (Thia)		Thiamine	50 µM	0, 5, 15, 20, 30 min	
	Sulbutiamine (SBT)		Sulbutiamine	0,5, 25 et 50 µM	0, 20, 40, 60 min	
	Thiamine (Thia)	MPT sans FBS	Thiamine 1 mM	1, 10 et 50 µM	2 h	HPLC
	Benfotiamine (BFT)		Benfotiamine 1 mM	1, 10 et 50 µM		
	Benfotiamine + PA (BPA)		Benfotiamine 1 mM + PA	1, 10 et 50 µM		
	Sulbutiamine (SBT)		Sulbutiamine 1 mM	0,5, 25 et 50 µM		
Métabolisation de la benfotiamine en présence et en absence de sérum	Contrôle (Con) avec/sans sérum	MPT avec/sans FBS	/	10 nM thiamine	2 h	HPLC
	Thiamine (Thia) avec/sans sérum		Thiamine 1 mM	10 µM		
	Benfotiamine (BFT) avec/sans sérum		Benfotiamine 1 mM			
	Sulbutiamine (SBT) avec/sans sérum		Sulbutiamine 1 mM	5 µM		
	Contrôle (Con) avec/sans sérum	MRT avec/sans FBS	/	10 µM thiamine	12 h	HPLC
	Thiamine (Thia) avec/sans sérum		Thiamine 1 mM	100 µM		
	Benfotiamine (BFT) avec/sans sérum		Benfotiamine 1 mM			
	Sulbutiamine (SBT) avec/sans sérum		Sulbutiamine 1 mM	50 µM		
	Benfotiamine (BFT) avec/sans sérum	MRT avec/sans	Benfotiamine 1 mM	100 µM	24 h	HPLC
Comparaison de la métabolisation de la benfotiamine en présence de sérum ou de phosphatase alcaline.	Benfotiamine (BFT) sans sérum	MPT sans FBS	Benfotiamine 1 mM	10 µM	2 h	HPLC
	Benfotiamine (BFT) avec sérum	MPT avec FBS	Benfotiamine 1 mM			
	Benfotiamine + PA (BPA)	MPT sans FBS	Benfotiamine 1 mM + PA			
Effet de la teneur en thiamine de milieu de culture sur l'expression de la GSK-3β et son niveau de phosphorylation dans les cellules N2a	Milieu riche en thiamine (MRT)	MRT	/	10 µM thiamine	7 jours	WB (pGSK3 et GSK3)
	Milieu pauvre en thiamine (MPT)	MPT	/	10 nM thiamine		
	Milieu déficient en thiamine (MDT)	MDT	/	0 nM thiamine	7 jours en MPT puis 12 h en MDT	
Effet de l'addition de thiamine et d'insuline pendant des temps courts sur des cellules N2a	MPT + Ins	MPT sans FBS	Insuline 344 µM	0, 10, 100 nM	15, 30, 60, 120 min	WB (p)GSK3 et (p)Akt
	MRT + Ins	MRT sans FBS				
	MPT + Ins + Thia 50 µM	MRT sans FBS	Insuline 344 µM	0, 10, 100 nM		
	MRT + Ins + Thia 50 µM	MRT sans FBS	Thiamine 1 mM	0, 50 µM		

2.1.3. Production de S-benzoylthiamine à partir de benfotiamine

2.1.3.4. Pour les expériences in vitro sur les cellules N2a

La quantité de phosphatase a été calculée pour hydrolyser la benfotiamine en moins d'une heure d'incubation. Le volume correspondant à une unité DEA de phosphatase alcaline (PA, P5521, Sigma-Aldrich) est nécessaire pour hydrolyser 10 nanomoles de benfotiamine en moins d'une heure. La benfotiamine (1 mL, 1 mM) est donc incubée 1 heure à 37 °C dans du TBS avec de la PA (2 mg/mL ou 100 unités DEA). Afin de vérifier que la benfotiamine est bien hydrolysée et libère un phosphate, la concentration en phosphate inorganique est mesurée par une méthode colorimétrique (Lanzetta et al. 1979). La concentration maximale en phosphate (75 µM) est atteinte après 5 minutes (Figure 26A, page 84), suggérant que 75% de la benfotiamine est hydrolysée en S-benzoylthiamine. La solution de BFT + PA est ensuite diluée très rapidement dans le milieu cellulaire et ajoutée sur les cellules à la concentration désirée (Tableau 2).

2.1.3.5. Pour l'analyse par le laboratoire de Chimie Pharmaceutique

La benfotiamine (350 mM) préparée dans 2 mL de tampon NaTAPS pH 8.5 a été incubée pendant 1 h avec de la PA (2 mg/mL) à 37°C. L'échantillon a ensuite été centrifugé à 693 g pendant 5 minutes : 1) le surnageant obtenu (ST1) a été transféré à 4 °C pendant 4 jours ; 2) le précipité obtenu a été dissous dans du méthanol puis de l'eau a été ajoutée progressivement jusqu'à obtenir une solution laiteuse, qui est ensuite soumise à centrifugation (693 g, 5 minutes), le surnageant résultant est transféré dans un tube (ST2) et le précipité subit à nouveau le cycle solubilisation/centrifugation. Cette opération est répétée 3 fois et à la fin de celles-ci, nous possédons un tube avec du surnageant (SNT2) et un tube avec un précipité (PP2). Après 4 jours à 4 °C, un dépôt est apparu dans le tube du surnageant SNT1. Après centrifugation, le surnageant est transféré dans un autre tube (SNT1) et le précipité subit la même procédure que décrite précédemment (solubilisation/précipitation) ce qui permet d'obtenir un surnageant (SNT1) et un précipité (PP1).

Ces échantillons ont ensuite été transmis au Laboratoire de Chimie Pharmaceutique de l'Université de Liège (Professeur Liégeois). Après application de différentes méthodes de préparation et de séparation, la composition des échantillons a été analysée par résonance

magnétique nucléaire (RMN ^{1}H et ^{13}C), par spectrométrie de masse (LC-MS), par spectroscopie infrarouge et par cristallographie aux rayons X.

2.2. EXPERIMENTATION *IN VIVO* : Etude de la neurogenèse chez des souris adultes normales traitées par la thiamine et ses précurseurs

2.2.1. Animaux

Les souris C57BL/6Rj mâles âgées de 12 semaines ± 3 jours (*n=80*) au moment de l'expérience ont été fournies par Janvier (France) ou par l'animalerie institutionnelle. Les souris ont été hébergées avec nourriture et eau à volonté dans des conditions contrôlées de température et d'humidité sous un cycle jour/nuit 12h-12h. Les souris, hébergées individuellement par cage, ont été habituées à l'animalerie locale pendant au moins 1 semaine avant toute expérience. Toutes les expérimentations animales ont été réalisées selon les directives du comité d'éthique pour le bien-être et l'utilisation des animaux de l'Université de Liège en accord avec le « European Communities Council Directive » du 24 novembre 1986 (86/609/EEC).

2.2.2. Design expérimental

Les souris ont été divisées aléatoirement en 4 groupes différents :

- Traité avec le véhicule (Con)
- Traité avec de la thiamine (Thia)
- Traité avec de la benfotiamine (BFT)
- Traité avec de la sulbutiamine (SBT)

Les souris, hébergées individuellement, ont donc été traitées pendant 20 jours par voie orale avec de la thiamine (200 mg/kg/jour), de la benfotiamine (200 mg/kg/jour) via des gaufrettes et avec de la sulbutiamine (100 mg/kg/jour, préparée dans du NaCl 0.9% stérile) via injection intrapéritonéale. Les souris traitées à la sulbutiamine recevaient également une gaufrette contrôle.

La première cohorte de souris (*n=10* par condition de traitement, Figure 8A), destinée aux études biochimiques (HPLC et WB), a été sacrifiée par décapitation 24 heures après le dernier traitement. Le sang du tronc a été récupéré dans des tubes coatés à l'EDTA. Le foie,

le cortex et l'hippocampe ont été disséqués à 4 °C. Le tout a été congelé instantanément dans de l'azote liquide.

La seconde cohorte de souris (*n=5* par condition de traitement et par groupe de BrdU, Figure 8B), destinée à l'étude de la neurogenèse, a reçu une injection intrapéritonéale de BrdU (150 mg/kg) 24 heures après le dernier traitement. Elles ont été ensuite sacrifiées et perfusées après 2 h, 24 h et 3 semaines afin d'étudier respectivement la prolifération, l'index de sortie de cycle cellulaire, la survie et la neurogenèse par immunohistochimie.

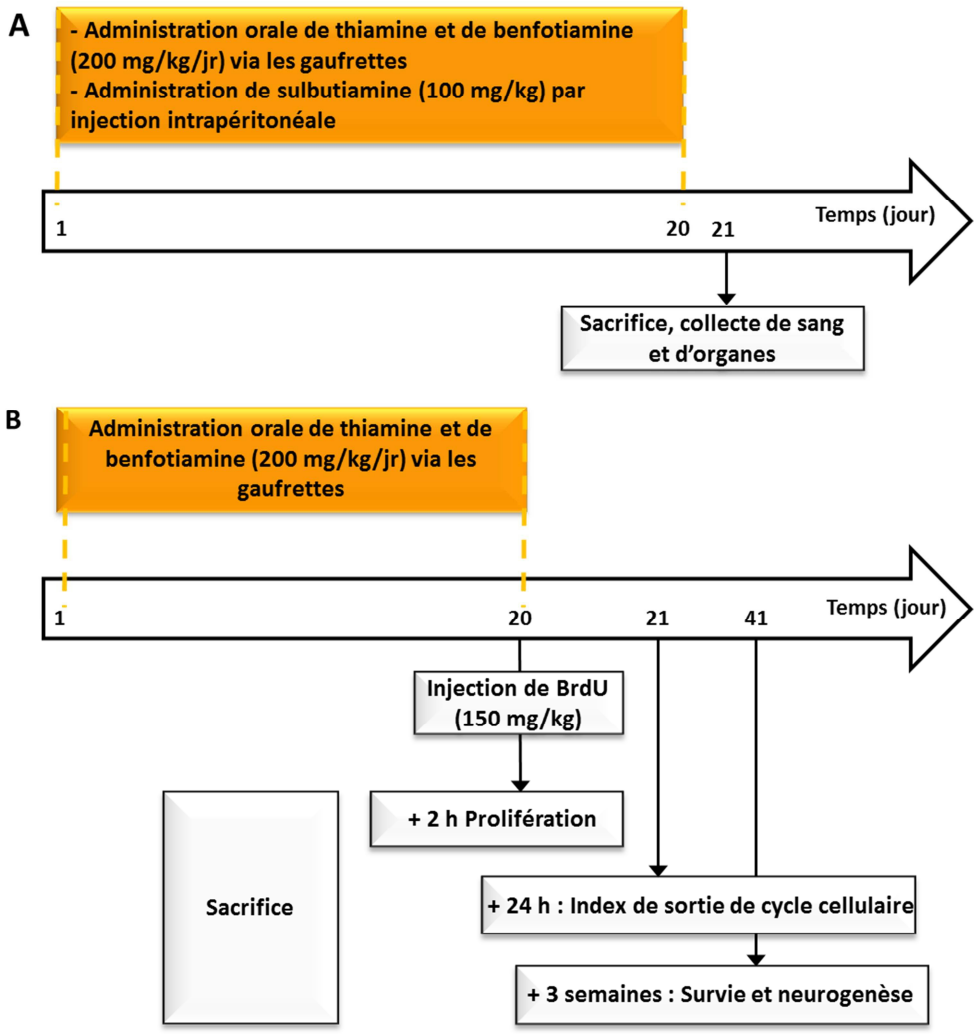


Figure 8 : **Design expérimental de l'étude de l'effet d'un traitement par la thiamine ou la benfotiamine sur des souris normales** (A) pour les analyses biochimiques par HPLC et WB et (B) pour l'analyse de la neurogenèse par immunohistochimie.

2.2.3. Préparation des solutions de traitement

Les solutions de thiamine et de benfotiamine ont été préparées dans du saccharose 50% et renouvelées tous les 7 jours. Le volume injecté dans les gaufrettes, ajusté chaque jour en fonction du poids individuel des souris, est d'environ 50 µL et doit contenir la dose quotidienne (200 mg/kg) de traitement. La concentration des solutions est calculée en fonction de la moyenne du poids des souris de chaque groupe des 3 jours précédant le renouvellement de la solution de traitement. Celle-ci est donc variable d'une préparation à l'autre (Tableau 3). Chaque composé requiert une préparation particulière mais toutes les solutions administrées oralement ont été ajustées au pH de 7.5-7.6. En effet, la thiamine engendre un pH acide lorsqu'elle est dissoute, qui est alors ramené à neutralité à l'aide de NaOH 15%. La dissolution complète de la benfotiamine requiert une basification progressive du solvant jusqu'à pH 9-10 sous agitation constante.

Quant à la sulbutiamine, administrée par des injections intrapéritonéales, elle est préparée dans une solution saline stérile 0.9%. La dissolution de la sulbutiamine résulte en un pH très acide (proche de 0.5) et ne peut être ajusté qu'à pH 4.3.

Tableau 3 : **Concentration et préparation des solutions de traitement utilisées dans l'étude *in vitro* sur les souris normales.**

	Solutions stock de traitement des souris normales	
	Concentration	**Préparation**
Contrôle / Véhicule	Saccharose 50 %, pH≈7,5	10 g de saccharose (Sigma-Aldrich) dans 20 mL d'eau du robinet
Thiamine	300-350 mM, pH≈7,5	500-600 mg de chlorhydrate de thiamine (T4625, Sigma-Aldrich) dans 5 mL de saccharose 50 %
Benfotiamine	225-245 mM, pH≈7,5	520-580 mg de benfotiamine (B9636, Sigma-Aldrich) dans 5 mL de saccharose 50 %
Sulbutiamine	14,2 mM	150 mg de sulbutiamine dans 15 mL de solution saline 0,9%

2.2.4. Immunohistochimie

2.2.4.1. Injection de Bromodeoxyuridine (BrdU)

Pour déterminer le nombre de cellules en phase S, les souris WT C57BL ont reçu une injection intrapéritonéale de bromodéoxyuridine (BrdU, 100 mg/kg, Sigma, Bornem, Belgique) deux heures, 24 heures et 3 semaines avant d'être sacrifiées afin d'étudier respectivement la prolifération, l'index de sortie de cycle cellulaire, la survie et la neurogenèse.

2.2.4.2. Préparation des tissus

Les souris ont été anesthésiées par une injection intrapéritonéale de 10 µL Nembutal® (Sodium pentobarbital 0.6 g/L, CEVA *Santé Animale*, Bruxelles) par gramme de souris. Ces souris ont ensuite reçu une injection intracardiaque (dans le ventricule gauche) de 0.1 mL d'héparine B (Heparine LEO 5000 U.I./mL, LEO Pharma, Ballerup, Danemark) afin de limiter les risques de thrombi intravasculaires pendant la fixation. Une perfusion intracardiaque de NaCl à 0.9% (pompe 101U/R, Watson Marlow, UK) dans le ventricule gauche a permis de purger les vaisseaux sanguins de leur contenu hématique. Une solution de paraformaldéhyde à 4% (PFA, Sigma Aldrich, St. Louis, USA) (4.3 g/L NaOH, 40 g/L de PFA ajoutée à chaud ; 18.8 g/L NaH_2PO_4, pH=7.4) a ensuite été perfusée, toujours par le ventricule gauche, pour fixer les tissus. Les cerveaux des souris perfusées ont ensuite été prélevés et postfixés dans une solution de PFA 4% à 4 °C pendant une nuit. Ils ont ensuite été incubés dans une solution cryo-protectrice de saccharose 20% préparée dans du tampon PBS 0.1 M (*phosphate buffer saline*, 140 mM NaCl ; 27 mM KCl ; 1.5 mM KH_2PO_4 $2H_2O$; 16 mM $NaHPO_4$ $2H_2O$, pH 7.4) pendant minimum 48 h à 4 °C. Les cerveaux ont ensuite été congelés dans une solution de 2-méthylbutane (Sigma Aldrich, St. Louis, USA) refroidie à -60 °C sur un lit de carboglace et conservés à -80 °C jusqu'à utilisation.

A l'aide d'un cryostat (Microm, Prosan, Ghent, Belgium), les cerveaux enchassés dans du tissu-tek® (SAKURA, Pays-Bas) ont été débités en coupes coronales de 40 µM d'épaisseur et conservées dans une solution d'antigel (solution préparée dans de l'eau distillée contenant 0.9 M de saccharose ; 0.1 M de PBS ; 30% d'éthyène glycol ; 10 g/L de polyvinylpyrrolidone) à 4 °C. Les coupes de cerveaux, dites « flottantes », ont été collectées du point Bregma -1.34 au point Bregma -2.54 (approximativement selon Paxinos and Franklin, 2001). Les manipulations quantitatives comparatives ont été réalisées en prenant 6 coupes à intervalle régulier.

2.2.4.3. Les immunomarquages

2.2.4.3.1. Immunomarquage BrdU-Ki67

Les coupes obtenues (BrdU 2 h et 24 h) sont traitées dans une solution de démasquage des épitopes (Target Retrieval Solution, Dako, Glostrup, Danemark) pendant 30 minutes à 90 °C. Elles sont ensuite rincées 2 x 10 minutes dans du TBS (*Tris Buffer Saline,* 6 g/L Tris Base, 9 g/L NaCl, pH 7.6) avant d'être incubées avec de l'acide chlorhydrique 2 N pendant 20 minutes à 37 °C. L'acide chlorhydrique engendre la décondensation de l'ADN lui permettant ainsi d'être accessible à l'anticorps anti-BrdU. L'incubation subséquente des sections avec un tampon borate neutralisant (acide borique 0.1 M pH 8.5) pendant 15 min permet le rinçage de l'acide chlorhydrique. Les coupes sont ensuite rincées 2 x 5 minutes dans du TBS avant d'être perméabilisées dans du TBS-T-T (TBS - 0.3% Triton – 0.1% Tween 20) pendant 10 minutes. Les anticorps primaires (anti-BrdU et anti-Ki67, voir Tableau 5) sont préparés dans du sérum d'âne 5% TBS-T-T et incubés avec les coupes pendant une nuit à 4 °C. La solution de dilution des anticorps est en fait une solution de blocage et de perméabilisation. En effet, le sérum d'âne permet de saturer les sites de fixation aspécifiques alors que le TBS-T-T permet de perméabiliser les membranes et donc l'accès des épitopes aux anticorps.

Les sections sont ensuite rincées 3 x 20 minutes dans du TBS avant d'être incubées en présence des anticorps secondaires (voir Tableau 5) dirigés contre les anticorps primaires et préparés dans du TBS-T-T sous agitation pendant 1 h à température ambiante et dans l'obscurité. Les anticorps secondaires sont couplés à des fluorochromes qui ont la propriété de posséder des longueurs d'onde d'excitation et d'émission suffisamment distinctes pour permettre leur analyse différentielle. A la fin du processus d'immunomarquage, les sections sont rincées 3 x 20 minutes dans du TBS avant d'être trasnférées sur une lame porte-objet et montées entre lame et lamelle à l'aide d'une goutte de milieu de montage VectaShield (Vector Laboratories Burlingame, CA, USA). Le Vectashield contient du DAPI, un composé fluorescent qui permet de marquer les noyaux cellulaires. Les lames sont stockées à 4 °C à l'abri de la lumière.

2.2.4.3.2. Immunomarquage BrdU-NeuN

L'immunomarquage réalisé sur les coupes BrdU 3 semaines est similaire au marquage BrdU/ Ki-67 à l'exception de l'étape de démasquage des épitopes et des anticorps utilisés. Les coupes sont d'abord incubées avec de l'acide chlorhydrique 2 N pendant 30 minutes à 37 °C. L'incubation subséquente des sections avec un tampon borate neutralisant (acide borique 0.1 M pH 8.5) pendant 30 min permet le rinçage de l'acide chlorhydrique. Les coupes sont ensuite rincées 2 x 5 minutes dans du TBS avant d'être perméabilisée dans du TBS-T-T (TBS - 0.3% Triton – 0.1% Tween 20) pendant 10 minutes. Les anticorps primaires (anti-BrdU et anti-NeuN, voir Tableau 5) sont préparés dans du sérum d'âne 5% TBS-T-T et incubés avec les coupes pendant une nuit à 4 °C. La solution de dilution des anticorps est en fait une solution de blocage et de perméabilisation. En effet, le sérum d'âne permet de saturer les sites de fixation aspécifiques alors que le TBS-T-T permet de perméabiliser les membranes et donc l'accès des épitopes aux anticorps.

Les sections sont ensuite rincées 3 x 20 minutes dans du TBS avant d'être incubées en présence des anticorps secondaires (voir Tableau 5) dirigés contre les anticorps primaires et préparés dans du TBS-T-T sous agitation pendant 1 h à température ambiante et dans l'obscurité. Les anticorps secondaires sont couplés à des fluorochromes qui ont la propriété de posséder des longueurs d'onde d'excitation et d'émission suffisamment distinctes pour permettre leur analyse différentielle. A la fin du processus d'immunomarquage, les sections sont rincées 3 x 20 minutes dans du TBS avant d'être trasnférées sur une lame porte-objet et montées entre lame et lamelle à l'aide d'une goutte de milieu de montage VectaShield (Vector Laboratories Burlingame, CA, USA). Le Vectashield contient du DAPI, un composé fluorescent qui permet de marquer les noyaux cellulaires. Les lames sont stockées à 4 °C à l'abri de la lumière.

2.2.4.4. Imagerie confocale et quantification

2.2.4.4.1. Imagerie confocale

Les coupes sont observées après immunomarquage à l'aide d'un microscope confocal inversé Nikon A1R Hybrid Resonant équipé du système confocal NIS-Element. Les images scannées sont traitées à l'aide des logiciels informatiques NIS-Element Viewer (Nikon) et ImageJ. L'acquisition des images de fluorescence a été effectuée grâce au microscope confocal inversé en utilisant un Z-scan avec une distance de 1.5 µM entre chaque plan confocal. Toutes les sections préparées pour comparaison ont été analysées au même moment, en utilisant les mêmes paramètres d'acquisition.

2.2.4.4.2. Quantification du nombre de cellules et calculs

Comptage cellulaire

Tous les comptages et quantifications ont été réalisés par un expérimentateur aveugle aux conditions expérimentales. Pour évaluer la prolifération cellulaire dans le gyrus denté (DG) de l'hippocampe, les cellules $BrdU^+$ et $Ki67^+$ ont été comptées manuellement de façon exhaustive dans la couche de cellules granulaires (CGL) et dans la zone sous-granulaire (SGZ), définie comme une couche d'une largeur de 2 cellules sur chaque côté de la frontière entre la CGL et le hile, sous un objectif 40× (Axiovert 135, Zeiss, Germany). Les cellules $BrdU^+$ et $Ki67^+$ ont également été comptées manuellement de façon exhaustive dans le hile.

Lorsque les souris sont sacrifiées 3 semaines après injection unique de BrdU, le marquage de celui-ci est plus dilué et apparait majoritairement comme segmenté ou sous forme de petits points (Mandyam et al. 2007). Dès lors, pour évaluer la neurogenèse, le comptage des cellules $BrdU^+$ et $NeuN^+$ a été réalisé sur les images scannées à grossissement 40X à l'aide du programme NIS-Element Viewer (Nikon) afin de vérifier la colocalisation du marquage BrdU et du marquage DAPI. Les cellules ont été comptées dans le DG comme décrit ci-dessus (= GCL + SGZ).

Les comptages de chaque condition expérimentale ont été réalisés sur une coupe toutes les 6 sections coronales de 40 µM (240 µm) selon l'axe rostro-caudal du DG (c.-à-d., du point bregma -1.34 mm au point -2.54 mm, Paxinos and Franklin, 2001). Pour chaque

section, le volume (surface x épaisseur de la section) du gyrus denté a été mesuré afin d'exprimer les résultats en nombre de cellules par mm^3 de CGL.

Calculs

L'index de sortie du cycle cellulaire, réalisé à partir du comptage des cellules $BrdU^+$ et $Ki67^+$ sur les coupes BrdU 24h, s'obtient en divisant le nombre de cellules sorties du cycle cellulaire (cellules $BrdU^+$ $Ki67^-$) par le nombre total de cellules marquées au BrdU, prolifératives ou non (cellules $BrdU^+$ = cellules $BrdU^+$ $Ki67^+$ + cellules $BrdU^+$ $Ki67^-$). Le taux de survie correspond au rapport du nombre de cellules $BrdU^+$ obtenu après 3 semaines sur le nombre de cellules $BrdU^+$ obtenu après 2 h.

La répartition des cellules (en pourcentage) dans les différentes couches a été calculée comme suit : le nombre de cellules BrdU positives des différentes couches (SGZ, CGL et Hile) a été additionné en un nombre total de cellules. Le nombre de cellules par couche a ensuite été divisé par le nombre total de cellules et multiplié par 100.

2.3. EXPERIMENTATION *IN VIVO* : Etude préliminaire de la neurogenèse chez des souris adultes stressées traitées ou non par la thiamine ou la benfotiamine.

Les parties "traitement et stress" de cette expérimentation se sont déroulées à la Medical Faculty of New Lisbon University (Portugal), en collaboration avec l'équipe du Dr Strekalova.

2.3.1. Animaux

Les souris C57BL/6Rj mâles âgées de 14 semaines (*n=40*) au moment de l'expérience ont été fournies par Charles River (Lisbonne, Portugal). Les rats Wistar, âgés de 10 semaines, ont été fournis par l'animalerie institutionnelle. Les souris ont été hébergées avec nourriture et eau à volonté dans des conditions contrôlées de température et d'humidité sous un cycle jour/nuit 12h-12h (éclairage de jour à partir de 21h). Les souris, hébergées individuellement par cage, ont été habituées à l'animalerie locale pendant au moins 1 semaine avant toute expérience. Toutes les expérimentations animales ont été réalisées selon les directives du comité d'éthique pour le bien-être et l'utilisation des animaux de l'Université de Lisbonne en accord avec le « European Communities Council Directive » du 24 novembre 1986 (86/609/EEC).

2.3.2. Design expérimental

Les souris ont été divisées aléatoirement en 4 groupes expérimentaux différents (Figure 9):

- Non stressé non traité (NS-NT)
- Stressé non traité (S-NT)
- Stressé traité avec de la thiamine (S-Thia)
- Stressé traité avec de la benfotiamine (S-BFT)

Les souris ont été hébergées à raison de 5 par cage et ont reçu de l'eau (véhicule), de la thiamine (200 mg/kg/jour) ou de la benfotiamine (200 mg/kg/jour) préparées dans l'eau et disponible à volonté. Les solutions de thiamine et de benfotiamine ont été remplacées tous les 7 jours.

La première cohorte de souris (*n=5* par groupe expérimental), destinée à l'étude de la neurogenèse, a reçu 4 injections intrapéritonéales de BrdU (50 mg/kg) à 2 h d'intervalle après 14 jours de traitement, avant la première session de stress. Entre les jours 15 et 20, les souris des groupes S-NT, S-Thia et S-BFT ont subi un protocole de stress par exposition à un prédateur. Ce protocole consiste à placer les souris, protégées dans des cylindres transparents, dans une cage avec des rats. Les cylindres, transparents (15 cm hauteur x 8 cm de diamètre) et percés de trous, permettaient un contact olfactif et visuel avec le prédateur. Les sessions de stress, d'une durée de 15 h (18 h à 9 h) ont été menées pendant 5 nuits consécutives, durant la phase de sommeil des souris. Les souris n'avaient accès ni à l'eau ni à la nourriture durant l'exposition au prédateur mais y avaient accès à volonté le reste du temps. Les souris ont ensuite été sacrifiées 24 heures après la dernière session de stress afin d'étudier la survie des neurones néoformés (marquage au BrdU) et la prolifération totale (marquage au Ki67) à court terme par immunohistochimie.

La seconde cohorte (*n=5* par groupe expérimental), destinée aux études biochimiques (HPLC, WB et RT-PCR), a été sacrifiée par décapitation 24 heures après le dernier traitement et 12 heures après la dernière session de stress. Le sacrifice a été effectué 12 heures après la dernière session de stress (au lieu de 24 h comme pour la neurogenèse) afin d'optimiser l'opportunité d'observer des changements dans les niveaux d'ARN messager du BDNF (Lakshminarasimhan & Chattarji 2012; Murakami et al. 2005) et dans les niveaux de

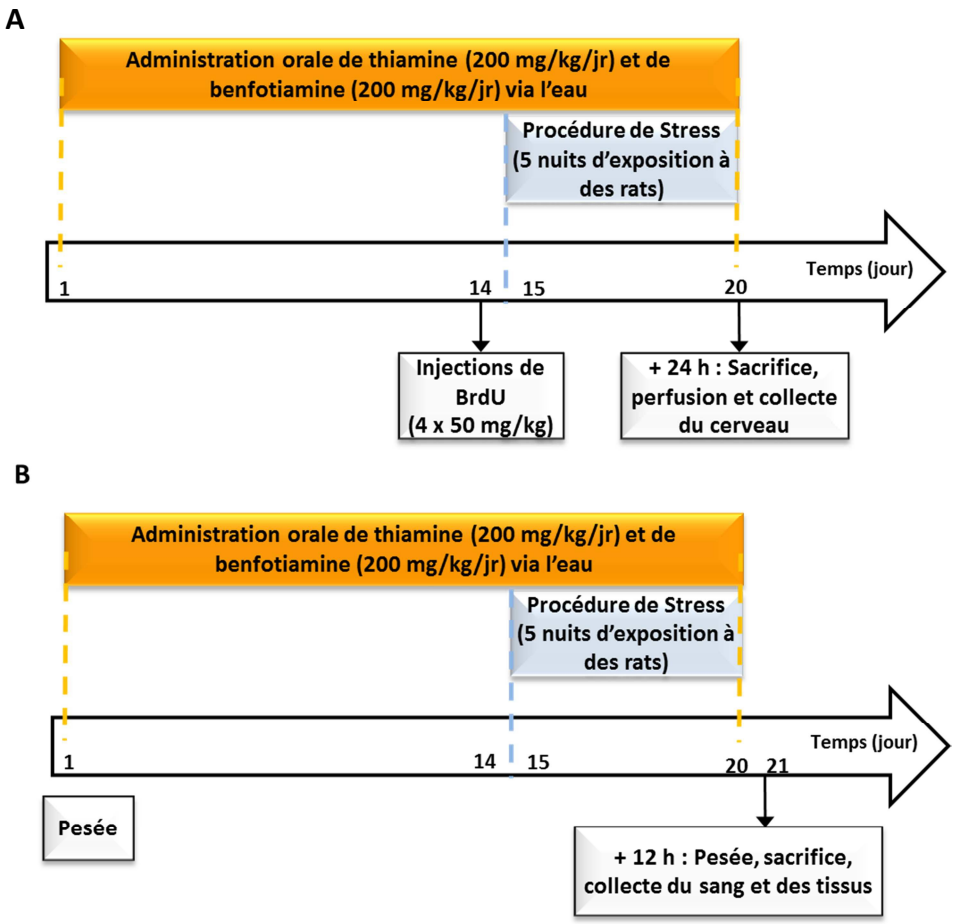


Figure 9 : **Design expérimental de l'étude de l'effet d'un traitement par la thiamine ou la benfotiamine sur des souris stressées** (A) pour les analyses biochimiques par HPLC et WB ainsi que (B) pour l'analyse de la neurogenèse par immunohistochimie.

phosphorylation des protéines étudiées. Le sang du tronc a été récupéré dans des tubes coatés à l'EDTA. Le foie, le cortex et l'hippocampe (hémisphères gauches et droits) ont été disséqués à 4 °C. Le cortex entourant l'hippocampe mais excluant celui-ci ainsi que le cortex préfrontal a été disséqué (5 mg) et placé dans du RNA *Later* (pour l'étude de l'expression du BDNF par RT-PCR). Le tout a été congelé instantanément dans de l'azote liquide.

Le poids des souris a été mesuré au jour 1 (avant le traitement) et au jour 20 (12h après la dernière session de stress).

2.3.3. Préparation des solutions de traitement

Pour des raisons pratiques, la thiamine et la benfotiamine ont été administrées via de l'eau disponible à volonté. Les concentrations des solutions de traitement sont calculées sur base d'une consommation d'eau de 3.5 mL par jour de sorte que chaque souris ingère au minimum une dose quotidienne de 200 mg/kg (Tableau 4). Les solutions sont renouvelées tous les 7 jours. Chaque composé requiert une préparation particulière mais toutes les solutions administrées oralement ont été ajustées au pH de 7-8. En effet, la thiamine, sous forme d'hydrochlorure, engendre un pH acide lorsqu'elle est dissoute. La dissolution complète de la benfotiamine requiert une eau alcaline (pH 8.5) et une agitation constante pendant 20 minutes.

Tableau 4 : **Concentration et préparation des solutions de traitement utilisées dans l'étude *in vitro* sur les souris stressées.**

	Solutions stock de traitement des souris stressées	
	Concentration	**Préparation**
Contrôle / Véhicule		Eau du robinet
Thiamine	5 mM, pH 7-8	0,34 g dans 200 mL d'eau du robinet
Benfotiamine	3,7 mM, pH 7-8	0,34 g dans 200 mL d'eau du robinet

2.3.4. Immunohistochimie

L'expérience sur les souris stressées a été réalisée en collaboration avec le Dr Strekalova, les étapes antérieures à l'immunomarquage ont été effectuées dans son laboratoire, sont un peu différentes de celles utilisées dans notre laboratoire et sont détaillées ci-dessous.

2.3.4.1. Injection de Bromodeoxyuridine

Pour déterminer le nombre de cellules en phase S, les souris WT C57BL ont reçu 4 injections intrapéritonéales de bromodeoxyuridine (BrdU, 50 mg/kg, Sigma, Bornem, Belgique) à deux heures d'intervalle avant d'être sacrifiées 5 jours plus tard afin d'étudier respectivement la prolifération et la survie des cellules marquées à court terme.

2.3.4.2. Préparation des tissus

Les souris ont été anesthésiées par une injection intrapéritonéale de 10 µL Nembutal® (Natrium pentobabrbital 0.6g/L, CEVA *Santé Animale*, Bruxelles) par gramme de souris. Ces souris ont ensuite reçu une injection intracardiaque (dans le ventricule gauche) de 0.1 mL d'héparine B (Heparine LEO 5000 U.I./mL, LEO Pharma, Ballerup, Danemark) afin de limiter les risques de thrombi intravasculaires pendant la fixation. Une perfusion intracardiaque de NaCl à 0.9% (pompe 101U/R, Watson Marlow, England) dans le ventricule gauche a permis de purger les vaisseaux sanguins de leur contenu hématique. Une solution de paraformaldéhyde à 4% (PFA, Sigma Aldrich, St. Louis, USA) (4.3 g/L NaOH, 40 g/L de PFA ajoutée à chaud ; 18.8 g/L NaH_2PO_4, pH=7.4) a ensuite été perfusée, toujours par le ventricule gauche, pour fixer les tissus. Les cerveaux des souris perfusées ont ensuite été prélevés et postfixés dans une solution de PFA 4% à 4 °C pendant une nuit. Ils ont ensuite été incubés dans une solution cryo-protectrice de saccharose 30% préparée dans du tampon PBS 0.1 M (*phosphate buffer saline*, 140 mM NaCl ; 27 mM KCl ; 1.5 mM KH_2PO_4 ; 16 mM Na_2HPO_4, pH 7.4) pendant minimum 12 h à 4 °C.

A l'aide d'un microtome MSE (UK) couplé à une table de congélation, les cerveaux ont été débités en coupes sagittales de 40 µM d'épaisseur. Une section sur 4 était récoltée dans du PBS avant d'être conservée dans une solution d'antigel (solution de 2 L préparée dans de l'eau distillée contenant 0.9 M de saccharose ; 1 L de PBS 0.1 M ; 30% d'éthyène glycol ; 10 g/L de polyvinylpyrrolidone) à -20 °C. Les coupes de cerveaux, dites « flottantes », ont été collectées du point Bregma latéral 3.5 au point Bregma latéral 0.3 (approximativement selon Paxinos and Franklin, 2001).

2.3.4.3. Immunomarquage BrdU-Ki67

Le protocole utilisé est le même que celui appliqué précédemment (voir section 3.4.3.1, page 12-13) à l'exception des anticorps secondaires (anti-rat ou anti-souris couplés soit à l'isothiocyanate de fluorescéine (FITC) soit à la rhodamine Red-X (Jackson ImmunoResearch, Europe Ltd, Suffolk, U.K.)) préparés à la dilution 1 :500 dans du TBS-T-T.

2.3.4.4. Microscopie confocale

L'acquisition des images de fluorescence a été effectuée grâce à un un microscope confocal inversé Nikon A1R Hybrid Resonant équipé du système confocal NIS-Element en utilisant un Z-scan avec une distance de 1.5 µM entre chaque plan confocal. Toutes les sections préparées pour comparaison ont été analysées au même moment, en utilisant les mêmes paramètres d'acquisition.

2.3.4.5. Quantification du nombre de cellules

Toutes les quantifications stéréologiques sont réalisées par un expérimentateur aveugle aux conditions expérimentales. Pour évaluer la prolifération et la survie cellulaire dans le gyrus denté (DG) de l'hippocampe, les cellules $BrdU^+$ et $Ki67^+$ ont été comptées manuellement de façon exhaustive dans la couche de cellules granulaires (CGL) et dans la zone sous-granulaire (SGZ), définie comme une couche d'une largeur de 2 cellules sur chaque côté de la frontière entre la CGL et le hile, sous un objectif 40× (Axiovert 135, Zeiss, Germany). Les cellules $BrdU^+$ et $Ki67^+$ ont également été comptées manuellement de façon exhaustive dans le hile. Les comptages de chaque condition expérimentale ont été réalisés sur une coupe toutes les 4 sections sagittales de 40 µM (560 µm) du début à la fin du DG (c.-à-d., de 0.36 mm à 2.52 mm du bregma latéral; environ 14 sections de 40 µM par animal). Pour chaque section, le volume du gyrus denté a été mesuré (mesure de l'aire x l'épaisseur de la section) afin d'exprimer les résultats en nombre de cellules par mm^3 de CGL.

La répartition des cellules (en pourcentage) dans les différentes couches a été calculée comme suit : le nombre de cellules BrdU positives des différentes couches (SGZ, CGL et Hile) a été additionné en un nombre total de cellules. Le nombre de cellules par couche a ensuite été divisé par le nombre total de cellules et multiplié par 100.

Tableau 5 : **Liste des anticorps primaires et secondaires utilisés lors des immunohistochimies ainsi que leurs conditions de dilution, de blocage et d'incubation.**

Anticorps primaires	Hôte	Dilution	Solution de dilution	Utilité	Conditions d'incubation	Références
Anti-BrdU	rat	1/500	Donkey serum 5% - TBS-T-T	Marqueur de phase S au moment de l'injection	Une nuit à 4°C	OBT0030, AbD Serotec
Anti-Ki67	souris	1/500	Donkey serum 5% - TBS-T-T	Marqueur de prolifération cellulaire	Une nuit à 4°C	550609, BD Pharmingen
Anti-NeuN	souris	1/500	Donkey serum 5% - TBS-T-T	Marqueur de neurone mature	Une nuit à 4°C	MAB377, Millipore
Anticorps secondaires	**Hôte**	**Dilution**	**Solution de dilution**	**Anticorps primaires**	**Conditions d'incubation**	**Références**
Alexa Fluor 488 Donkey anti-rat (âne contre rat)	âne	1/2000	TBS-T-T	BrdU	1h à température ambiante dans l'obscurité	A21208, Molecular Probe®
Alexa Fluor 555 Donkey anti-mouse (âne contre souris)	âne	1/1000	TBS-T-T	Ki67, NeuN		A31570, Molecular Probe®

2.3.5. RT-PCR

Pour l'analyse de l'expression génique du BDNF, l'ARN messager (ARNm) a été extrait à l'aide du kit RNA isolation Nucleospin® RNA XS (Macherey-Nagel, Filter Service, Belgium). La synthèse du premier brin complémentaire a été réalisée sur 1 µg d'ARN total en utilisant des amorces random et le kit *ProtoScript® II First Stand cDNA Synthesis* (Bioké). La RT-PCR quantitative a été réalisée en utilisant le kit *FastStart SYBR Green Master (*Roche) et le thermocycleur Light Cycler 480 II System (Roche). Les séquences des primers utilisés étaient les suivantes: pour l'actine, 5'-GACGGCCAGGTCATCACTAT-3' (sens) and 5'-ATGCCACAGGATTCCATACC-3' (antisens); pour l'hypoxanthine-guanine phosphoribosyltransférase (HPRT), 5'-GGTGGATTACATTAAAGCACTGAAT-3' (sens) et 5'-AAAGTTTGCATTGTTTTACCAGTGT-3' (antisens); et pour BDNF 5'-CGGCGCCCATGAAAGAAGTA-3' (sens) and 5'-AGACCTCTCGAACCTGCCCT-3' (antisens). Toutes les amorces proviennent d'Integrated DNA technology (Leuven, Belgium). La quantification relative a été effectuée selon la méthode comparative Ct (méthode ΔΔ Ct). Le gène HPRT a été choisi comme gène de référence pour l'hippocampe tandis que l'actine a été choisie pour le cortex. En effet, bien que ces 2 gènes de référence aient été utilisés dans la RT-PCR pour les 2 structures, seul le gène HPRT a rempli les conditions de validation de l'expérience dans l'hippocampe alors que seul le gène l'actine les a remplies pour le cortex (valeur absolue de la pente du Δ Ct = f (dilution de l'éhantillon) doit être inférieure à 0.1; voir « guide to performing quantitation of gene expression using qRT-PCR » (Applied Biosystem).

Les données sont exprimées de façon relative par rapport à la valeur moyenne d'expression du BDNF des souris non-stressées non-traitées (NS-NT) (Guide to performing quantitation of gene expression using qRT-PCR, Applied Biosystem).

2.4. METHODES GENERALES (utilisées dans les différentes études *in vitro/in vivo*

2.4.1. Détermination des niveaux relatifs de phosphorylation et d'expression protéique par Western Blotting

2.4.1.1. Préparation d'extraits cellulaires et tissulaires et dosage protéique

Les cellules, préalablement rincées avec du tampon phosphate froid (PBS, Lonza) afin d'éliminer le milieu/la solution d'incubation, sont incubées avec du tampon de lyse (20 mM Tris-HCl pH 7.5 ; 150 mM NaCl; 1% Triton X-100 ; 1 mM EDTA; 1 mM NaF; 1 mM Na_3VO_4 ; inhibiteur de protéase (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics, Belgium)) à raison de 200 µL par puits sous agitation pendant 30 minutes à 4 °C. Les cellules sont ensuite récupérées par scrapping et centrifugées à 16000 g pendant 15 minutes, toujours à 4 °C. Le surnageant obtenu est congelé et conservé à -20 °C.

Les protéines sont extraites par sonication (20 s) des tissus cérébraux collectés (hippocampe et cortex) incubés avec du tampon de lyse (20 mM Tris-HCl pH 7.5 ; 450 mM NaCl; 1% Triton X-100 ; 1 mM EDTA; 1 mM NaF; 1 mM Na_3VO_4 ; inhibiteur de protéase (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics, Belgique) à raison de 20 µL pour 1 mg de tissu. Les échantillons sont ensuite centrifugés à 16000 g pendant 15 minutes, à 4 °C. Le surnageant obtenu est congelé et conservé à -20 °C.

La quantité de protéines présentes dans le surnageant est dosée par la méthode BCA Protein Assay Kit (Fischer Scientific).

2.4.1.2. Electrophorèse SDS-PAGE et transfert de protéines

Les extraits protéiques sont préparés dans du tampon de chargement concentré 2x (glycérol 20% ; Tris-HCl 125 mM pH 6.8 ; SDS 4% ; bleu de bromophénol 0.02% ; dithiothréitol (DTT) 200 mM) et dans de l'eau de sorte que 25 µg de protéines soient présents dans 35 µL d'échantillon. Les échantillons ainsi préparés sont ensuite déposés à

raison de 35 µL dans un gel de polyacrylamide-SDS 10% (1.5 mm d'épaisseur où le gel de concentration est de polyacrylamide-SDS 5%). Les protéines sont séparées en fonction de leur taille par électrophorèse à ampérage constant (30 mA/gel) pendant 2 h dans du tampon de migration (Tris-Base 25 mM, Glycine 192 mM, SDS 0.1% pH 8.3). Les protéines présentes sur le gel sont ensuite transférées sur une membrane de polyfluorure de vinylidène (PVDF) (HYBOND-P PVDF Membrane 30cm, Fischer Scientific), préalablement activée par du méthanol 100%, par champ électrique à voltage constant (80 V, 2 h) dans du tampon de transfert (Tris-Base 25 mM, Glycine 192 mM, méthanol 20% pH 8.3). La membrane de PVDF est ensuite récupérée afin d'être incubée avec les anticorps primaires reconnaissant les protéines d'intérêt.

2.4.1.3. Incubation des anticorps pour la détection des protéines

Dans un premier temps, la membrane de PVDF sur laquelle les protéines ont été transférées, est mise en présence d'une solution de blocage, qui permet de saturer les sites de fixation aspécifiques, pendant 1 heure à température ambiante. La solution de blocage utilisée est une solution de TBS- Tween (NaCl 150 mM ; Tris Base 20 mM ; pH 7.5 ; Tween 20 0.01% (Sigma Aldrich, St. Louis, USA)) dans laquelle est dissous soit du lait écrémé en poudre (5% m/V, Nestlé), soit de la BSA (3-5% m/V, (Sigma Aldrich, St. Louis, USA)) en fonction des anticorps primaires qui seront utilisés. Les membranes sont ensuite incubées avec 10 mL de solution contenant l'anticorps primaire dirigé contre la protéine d'intérêt dans les conditions suivantes (voir Tableau 6). Elle est ensuite rincée 3 x 5 minutes dans du TBST-T afin d'éliminer le surplus d'anticorps primaire. La membrane est alors incubée avec l'anticorps secondaire, couplé à de la peroxydase de raifort et dirigé contre l'anticorps primaire, pendant 1 heure avant d'être à nouveau rincée 3 x 5 minutes dans du TBS-T.

2.4.1.4. Révélation

La révélation des bandes de protéines d'intérêt est effectuée par la méthode de chemiluminescence grâce au kit Western Bright ECL HRP (Advansta - Isogen Life Science). Un substrat, mélange de 500 µl de luminol et 500 µl de peroxyde d'hydrogène, déposé sur la membrane, est oxydé par la peroxydase de raifort présente sur les anticorps secondaires, ce qui résulte en une émission de lumière détectable grâce à une caméra de type CCD (ImageQuant^{TM} LAS 4000, GE Healthcare Life Sciences). Des marqueurs de taille (MagicMark

(Life technologies) et PageRuler (Thermoscientific) permettent de vérifier que les protéines révélées ont une masse moléculaire apparente correspondant à la masse théorique.

2.4.1.5. Stripping

Le « stripping » est une méthode qui permet d'enlever les anticorps primaires et secondaires liées aux protéines afin de réutiliser la même membrane pour détecter d'autres protéines d'intérêt. Cette étape est ici utilisée entre la détection des protéines phosphorylées et des protéines totales. Après la révélation, les membranes sont rincées 1 x 5 minutes dans du TBS-T avant d'être incubées pendant 30 minutes à 70 °C dans 10 mL de solution de stripping (glycine 0.2 M pH 2.5) et d'être à nouveau lavées 3 x 5 minutes dans du TBS-T. La membrane est alors de nouveau soumise aux étapes de blocage, d'incubation avec les anticorps et de révélation.

2.4.1.6. Quantifications relatives des bandes présentes sur la membrane

Les bandes obtenues sur les membranes sont quantifiées en utilisant le programme ImageJ. Les bandes du Western blot sont sélectionnées individuellement et leur densité optique est mesurée. La densité optique de la bande d'intérêt est ensuite comparée à la densité optique de la bande de référence (échantillon « contrôle » ou échantillon choisi comme référence), ce qui nous donne une densité relative exprimée en fonction du contrôle. La densité relative de chaque bande d'intérêt est ainsi déterminée pour les différentes protéines étudiées (Akt, pAkt, β-actine,…). On réalise ensuite le ratio des densités relatives de 1) la protéine totale sur la β-actine, et 2) la protéine phosphorylée sur la protéine totale. On obtient alors 1) l'expresssion de la protéine d'intérêt et 2) le niveau de phosphorylation de la protéine d'intérêt. La β-actine permet en effet de savoir si la quantité de protéine chargée dans chaque puits est la même. Les valeurs relatives ainsi obtenues vont être transférées dans le programme GraphPad Prism 5.0.

L'analyse statistique de ces graphes est réalisée en utilisant les tests d'analyse de la variance à deux entrées (ANOVA 2). Le deuxième test utilisé est le test de Tukey permettant de comparer les différentes colonnes entre elles.

Tableau 6 : **Liste des anticorps primaires et secondaires utilisés lors des Western blots ainsi que leurs conditions de dilution, de blocage et d'incubation.**

Anticorps primaires	**Abréviation**	**Dilution**	**Solution de dilution**	**Solution de Blocage**	**Conditions d'incubation**	**Références**
Anti-GSK3β phosphorylé en sérine 9	pGSK3β(Ser9)	1/1000	BSA 3% TBS-T	BSA 5% TBS-T	Une nuit à 4°C	9336, Bioké (Cell Signaling)
Anti-GSK3β total	GSK3β	1/1000	Lait 5% ou BSA 3% TBS-T	Lait 5%-TBS-T	Une nuit à 4°C	sc-53931,Tebu-bio (Santa Cruz Biotechnology)
Anti-Akt phosphorylé en thréonine 308	pAkt(Thr308)	1/1000	BSA 3% TBS-T	BSA 5% TBS-T	Une nuit à 4°C	4056, Bioké (Cell Signaling)
Anti-Akt phosphorylé en thréonine 308	pAkt(Thr308)	1/2000	BSA 3% TBS-T	BSA 5% TBS-T	Une nuit à 4°C	13038S XP, Bioké (Cell Signaling)
Anti-Akt phosphorylé en sérine 473	pAkt(S473)	1/2000	Lait 5%-TBS-T	Lait 5%-TBS-T	Une nuit à 4°C	4060, Bioké (Cell Signaling)
Anti-Akt	Akt	1/5000	Lait 5%-TBS-T	Lait 5%-TBS-T	Une nuit à 4°C	9272, Bioké (Cell Signaling)
Anti-Aquaporine 4	AQP4	1/1000	Lait 5%-TBS-T	Lait 5%-TBS-T	Une nuit à 4°C	sc-9888, Bio-connect (Santa Cruz Biotechnology)
Anti-β-actine	β-actine	1/5000	Lait 5%-TBS-T	Lait 5%-TBS-T	1h à température ambiante	A5441, Sigma Aldrich
Anticorps secondaires	**Abréviation**	**Dilution**	**Solution de dilution**	**Anticorps primaires**	**Conditions d'incubation**	**Références**
Goat anti-rabbit HRP (chèvre contre lapin)	GAR	1/10 000	Lait 5%-TBS-T	pGSK3β(Ser9), GSK3β, pAkt(Thr308), Akt	1h à température ambiante	ab 6721, Abcam®
Goat anti-rabbit HRP (chèvre contre lapin)	GAR	1/2000	Lait 5%-TBS-T			7074, Bioké (Cell Signaling)
Goat anti-mouse HRP (chèvre contre souris)	GAM	1/10 000	Lait 5%-TBS-T	pAkt(S473), β-actine		A4416, Sigma Aldrich
Goat anti-donkey HRP (chèvre contre âne)	GAD	1/10 000	Lait 5%-TBS-T	AQP4		sc-2020, Bio-connect (Santa Cruz Biotechnology)

2.4.2. Détermination de la concentration en thiamine et ses dérivés phosphorylés par HPLC

2.4.2.1. Préparation d'extraits cellulaires et tissulaires

Extraits cellulaires.

Les cellules, préalablement rincées avec du tampon phosphate froid (PBS, Lonza) afin d'éliminer les conditions d'incubation, sont récoltées par scrapping avec du PBS et centrifugées pendant 2 minutes à 1000 g. Afin de précipiter les protéines, le culot de cellules est dissous dans 500 µL d'acide trichloracétique 12% (TCA 12%) pour les boîtes T75/de Petri et 250 µL/puits pour les plaques 6 puits avant d'être centrifugé à 5000 g pendant 15 minutes, à 4 °C. Le surnageant est récupéré et rincé 4 fois avec 2 mL d'éther diéthylique afin d'en éliminer le TCA. Les surnageants sont conservés dans des tubes Eppendorf à -20 °C jusqu'au moment de leur dosage pour leur contenu en thiamine par HPLC. Le culot, resuspendu dans 1 mL de NaOH 0,8 N et conservé à 4 °C, est dosé pour son contenu en protéines par la méthode de Peterson (Peterson 1977).

Extraits tissulaires.

Le sang (200 µL) est mélangé à 2 volumes de TCA 18% dans un tube Eppendorf. Des pièces de foie (70-90 mg), d'hippocampe (8-20 mg) et de cortex (20-25 mg) sont homogénéisés dans 10 volumes de TCA 12% dans un Potter–Elvehjem de type verre-verre. Après centrifugation, les échantillons (5000 g, 15 minutes), le surnageant est extrait à l'éther diéthylique (4 x 2 mL) et les dérivés de la thiamine sont déterminés par HPLC. Le culot, resuspendu dans 1 mL de NaOH 0,8 N et conservé à 4 °C, est dosé pour son contenu en protéines par la méthode de Peterson (Peterson 1977).

2.4.2.2. Détermination de la thiamine et de ses dérivés phosphorylés

Les concentrations en thiamine et ses dérivés phosphorylés sont déterminées par chromatographie liquide à haute performance (HPLC) après oxydation en thiochromes fluorescents (Bettendorff et al. 1991). L'échantillon (40 µL) est mélangé à une solution d'oxydation (25 µL ; composée de 500 µL de ferricyanure de potassium ($K_3Fe(Cn)_6$) 1% (m/v) et de 2.5 mL de NaOH 15% (m/v)) est injecté dans la boucle d'injection (20 µL) de l'HPLC. La

séparation des dérivés se fait alors sur une colonne PRP-1 sur base de formation de paires d'ions en phase inverse. Cette colonne (150 x 4,1 mm Ø, 5 µm, Hamilton, Reno, USA) est composée de poly(styrène-divinylbenzène) et est protégée par une précolonne (25 x 2.3 mm Ø). La phase mobile (flux = 0.5 ml/min) est une solution de pH 9.5, contenant 50 mM de Na_2HPO_4, 25 mM de tétrabutylammonium et 4% de tétrahydrofurane. La colonne est couplée à un détecteur de fluorescence (365 nm pour l'excitation et 433 nm pour l'émission ; KONTRON, SFM 25) qui transmet les données à l'ordinateur sous forme de signaux électriques pour la reconstitution des chromatogrammes (Logiciel Kromasystem 2000). La concentration en thiamine et dérivés des échantillons est calculée sur base de l'aire des pics obtenus par comparaison aux aires des pics obtenus pour une solution standard dont la concentration de chaque dérivé (thiamine, ThMP, ThDP et ThTP) est connue (0.25 µM). La concentration des dérivés de la thiamine est ensuite exprimée par rapport à la concentration protéique de l'échantillon (pmol/mg de protéines).

2.4.3. Analyses statistiques

Toutes les analyses statistiques ont été réalisées grâce au programme GraphPad Prism version 5.03 pour Windows (San Diego California, USA). Tous les résultats sont indiqués sous forme de moyenne ± écart-type sauf indication contraire. Les analyses statistiques ont été réalisées en effectuant des tests t de student lors de la comparaison de 2 groupes alors que des tests de variance à un ou 2 facteurs (ANOVA à 1 ou 2 facteurs) ont été effectués lors de la comparaison de plusieurs groupes. Lorsque le résultat était significatif, des tests post-hoc ont été utilisés afin de déterminer où se trouvait la différence entre plusieurs groupes. Les tests utilisés sont décrits dans la légende de chaque résultat.

RESULTATS ET DISCUSSION

1. EXPERIMENTATION *IN VITRO*

Au départ, notre projet de recherche était de découvrir par quels mécanismes le traitement par la benfotiamine (un précurseur de la thiamine à plus haute biodisponibilité) exerçait des effets bénéfiques dans des modèles murins de la neurodégénération. Une telle étude est particulièrement complexe chez l'animal entier : le premier problème est que la benfotiamine est une substance polaire qui ne diffuse pas à travers les membranes ; on ne la trouve donc pas dans le sang (ni, à fortiori, dans le parenchyme cérébral) lorsqu'elle est administrée oralement. On suppose que, au niveau des bordures en brosse de l'épithélium intestinal, la benfotiamine est déphosphorylée en S-benzoylthiamine par la phosphatase alcaline (une ectoenzyme). La S-benzoylthiamine, très lipophile, traverse facilement l'épithélium intestinal et, dans le sang et le foie, elle est rapidement hydrolysée par des thioestérases pour régénérer la thiamine, dont la concentration plasmatique peut alors atteindre des valeurs élevées (10 à 50 µM). Le problème est que la thiamine ne franchit que lentement la barrière hémato-encéphalique (BHE) et, même après traitement oral par des doses élevées de benfotiamine, la teneur en thiamine dans le cerveau passe, au mieux, de 1 à 2 µM tandis que la teneur en coenzyme ThDP ne présente aucune augmentation significative. La question se pose donc : quelle est la substance active qui, après traitement de l'animal par la benfotiamine, est responsable des effets neuroprotecteurs observés dans le parenchyme cérébral ?

Nous avons donc abordé le problème en utilisant d'abord un système plus simple : des cellules de neuroblastome (N2a) en culture. Ces cellules ont des propriétés d'excitabilité analogues à celles des neurones matures. Il a été démontré précédemment (Bettendorff & Wins 1994; Volvert et al. 2008) que ces cellules ont une croissance pratiquement normale lorsque le milieu de culture a une teneur très réduite en thiamine (de l'ordre de 10^{-8} M). Il est donc aisé d'étudier comment ces cellules réagissent à l'addition subséquente de concentrations plus élevées en thiamine ou de précurseurs tels que la benfotiamine et la sulbutiamine. La comparaison avec la sulbutiamine est intéressante, car il s'agit d'un composé lipophile diffusant rapidement à travers les membranes cellulaires ; il est rapidement transformé en thiamine libre dans le cytoplasme. L'incubation des cellules N2a aboutit donc à une accumulation importante et rapide de thiamine dans les cellules, ce qui n'est pas le cas si les cellules sont traitées par la thiamine libre ou la benfotiamine

(Bettendorff 1994; Volvert et al. 2008). En comparant les résultats obtenus avec la thiamine et 2 précurseurs différents, nous pourrons distinguer les effets liés à l'augmentation de la thiamine intracellulaire et des effets liés spécifiquement à des métabolites de la benfotiamine.

Enfin, nous examinerons la question de savoir si la thiamine et la benfotiamine peuvent exercer des effets bénéfiques sur la survie et la croissance cellulaire via la cascade « pro-survival » bien connue : insuline (ou IGF-1)-RTK-PI3K-Akt-GSK-3β.

1.1. Teneur en thiamine et dérivés phosphorylés dans des cellules N2a cultivées en milieu riche ou pauvre en thiamine

Le milieu de culture commercial normal DMEM contient des concentrations élevées de diverses vitamines. Sa concentration en thiamine (environ 10 µM) représente 4 à 400 fois le K_m pour les transporteurs connus de la thiamine. Dans les cellules N2a, le K_m pour le transport saturable de haute affinité a été estimé à 35 nM (Bettendorff & Wins 1994) ce qui suggère qu'il s'agit d'un transporteur THTR-2, celui qui a la plus forte affinité pour la thiamine.

En plus du milieu DMEM normal riche en thiamine, nous avons également utilisé un milieu commercial déficient en thiamine. Ce milieu ne contient pas du tout de thiamine mais lorsqu'on y ajoute les 10% de sérum fœtal de veau nécessaires à la croissance et contenant la vitamine, la concentration finale en thiamine dans ce milieu pauvre (MPT) est d'environ 6-10 nM. Remarquons que les concentrations physiologiques dans le plasma sanguin des mammifères sont un peu plus élevées mais ne dépassent généralement pas 20 nM.

La culture des cellules dans un milieu pauvre en thiamine (MPT) induit une diminution progressive des concentrations intracellulaires en thiamine, thiamine monophosphate (ThMP) et thiamine diphosphate (ThDP) (Figure 10). Dès le 4ème jour, la thiamine libre devient indétectable. La teneur en ThMP atteint un minimum (1 pmol/mg de protéine) après 7 jours et reste sensiblement constante par la suite. La concentration intracellulaire en ThDP atteint également un minimum après 7 jours, environ 20 pmol/mg de protéine. Ces concentrations, comme la teneur en thiamine totale, restent sensiblement constantes entre 7 et 42 jours de culture en milieu pauvre en thiamine (Fig. 10). Il a été estimé précédemment

(Bettendorff & Wins 1994) que, pour les cellules N2a, le volume cellulaire est d'environ 4.5 µL par mg de protéine. Les concentrations intracellulaires en thiamine, ThMP et ThDP après 7 jours de culture en milieu riche ou pauvre en thiamine sont données en molarités dans le Tableau 7 :

Composé	Concentration intracellulaire (µM)	
	MRT	MPT
Thiamine	0,078 ± 0,22	n.d.
ThMP	0,89 ± 0,15	0,22 ± 0,07
ThDP	33,3 ± 3,5	4,4 ± 1,2
ThTP	n.d.	n.d.

n.d., non détectable

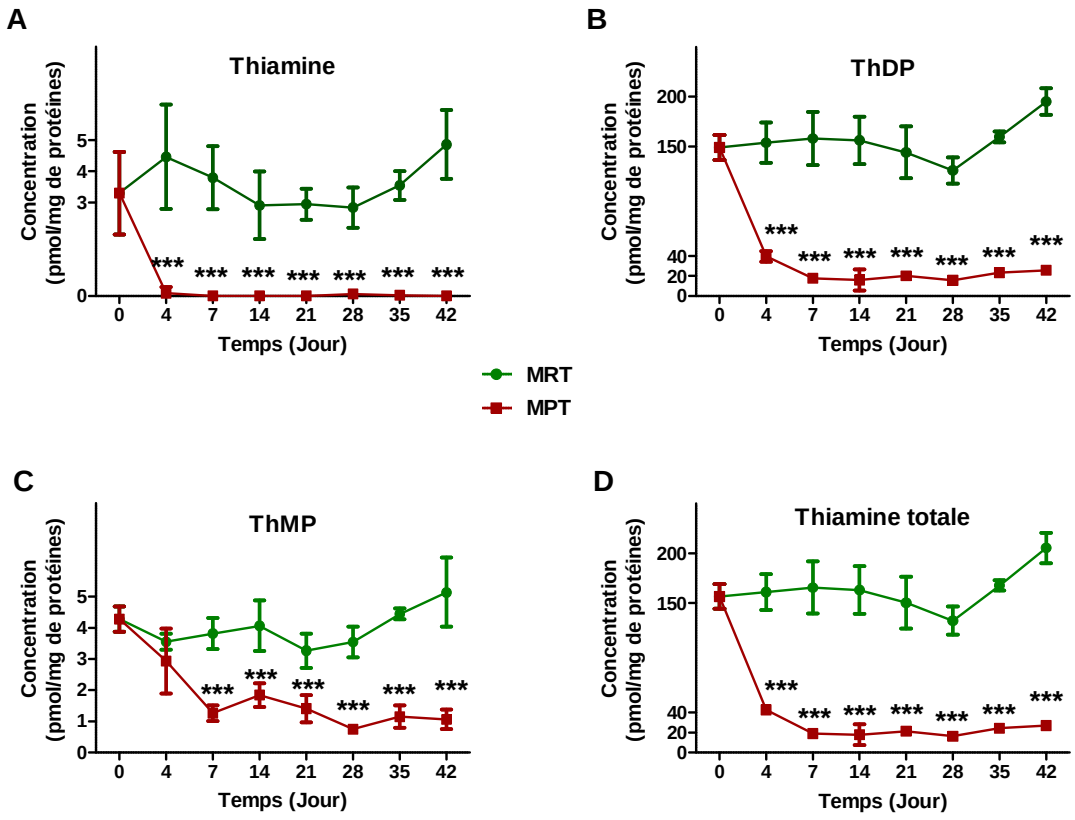


Figure 10 : **Evolution des teneurs en (A) thiamine (B) ThDP, (C) ThMP et (D) thiamine totale dans les cellules N2a cultivées en milieu pauvre en thiamine (MPT, 10 nM) ou en milieu riche (MRT, 10 µM).** Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; ***p<0.001 par comparaison avec la condition milieu riche, ANOVA à 2 entrées suivie du test post-hoc de Bonferroni).

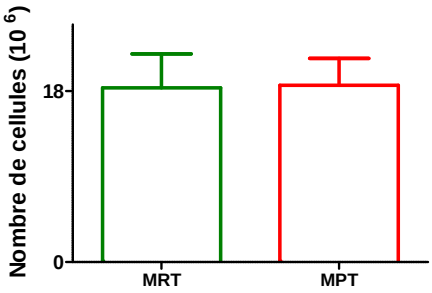


Figure 11 : **Nombre de cellules N2a mesuré 5 jours après ensemencement à la même densité et culture dans un milieu pauvre (MPT) ou riche (MRT) en thiamine.** Les données sont présentées sous forme de moyenne ± écart-type (n=20).

Ces résultats concordent avec ceux obtenus précédemment (Bettendorff 1995) concernant l'évolution du contenu en ThDP des cellules N2a cultivées en MPT, mais on note des différences concernant les autres dérivés : après 7 jours, la concentration en thiamine restait mesurable (≥ 0.1 µM) alors que la concentration en ThMP tombait beaucoup plus bas. En outre, les cellules utilisées par Bettendorff (1995) contenaient toujours des concentrations mesurables en ThTP (environ 1% de la thiamine totale) alors que nous n'avons jamais détecté de dérivés triphosphorylés dans nos cellules. L'ensemble de ces résultats indique donc que seul le ThDP est essentiel pour la survie et la croissance des cellules N2a, la limite inférieure étant de 5 µM dans les cellules. Ce point est confirmé par la mesure du nombre de cellules obtenues après 5 jours de culture en milieu riche ou pauvre en thiamine (Figure 11). Aucune différence significative n'a été obtenue, ce qui indique que le taux de division cellulaire ne varie pas lorsque la concentration externe en thiamine passe de 10^{-5} à 10^{-8} M.

Il est donc très vraisemblable qu'en milieu pauvre en thiamine, les enzymes ThDP dépendantes (TK, PDH et OGDH) restent pratiquement saturées en ThDP (excepté peut-être la PDH dont l'activité n'est pas un facteur limitant pour le métabolisme énergétique). L'excès de ThDP intracellulaire même en milieu riche est probablement accumulé dans la matrice mitochondriale.

1.2. Evolution de la teneur en thiamine, ThMP et ThDP lorsque les cellules N2a cultivées en milieu pauvre en thiamine sont incubées en présence de thiamine et de différents précurseurs.

Après 7 jours de culture en milieu pauvre en thiamine, les cellules sont incubées pendant 2, 4 et 6 heures dans le milieu de culture déficient en thiamine ne contenant pas de sérum mais auquel on ajoute différentes concentrations de thiamine (1, 10 et 50 µM), benfotiamine (1, 10 et 50 µM) ou sulbutiamine (0.5, 5 et 50 µM). On utilise des concentrations 2 fois plus basses en sulbutiamine (SBT) car après diffusion dans le cytoplasme, le pont disulfure de la SBT est réduit (sans doute principalement par le glutathion) et 2 molécules de thiamine libre sont formées.

Les résultats présentés dans la Figure 12 vont nous révéler que les profils d'évolution des concentrations intracellulaires en thiamine et dérivés posphorylés diffèrent fortement en fonction des composés testés (thiamine ou précurseurs).

Considérons d'abord l'absorption de la thiamine libre en fonction du temps (Fig. 12Aa). Au temps zéro, les cellules ne contiennent pas de concentrations détectables en thiamine, en accord avec les résultats montrés dans la Figure 1. Après 4 heures, en l'absence de thiamine ajoutée à l'extérieur, on note l'apparition d'une faible quantité de thiamine, provenant vraisemblablement d'une hydrolyse du ThMP (cf. Fig. 12Ac).

Si de la thiamine 1 µM est ajoutée dans le milieu, nous constatons que la concentration intracellulaire en thiamine n'augmente que très peu, même après 6 heures d'incubation. Nous savons pourtant que l'entrée de la thiamine dans les cellules N2a se fait via un transporteur à haute affinité, probablement THTR-2, dont le K_m pour la thiamine est très inférieur à 1 µM, environ 20 à 50 nM (Bettendorff & Wins 1994). Donc, en présence de 1 µM de thiamine externe, les sites de liaison du transporteur doivent être pratiquement saturés et la vitesse maximum de transport est atteinte. Le fait que nous n'observons pas d'augmentation de la teneur des cellules en thiamine après plusieurs heures s'explique probablement par le fait que, dans le cytosol, la thiamine est transformée en ThDP par la pyrophosphokinase (TPK). Cette enzyme a une haute affinité pour la thiamine (K_m de l'ordre de 10^{-7} M) mais la réaction est lente.

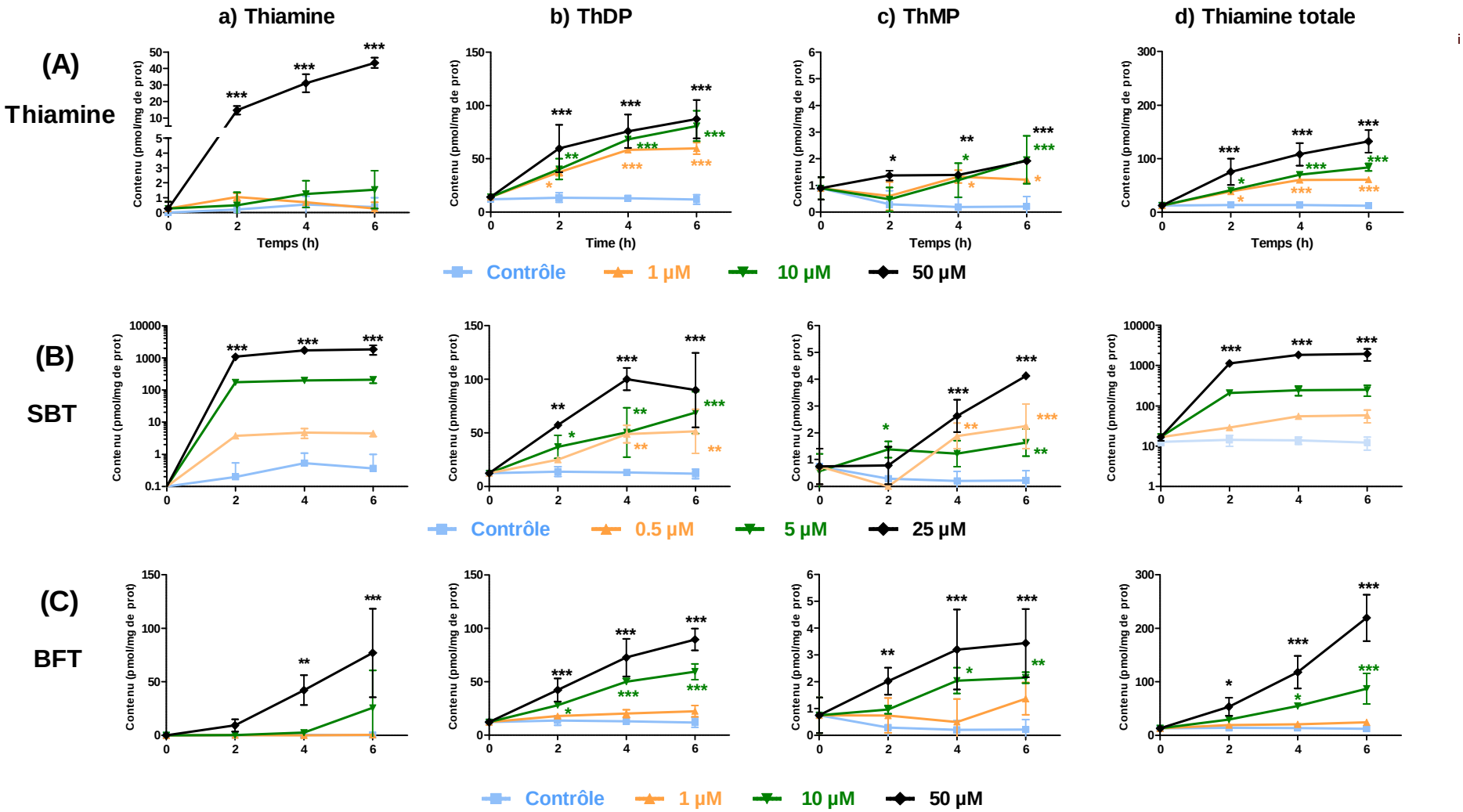
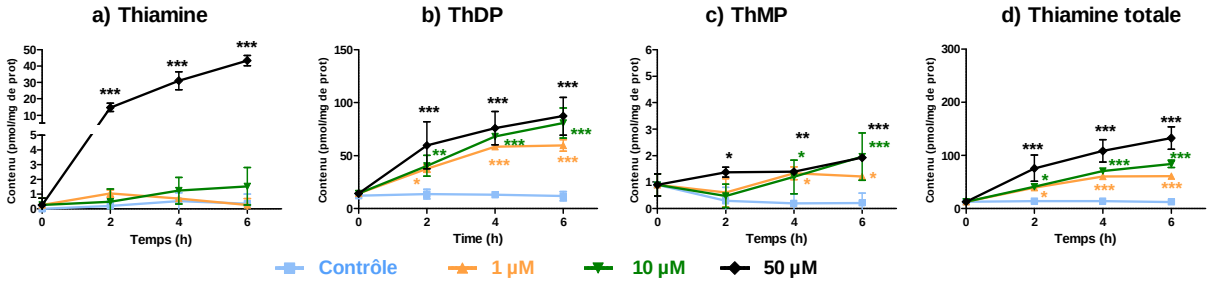


Figure 12 : **Evolution du contenu intracellulaire (pmol/mg de protéines) en a) thiamine, b) ThDP, c) ThMP et d) de la thiamine totale au cours du temps après ajout de différentes concentrations de (A) thiamine (10 nM = Contrôle ; 1, 10 et 50 µM = Thiamine), de (B) sulbutiamine (SBT, 0.5, 5 et 25 µM) et de (C) benfotiamine (BFT, 1, 10 et 50 µM) en milieu extracellulaire.** Les données sont représentées sous la forme moyenne ± l'écart-type (n=3 ; *p<0.05, **p<0.01, ***p<0.001 vs contrôle, ANOVA à 2 entrées suivie du test posthoc de Bonferroni). Pour les dérivés thiamine et thiamine totale lors de traitement par la sulbutiamine, les échelles de concentrations sont logarithmiques.

Il n'y a pas d'accumulation de thiamine libre car celle-ci n'est pas transportée activement (THTR-2 est seulement un échangeur thiamine/H^+). L'équilibre de la réaction ATP + thiamine ⇆ ThDP + AMP n'est pas en faveur de la formation de ThDP mais l'équilibre est déplacé car une partie du ThDP se lie à la transcétolase cytosolique avec une haute affinité et le reste s'accumule dans la matrice mitochondriale par échange ATP/ThDP.
Les résultats montrés dans la Figure 3Ab sont en accord avec ces interprétations : on voit que le ThDP intracellulaire augmente progressivement jusqu'à atteindre les mêmes concentrations que celles mesurées en milieu riche (cf. Fig. 11). On peut estimer que la concentration intracellulaire globale en ThDP est de 30 à 50 µM en milieu riche et 5 µM en milieu pauvre, comme nous l'avions estimé précédemment (Tableau 7).
Il est intéressant de constater que la concentration intracellulaire en ThDP ne dépasse jamais 50 µM, quelles que soient les conditions : même si la concentration en thiamine externe est élevée (50 µM) et que l'incubation est de 6 heures, ce plafond n'est pas dépassé. Il pourrait correspondre à la capacité maximum des mitochondries à accumuler le ThDP, les cellules N2a étant relativement pauvres en mitochondries.

Fig. 12A : Thiamine



Enfin, les résultats de la Figure 3Aa nous indiquent une dernière propriété intéressante : lorsque la concentration externe en thiamine passe de 1 à 50 µM, l'accumulation de thiamine non phosphorylée augmente assez fortement, jusqu'à atteindre environ 20 µM après 6 h. Comme le transport de la thiamine via le transporteur de haute affinité a déjà atteint sa vitesse maximum lorsque la concentration externe est de 1 µM (voir plus haut), il est probable qu'un autre système de transport à plus basse affinité est activé lorsque la concentration en thiamine dépasse 10 µM. La présence d'un tel transporteur a été suggérée et son K_m a été estimé à 0.8 µM dans les cellules N2a (Bettendorff & Wins 1994) mais il n'a pas été caractérisé sur le plan moléculaire.

2. EXPERIMENTATION *IN VIVO*

2.1. Introduction

De plus en plus d'études suggèrent que la thiamine (vitamine B1) et ses précurseurs possédant une biodisponibilité plus élevée ont la capacité d'exercer de remarquables effets neuroprotecteurs au niveau du cerveau mammalien. Le diphosphate de thiamine (ThDP) est bien connu pour son rôle essentiel dans le métabolisme du glucose comme cofacteur de 3 enzymes essentielles : la transcétolase dans le cytoplasme catalyse une étape limitante dans la partie non-oxydative du cycle des pentoses-phosphates. Dans les mitochondries, le ThDP est un coenzyme de la pyruvate déshydrogénase et de l'oxoglutarate déshydrogénase. Cette dernière catalyse une réaction qui est limitante dans le cycle de Krebs, d'où l'importance du ThDP pour le métabolisme énergétique oxydatif. Il n'est dès lors pas surprenant que la déficience en thiamine (TD) ait des répercussions délétères sur l'activité cérébrale, vu que cette dernière dépend fortement du métabolisme oxydatif du glucose (Gibson & Blass 2007). Cependant, une altération générale du métabolisme énergétique cérébral ne peut pas expliquer à elle seule la spécificité des lésions diencéphaliques apparaissant lors d'une déficience en thiamine. Il a dès lors été suggéré que la thiamine pourrait exercer des actions neuromodulatrices ou neuroprotectrices à travers des mécanismes indépendants de son rôle de coenzyme (Nghiêm et al. 2000; Bettendorff 2013; Bettendorff et al. 2007; Mkrtchyan et al. 2015).

La sensibilité élevée du cerveau à la TD est due à l'absorption lente de la thiamine à travers l'épithélium intestinal et à travers la barrière hémato-encéphalique (BHE) (Greenwood et al. 1982). En conséquence, des précurseurs lipophiles possédant une biodisponibilité plus élevée ont été développés dans le but d'améliorer l'absorption de la vitamine. La benfotiamine (S-benzoylthiamine-O-monophosphate) n'est pas lipophile mais, après administration orale, elle est déphosphorylée par une ecto-phosphatase alkaline en S-benzoylthiamine qui est liposoluble et qui, à son tour, peut être convertie en thiamine dans le foie et le sang (Volvert et al. 2008). Chez un modèle murin de la maladie d'Alzheimer (souris APP/PS1), il a été récemment montré qu'un traitement oral par la benfotiamine fait régresser à la fois les dépôts amyloïdes et les déficits cognitifs (Pan et al. 2010). Dans cette étude, la benfotiamine était plus efficace qu'un autre précurseur lipophile, la fursultiamine (voir Figure 4, p. 16). Il se pourrait donc que des métabolites non identifiés autres que la

thiamine soient impliqués dans les effets neuroprotecteurs, bien que les mécanismes impliqués soient inconnus à l'heure actuelle. Les effets observés pour la benfotiamine semblent donc indépendants d'une activation des enzymes ThDP-dépendantes et d'une stimulation du métabolisme énergétique cérébral.

Etant donné que la TD est associée à des troubles de la mémoire bien avant l'apparition des lésions diencéphaliques (Vetreno et al. 2011) et que les altérations cognitives sont généralement associées à un dysfonctionnement hippocampique, Zhao et al. (2008) ont étudié si une telle altération était possible dans les souris mises en déficience en thiamine à des stades pré-pathologiques où il n'y a pas de lésions visibles (Zhao et al. 2008). Chez ces souris, les capacités mnésiques étaient fortement réduites et ce, de façon concomitante à une altération de la prolifération cellulaire des progéniteurs et de la neurogenèse dans le gyrus denté. Ces résultats ont récemment été confirmés chez des rats traités à la pyrithiamine, un antagoniste très efficace bloquant l'absorption de la vitamine et sa transformation en ThDP (Hazell et al. 2014). Les troubles de la neurogenèse sont connus pour être impliqués dans les altérations cognitives. Des troubles de la neurogenèse ont également été observés dans la maladie d'Alzheimer, dans les modèles murins, ou chez les humains (Lazarov & Marr 2013). Nous avons dès lors émis l'hypothèse que les effets bénéfiques sur la mémoire observés avec la benfotiamine dans les différents modèles murins pourraient être liés, au moins en partie, à une stimulation de la neurogenèse.

En dehors de l'apprentissage, la formation hippocampique est également impliquée dans le contrôle de l'humeur et l'adaptation au stress (Figure 27). En effet, il s'agit d'un important modulateur de l'axe hypothalamo-hypophyso-surrénalien (HPS), également appelé l'axe du stress (Jacobson & Sapolsky 1991; Pariante & Lightman 2008; Lucassen et al. 2014). Plusieurs études ont montré que l'exposition de rongeurs ou de mammifères au stress engendre d'importantes altérations de la neurogenèse hippocampique adulte (NHA) (Gould et al. 1998; Malberg & Duman 2003). Les antidépresseurs protègent la NHA contre les effets néfastes du stress (Warner-Schmidt & Duman 2006; Miller & Hen 2015). D'autres facteurs ont également cet effet bénéfique sur la neurogenèse, notamment l'enrichissement environnemental, l'exercice et la thérapie électroconvulsive (Stranahan et al. 2006; Ming & Song 2011). Le fait que la NHA semble contrôlée par un éventail de facteurs différents suggère que plusieurs sortes de molécules (en plus des antidépresseurs) pourraient être

utilisées pour protéger ou favoriser la neurogenèse quand celle-ci est affectée par des évènements stressants.

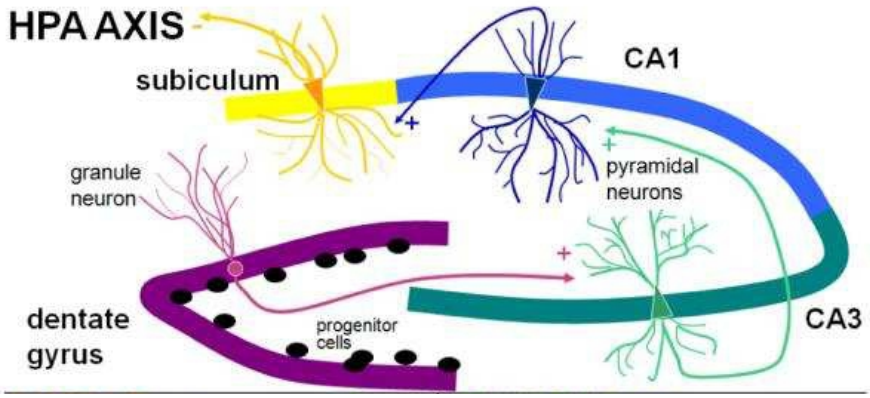


Figure 27 : **Diagramme schématique de l'effet du stress et des glucocorticoïdes sur les types cellulaires principaux de l'hippocampe.** Le stress induit la libération de glucocorticoïdes qui se lient aux récepteurs des différents types de cellules présents dans l'hippocampe. Le gyrus denté contient des cellules granulaires qui ont des connections excitatrices avec les cellules pyramidales de la région CA3 (Corne d'Ammon). Les cellules pyramidales de la région CA3 ont des connections excitatrices avec les cellules pyramidales région CA1, qui ont des connections excitatrices avec les cellules pyramidales dans le subiculum, la principale population efférente dans l'hippocampe. Les cellules pyramidales dans le subiculum exercent une action inhibitrice sur l'axe hypothalamo-hypophyso-surrénalien (HPS, HPA sur le schéma), permettant un retour à la normale du système après le stress. [Tiré de (Schoenfeld & Gould 2012)].

2.2. Etude de la concentration en thiamine et dérivés dans des cerveaux de souris P301S traitées par la benfotiamine.

L'équipe du professeur Beal (Weill Cornell Medical College, New-York) a étudié l'effet d'un traitement par la benfotiamine sur des souris transgéniques avec la mutation P301S, une mutation de la protéine tau responsable de la démence frontotemporale chez l'homme. Ces souris ont reçu de la benfotiamine (1250 mg/kg/jour) par voie orale *ad libitum* pendant 9 mois. Après le traitement, on constate une diminution spectaculaire de l'hyperphosphorylation de tau et une augmentation de la longévité des animaux (résultats en cours de publication). La collaboration mise en place avec cette équipe nous a permis d'analyser les contenus en thiamine et ses dérivés dans le sang, le foie, le cortex cérébral et l'hippocampe des souris wild-type (Wt) et transgéniques (Tg) traitées ou non à la benfotiamine (Figure 28).

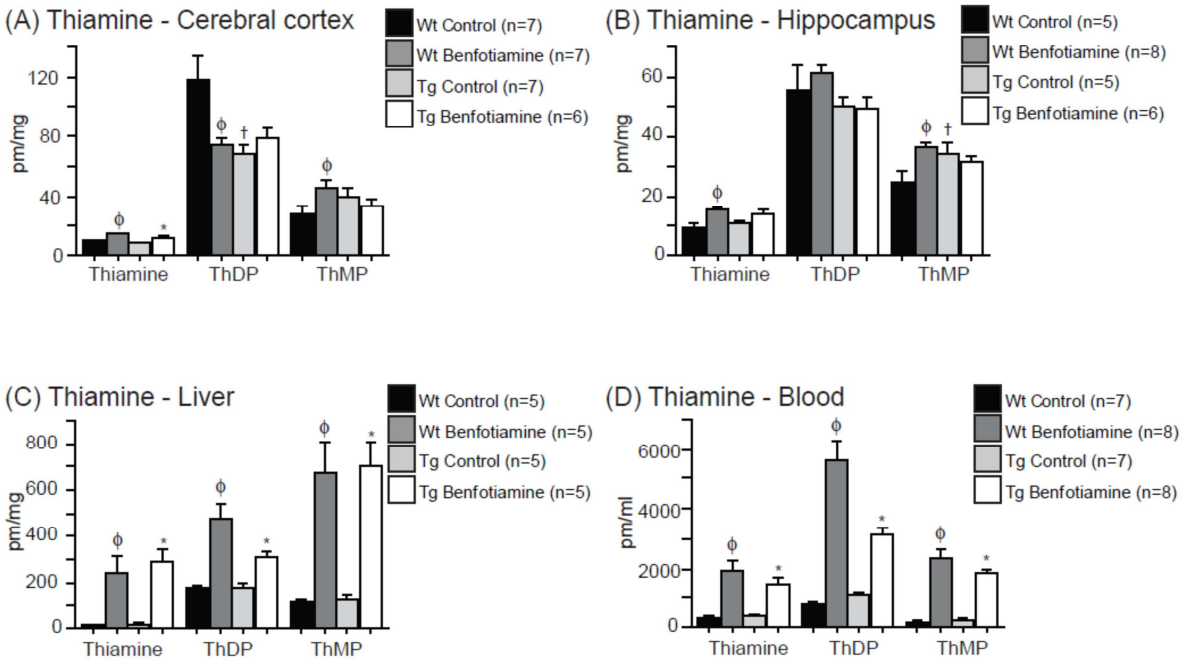


Figure 28 : **Contenu en thiamine, thiamine monophosphate (ThMP) et thiamine diphosphate (ThDP) (pmol/mg de protéines) dans le cerveau de souris wild-type (Wt) et de souris transgéniques (Tg) traitées ou non à la benfotiamine** dans le (A) cortex cérébral, (B) l'hippocampe, (C) le foie et (D) le sang. Données présentées sous forme de moyenne ± SEM. (ϕ Wt control vs Wt benfotiamine, † Wt control vs Tg control, * Tg control vs Tg benfotiamine $p<0.05$, ANOVA, suivie d'un post hoc test Fischer's PLSD).

L'administration de benfotiamine induit une augmentation des niveaux de la thiamine et de tous ses dérivés dans le sang et le foie dans les souris Wt et Tg. Chez les souris Wt, les niveaux cérébraux de thiamine et de ThMP sont augmentés significativement par le traitement. Les niveaux de ThDP sont diminués dans le cortex et ne varient pas dans l'hippocampe. Dans le cortex et l'hippocampe des souris P301S, on constate une diminution des niveaux de ThDP et une augmentation des niveaux de ThMP par rapport aux souris Wt. Le traitement à la benfotiamine n'engendre qu'une faible élévation du contenu cérébral en thiamine chez les souris Tg. Nous pouvons donc conclure que dans ce modèle de neurodégénérescence (tauopathie), les effets bénéfiques de la benfotiamine ne sont pas dus à une augmentation du contenu en ThDP du cerveau et à la stimulation du métabolisme énergétique qui en résulterait. Pan et al. (2010) arrivaient à la même conclusion avec leur modèle murin de la maladie d'Alzheimer. Nous reviendrons sur cette conclusion par la suite.

2.3. Effets du traitement de souris adultes normales par la thiamine et ses précurseurs.

2.3.1. Validité de l'utilisation de la sulbutiamine « complément alimentaire »

La sulbutiamine a été développée comme médicament par la firme Servier dans les années 80 pour le traitement des asthénies fonctionnelles. Le mécanisme d'action de ce précurseur a été étudié dans notre laboratoire (Bettendorff 1994), en utilisant la substance fournie par la firme, désignée sous le nom de « sulbutiamine à usage clinique ». C'est cette forme que nous avons d'abord utilisée mais, comme elle n'est plus disponible actuellement, nous avons acheté de la sulbutiamine sous forme de complément alimentaire (Mind Nutrition®). Nous avons comparé l'effet de l'addition de sulbutiamine à usage « clinique » et à usage « complément alimentaire » aux mêmes concentrations sur les contenus intracellulaires en thiamine et dérivés de cellules N2a cultivées en milieu pauvre en thiamine. La sulbutiamine « complément alimentaire » s'est révélée plus efficace que la sulbutiamine « clinique » pour augmenter la concentration intracellulaire en thiamine totale (Figure 29, ***p<0.001). Nous avons donc considéré que nous pouvions l'utiliser pour nos expériences.

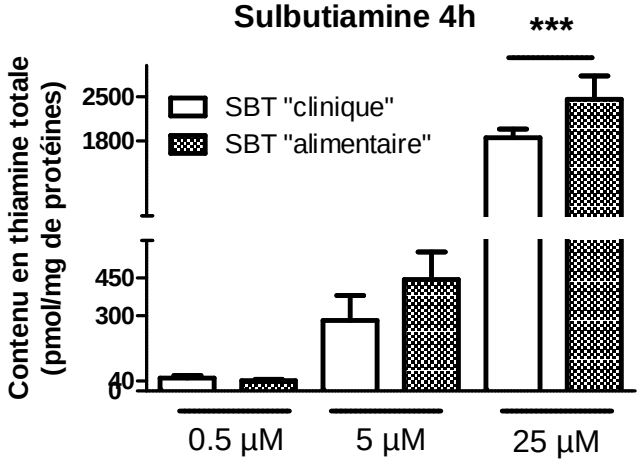


Figure 29 : **Contenu intracellulaire en thiamine totale (pmol/mg de protéines) dans des cellules de neuroblastomes N2a pauvres en thiamine après 4 heures d'incubation avec de la sulbutiamine « clinique » (Servier®) et de la sulbutiamine « alimentaire » (Mind Nutrition®) à différentes concentrations (0.5, 5 et 25 µM).** Les données sont présentées sous forme de moyenne ± écart-type (n=3-5 ; ***p<0.001 par comparaison avec la condition SBT « clinique », ANOVA à 2 entrées, suivie d'un test post-hoc de Bonferroni).

2.3.2. Mise au point de l'administration du traitement par voie orale (eau de boisson, gelée ou gaufrettes) et intrapéritonéale

Nous avons tenté de trouver une méthode d'administration orale qui pourrait être utilisée à la fois pour la thiamine, la benfotiamine et la sulbutiamine, qui possèdent des propriétés chimiques fort différentes. Nous avons pour cela essayé dans un premier temps de dissoudre ces composés dans de l'eau, car il a été montré que le gavage engendre un stress (Brown et al. 2000) et nous voulions étudier l'effet de ces composés sur la neurogenèse et non sur la neurogenèse influencée par le stress. Bien que la dissolution des composés dans l'eau n'ait pas posé de problème (grâce à la modification du pH, voir méthodes), les souris ont montré une répulsion à consommer volontairement de l'eau contenant de la sulbutiamine. Il s'est avéré que la sulbutiamine présentait une amertume élevée. Nous avons donc tenté de l'administrer via des gaufrettes (Croustifondant Vanille de Delacre®) ou même préparée dans du sucralose (pouvoir sucrant 600 fois supérieur au saccharose) via des bonbons en gelée mais sans succès. Par conséquent, les souris ont été traitées pendant 20 jours par voie orale avec de la thiamine (200 mg/kg/jour) ou de la benfotiamine (200 mg/kg/jour) via des gaufrettes, et par injection intrapéritonéale avec de la sulbutiamine (100 mg/kg/jour, préparée dans du NaCl 0.9% stérile). Les souris traitées à la sulbutiamine recevaient également une gaufrette contrôle. Les souris étant friandes des gaufrettes à la vanille, celles-ci étaient généralement consommées dans les 2 h suivant leur dépôt dans la cage.

La stabilité des composés dissous dans du saccharose, ou en tout cas, leur non-décomposition en dérivés de la thiamine a été étudiée par HPLC (Figure 30). La thiamine et la sulbutiamine sont stables en solution, et ce, pendant au moins 7 jours. La benfotiamine quant à elle contient dès le départ 6-9 % de thiamine, principalement sous forme monophosphate. Cette proportion de ThMP reste constante au cours du temps.

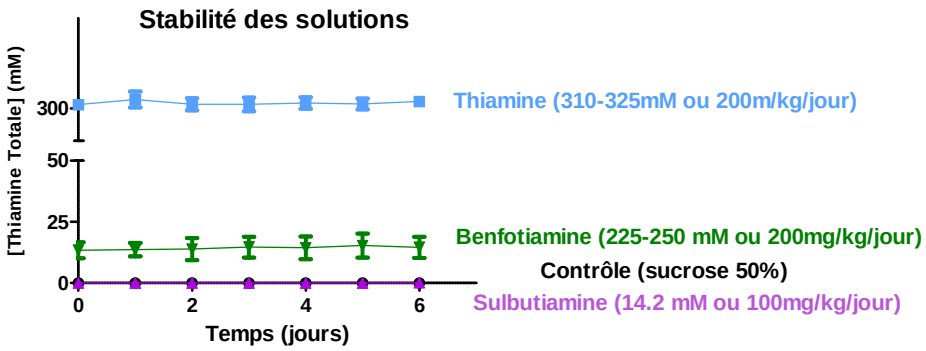


Figure 30 : **Concentration en thiamine totale au cours du temps dans les solutions de traitement, c'est-à-dire de la thiamine, de la benfotiamine et de la sulbutiamine préparées dans du saccharose 50% (m/v, contrôle).** Les données sont présentées sous forme de moyenne ± écart-type (n=5-9).

2.3.3. Effet du traitement par les gaufrettes sur le métabolisme des souris

2.3.3.1. Effet du traitement par les gaufrettes sur le poids des souris

Afin d'étudier l'impact des méthodes d'administration utilisées et des différents traitements sur le poids, les souris ont été pesées quotidiennement. Les souris ayant reçu leur traitement via des gaufrettes présentent toutes une augmentation du poids entre le premier et le dernier jour de traitement (Figure 31). Seules les souris ayant reçu une injection intrapéritonéale (traitement à la sulbutiamine) simultanée à la gaufrette ne présentent pas une augmentation de poids (Fig. 31B). Ces données suggèrent que soit la sulbutiamine, soit les injections intrapéritonéales, soit les deux sont responsables d'une non-prise de poids.

2.3.3.2. Effet du traitement sur le contenu en thiamine et dérivés dans le sang, le foie et le cerveau des souris

Vingt-quatre heures après avoir reçu le dernier traitement, les souris ont été sacrifiées et le sang, le foie, le cortex et l'hippocampe ont été récupérés afin d'en analyser les contenus en thiamine et dérivés (Figure 32).

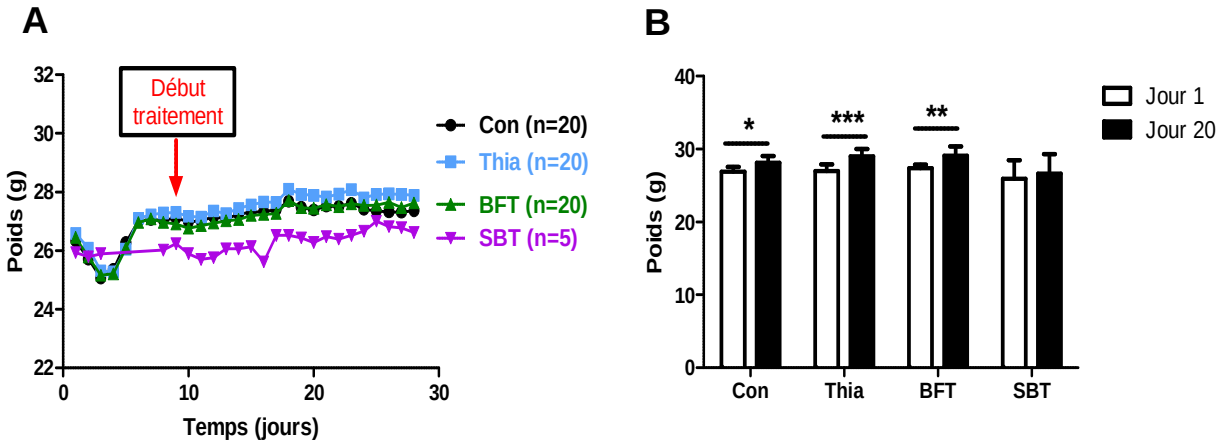


Figure 31 : **Evolution du poids des souris au cours du traitement.** (A) Evolution du poids des souris depuis leur arrivée jusqu'à la fin du traitement (début du traitement au jour 9). Les données sont présentées sous forme de moyenne ± écart-type (n=4-6). (B) Poids des souris au début (jour 1) et à la fin (jour 20) du traitement avec le contrôle, la thiamine, ou la benfotiamine dans des gaufrettes et avec la sulbutiamine par injection i.p. Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; *p<0.05, **p<0.01, ***p<0.001 vs jour 1, ANOVA à 2 entrées suivie d'un test post hoc de Bonferroni,).

2.3.3.2.1. Effet du traitement sur la concentration en thiamine et dérivés dans le sang

Un traitement chronique avec de la sulbutiamine en injection intrapéritonéale pendant 20 jours augmente la concentration en thiamine dans le sang (Fig. 32A) mais pas dans le foie (Fig. 32C). Cependant, Bettendorff et al. (1990) ont montré que l'administration d'une dose inférieure de sulbutiamine (52 mg/kg) à des rats pendant 14 jours était responsable d'une augmentation nettement plus importante des niveaux plasmatiques de thiamine par rapport au contrôle (6 fois supérieure pour la thiamine, 3 fois supérieure pour le ThMP et 2 fois supérieure pour le ThDP).

Bien que le sacrifice des souris ait aussi été effectué 24 h après le dernier traitement, l'augmentation observée dans notre étude, qui n'a lieu que pour les niveaux de thiamine, n'est que de 1.3 fois supérieure au contrôle. Nous avons vérifié que la sulbutiamine était bien injectée et/ou métabolisée, en mesurant les taux de thiamine et dérivés dans le sang et le foie 1 h après injection unique d'une dose de 100 mg/kg (Figure 33). Dans le sang, seuls les niveaux de thiamine augmentent (de 20 fois par rapport au contrôle) alors que dans le foie, la thiamine, le ThMP et le ThDP sont significativement augmentés par rapport au contrôle. Ces données suggèrent que la métabolisation de la sulbutiamine pourrait différer entre les rats et les souris.

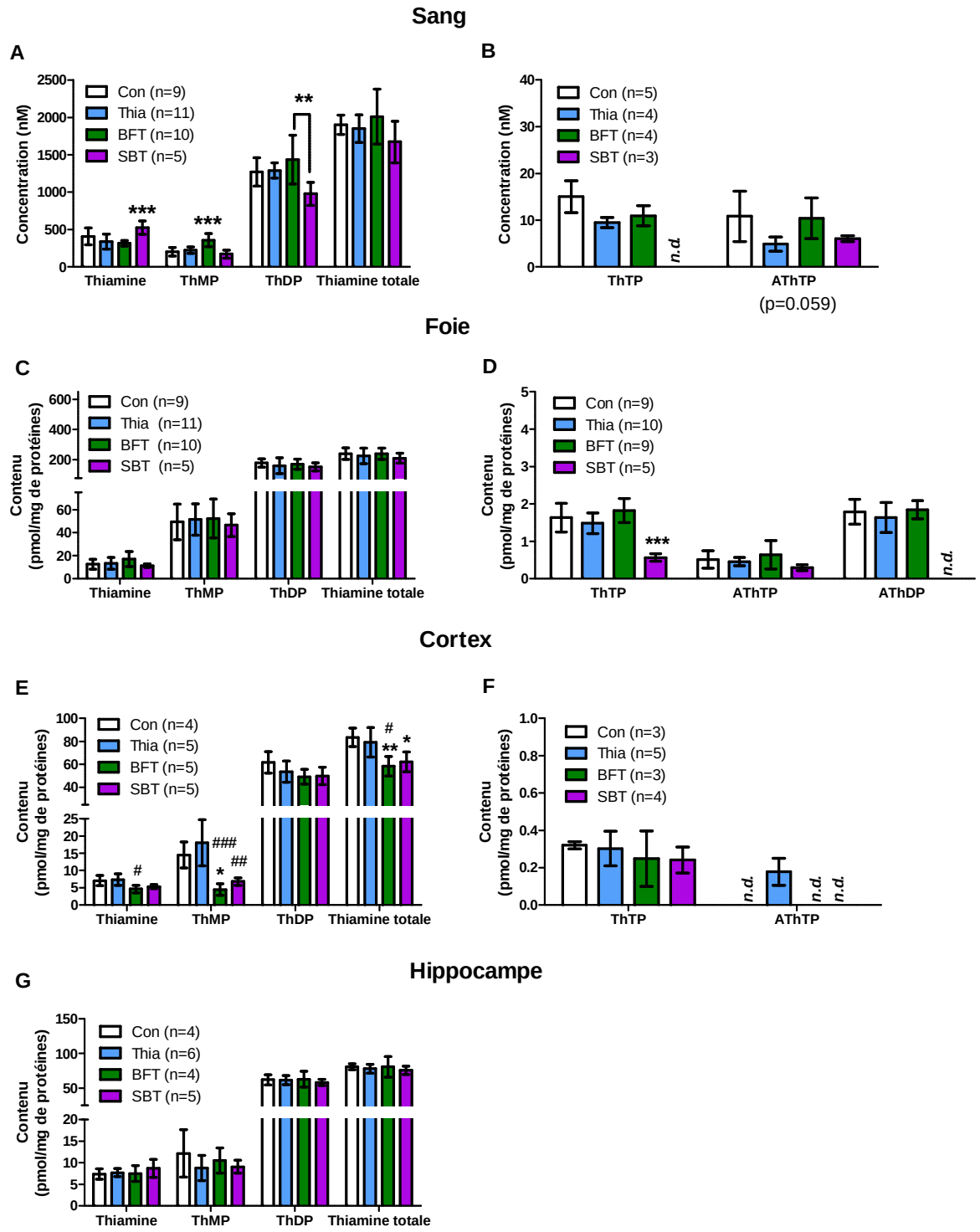


Figure 32 : **Contenu en thiamine et ses dérivés phosphorylés dans le sang (A, B), le foie (C, D), le cortex (E, F) et l'hippocampe (G) après traitement oral à la thiamine (200 mg/kg/jour), à la benfotiamine (200 mg/kg/jour) et traitement intrapéritonéal à la sulbutiamine (100mg/kg/jour) pendant 20 jours.** Les souris ont été sacrifiées 24 h après le dernier traitement. La benfotiamine augmente les concentrations de ThMP dans le sang tandis que la sulbutiamine augmente les concentrations de thiamine (*p<0.05, **p<0.01, ***p<0.001 vs autres). La benfotiamine et la sulbutiamine induisent une diminution significative de la thiamine et du ThMP dans le cortex (*p<0.05 ; **p<0.01 vs contrôle ; #p<0.05 ; ##p<0.01, ###p<0.001 vs thiamine, ANOVA à une entrée suivie d'un test posthoc de Tukey,). Les barres représentent la moyenne ± écart-type. *N.d. = non détecté.*

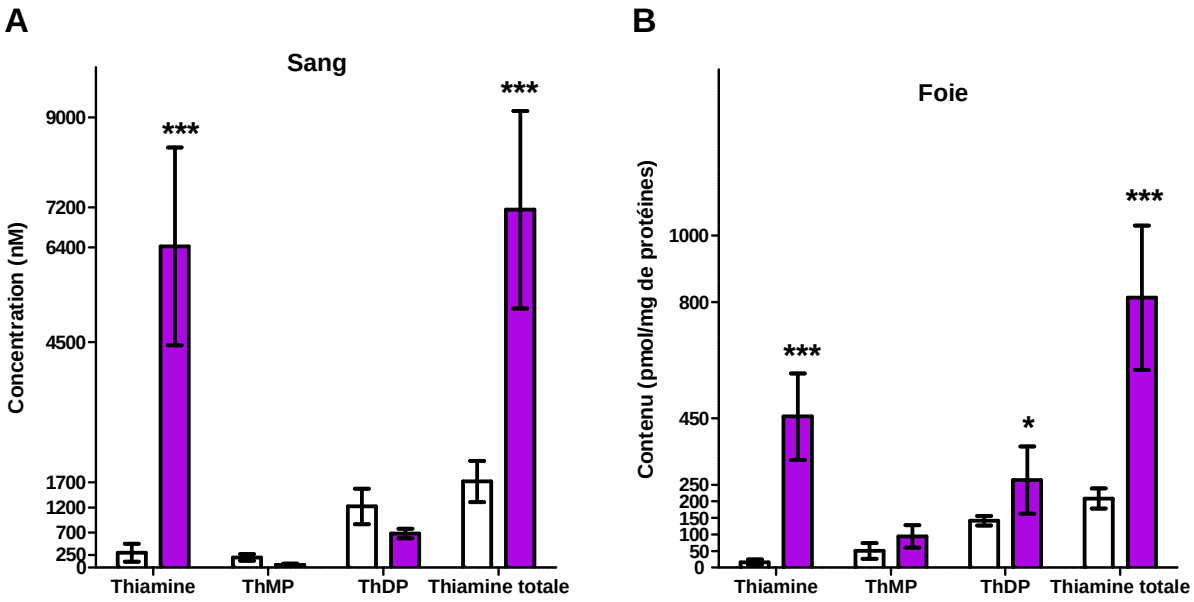


Figure 33 : **Contenu des différents dérivés de la thiamine dans le sang (A) et le foie (B) 1 h après injection intrapéritonéale d'une dose unique de sulbutiamine (100 mg/kg).** La sulbutiamine (n=5) est responsable d'une augmentation drastique des niveaux de thiamine par rapport au contrôle (n=8). (*p<0.05 ; ***p<0.001 vs contrôle, ANOVA à 2 entrées suivie d'un test post hoc de Bonferroni). Les barres représentent la moyenne ± écart-type.

La benfotiamine, quant à elle, entraîne une augmentation des niveaux sanguins de ThMP (Fig. 32A). Plusieurs études ont montré qu'une dose unique de benfotiamine (100-300 mg) engendre une augmentation importante de la thiamine (jusque 50 µM) et une augmentation moins importante du ThMP et du ThDP lorsque les souris sont sacrifiées une heure après administration (Volvert et al. 2008; Pan et al. 2010; Hurt et al. 2012). L'administration chronique de benfotiamine engendre également une augmentation de la thiamine et de ses dérivés dans le sang (voir point 2.2, p. 88). Par ailleurs, Xie et al. (2014) ont montré que 24 h après l'administration unique ou chronique de benfotiamine, le ThMP reste plus élevé dans le sang chez l'homme. Dès lors, il est pertinent de se demander pourquoi 24 h après administration de benfotiamine, les niveaux de ThMP restent élevés dans le sang. Plusieurs études ont montré que l'absorption de benfotiamine dans l'organisme était plus rapide mais aussi que cette molécule ou ses dérivés restaient plus longtemps dans les tissus (Wada et al. 1961; Loew 1996; Greb & Bitsch 1998; Hilbig & Rahmann 1998; Volvert et al. 2008; Pan et al. 2010; Hurt et al. 2012; Xie et al. 2014).

Dès lors, l'augmentation du ThMP dans le sang peut s'expliquer de plusieurs manières. Soit elle est liée à l'action d'un métabolite non identifié de la benfotiamine, soit elle est directement liée à la présence de ThMP dans la solution de traitement. La première hypothèse est peu probable étant donné que la benfotiamine rentre dans les cellules sous forme déphosphorylée (Shindo et al. 1967). Pour rappel, nous avons observé que la solution de benfotiamine contient dès le départ 6 à 9% de ThMP, ce qui correspond à 9-12 mM de ThMP (Figure 30). Le ThMP présente une liaison phosphoester suffisamment stable pour ne pas être rompue pendant le transit intestinal (estomac-intestin) et le ThMP pourrait donc être transporté tel quel à travers les entérocytes jusque dans le sang. Une autre hypothèse, moins probable, est que la benfotiamine donne lieu à une augmentation importante de la thiamine et à une augmentation de sa transphosphorylation en ThMP, une réaction qui peut être catalysée par les phosphatases alcalines (Rindi et al. 1995). Cette transphosphorylation ne serait pas observée pour la thiamine à cause de son absorption trop lente et non plus pour la sulbutiamine, car elle est administrée par i.p. et donc ne passe pas par les entérocytes. Une dernière hypothèse est que l'augmentation de ThMP provient de l'hydrolyse du ThDP suite à son augmentation dans le sang, ce qui expliquerait le délai observé.

2.3.3.2.2. Effet du traitement sur la concentration en thiamine et dérivés dans le foie

Aucun traitement n'induit des variations de la teneur en thiamine et dérivés dans le foie des souris. Par ailleurs, Volvert et al. (2008) ont montré que des souris traitées pendant 14 jours avec une dose de 100 mg/kg présentent une légère augmentation des niveaux de thiamine, de ThMP et de ThDP dans le foie 24 h après le dernier traitement. Beal et al. (voir point 2.2, Fig. 28C, p. 88) ont également montré que l'administration chronique de benfotiamine (1250 mg/k/jour) est responsable d'une augmentation des différents dérivés de la thiamine dans le foie. Cependant dans l'étude de Volvert et al. (2008), le véhicule utilisé pour dissoudre la benfotiamine est l'hydroxypropyl-β-cyclodextrine (HP-β-CD), un polysaccharide cyclique présentant une cavité intérieure apolaire et des groupements hydroxyles à l'extérieur, permettant ainsi l'inclusion de molécules hydrophobes et leur solubilisation en milieu aqueux. Or, la libération des molécules incluses dans l'HP-β-CD est plus lente et pourrait expliquer pourquoi 24 h après administration, on observe encore une augmentation de tous les dérivés de la thiamine dans le foie. Dans l'étude de Beal et al.

(point 2.2, p. 88), les souris avaient accès à la nourriture ad *libitum* donc il est impossible de déterminer si l'augmentation observée est due à la dernière dose ou à l'administration chronique. D'autre part, Hurt et al. (2012), dans la seule étude à utiliser l'eau comme mode d'administration de la thiamine, ont montré que seuls les niveaux de ThMP et de thiamine étaient augmentés dans le foie 1 h après administration ; ces augmentations sont nettement moindres que celles observées dans les autres études (Volvert et al. 2008). Dès lors, ces données suggèrent que le véhicule utilisé ainsi que le mode d'administration (gavage ou *ad libitum,* dosage ponctuel ou continu) de la benfotiamine par voie orale a un impact sur la métabolisation de celle-ci.

Par ailleurs, la sulbutiamine induit une diminution significative du ThTP dans le foie (Fig. 32D).

2.3.3.2.3. Effet du traitement sur la concentration en thiamine et dérivés dans le cerveau

Comme dans le cas des modèles murins de neurodégénérescence (voir plus haut), le traitement par la benfotiamine n'augmente pas la teneur du cerveau en ThDP (Fig. 28A et D). C'est également le cas si nous utilisons la sulbutiamine (Fig. 32E, F, G). Etonnamment, au niveau du cortex, la benfotiamine et la sulbutiamine engendrent une diminution significative de tous les dérivés (excepté le ThDP). Bien que non significative, cette diminution, suite à l'administration à court terme de benfotiamine, a déjà été observée dans une autre étude (Volvert et al. 2008). Cependant, dans d'autre études (Pan et al. 2010; Hurt et al. 2012), 1 h après administration d'une dose unique ou de doses multiples (pendant 10 jours), les niveaux de thiamine seule sont augmentés dans le cerveau.

Par ailleurs, lors du passage des échantillons en HPLC, le pic d'un composé inconnu est apparu après 22 minutes sur le chromatogramme pour le sang, le foie et le cortex. Dans le sang et le foie, le pic est sensiblement le même pour les 3 traitements mais dans le cortex, la benfotiamine et la sulbutiamine induisent une plus forte augmentation. Malheureusement, nous n'avons pas eu l'opportunité d'explorer la nature de ce composé inconnu.

2.3.3.2.4. Effet du traitement sur des protéines impliquées dans la neuroprotection (Akt et GSK-3β) présentes dans l'hippocampe et le cortex

Les différents traitements n'ont eu aucun effet sur la phosphorylation et l'expression des protéines GSK-3β et Akt dans l'hippocampe et le cortex (résultats non montrés).

2.3.4. Effet du traitement sur la neurogenèse hippocampique en conditions non pathologiques

Nous nous sommes ensuite intéressés à l'effet de la thiamine et de la benfotiamine sur différents aspects de la neurogenèse hippocampique. La sulbutiamine a été écartée de l'étude car elle aurait nécessité un nombre de souris supplémentaire inutile pour une étude préliminaire. Les souris ont donc été traitées avec de la thiamine et de la benfotiamine (via les gaufrettes) pendant 20 jours avant de recevoir une injection intrapéritonéale unique de bromodéoxyuridine (BrdU, 150 mg/kg). Pour rappel, le bromodéoxyuridine est un nucléoside synthétique qui va s'intercaler dans l'ADN des cellules durant la phase S de la réplication. Le BrdU incorporé peut être ensuite détecté grâce à des anticorps spécifiques. L'évolution et le destin des cellules progénitrices peuvent être ainsi étudiés au cours du temps. Les souris ont ensuite été sacrifiées 2 h, 24 h et 3 semaines après l'injection de BrdU afin d'étudier respectivement la prolifération, l'index de cycle cellulaire, la survie et enfin, la neurogenèse complète (Figure 8, p. 33). Les résultats obtenus mettent en évidence l'absence d'effet de la thiamine et de la benfotiamine sur la neurogenèse lors d'un traitement à court terme chez des souris adultes normales (résultats non montrés). Les paramètres étudiés ne sont donc pas influencés par les conditions et les durées de traitement utilisées dans la présente étude.

2.4. Etude de la neurogenèse chez des souris adultes stressées traitées ou non par la thiamine ou la benfotiamine.

Etant donné que nous n'avons pas obtenu d'effets de la thiamine et de la benfotiamine sur la neurogenèse chez des souris normales, nous avons tenté d'étudier l'effet de ces traitements en conditions pathologiques. En collaboration avec le Dr Strekalova (Departement of Neurosciences, School for Mental Health and Neuroscience, Maastricht University), nous avons eu l'opportunité de tester l'effet d'un traitement de 20 jours avec ces composés sur la prolifération des progéniteurs (cellules Ki67+) et sur la survie à court

terme (5 jours) des néoneurones (cellules BrdU+) chez des souris stressées (Fig. 34A), la procédure de stress utilisée consistant à exposer les souris à un prédateur. Pour ce faire, les souris sont placées dans des cylindres transparents permettant un contact visuel et olfactif à l'intérieur d'une cage en présence de rats. Dans une expérience parallèle (Fig. 34B), les souris ont été soumises au même protocole expérimental afin d'étudier l'impact du traitement sur des paramètres biochimiques dans le sang, le foie, le cortex et l'hippocampe (contenu en thiamine par HPLC, niveau de phosphorylation et d'expression des protéines Akt et GSK-3β par western blot et niveau d'expression du BDNF par RT-PCR).

2.4.1. Effet du stress et du traitement à la thiamine et à la benfotiamine sur la prolifération des progéniteurs et sur la survie à court terme (5 jours) des neurones immatures

Comme le montre la Figure 35A, le nombre de cellules $BrdU^+$, représentatif de la survie des neurones immatures nouvellement formés dans la couche cellulaire granulaire (CGL) du gyrus denté (DG), est significativement diminué par le stress. Le nombre de cellules $Ki67^+$ (Fig. 35B), indicatif de la prolifération cellulaire totale, pourrait également être réduit par l'exposition au prédateur mais la différence n'est pas significative au stade actuel. Le traitement des souris par la thiamine et la benfotiamine (200 mg/kg/jour, 20 jours) prévient cette diminution de la survie et de la prolifération due au stress. Lorsque les souris stressées sont traitées à la benfotiamine, on observe même un niveau supérieur de cellules $BrdU^+$ et $Ki67^+$ en comparaison à celui des souris non stressées (NS-NT). Cet effet plus important de la benfotiamine peut s'expliquer par le fait que celle-ci augmente drastiquement les niveaux sanguins de thiamine (Volvert et al. 2008) alors qu'un traitement à la thiamine, même avec des doses supérieures, est beaucoup moins efficace (Bitsch et al. 1991; Greb & Bitsch 1998; Hilbig & Rahmann 1998; Loew 1996; Xie et al. 2014).

Dans la littérature, il a été montré que l'exposition nocturne de souris à des rats pendant 5 nuits engendre un stress important chez la souris (Strekalova et al. 2015) et engendre une diminution importante de la survie (Cellules $BrdU^+$) et du nombre de progéniteurs totaux (Cellules $Ki67^+$). Déjà en 2001, Tanapat et al. ont montré que l'exposition de rats à l'odeur de renard induit une diminution de la prolifération cellulaire hippocampique.

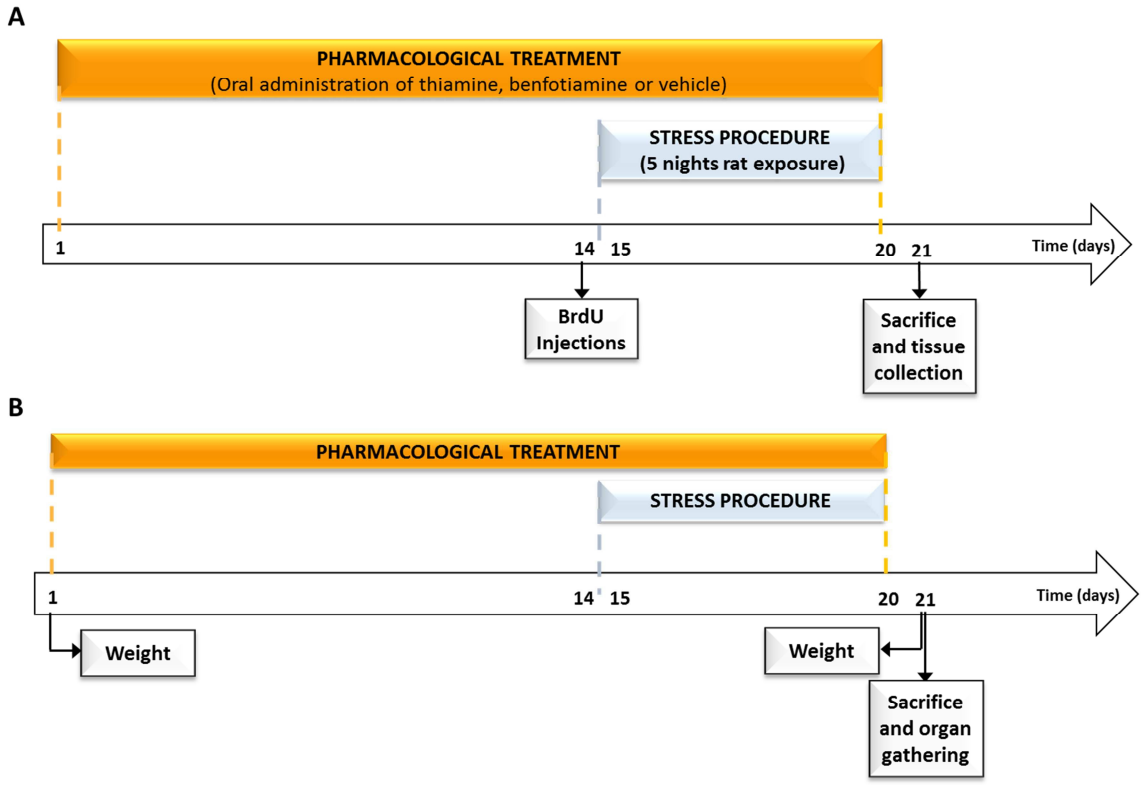


Figure 34 : **Ligne du temps schématique du protocole expérimental.** (A) Expérience de stress avec la première cohorte : impact de l'exposition à un prédateur (avec ou sans traitement à la thiamine ou à la benfotiamine) sur la proliferation des progéniteurs (marquage Ki67) et la survie des néoneurones immatures (marquage BrdU) dans la couche sous-granulaire du DG. Du jour 1 à 20, les souris C57BL/6J ont reçu de la thiamine (200 mg/kg/jour), de la benfotiamine (200 mg/kg/jour) ou le véhicule (eau normale) via l'eau ad libitum. Le 14ème jour, les souris ont reçu 4 injections i.p. (50 mg/kg, 2 h entre chaque injection). Elles ont ensuite été exposées aux rats pour les 5 dernières nuits du traitement et ont été sacrifiées 24 h après la fin du traitement pharmacologique et de la période de stress. La prolifération cellulaire et la survie ont été analysées comme décrit dans Matériel et Méthodes. (B) Expérience de stress avec la deuxième cohorte: impact de l'exposition à un prédateur (avec ou sans traitement à la thiamine ou à la benfotiamine) sur les paramètres biochimiques (concentrations en thiamine et dérivés, niveau de phosphorylation et d'expression des protéines Akt, GSK-3β, et expression du BDNF). Les souris ont été soumises au même traitement que la première cohorte sauf qu'elles n'ont pas reçu d'injection de BrdU avant l'exposition au prédateur. Les souris ont été sacrifiées 24 h après le remplacement du traitement par de l'eau et 12 h après la dernière procédure de stress. Le sang, le foie, le cortex et l'hippocampe ont été collectés et congelés rapidement (ou placés dans du RNAlater®) jusqu'aux analyses biochimiques comme décrit dans Matériel et Méthodes.

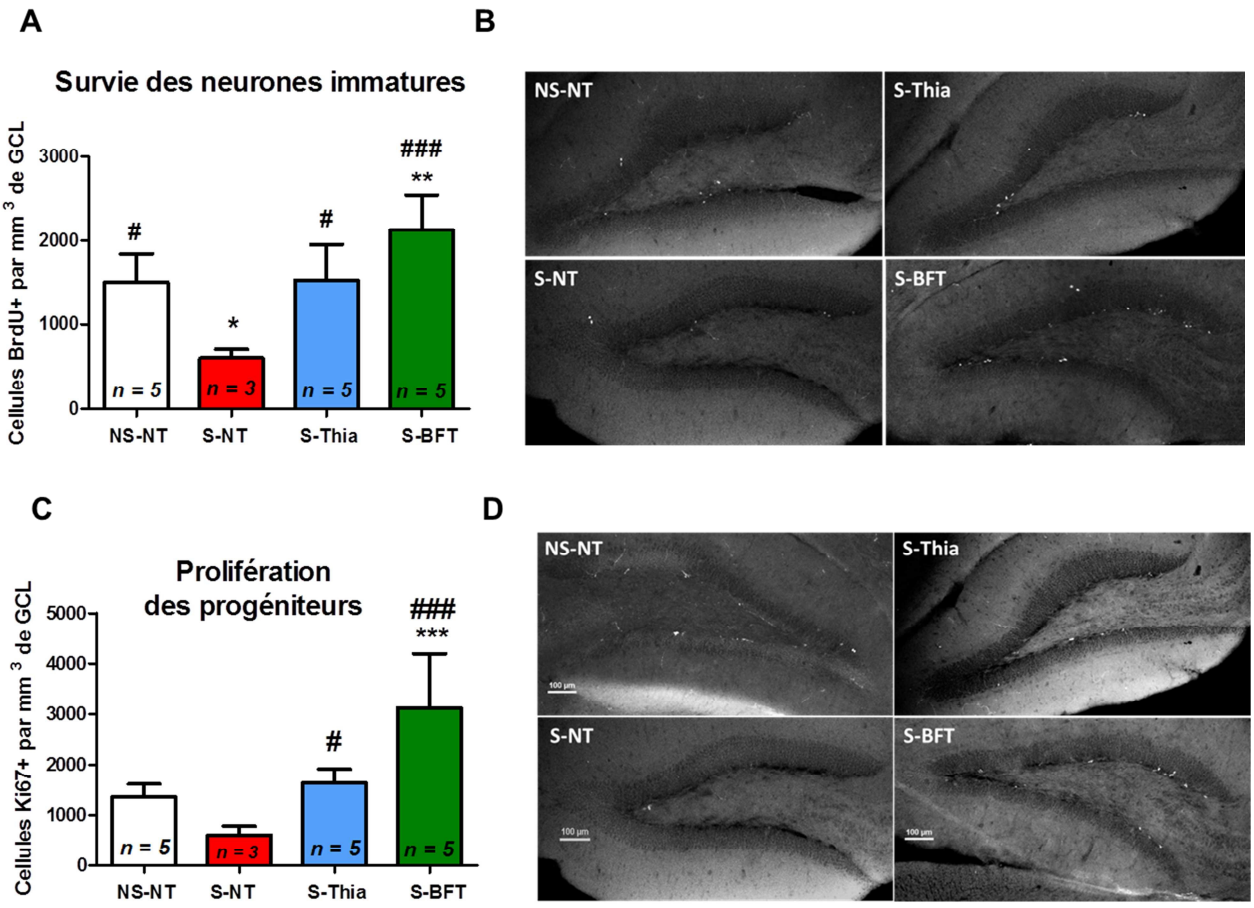


Figure 35 : **Etude de l'impact d'un traitement oral avec de la thiamine (200 mg/kg/jour) et de la benfotiamine (200 mg/kg/jour) sur la prolifération de progéniteurs et sur la survie à court terme des neurones nouvellement formés.** (A) Représentation graphique du nombre de cellules BrdU positives par mm^3 de couche cellulaire granulaire (CGL) dans les souris non stressées non traitées (NS-NT, n=5), stressées non traitées (S-NT, n=3), stressées et traitées à la thiamine (S-Thia, n=5) et stressées et traitées à la benfotiamine (S-BFT, n=5). (B) Photographies représentatives du marquage BrdU dans les groupes NS-NT, S-NT, S-Thia et S-BFT. (C) Représentation graphique du nombre de cellules Ki67 positives par mm^3 de couche cellulaire granulaire (CGL) dans les mêmes groupes qu'en A. (D) Photographies représentatives du marquage Ki67 dans les mêmes groupes qu'en B. Les données sont présentées sous forme de moyenne ± écart-type (*p<0.05, **p<0.01 vs NS-NT; #p<0.05, ###p<0.001 vs S-NT, ANOVA à une entrée suivie d'un test posthoc de Tukey pour comparaison multiple).

Plusieurs études ont montré que différentes formes de stress, capables de précipiter ou d'empirer la dépression, peuvent avoir de profondes conséquences sur la neurogenèse dans l'hippocampe adulte, par exemple une réduction importante de la prolifération des neurones immatures (Duman 2004). Par ailleurs, d'autres expériences ont montré que les antidépresseurs (AD) contrecarrent les effets du stress et améliorent la NHA (Warner-Schmidt & Duman 2006; Miller & Hen 2015). Les effets observés pour la thiamine et la

benfotiamine semblent être fort similaires à ceux observés pour les traitements antidépresseurs, ainsi que pour d'autres traitements (thérapie électroconvulsive, exercice physique ou environnement enrichi). En particulier, les effets observés pour la benfotiamine sur la prolifération et la survie de cellules néoformées après le stress d'exposition au prédateur rappellent les effets de la fluoxétine sur la NHA chez les souris traitées à la corticostérone (David et al. 2009).

2.4.2. Effet du stress et du traitement à la thiamine ou à la benfotiamine sur le poids des souris

Avant le traitement, le poids moyen des souris (Figure 36) est similaire entre les différents groupes. D'une part, la procédure de stress induit une diminution du poids des souris, qui est empêchée par les traitements à la thiamine et à la benfotiamine. Ces résultats confirment que la thiamine et la benfotiamine ont un effet protecteur contre le stress. Des études précédentes ont montré que 3 jours de stress de contention (Jeong et al. 2013; Chotiwat et al. 2010) ou l'exposition à une odeur de prédateur (Calvo-Torrent et al. 1999) conduisent également à une perte de poids ainsi qu'à une diminution de la prise de nourriture.

D'autre part, le poids moyen des souris traitées à la thiamine et à la benfotiamine à la fin de l'étude est supérieur à celui des groupes contrôles stressés et non stressés.

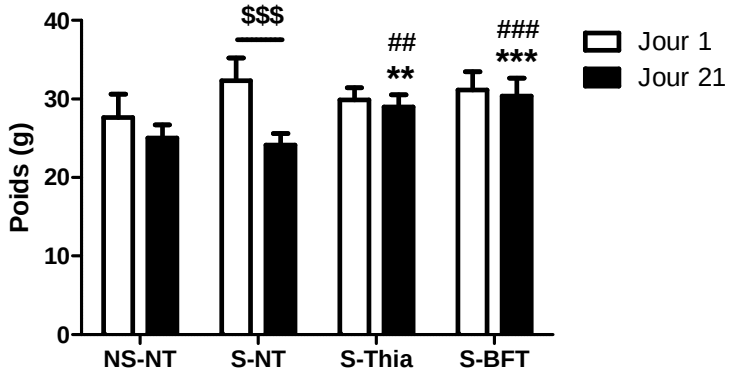


Figure 36 : **Poids (g) des souris non-stressées non-traitées (NS-NT, n=5), stressées non-traitées (S-NT, n=5), stressées traitées avec de la thiamine (S-Thia, n=5) ou de la benfotiamine (S-BFT, n=5).** Les souris sont pesées au début (jour 1) et à la fin (jour 21) du traitement. Les données sont présentées sous forme de moyenne ± écart-type (*p<0.05 vs NS-NT; #p<0.05 vs S-NT le jour 21, ANOVA à une entrée suivie d'un test posthoc de Tukey pour comparaison multiple; $$$ p<0.001 jour 21 vs jour 1, ANOVA à 2 entrées suivie d'un test posthoc de Bonferroni).

2.4.1. Effet du stress et du traitement à la thiamine ou à la benfotiamine sur les contenus en thiamine et en dérivés phosphorylés dans le sang et le foie

La benfotiamine administrée dans l'eau de boisson à des souris stressées induit une augmentation du ThMP dans le sang (Fig. 37A), comme le faisait le traitement à la benfotiamine via les gaufrettes chez les souris normales. Dans le foie, bien que le stress tende à diminuer les niveaux de thiamine, de ThDP, et de thiamine totale, cette tendance graphique n'est pas significative ($p=0.084$, $p=0.079$, et $p=0.072$). De façon inattendue, la benfotiamine administrée à des souris stressées engendre une réduction des niveaux d'AThTP et d'AThDP dans le foie. La thiamine, en plus de diminuer le niveau de ThTP, diminue également les niveaux d'AThTP et d'AThDP et ce, de façon plus importante que la benfotiamine. Ces résultats sont en contradiction avec ceux observés pour les souris normales. Il est difficile d'émettre des hypothèses ou de tirer des conclusions quant aux différences de résultats obtenus entre les souris normales et stressées. En effet, il n'existe pas de données dans la littérature concernant l'effet du stress sur les niveaux de thiamine et, d'autre part, les niveaux d'AThD(T)P et de ThTP ont rarement été étudiés.

2.4.2. Effet du stress et du traitement à la thiamine ou à la benfotiamine sur le contenu en thiamine et en dérivés phosphorylés dans le cerveau (hémisphères gauches et droits du cortex et de l'hippocampe).

La procédure de stress appliquée aux souris n'engendre pas de variation des niveaux cérébraux de la thiamine libre ou du ThDP (Figure 38). Par contre, il semblerait que le stress soit responsable d'une diminution du ThMP dans le cortex (Fig. 38A). Ces résultats sont interpellants car les niveaux de ThMP corticaux sont également réduits chez les souris normales traitées à la benfotiamine. Le traitement à la thiamine semble prévenir cette diminution. Pourtant, seule la benfotiamine augmente les niveaux sanguins de ThMP (Fig. 37A).

Les traitements à la thiamine et à la benfotiamine engendrent une augmentation significative de la teneur en thiamine libre dans le cortex et l'hippocampe (Fig. 38B), confirmant les résultats obtenus par Pan et al., 2010 et Hurt et al., 2012. Seul le traitement à la thiamine induit une augmentation significative de la thiamine totale.

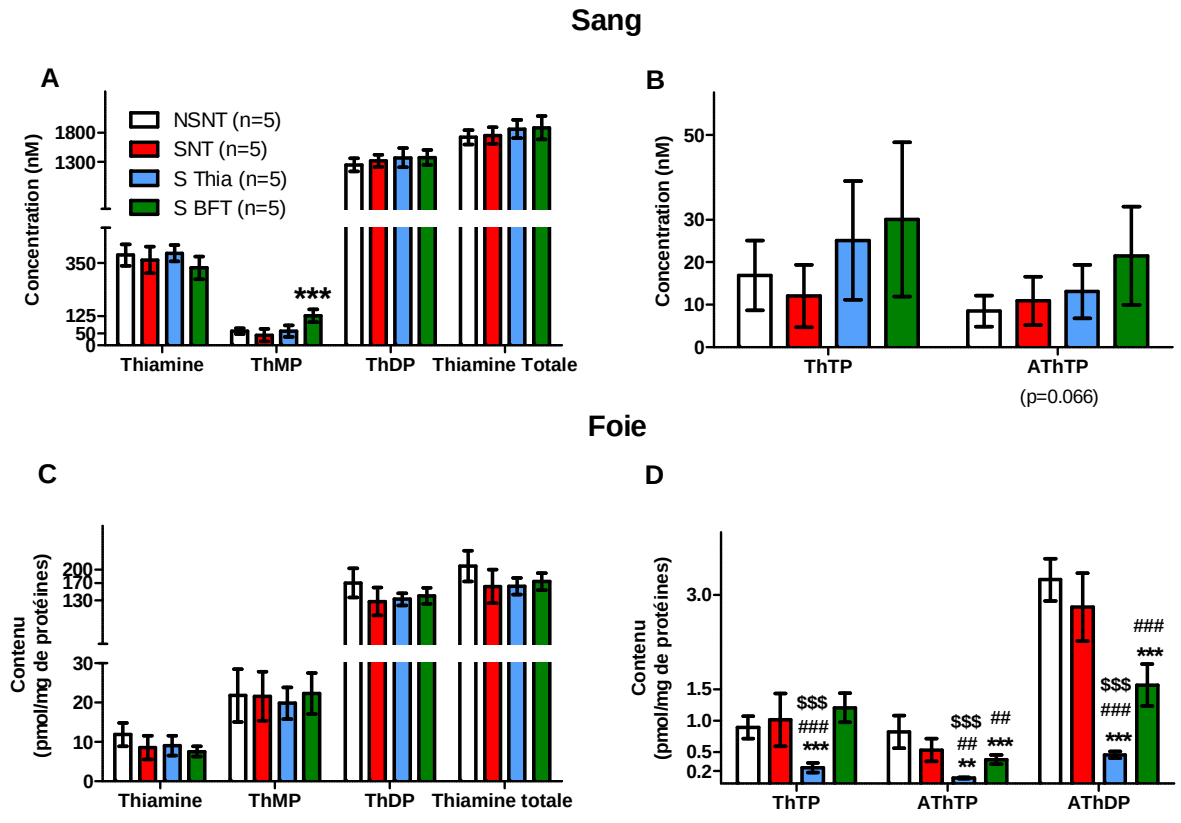


Figure 37 : **Concentration de la thiamine et de ses dérivés phosphorylés dans le sang (A, B) et le foie (C, D) des souris après exposition au stress et traitement oral avec le véhicule, la thiamine ou la benfotiamine.** Les souris ont été sacrifiées 24 h après le dernier traitement pharmacologique. La benfotiamine augmente les niveaux de ThMP dans le sang. Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; *p<0.05 vs NS-NT; #p<0.05 vs S-NT, $p<0.05 vs S-BFT ANOVA à une entrée, suivie d'un test posthoc de Tukey pour comparaison multiple).

Il s'agit ici de la première étude qui adresse les effets du stress sur les niveaux cérébraux de thiamine et de ses dérivés. Il a longtemps été considéré (Gibson & Blass 2007) que les effets bénéfiques observés suite à une augmentation de la thiamine dans le sang étaient dus à une augmentation de l'activité du métabolisme cérébral résultant d'une augmentation des contenus en ThDP du cerveau. Cependant, aucun des traitements utilisés ici n'engendrent d'augmentation des niveaux de ThDP dans le cortex ou l'hippocampe. Les effets bénéfiques observés seraient donc indépendants du rôle de cofacteur du ThDP. Par contre les niveaux de thiamine libre sont augmentés significativement, suggérant un rôle potentiel propre à la thiamine libre.

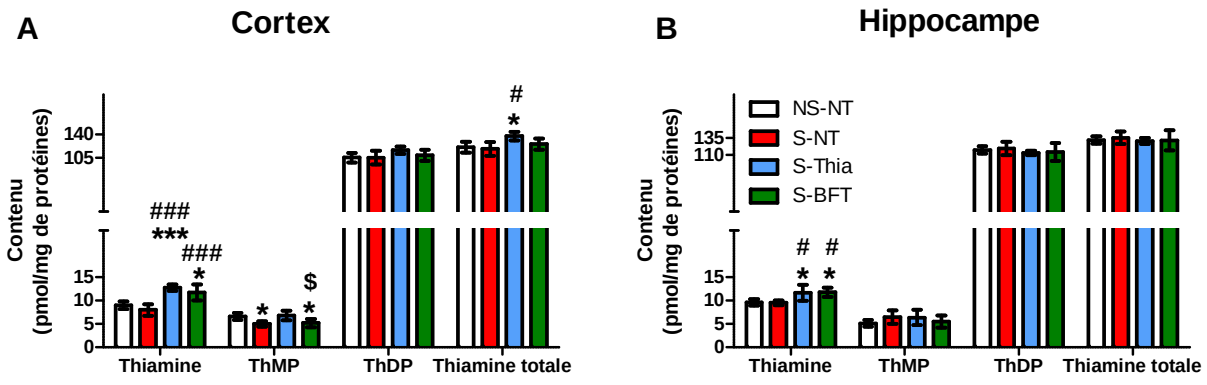


Figure 38 : **Contenu en thiamine et ses dérivés phosphorylés dans le cortex (A) et l'hippocampe (B) des souris après exposition au stress et traitement oral avec le véhicule, la thiamine ou la benfotiamine.** Les souris ont été sacrifiées 24 h après le dernier traitement pharmacologique. Les traitements à la thiamine et à la benfotiamine augmentent les niveaux de thiamine libre dans le cortex et l'hippocampe. Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; *p<0.05 vs NS-NT; #p<0.05 vs S-NT, $p<0.05 vs S-Thia, ANOVA à une entrée suivie d'un test posthoc de Tukey pour comparaison multiple).

Comme des études précédentes ont mis en évidence des différences de plasticité synaptique, de prolifération cellulaire et d'expression protéique (Shipton et al. 2014; Czéh et al. 2008; Cerqueira et al. 2008) entre les hémisphères gauches et droits des cortex de souris stressées, nous avons étudié le contenu en thiamine des cortex et hippocampes gauches et droits des souris traitées.

Les résultats obtenus sont assez prometteurs. En effet, il semblerait que les niveaux corticaux (Figure 39) de thiamine, de ThMP, de ThDP et de thiamine totale soient différents entre les hémisphères gauches et droits des souris stressées et non stressées mais cette assymétrie semble disparaître lorsque les souris sont traitées à la thiamine et à la benfotiamine.

Au niveau de l'hippocampe, les hémisphères gauches et droits ne présentent aucune différence sauf lorsque les souris sont stressées (Figure 40). Il y a alors en effet une différence significative entre hémisphère droit et gauche pour la thiamine et le ThMP (Fig. 40A et B). Il est cependant difficile de déterminer si cela est dû à une augmentation ou à une diminution de niveau dans un hémisphère ou l'autre. Toutefois, le ThMP dans l'hippocampe gauche est augmenté par rapport au contrôle (résultats non montrés).

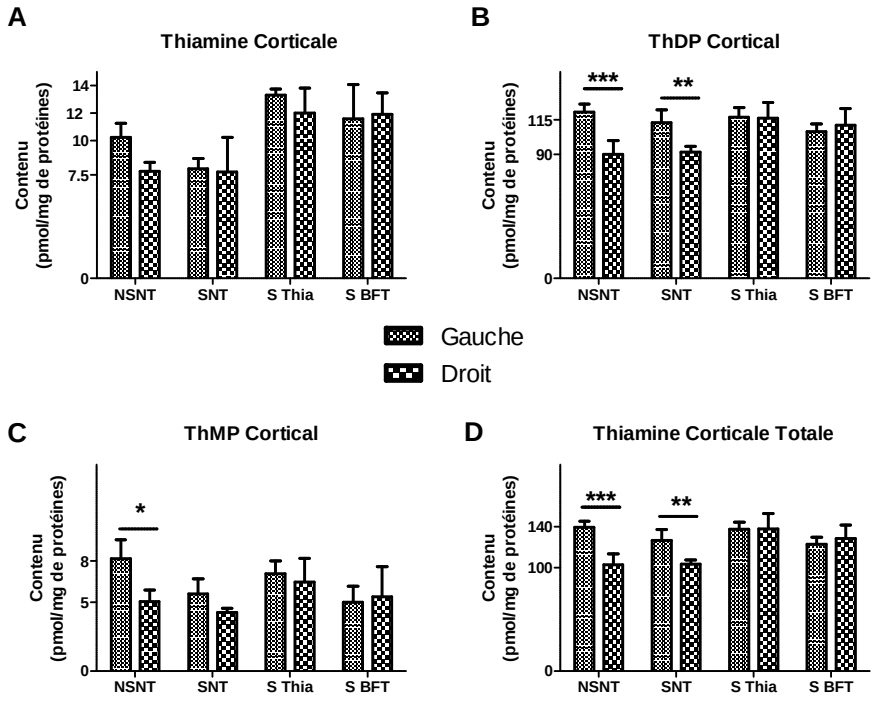


Figure 39 : **Contenu en thiamine (A) et ThDP (B), ThMP (C) et thiamine totale (D) dans les hémisphères gauche et droit du cortex des souris après exposition au stress et traitement oral avec le véhicule, la thiamine ou la benfotiamine.** Les souris ont été sacrifiées 24 h après le dernier traitement pharmacologique. Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; *p<0.05 gauche vs droite, ANOVA à 2 entrées suivie d'un test posthoc de Bonferroni).

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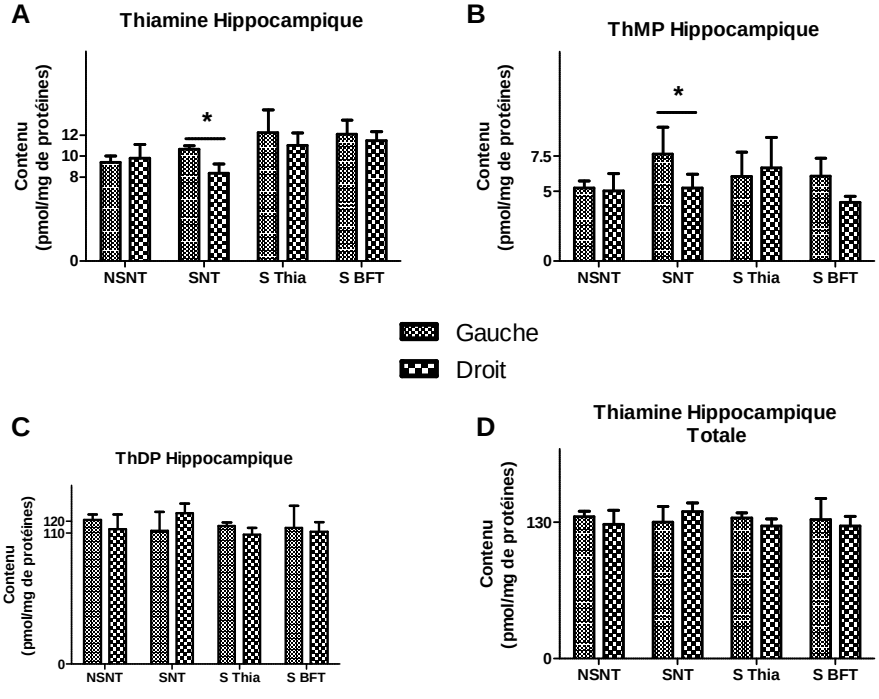

Figure 40 : **Contenu en thiamine (A) et ThDP (B), ThMP (C) et thiamine totale (D) dans hippocampe gauches et droits des souris après exposition au stress et traitement oral avec le véhicule, la thiamine ou la benfotiamine.** Les souris ont été sacrifiées 24 h après le dernier traitement pharmacologique. Les données sont présentées sous forme de moyenne ± écart-type (n=5 ; *p<0.05 gauche vs droite, ANOVA à 2 entrées suivie d'un test posthoc de Bonferroni).

2.4.3. Effet du stress et du traitement à la thiamine ou à la benfotiamine sur les niveaux de phosphorylation et d'expression d'Akt et de GSK-3β dans les hémisphères gauches et droits du cortex et de l'hippocampe.

Dans le cortex, on n'observe aucun effet du stress ou des traitements sur les niveaux de phosphorylation et d'expression des protéines étudiées (résultats non montrés).

Par contre, dans l'hippocampe, on observe que le stress engendre une augmentation significative de la phosphorylation de GSK-3β en sérine 9. Cette augmentation semble être contrecarrée chez les souris traitées à la thiamine et à la benfotiamine (Figure 41). Il est intéressant de comparer ces résultats avec ceux rapportés par Pan et al. (2010), qui suggéraient que, dans un modèle murin de la maladie d'Alzheimer, le traitement par la benfotiamine augmente le niveau de phosphorylation de GSK-3β. D'après ces auteurs, cela pourrait expliquer partiellement l'effet bénéfique de la benfotiamine car la phosphorylation de GSK-3β sur la sérine 9 diminue son activité ; on sait qu'une trop forte activité de GSK-3β peut induire l'hyperphosphorylation de la protéine tau et la formation des pelotes neurofibrillaires favorisant la mort neuronale. Dans notre modèle, le traitement à la benfotiamine semble, à l'inverse, diminuer la phosphorylation de GSK-3β et, par conséquent, augmenter son activité, ce qui ne favoriserait pas la survie cellulaire. Il faut toutefois remarquer que la phosphorylation de GSK-3β est significativement augmentée chez les souris stressées, ce qui peut être une réaction spontanée ayant un effet neuroprotecteur. Le traitement par la thiamine et la benfotiamine ne produirait pas d'augmentation de phosphorylation car celle-ci a déjà été stimulée par le stress. D'après les résultats de la Figure 41B, la procédure de stress semble également stimuler la phosphorylation de la protéine kinase B (ou Akt), bien que l'augmentation ne soit pas statistiquement significative ($p=0.09$). La phosphorylation d'Akt sur la thréonine 308 active l'enzyme, qui est la principale kinase responsable de la posphorylation de GSK-3β. Nos résultats suggèrent donc que dans nos conditions, le stress stimule la voie cytoprotectrice RTK - PI3K – Akt - GSK-3β.

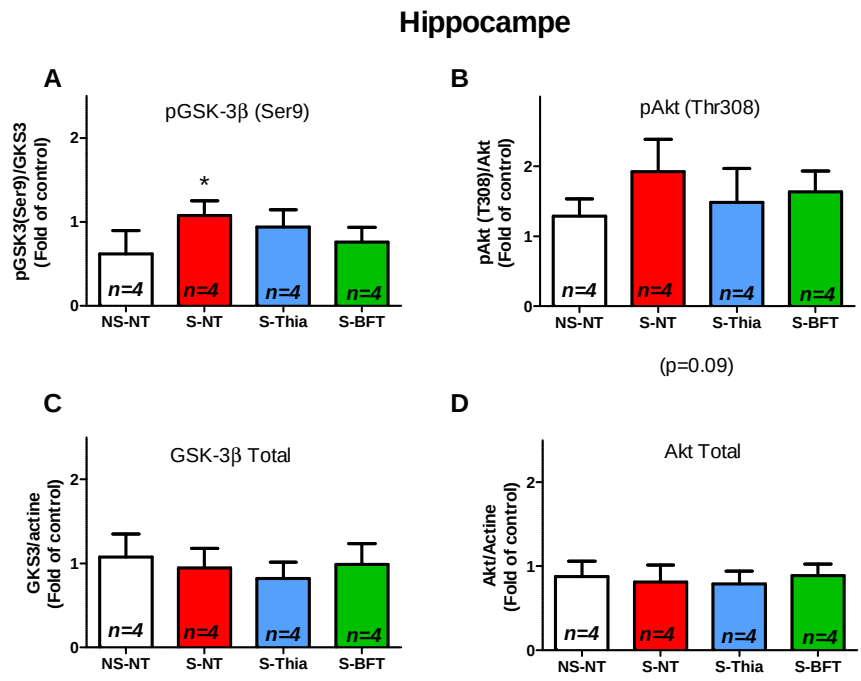

Figure 41 : **Etat de phophorylation de (A) GSK-3β(Ser9) et (B) d'Akt(Thr308) et niveaux d'expression de (C) GSK-3β et (D) d'Akt dans l'hippocampe de souris non traitées non stressées (NS-NT, n=4), de souris stressées non-traitées (S-NT, n=4), de souris stressées traitées à la thiamine (S-Thia, n=4) ou à la benfotiamine (S-BFT, n=4).** Les données sont exprimées sous forme de moyenne ± écart-type (*p<0.05 vs NS-NT, ANOVA à une entrée, suivie d'un test posthoc de Tukey pour comparaison multiple).

2.4.4. Effet du stress et du traitement à la thiamine ou à la benfotiamine sur les niveaux d'ARN messager du BDNF.

Pour mesurer l'expression du BDNF (« Brain-Derived Neurotrophic Factor »), un facteur de croissance spécifique pour les neurones cérébraux, nous avons utilisé la méthode RT-qPCR. Nous avons utilisé la méthode comparative (méthode ΔΔCt) pour comparer l'expression du BDNF dans différentes conditions, exprimée par rapport à la condition contrôle, par rapport à 2 gènes rapporteurs (HPRT et actine). Les expériences de validation ont montré que l'HPRT était le gène rapporteur le plus fiable et le plus sensible pour repérer des différences d'expression dans l'hippocampe, tandis que l'actine est préférable pour le cortex. Les résultats sont donc présentés ici par rapport à l'actine pour le cortex et par rapport à l'HPRT pour l'hippocampe.

De manière inattendue, nos résultats (Figure 42) ne montrent pas de diminution significative de l'expression du BDNF dans l'hippocampe des animaux stressés. Dans l'hippocampe droit (Fig. 42D), on voit même une augmentation significative de l'expression du BDNF chez les souris stressées non traitées. Ce résultat est en désaccord avec des données de la littérature, qui montrent que le stress chronique imprévisible diminue l'expression du BDNF dans l'hippocampe (Autry et al. 2009; Bath et al. 2013), alors que le traitement par les antidépresseurs augmente à la fois la neurogenèse et l'expression du BDNF (Duman & Monteggia 2006; Castrén et al. 2007). Ces données suggèrent donc une corrélation entre la NHA et la présence de niveaux élevés de BDNF dans l'hippocampe. Il n'est toutefois pas certain que la diminution de la prolifération des cellules progénitrices dans le DG est causée par une diminution du niveau de BDNF. Il a été montré (Choi et al. 2009) qu'une délétion conditionnelle de l'expression du BDNF dans l'hippocampe de souris diminue la survie mais pas la prolifération des cellules progénitrices. Une autre étude (Chan et al. 2008) a montré qu'une inhibition de l'expression du BDNF induisait une augmentation de prolifération dans le DG sans affecter la survie cellulaire chez la souris (mais la maturation des nouveaux neurones était bloquée). Ces données suggèrent que le BDNF est surtout essentiel pour la différenciation terminale des neurones, pas pour la prolifération et la survie des progéniteurs. Ceci est en accord avec une étude (Donovan et al. 2008) qui a montré que seulement une faible proportion de cellules proliférant dans le DG expriment le TrkB (récepteur de haute affinité pour le BDNF).

On constate par ailleurs que le stress engendre une diminution significative du BDNF dans les cortex gauche et droit ainsi que dans le cortex total (Fig. 42A, C, D). Seul le traitement à la benfotiamine prévient cette altération des niveaux de BDNF. D'autres études ont également montré que différents types de stress étaient responsables d'une diminution du BDNF dans le cortex (Bath et al. 2013). Par ailleurs, une étude (Balu et al. 2008) a montré qu'un traitement avec différentes classes d'antidépresseurs pendant 21 jours induisait une augmentation des niveaux protéiques de BDNF dans le cortex mais pas dans l'hippocampe ou dans d'autres régions cérébrales. De plus, d'autres études ont montré que, bien qu'un traitement à la fluoxétine n'engendrait pas d'augmentation de BDNF dans l'hippocampe (Coppell et al. 2003; Dias et al. 2003; Altieri et al. 2004; Balu et al. 2008), celle-ci avait un

impact positif sur la prolifération et sur la survie à court ou à long terme des neurones néoformés dans l'hippocampe (Hanson et al. 2011).

Il n'est donc finalement pas surprenant que nos résultats ne montrent pas de diminution de l'expression du BDNF dans l'hippocampe lorsque la prolifération et la survie des cellules progénitrices sont affectées par l'exposition à un stress intense. Les effets protecteurs et la stimulation importante de la prolifération et de la survie des progéniteurs par la thiamine et la benfotiamine ne sont donc probablement pas liés à une augmentation de l'expression du BDNF dans l'hippocampe.

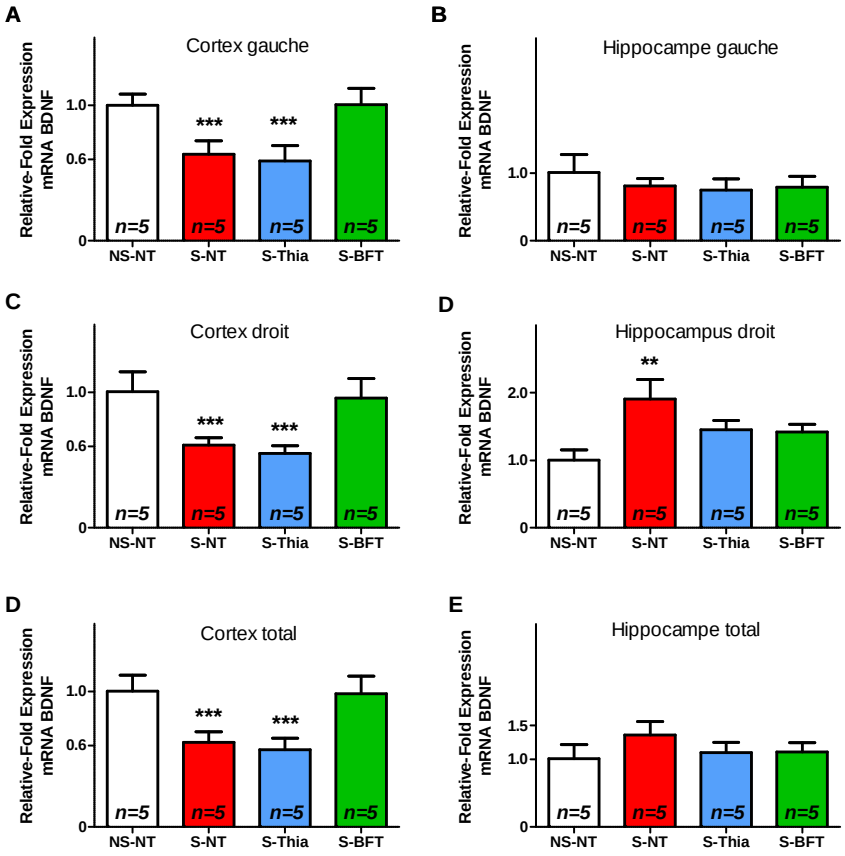

Figure 42: **Effets du stress et du traitement par la thiamine ou la benfotiamine sur l'expression du BDNF dans le cortex et l'hippocampe des souris C57BL.** Niveaux d'ARNm du BDNF obtenus par qRT-PCR dans le cortex et l'hippocampe des hémisphères droits et gauches, exprimés en fonction des niveaux d'ARNm du BDNF dans les souris non traitées non stressées (NS-NT). Les groupes représentés sont les souris non traitées non stressées (NS-NT, n=5), les souris stressées non traitées ((S-NT, n=5), les souris stressées traitées à la thiamine (S-Thia, n=5) ou à la benfotiamine (S-BFT, n=5). Les données sont exprimées sous forme de moyenne ± écart-type (*p<0.05 vs NS-NT, ANOVA à une entrée suivie d'un test posthoc de Tukey pour comparaison multiple).

CONCLUSIONS

Lorsque nous avons commencé notre travail, il avait été démontré par plusieurs auteurs que, chez l'animal entier, la thiamine et certains précurseurs à plus grande biodisponibilité exerçaient des effets bénéfiques sur le cerveau, en particulier lorsque celui-ci était soumis à des traitements simulant des processus neurodégénératifs.

L'un de ces précurseurs, la benfotiamine, était remarquablement efficace dans un modèle murin de la maladie d'Alzheimer, mais son mécanisme d'action était inconnu. Les effets de la benfotiamine avaient aussi été étudiés *in vitro* sur des cellules endothéliales en culture en situation d'hyperglycémie mais là encore, le mécanisme d'action de la benfotiamine n'était pas clair. Nos recherches ont donc visé à préciser le mécanisme d'action de la thiamine et de ses précurseurs, d'abord *in vitro* sur des cellules de neuroblastome en culture, puis *in vivo* sur des souris soumises à un stress intense. Les résultats obtenus ont permis de tirer les conclusions suivantes :

1. EXPERIMENTATION *IN VITRO*

1.1. Effets de la thiamine et de ses précurseurs sur le métabolisme des dérivés phosphorylés de la thiamine

La culture de cellules de type neuronal en milieu pauvre en thiamine engendre une diminution des contenus intracellulaires en thiamine et dérivés phosphorylés. Bien que la teneur en thiamine libre devienne indétectable dans ces cellules, la croissance cellulaire n'est pas affectée. Il semblerait donc que seul le ThDP soit essentiel pour la survie et la croissance des cellules de type neuronal en culture.

L'addition de la thiamine et des précurseurs (benfotiamine et sulbutiamine) à différentes concentrations sur les cellules en culture nous a permis de confirmer des résultats précédemment obtenus (Bettendorff 1994a; Volvert et al. 2008) et d'acquérir de nouvelles connaissances sur à la métabolisation de ces précurseurs :

- Le transport de la thiamine dans les cellules N2a est un processus lent essentiellement médié par un transporteur de haute affinité et est couplé à la conversion de la thiamine en ThDP. En présence de concentrations élevées en thiamine, un transporteur de basse affinité est également impliqué.

- La sulbutiamine est un composé lipophile engendrant une accumulation rapide et importante de thiamine intracellulaire. Mais cette accumulation, contrairement à ce que l'on aurait pu croire, est rapidement limitée et ceci n'est pas dû à un épuisement de la sulbutiamine extracellulaire. En effet, nous avons pu déterminer que, peu importe la concentration en SBT externe, seul 1 à 2 % de celle-ci est transformée en thiamine à l'intérieur de la cellule. Il s'agit probablement d'un état stationnaire (plutôt qu'un équilibre) où l'efflux de thiamine compense la production de thiamine intracellulaire à partir de la sulbutiamine absorbée. Etant donné que l'efflux de thiamine semble supérieur à celui observé dans des expériences précédentes, nous avons émis l'hypothèse que l'expression des transporteurs de la thiamine était augmentée dans les cellules cultivées dans un milieu pauvre en thiamine par rapport à un milieu riche où elle serait plus réprimée.

- La benfotiamine induit également une augmentation lente de la thiamine intracellulaire totale jusqu'à un niveau relativement similaire à celui obtenu après l'ajout de thiamine extracellulaire. La benfotiamine possède une biodisponibilité plus élevée in *vivo* mais nous savons que la molécule est hydrophile et a besoin d'être hydrolysée en S-benzoylthiamine par des phosphatases alcalines pour pouvoir pénétrer les cellules. Nous avons supposé que des ectophosphatases présentes sur les N2a pourraient être responsables de son hydrolyse avant sa pénétration. Ce qui explique le délai observé avant l'augmentation de la teneur en thiamine et en ThDP intracellulaire observée en présence de ce composé. De fait, nous avons confirmé que l'incubation de la benfotiamine avec de la phosphatase alcaline purifiée avant son addition sur les cellules permettait une pénétration beaucoup plus rapide de celle-ci. Cette augmentation rapide de la thiamine intracellulaire est moins élevée que celle observée lors de l'ajout de sulbutiamine externe à même concentration. Par ailleurs, expérimentalement, nous avons également constaté que la présence de sérum dans le milieu influençait la métabolisation de la benfotiamine et accélérait la pénétration de benfotiamine dans les cellules alors que ce n'est pas le cas pour les autres précurseurs. Il existe très probablement dans le sérum un élément qui accélère/favorise la

déphosphorylation de la benfotiamine par l'ectophosphatase présente dans les N2a.

1.2. Effet de la thiamine et de ses précurseurs sur l'expression et la phosphorylation de GSK-3β et Akt en culture cellulaire

On sait que la glycogène synthase kinase-3β (GSK-3β) est impliquée dans de nombreuses affections psychiatriques ou neurodégénératives. La culture de cellules N2a pendant 7 jours dans un milieu pauvre en thiamine (à concentration physiologique), par rapport à un milieu riche en thiamine, est responsable d'une diminution de la phosphorylation de GSK-3β en sérine-9 et donc d'une activation de celle-ci. Mais si l'on rajoute de la thiamine ou des précurseurs après 7 jours de culture en milieu pauvre, on ne trouve pas d'augmentation significative de la phosphorylation de GSK-3β, du moins si le temps de l'incubation est inférieur à 6 heures.

Nous avons également étudié l'effet de la présence de thiamine à haute concentration en présence d'un activateur de la voie « pro-survie » PI3K-Akt- GSK-3β, l'insuline (0 – 100 nM) pendant des temps courts (15- 120 minutes) dans les N2a pauvres en thiamine. Comme pour GSK-3β, la culture en milieu pauvre engendre une diminution de la phosphorylation d'Akt en thréonine-308. La thiamine engendre une potentialisation de la voie PI3K-Akt qui se traduit par une augmentation plus rapide de la phosphorylation d'Akt en thréonine-308 et également une déphosphorylation plus rapide. La thiamine n'a aucun effet sur l'expression de GSK-3β et d'Akt.

La conclusion essentielle de ces expériences est que, lorsque les cellules ont été cultivées dans un milieu pauvre en thiamine, le degré de phosphorylation des kinases Akt et GSK-3β est plus bas que si les cellules ont été cultivées en milieu riche. De plus, lorsque les cellules cultivées en milieu pauvre sont incubées pendant un temps court en présence d'insuline et de thiamine, on constate une augmentation modeste mais significative de la phosphorylation d'Akt, ce qui correspond à une activation de cette kinase. Nos résultats suggèrent donc une potentialisation par la thiamine de la cascade :

Insuline (ou IGF-1) → RTK → PI3K →Akt → GSK-3β

L'activation de cette voie de signalisation « pro-survie » peut expliquer en partie les effets neuroprotecteurs de la benfotiamine observés *in vivo* dans les modèles de neurodégénération. De plus, comme la culture en milieu riche en thiamine induit une phosphorylation accrue de GSK-3β et donc une diminution de son activité, ce mécanisme peut aboutir à une moindre phosphorylation des protéines tau et un ralentissement de la formation des lésions neurofibrillaires dans les modèles de la maladie d'Alzheimer : la GSK-3β est en effet une des principales kinases responsables de l'hyperphosphorylation de tau.

2. EXPERIMENTATION *IN VIVO*

Les études réalisées *in vivo* ont confirmé que la benfotiamine est plus efficace que la thiamine pour induire une augmentation de la concentration en thiamine dans le sang de souris. D'une part, cela confirme la biodisponibilité plus élevée de la benfotiamine par rapport à la thiamine et d'autre part, cela nous indique que le mode d'administration utilisé est important et peut avoir un impact sur la métabolisation de la benfotiamine.

Toutefois, les mesures que nous avons réalisées sur des souris transgéniques utilisées comme modèle de tauopathie ont montré que le traitement par la benfotiamine n'augmente pas les concentrations en coenzyme ThDP dans le cerveau (Collaboration avec l'équipe de F. Beal, Weill Medical College of Cornell, New York, résultats non publiés). Des résultats similaires avaient été obtenus dans notre laboratoire (Volvert et al. 2008) ainsi que par Pan et al. (2010) avec des souris WT. Il est donc peu probable que les effets bénéfiques du traitement par la benfotiamine puissent être liés à une stimulation des enzymes ThDP-dépendantes (et la stimulation du métabolisme énergétique qui en résulterait) dans le cerveau. Les effets bénéfiques de la benfotiamine seraient donc dus à des effets « non-cofacteurs » de la thiamine ou de dérivés non-identifiés.

Chez les souris, le stress induit par l'exposition à un prédateur engendre une diminution de la prolifération des progéniteurs ainsi que de la survie des cellules néoformées, et une réduction du poids. Le traitement de ces souris stressées avec de la thiamine et de la benfotiamine contrecarre les effets du stress précédemment mentionnés, la benfotiamine ayant un effet plus marqué que la thiamine. Ces résultats confirment les effets neuroprotecteurs de la thiamine et de la benfotiamine en conditions pathologiques (puisqu'il n'existe aucun effet de ces traitements en condition non pathologique). Notre

étude a permis de déterminer que la prolifération et la survie des progéniteurs neuronaux est, au moins en partie, impliquée dans les effets neuroprotecteurs de la benfotiamine. Bien que le(s) mécanisme(s) d'action exacte(s) reste(nt) à déterminer, nous avons d'ores et déjà identifiés des acteurs potentiels tels que le BDNF : la diminution de l'expression du BDNF observée dans le cortex suite au stress est contrecarrée par la benfotiamine.

En conclusion, les résultats obtenus confirment que les effets neuroprotecteurs observés avec la thiamine ou la benfotiamine dans des conditions neuropathologiques ne sont pas liés au rôle cofacteur du ThDP. L'existence d'un rôle de la thiamine ou d'un de ses dérivés indépendamment du rôle de cofacteur du ThDP est une vieille hypothèse récemment remise au goût du jour (Mkrtchyan et al. 2015). Notre étude est la première qui compare directement les effets de la thiamine et de la BFT au niveau du système nerveux central. Si dans certains cas la benfotiamine semble plus efficace que la thiamine, les différences sont rarement significatives (Fig. 35, p. 102, par exemple). Il est donc vraisemblable que la plus grande efficacité de la BFT par rapport à la thiamine, mise en évidence dans la littérature scientifique après administration orale, soit due à sa capacité d'augmenter les concentrations sanguines en thiamine et donc à sa meilleure biodisponibilité (Xie et al. 2014).

Dans tous les modèles étudiés, nous avons observé une forte augmentation de la thiamine dans le sang (Fig. 28, p. 90 et Volvert et al. 2008) et une augmentation modeste de la thiamine libre dans le cerveau (Fig. 38, p. 106). Nous pouvons donc émettre deux hypothèses :

- Soit une augmentation de l'ordre de 50% du contenu en thiamine du parenchyme cérébral est suffisante pour induire des effets pharmacologiques ;

- Soit l'augmentation de la concentration en thiamine dans le sang qui passe de l'ordre de 0,1 µM à 50 µM (une heure après l'administration de BFT, Volvert et al. 2008) a des effets sur les cellules endothéliales, par exemple via l'activation de eNOS (Margaux Sambon, résultats non publiés).

Remarquons que même une forte augmentation des concentrations circulantes en thiamine n'entraine qu'une faible augmentation du contenu en thiamine du cerveau

suggérant l'existence de mécanismes puissants, et inconnus à l'heure actuelle, régulant le transport de thiamine à travers la barrière hémato-encéphalique. Nous avons également mis en évidence l'existence en faibles quantités d'autres dérivés de la thiamine dans le cerveau des souris tels que le ThTP et l'AThTP (Fig. 32, p. 95). Leur contenu ne semble pas affecté par les traitements et il est donc peu vraisemblable qu'ils jouent un rôle dans les effets pharmacologiques observés.

Finalement, nos résultats suggèrent que, chez les souris stressées, le traitement par la thiamine et, surtout, par la benfotiamine a des caractéristiques similaires aux antidépresseurs, un résultat original et nouveau. Dans la mesure où ni l'administration de thiamine ni celle de BFT ne présentent de toxicité ou d'effets secondaires, il serait dès lors intéressant d'investiguer les effets de précurseurs plus actifs dans des modèles de dépression.

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ANNEXES

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Dimebon enhances hippocampus-dependent learning in both appetitive and inhibitory memory tasks in mice

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ABSTRACT

Pre-clinical and clinical studies on dimebon (dimebolin or latrepirdine) have demonstrated its use as a cognitive enhancer. Here, we show that dimebon administered to 3-month-old C57BL6N mice 15 min prior to training in both appetitive and inhibitory learning tasks via repeated (0.1 mg/kg) and acute (0.5 mg/kg) i.p. injections, respectively, increases memory scores. Acute treatment with dimebon was found to enhance inhibitory learning, as also shown in the step-down avoidance paradigm in 7-month-old mice. Bolus administration of dimebon did not affect the animals' locomotion, exploration or anxiety-like behaviour, with the exception of exploratory behaviour in older mice in the novel cage test. In a model of appetitive learning, a spatial version of the Y-maze, dimebon increased the rate of correct choices and decreased the latency of accessing a water reward after water deprivation, and increased the duration of drinking behaviour during training/testing procedures. Repeated treatment with dimebon did not alter the behaviours in other tests or water consumption. Acute treatment of water-deprived and non-water-deprived mice with dimebon also did not affect their water intake. Our data suggest that dimebon enhances hippocampus-dependent learning in both appetitive and inhibitory tasks in mice.

1. Introduction

Dimebon is a candidate for therapeutics against Alzheimer's disease, and its clinical activity is currently under investigation. It was originally developed in 1983 in Russia where it was used as an antihistamine. Ongoing clinical trials are re-assessing contradictory results (Miller, 2010) concerning the previously shown efficacy of dimebon in the improvement of thinking processes and functioning in patients with mild to moderate Alzheimer's disease (Bachurin et al., 2001; Doody et al., 2008; O'Brien, 2008; Gura, 2008) and its curative effects in patients with Huntington's disease (Kieburtz et al., 2010), also during co-application with other therapeutics. Evaluation of dimebon against a set of biochemical targets indicated that dimebon inhibits alpha-adrenergic receptors (alpha1A, alpha1B, and alpha1D, and alpha2A, alpha 2B, and alpha 2C), histamine H1 and H2 receptors and serotonin receptors (5-HT-2A, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6, and 5-HT7), dopamine receptors (D, D2S, and D3), and imidazoline I2 receptors (Schaffhauser et al., 2009; Giorgetti et al., 2010). At low concentrations, dimebon potentiates the activity of AMPA-receptors and blocks NMDA-receptors in neurons (Grigorev et al., 2003).

Pre-clinical studies revealed a number of activities of dimebon in *in vitro* and *in vivo* assays. Dimebon prevents the opening of mitochondrial pores induced by neurotoxins, which is regarded as the major pathogenetic factor of neurodegeneration (Bachurin et al., 2003; Hung, 2008), elevates extracellular levels of amyloid beta in cell culture and the hippocampus of freely moving Tg2576 mice (Steele et al., 2009), promotes neurite outgrowth in cultured hippocampal and cortical neurons (Protter et al., 2009; Bernales et al., 2009), and enhances hippocampal neurogenesis (Pieper et al., 2010).

Bolus administration of dimebon in rats resulted in enhancement of short-term learning in the social recognition paradigm (10 mg/kg, i.p.;

Abbreviations: H1, H2 receptors, histamine type 1 and 2 receptors; 5-HT-2A, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6 and 5-HT7 receptors, 5-hydroxy-tryptamine (serotonin) receptors types 2A, 2B, 2C, 5A, 6 and 7; D, D2S and D3 receptors, dopamine receptors types 2, 2S (short form) and 3; I2, imidazoline receptor type 2; AMPA, alfa-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; NMDA, N-Methyl-D-Aspartate; AF64A, ethylcholine aziridinium; Tg2576 mice, transgenic mouse with K670N/M671L mutation in APP; C57BL/6N, inbred mouse inbred mouse strain; ANOVA, analysis of variance; LTP, long-term potentiation; CA1, Cornu Ammonis 1; K1, constant of association of the drug with receptor; Ach, acetylcholine; AChE, acetylcholinesterase; BrdU, bromodeoxyuridine.

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Schaffhauser et al., 2009) and the novel object recognition task (0.05, 0.5 and 5 mg/kg, p.o.; Giorgetti et al., 2010). In rats treated with the neurotoxin AF64A, which selectively lesions cholinergic neurons and impairs active avoidance, 10-day administration of dimebon reversed these deficits (1 mg/kg/day, i.p.; Lermontova et al., 2000). Similar results were obtained in the Morris water maze in rats subjected to intracerebroventricular administration of AF64: chronic administration of dimebon at the dose of 0.05 mg/kg rescued spatial learning, which was disrupted by chemical lesion of the dorsal hippocampus (Bachurin et al., 2006). A single administration of dimebon increased the memory for new object localization in young C57BL/6N mice at the dose of 0.1 mg/kg, while in a separate experiment, this treatment did not alter the animals' exploratory activity estimated under the same testing conditions (Bolkunov et al., in preparation). Performance in a new object localization model in rodents was shown to be disrupted by selective lesions of the hippocampus (Galani et al., 1998). Thus, dimebon has been shown to facilitate learning in various animal models, including paradigms of hippocampus-dependent learning at a range of concentrations.

Here, we studied whether dimebon applied at the dose that is efficient in a mouse model of new object localization memory, interferes with the appetitive learning of mice in the Y-maze. Studies that assessed dimebon's activity in assays of this type have not been reported to date. We used a protocol of the Y-maze in mice that was previously validated as a test for spatial learning (Dolgov et al., 2005; Gorenkova et al., 2005). In this paradigm, water-deprived C57BL/6N mice were allowed to orientate themselves by distant visual cues when selecting the arm of the maze with a filled bottle. As a model of spatial learning, the Y-maze paradigm is considered to be a test for hippocampus-dependent memory (Gerlai, 2001; Liu et al., 2001; Finger et al., 2010; Alhassan et al., 2009; Tan et al., 2010). We also tested whether dimebon affects memory in a single trial inhibitory learning paradigm using the step-down avoidance paradigm, another model for hippocampus-dependent memory (Lorenzini et al., 1996; Izquierdo and Medina, 1997; Strekalova et al., 2001, 2001) in young and middle-aged mice. Additionally, we investigated the potential effects of the drug treatment applied in both memory tests on parameters of anxiety, exploration and water intake.

The selection of dimebon doses used in this study are based on previous reports, which demonstrate that a single peripheral administration of the drug at a dose of 0.05–0.5 mg/kg evokes a memory-enhancing effect in rats, and results in an effective dimebon brain concentration of 1.7–14 nM/g, that was suggested to trigger neurochemical processes associated with cognitive function (Bachurin et al., 2006; Giorgetti et al., 2010). Recent studies provide evidence for a stimulatory effect by dimebon on neurogenesis in the dentate gyrus of the hippocampus in rats, observed after 1-week of intraperitoneal injections of the drug applied at the same range of concentrations: 0.1 MKM/kg (0.32 mg/kg; Pieper et al., 2010). Therefore, we anticipated that repeated dosing with dimebon at a dose of 0.1 mg/kg, and single administration of a dose of 0.5 mg/kg, would evoke similar neurobiological effects as described in the literature.

We demonstrated that administration of dimebon shortly before training enhances hippocampus-dependent learning in both appetitive and inhibitory learning tasks in C57BL/6N mice. Acute treatment with dimebon was also shown to enhance inhibitory step-down avoidance in older animals. In the Y-maze, dimebon increased the duration of drinking behaviour. Together, our findings suggest the memory-enhancing effects of dimebon in two memory paradigms, which are based on the biologically opposite motivations of positive reward and aversive stimulation.

2. Methods

2.1. Animals and general conditions of testing

Male C57 BL/6N mice aged 3 and 7 months were used. After transportation to the experimental facilities, the animals were housed individually for ten days before the start of experiments under a reverse 12 h:12 h light–dark cycle (lights on: 22:00 h) in standard laboratory conditions. Mice were tested during the dark period of the light cycle in a lab protected from noise. Experimenters were blinded to the treatment. In order to minimize the possible influence of the environment, animals from vehicle- and drug-treated groups were tested alternately. All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals following approval by the local governmental bodies for animal care and welfare.

2.2. Study design

2.2.1. Effects of repeated dimebon administration in the Y-maze

In the first study, we investigated the effects of repeated administration of dimebon on learning to access a bottle with water after drinking deprivation in a spatial version of the Y-maze. The dislocation of visual cues impaired the performance of previously trained mice in the Y-maze protocol, which validated this test as a model of spatial learning (Gorenkova et al., 2005; Gorenkova and Strekalova, unpublished results). Naïve 3-month-old male mice were treated daily either with dimebon (0.1 mg/kg/day) or vehicle ($n=8$ and $n=8$, respectively) and 15 min thereafter were trained in two consequent trials spaced one hour apart; the training lasted 5 days (Fig. 1A); their latencies for reaching the bottle of water, the percentage of correct choices and the duration of drinking were scored (*see below*). Additionally, the potential effects of acute and 3-day repeated treatment of dimebon on behaviour in the O-maze and novel cage were subsequently evaluated 15 min after the injection of 0.1 mg/kg of dimebon ($n=8$) or vehicle ($n=8$). On the same day, a 24-h long water consumption test was carried out in repeatedly dosed animals (Fig. 1C). In a separate experiment, we further addressed the question of whether dimebon increases thirst in water-deprived animals (which would elevate their reward during training and explain the observed accelerated memory acquisition in the Y-maze) (Fig. 1D). Mice were either not deprived from water or deprived from drinking for 18 h prior to the test. For each condition, 10 animals were treated with dimebon at a dose of 0.1 mg/kg 15 min before the onset of the test and, respectively, 10 and 9 mice were treated by a vehicle. The amount of consumed water was evaluated 1, 3 and 24 h after the beginning of the test.

2.2.2. Effects of acute treatment with dimebon in the step-down avoidance model

As repeated administration of dimebon (0.1 mg/kg) was found to enhance performance in the Y-maze (*see below*), we addressed the question of whether acute treatment with dimebon at this dose affects hippocampus-dependent learning in a single trial paradigm. Besides, behavioural changes in dimebon-treated mice were observed in the Y-maze during the very first hours after drug administration. Therefore, naïve 3-month-old male mice were treated either with dimebon (dose 0.1 mg/kg) or vehicle and 15 min later were subjected to a training session in the step-down avoidance model ($n=9$ and $n=8$, respectively); the test for recall was carried out 1 and 24 h later (Fig. 1B, *also see below*). Because of the lack of any effects in this experiment, a higher dose of dimebon (0.5 mg/kg: $n=16$ and $n=17$) was tested in the same study design. Since in this assay, in which young animals were used, the higher dose of dimebon enhanced memory, the efficacy of dimebon at a dose of 0.5 mg/kg was further tested in 7-month-old mice, which received a bolus i.p. injection of dimebon or vehicle ($n=13$ in each group) and were tested in the same protocol of step-down avoidance as used for the younger animals. In a separate study, 3- and 7-month-old mice were given an i.p. injection of dimebon at a dose of 0.5 mg/kg ($n=8$ and $n=8$ for each age group, respectively) and 15 min later were tested in the O-maze and novel cage tests (Fig. 1C; *see below)* to address the possible

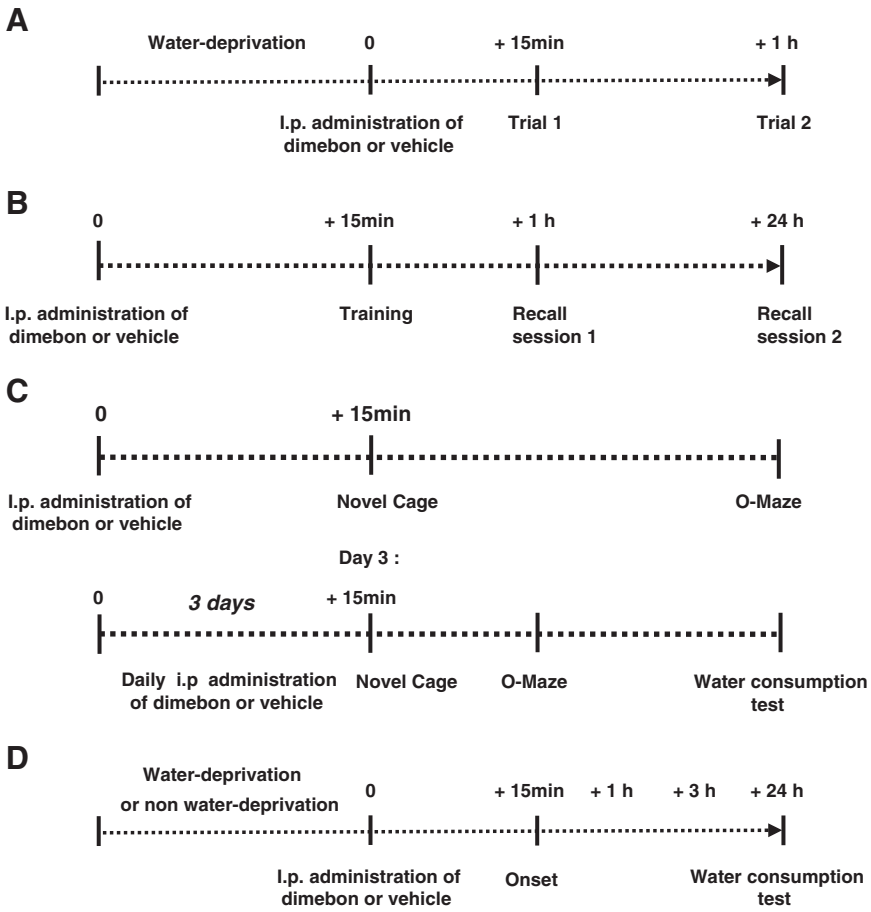

Fig. 1. Schemes of treatment and behavioural testing. (A) In the Y-Maze paradigm, two-trial training protocol was applied 15 min after an i.p. administration of dimebon or vehicle during 5 consecutive days. (B) In the step-down avoidance test, single training session was carried out 15 min after an i.p. administration of dimebon or vehicle; mice were tested for memory recall 1 and 24 h thereafter. (C) Timeline of testing of anxiety-like and exploratory behaviours, and water intake after bolus or repeated administration of dimebon or vehicle. (D) Timeline of testing the potential effects of bolus dimebon administration on 1-, 3- and 24-h water intake in water-deprived and non-water-deprived mice.

non-specific effects of dimebon on parameters of anxiety and exploration/locomotion.

2.2.3. Apparatus and experimental procedures

2.2.3.1. Y-maze. The apparatus used was a symmetrical Y-shaped construction made from black Plexiglas, which consisted of three arms (40×6×10 cm) with an angle of 120° between each arm (Technosmart, Rome, Italy). Figures of different shapes (approx. size 20×40 and 20×30 cm) were placed on the walls of the room to allow spatial orientation. Validation studies showed that the 180°-rotation of cues around the Y-maze apparatus disrupted the performance of previously trained mice (Gorenkova et al., 2005; Gorenkova and Strekalova, unpublished results). Two bottles, one filled with water and another empty, were placed at the ends of the arms in a position adjusted to allow drinking. The illumination strength was 25 lx.

Before the first training session, mice were water-deprived for 18 h. Two 15-min trials per day spaced one hour apart were carried out for 5 days in a row. The administration of either dimebon or vehicle was performed 15 min prior to the first training session on each day. During the trial, a mouse was placed at the starting point of the apparatus (where no bottle was presented) and allowed to access either arm of the maze containing the bottles, one of which was filled with water. Half of each experimental group was trained to receive a water reward from either the lefthand or righthand bottle. Each mouse was allowed to drink for up to 15 min in each trial; when no drinking behaviour was observed by the end of the training day, free access to water in the home cages was allowed for 30 min one hour after the termination of behavioural testing. The animals' body weight was monitored throughout the testing period; previous studies showed this Y-maze protocol to be optimal for training and demonstrated a lack of negative effects of the drinking schedule on body weight (Gorenkova et al., 2005, Gorenkova and Strekalova, unpublished results). The latency to reach the filled bottle and the percentage of correct choices for the arm containing this bottle were taken as indicators of learning of the task. The total duration of drinking during each trial was also measured.

2.2.3.2. Step-down avoidance test. Fifteen minutes after the i.p. administration of drug or vehicle, mice were trained in a step-down avoidance paradigm. The step-down apparatus (Evolocus LLC Tarrytown, NY, USA and Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 cm×25 cm×50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden platform (7 cm×7 cm×1.5 cm) was placed. A shocker was used to deliver an alternating electric current (AC, 50 Hz). The illumination strength was 25 lx. In this paradigm, animals will be trained not to step down from a platform onto a grid floor to avoid an electric shock.

During the training session, mice were placed onto the platform inside a transparent cylinder for 30 s to prevent them from stepping down immediately. After removal of the cylinder, the time until the animal left the platform with all four paws was measured as baseline latency of step down. Immediately after step down, mice received a single electric foot-shock (0.5 mA, 2 s) and returned to their home cages. One hour and twenty-four hours later, during the recall trial

session, animals were exposed to the apparatus again by being handled the same way as in the training session; no foot shock was delivered. Latency of step down with all four paws was measured until 180 s elapsed. Accordingly to a previously validated criterion of the acquisition of the step down avoidance task (Strekalova and Steinbusch, 2009, 2010), animals that showed latencies of more than 30 s in the recall session were considered as good learners. The behaviour of individual mice, which showed during a recall session the escape latency 1 s or lower while baseline latency exceeded 5 s, was regarded as panic response to aversive context, these animals were discarded from the experiment.

2.2.3.3. O-maze. The apparatus, which consisted of a circular path (runway width 5.5 cm, diameter 46 cm) was placed 50 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 25 lx. The apparatus was placed on the dark surface in order to reduce a reflection and keep a control over lighting conditions during testing. The anxiety-like behaviour was assessed in applied protocol of the O-maze test using parameters, which were validated earlier (Strekalova et al., 2004, 2005). Mice were placed in one of the close arms compartments of the apparatus. The latency of the first exit to the anxiety-related open compartments of the maze and total duration of time spent therein were scored during a 5 min observation period.

2.2.3.4. Novel cage test. The novel cage test was performed to assess exploration of a new environment (Strekalova et al., 2001, 2004). Mice were introduced into a standard plastic cage filled with fresh sawdust. The number of exploratory rearings was counted under red light during a 5 min period.

2.2.3.5. Water consumption test in repeatedly treated mice. A 24-h drinking test was carried out to assess potential effects of repeated administration of dimebon on water intake (17.00–17.00). Consumption of regular drinking water was evaluated by weighing bottles before and after the test.

2.2.3.6. Water consumption test in acutely treated mice. To further evaluate the possible effects of acute dimebon administration on water intake in water-deprived animals, mice were either not deprived of water or were deprived from drinking for 18 h. Dimebon or vehicle was administrated 15 min prior to the test, which was started at the onset of the dark phase of the light cycle (10.00). Intake of regular drinking water was measured by weighing bottles 1, 3 and 24 h after the beginning of the test.

2.2.4. Drugs and drug administration

Dimebon (obtained from the Institute of Physiologically Active Compounds, Chernogolovka, Russia) was dissolved in isotonic NaCl solution for delivery in a volume of 0.01 ml/g of body weight by intraperitoneal injection at doses of 0.1 mg/kg and 0.5 mg/kg; vehicle was administered in the same volume.

2.2.5. Statistical analysis

Data were analyzed with a statistical software package (Prizm 3, Chicago, IL). Values of latencies to reach water reward in the Y-maze had normal distribution, therefore, repeated measurements data were compared by repeated measures ANOVA test followed by the Tukey' post-hoc test, independent measurements were treated by one way ANOVA analysis followed by unpaired *t*-test; differences of variances were assessed by the F test. Since values of the duration of drinking behaviour in the Y-maze model in many cases were assigned to a period of observation, these data were considered as arbitrarily measured and, therefore, treated by the non-parametric analysis: Friedman test followed by the Dunn's multiple comparison test was applied for repeated measurements; Kruskal–Wallis test followed by Mann–Whitney *U*-test was used for a group comparison. Percentage of correct choices was analyzed by a Fischer's exact test. Independent data sets obtained in the step-down avoidance and O-maze test were treated with non-parametric analysis, even though the populations were Gaussian, since in essential percentage of animals behavioural parameters were measured arbitrary. This was due to the cut off in the behavioural scoring: several values were assigned to the observation periods elapsed. Mann–Whitney test was used for the analysis of independent data sets. Data obtained in the novel cage and water intake tests had normal distribution and were analyzed by the *t*-test. The level of confidence was set at 95% ($p<0.05$).

3. Results

3.1. Repeated dimebon administration enhances learning in the Y-maze

The effects of daily administration of dimebon on the selection of the correct arm of the Y-maze baited with a filled bottle and the latency to reach the water reward were measured to assess spatial learning. A repeated ANOVA revealed significant changes in the latter parameter in the course of the 5-day training period both in the control ($p=0.006$, $F=2.92$, $R^2=0.29$) and dimebon-treated groups ($p<0.0001$, 10.64, $R^2=0.60$); a decrease in the latency to reach the water reward (Fig. 2A,B) suggested that mice from both groups acquired the task. Compared to the mean latency to reach the water reward measured on day 1 (trial 1), the dimebon-treated group had a significant reduction in this parameter on day 2, while vehicle-treated mice showed this effect of training only on day 5 ($p<0.05$; Fig. 2A,B). ANOVA revealed significant differences between dimebon- and vehicle-treated groups in the overall comparison of latencies to reach the reward ($p<0.0001$, $F=3.77$, $R^2=0.34$). The percentage of correct choices in the course of nine testing trials (the training trial 1 on day 1 was excluded from the analysis), was significantly higher in the dimebon-treated group (77.8%) than in vehicle-treated mice (52.78%, $p=0.001$, Fisher's exact test, Fig. 2C) and chance values ($p=0.0004$); the vehicle-treated group showed no significant difference from the chance values for correct choices ($p=0.43$). Together, repeated treatment with dimebon accelerated the acquisition of the Y-maze memory task.

The Friedman test revealed significant differences in the duration of drinking between the trials both in dimebon- and vehicle-treated mice which spent more time drinking as the experiment progressed from day 1 to day 5 ($p<0.0001$ for each group; Fig. 3A,B). This suggests that animals gradually learnt the location of the water source and became less anxious in the course of training. The duration of drinking was significantly higher in the dimebon-treated group than in the vehicle-treated group (on day 1, trial 1: $p=0.01$, $U=11.0$; day 2, trials 1 and 2: $p=0.003$, $U=7.5$ and $p=0.0005$, $U=3.0$, respectively; on day 3, trial 2: $p=0.01$, $U=10.5$; Mann–Whitney *U* test; Fig. 3B). A tendency to increased time spent drinking in the dimebon-treated mice compared to control animals was revealed in trial 1, day 3 ($p=0.06$, $U=17.0$) and in trial 1, day 4 ($p=0.06$, $U=17.5$). Starting from day 4, trial 2 and until the end of the training period, the duration of drinking did not differ between groups ($p>0.05$, Mann–Whitney *U* test). Thus, in a novel environment, which is well documented to induce anxiety and suppress consummatory behaviour (Loiseau et al., 2003; Dulawa and Hen, 2005), mice repeatedly treated with dimebon showed elevated water intake. The latter finding suggested that drug administration might induce an anxiolytic-like effect and/or change the physiological need for water. To rule out these factors, additional experiments were carried out.

Since significant effects of dimebon on the latency to reach a reward and the duration of drinking behaviour were revealed both after repeated treatments, the potential effects of repeated dimebon administration was assessed in additional tests. It was found that the 3-day treatment with dimebon at a dose of 0.1 mg/kg, compared to a

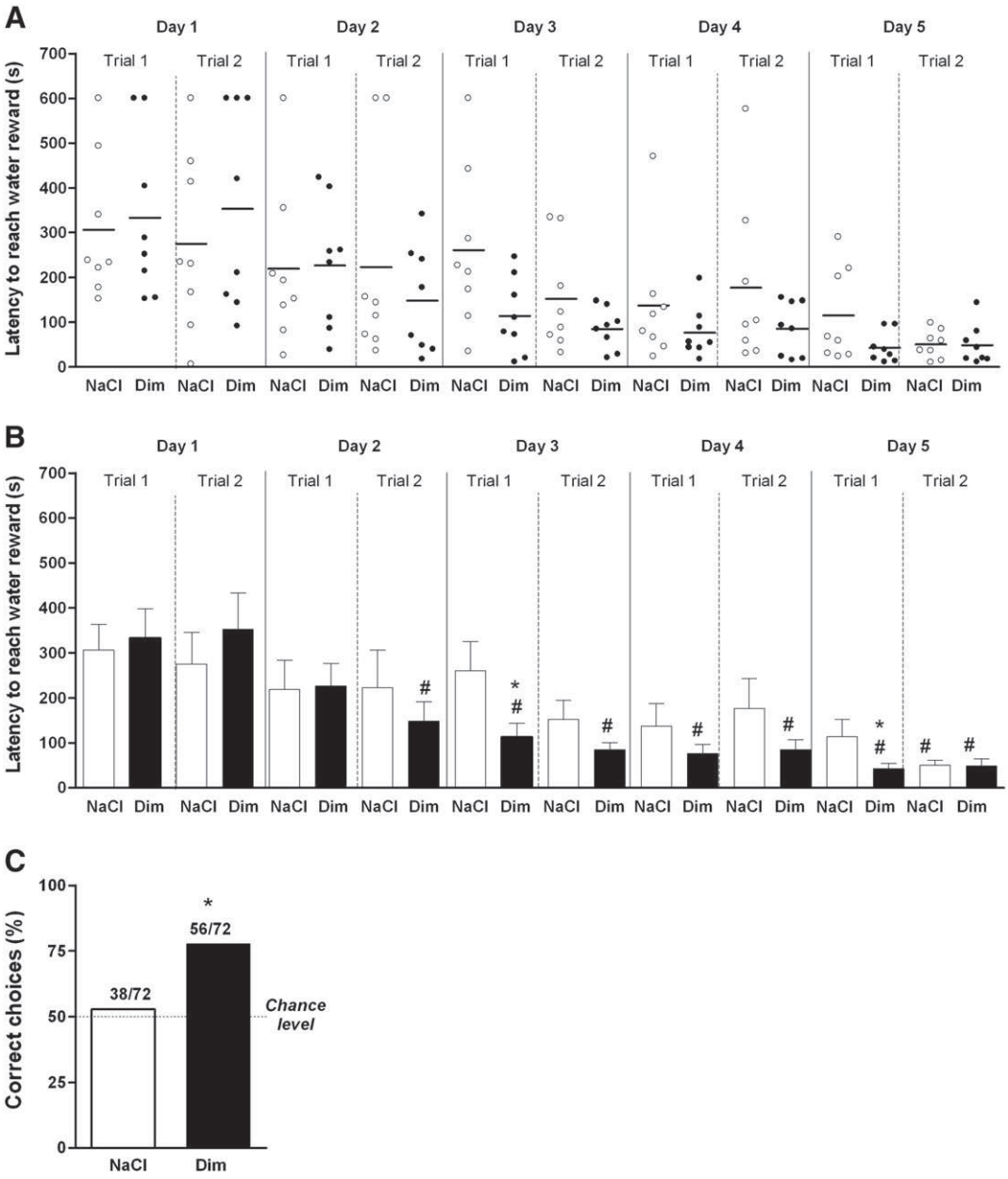

Fig. 2. Repeated dimebon administration accelerates learning in the Y-Maze. (A) Individual and (B) group data demonstrate a significant reduction of the latency to reach the water reward in dimebon-treated group on days 2–5 of training, while vehicle-treated group showed such difference on day 5 ($^{\#}p<0.05$ vs. training values on Day 1 Trial 1, Tukey's posthoc test). On days 3 and 5, this parameter was significantly lower in dimebon-treated animals than in vehicle-treated mice ($^{*}p<0.05$, unpaired *t*-test). Bars represent the means; each column represents the mean ± SEM. (C) Percentage of correct choices for the arm with a filled bottle from nine testing trials overall carried out for 8 animals was significantly higher in dimebon-treated group than in control group ($^{*}p<0.05$, Fischer's exact test). Absolute numbers of correct choices are indicated above the bars. NaCl: vehicle-treated group; and Dim: dimebon-treated group.

vehicle-treated group, did not change the latency of exit, the time spent in the open arms or the number of exits in the O-maze ($p>0.05$ and $p>0.05$ respectively, Mann–Whitney *U* test; Fig. 3C). In particular, the difference in time spent in the open arms of the O-maze between the dimebon- and vehicle-treated group was far from statistically significant ($p=0.79$, $U=29$). No difference was found between vehicle- and dimebon-treated groups in the number of exploratory rearings in the novel cage either ($p>0.05$ and $p>0.05$ respectively, unpaired *t*-test; Fig. 3D).

Repeated dosing with dimebon did not alter the 24-h water intake ($p>0.05$, unpaired *t*-test; Fig. 3E). In addition, in order to determine whether acute administration of dimebon can increase thirst in water-deprived animals, we studied the effects of dimebon on mice under normal and water-deprivation conditions. Bolus injection of dimebon did not affect 1-h, 3-h or 24-h water consumption regardless of previous water deprivation ($p>0.05$, two-way ANOVA; Fig. 3F). The amount of water consumed did not differ between vehicle-treated and dimebon-treated mice not deprived of water, or between animals previously deprived of water ($p>0.05$ and $p>0.05$ respectively, Mann–Whitney *U* test; Fig. 3F). A two-way ANOVA showed that water-deprivation increased water intake at three time points ($p<0.05$, two-way ANOVA). Together, our results suggest that treatment with dimebon at a dose of 0.1 mg/kg accelerates spatial learning of the task based on water reward without affecting the metabolic requirement for water, locomotion or exploration in mice. However, dimebon seemed to have an anxiolytic-like effect in the model of spatial learning, which may have contributed to the improvement of performance under the testing conditions employed.

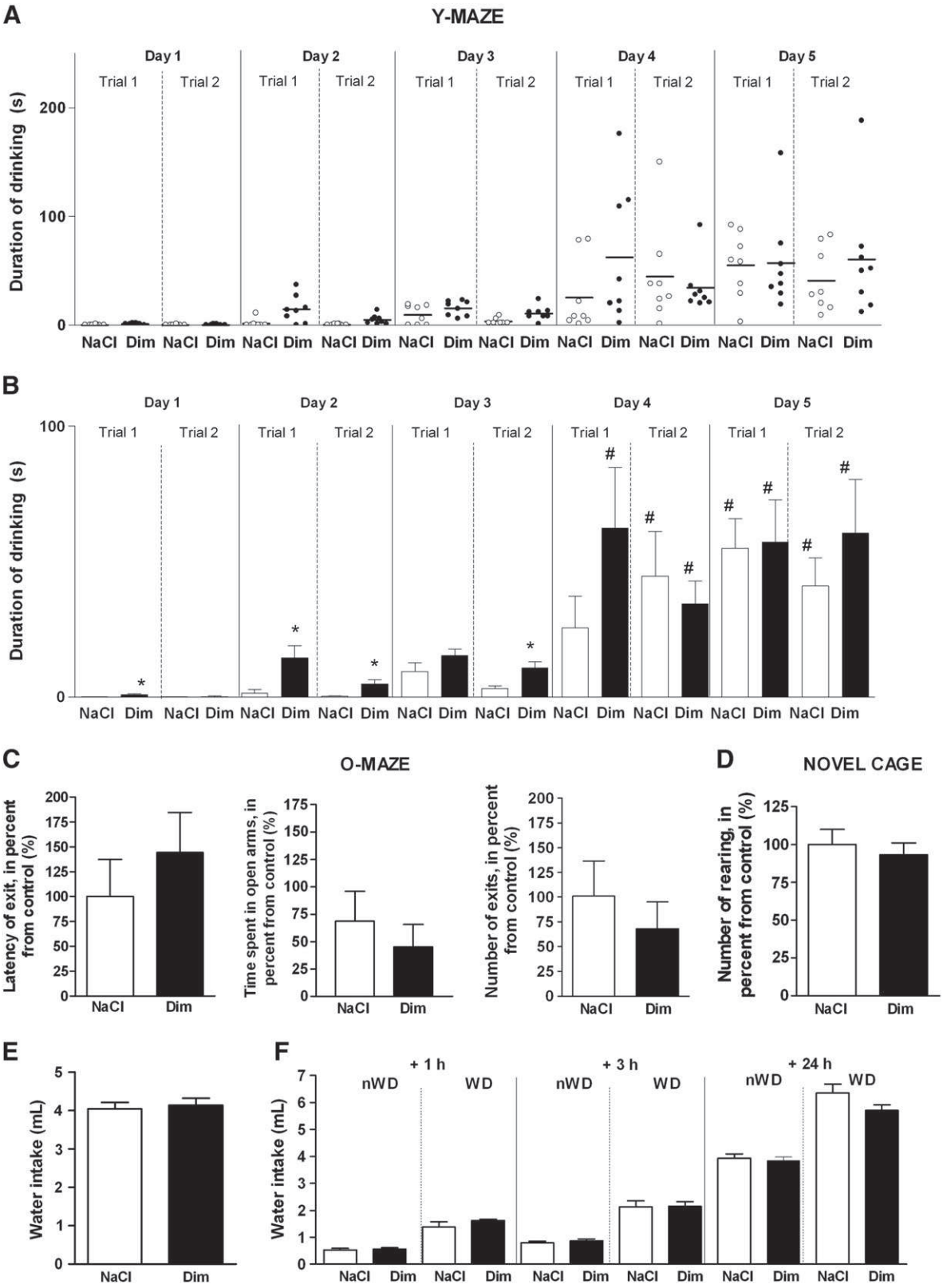

Fig. 3. Effects of repeated administration of dimebon on parameters of anxiety, locomotion, exploration and drinking. (A) Individual and (B) group data evidence significant increase of drinking behaviour in a course of training experiment in the Y-Maze ($^{*}p<0.05$ vs. training values on Day 1 Trial 1, Dunn's multiple comparison test). Duration of drinking was significantly higher in dimebon-treated mice than in control group on days 1–3 ($^{*}p<0.05$, Kruskal–Wallis test). Bars represent the medians. Each column represents the mean ± SEM. Mice, repeatedly treated with dimebon did not differ from vehicle-treated animals in (C) latency of exit to open arms and time spent therein, as well as number of exits in the O-maze, (D) number of exploratory rearings in the novel cage test, (E) 24-h water intake; $p>0.05$, Mann–Whitney *U*-test. (F) Bolus treatment with dimebon does not affect 1-, 3- and 24-h water intake, regardless of preceding water deprivation ($p>0.05$, two-way ANOVA), which increased 24-h water consumption both in vehicle- and dimebon-treated groups (*see text*). Each column represents the mean ± SEM. NaCl: vehicle-treated group; Dim: dimebon-treated group; WD: water-deprived; and nWD: non-water-deprived.

3.2. Acute treatment with dimebon increases performance in the step-down avoidance model

In the above-described experiment, dimebon-treated mice showed behavioural changes during the course of a repeated training/dosing protocol and also immediately after the very first drug administration (within a 2-h period), comprising a significant increase in the duration of drinking behaviour (Figs. 2,3). Therefore, we addressed the question of whether a bolus injection of dimebon at the dose of 0.1 mg/kg can affect learning in a single trial memory model in 3-month-old mice using the step-down avoidance paradigm. The vehicle- and dimebon-treated groups showed similar baseline latencies ($p = 0.35$, U = 21.00, Mann–Whitney *U* test) suggesting that dimebon injection is unlikely to affect locomotion, exploration or anxiety-like behaviours in the step-down avoidance test. In comparison to the baseline values, the latencies of step down were similarly increased in vehicle- and dimebon-treated groups during the first recall session ($p = 0.02$ and $p = 0.04$, respectively, Wilcoxon test; Fig. 4A,B) and the second recall session ($p = 0.01$ and $p = 0.01$ respectively), indicating that both experimental groups acquired the task.

We found no differences between the groups in the latency of step down in either of the two recall sessions, carried out 1 or 24 h after training ($p = 0.28$, U = 19.0 and $P = 0.45$, U = 23.5 respectively, Mann–Whitney *U* test; Fig. 4B). According to our exclusion criteria (*see Methods section*) three mice were discarded from the experiment (one from the control group and two from the dimebon-treated group). The number of animals that were classified as good learners according to the 30-sec criterion of the task acquisition (*see Methods section*) among vehicle- and dimebon-treated mice was similar during

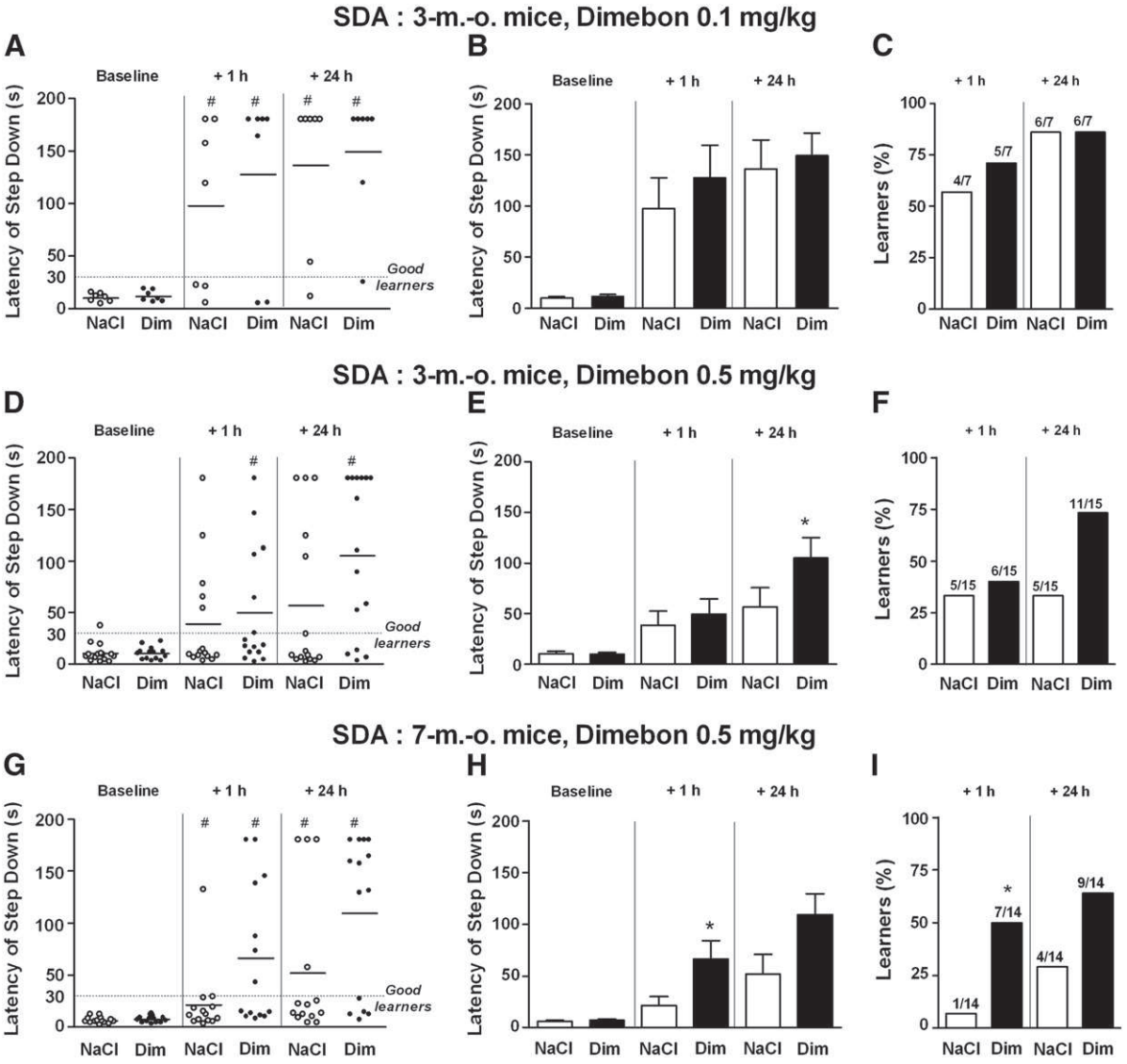

Fig. 4. Acute treatment with dimebon enhances performance in the step-down avoidance paradigm: effects in 3- and 7-month old mice. (A, D, G) Individual and (B, E, H) group data show an increase of the latencies of step down measured during two recall sessions(+1 h and +24 h) in comparison to the training session (baseline) in vehicle and dimebon-treated group suggesting that mice acquired the task ($^{\#}p<0.05$ vs. training values, Wilcoxon test). Three- and seven-month old mice treated with dimebon at the dose of 0.5 mg/kg revealed significant increase in the latency of step down evaluated 24 h after training as compared to vehicle-treated group ($^{*}p<0.05$, Mann–Whitney *U*-test); no such difference was observed in 3-month old mice treated by 0.1 mg/kg of dimebon ($p>0.05$, Mann–Whitney *U*-test). Seven-month old mice treated with dimebon showed also a significant increase of this parameter 1 h after training, as compared to vehicle-treated animals ($^{**}p<0.05$, Mann–Whitney *U*-test). (C, F, I) Three- and seven-month old mice treated with dimebon at the dose of 0.5 mg/kg had significantly higher percentage of good learners estimated 24 h after training as compared to vehicle-treated group ($^{*}p<0.05$, Fischer's exact test); no such difference was observed in 3-month old mice treated by 0.1 mg/kg of dimebon ($p>0.05$, Fischer's exact test). Seven-month old mice treated with dimebon showed also a significant increase of this parameter 1 h after training, as compared to vehicle-treated animals ($^{**}p<0.05$, Fischer's exact test). Each column represents the mean ± SEM. NaCl: vehicle-treated group; and Dim: dimebon-treated group.

the first and second recall sessions ($p = 0.50$ and $p = 0.77$ respectively, Fischer's exact test; Fig. 4C). Thus, acute injection of dimebon at the dose of 0.1 mg/kg does not alter short-term and contextual learning in mice trained in the step-down avoidance task.

In the next experiment, we studied whether a higher dose of dimebon (0.5 mg/kg) would change the performance in the step-down avoidance model assessed in the same experimental design. As in a previous study, drug administration did not affect the baseline latencies of step down, which were similar in vehicle-treated and dimebon-treated mice ($p > 0.05$, Mann–Whitney *U* test; Fig. 4D,E). The latencies of step down were increased during recall sessions in both groups in comparison to baseline values, both during the first recall trial ($p = 0.05$ and $p = 0.001$ for vehicle- and dimebon-treated groups, respectively, Wilcoxon; Fig. 4D,E) and during the second recall session ($p = 0.06$ and $p = 0.01$, respectively). Whereas no difference in the latency of step down between the groups was observed during the first recall session ($p = 0.18$, U = 90.0, Mann–Whitney *U* test; Fig. 4D, E), the dimebon-treated group showed a significantly longer latency of step down during the second recall session compared to vehicle-treated mice ($p = 0.03$, U = 67.5, Mann–Whitney *U* test). In addition, the percentage of good learners was significantly higher among dimebon-treated animals than in vehicle-treated mice during the second recall session ($p = 0.03$, Fischer's exact test, Fig. 4F). Three mice were discarded from the experiment (one from the control group and two from the dimebon-treated group), according to the exclusion criteria (*see Methods section*). The results of this experiment demonstrate improved scores of long-term memory of mice treated with a bolus i.p. injection of dimebon at a dose of 0.5 mg/kg in a step-down avoidance paradigm.

To verify these effects of dimebon in slightly different conditions, we used 7-month-old mice in the same study design. The administration of dimebon did not alter the baseline latencies of step down, which did not differ significantly between the vehicle and dimebon-treated mice ($p > 0.05$, Mann–Whitney *U* test; Fig. 4G,H). In vehicle- and dimebon-treated groups, the latencies of step down were elevated during recall sessions in comparison to baseline values, both during the first recall trial ($p = 0.0008$ and $p < 0.0001$ for respectively, Wilcoxon; Fig. 4D,E) and during the second recall session ($p < 0.0001$ and $p = 0.0004$, respectively; Fig. 4G,H). The dimebon-treated group showed a significantly higher latency of step down during the second recall session than vehicle-treated mice ($p = 0.03$, U = 63.0; Mann–Whitney *U* test; Fig. 4G,H) and during the first recall session ($p = 0.03$, U = 67.0). The percentage of good learners was significantly higher in the dimebon-treated than control mice during the first and second recall sessions ($p = 0.04$ and $p = 0.01$, respectively; Fisher's exact test, Fig. I). According to the exclusion criteria (*see Methods section*) five mice were excluded from the experiment (one from the control group and four from the dimebon-treated group). Thus, bolus i.p. injection of dimebon at a dose of 0.5 mg/kg also elevates memory scores in a step-down avoidance paradigm in 7-month-old mice. These results further demonstrate the memory-enhancing effects of a single administration of dimebon at a dose of 0.5 mg/kg in a step-down avoidance model.

To rule out the possibility that dimebon, at a dose that affects performance in the step-down avoidance model, interferes with anxiety-like behaviour and exploration, supplementary tests were carried out in young and middle-aged mice. Application of a battery of tests 15 min after intraperitoneal administration of dimebon and vehicle in 3-month-old and 7-month-old mice at a dose of 0.5 mg/kg revealed a lack of differences between the groups in the latency of exit, time spent in the open arms and the number of exits in the O-maze ($p > 0.05$, Mann–Whitney *U* test, Fig. 5A,D). Of note, while 3- and 7-month-old dimebon-treated mice show a graphical trend towards decreased time spent in the open arms of the O-maze, the difference in this parameter from the vehicle-treated group is far from statistically significant ($p = 0.88$, U = 30 and $p = 0.28$, U = 21.5, respectively).

The number of exploratory rearings in the novel cage was unchanged in young mice treated with dimebon ($p > 0.05$, unpaired *t*-test; Fig. 5B) and was significantly lower in older mice after dimebon administration ($p = 0.04$, unpaired *t*-test; Fig. 5E). Thus, the statistically significant increase in the latency of step down in 7-month-old mice treated with dimebon during the first recall session can be accounted for by the general suppressive effect of the treatment on exploration/locomotion; these effects were observed in 3-month-old animals. Together, our results suggest that similar to the experiment on young animals, bolus treatment with dimebon at a dose of 0.5 mg/kg enhances long-term contextual learning in the step-down model in middle-aged mice.

4. Discussion

The present data suggest that the administration of dimebon 15 min prior to training in the Y-maze and step-down avoidance via repeated (0.1 mg/kg) and acute (0.5 mg/kg) i.p. injections, respectively, increases learning scores in C57BL/6N mice while affecting other behaviours in some test situations, as well (Table 1). Bolus administration of dimebon at the dose of 0.1 mg/kg did not alter the learning of the step-down avoidance task in 3-month-old mice. Acute treatment with dimebon was also shown to enhance inhibitory learning in the 7-month-old mice. Bolus administration of dimebon did not affect locomotion, exploration or anxiety-like behaviour in additional experiments in young and old mice, except rearing activity in 7-month-old mice in a novel cage test, which was decreased by the treatment. No effects of 3-day treatment with dimebon on locomotion, exploration, O-maze behaviour or water consumption were found either. Repeated administration of dimebon increased the duration of drinking behaviour in the Y-maze. Together, our data suggest that in mice, dimebon increases hippocampus-dependent learning in both appetitive and inhibitory tasks. Thus, dimebon enhances memory based on the biologically opposite situations of positive reward and aversive stimulation.

In the course of training in the Y-maze, dimebon-treated mice showed overall shorter latencies of reaching the water reward than control mice (Fig. 2A,B). Dimebon-treated mice revealed a significant reduction in this parameter compared to the values measured on day 1, trial 1, starting from day 2, trial 2 of the training procedure, while control animals demonstrated such an effect of training only on day 5. On days 3 and 5, the dimebon-treated group had significantly shorter latencies to reach the water reward than the vehicle-treated group. The percentage of correct choices was significantly higher in the dimebon-treated group than in control mice (Fig. 2D). Together, our data suggest that repeated treatment with dimebon accelerated the acquisition of the spatial task in the Y-maze test.

Importantly, acute and chronic administrations of dimebon significantly increased the duration of drinking during the first experimental session and in the course of daily training (Fig. 3A,B). As anxiolytic drugs are well-documented to increase novelty-suppressed consummatory behaviour (Loiseau et al., 2003; Dulawa and Hen, 2005) and the anxiolytic effects of dimebon were observed in two classical tests for anxiety-like behaviour and open field tests (Bachurin and Grigoriev, 2009), our findings might be considered to be an indication of the anti-anxiety properties of dimebon. In the latter study, bolus intraperitoneal administration of dimebon at a dose of 2 mg/kg, 40 min prior to testing was found to induce an anxiolytic effect in a rat Vogel conflict model, dark/light box paradigm, and an open field test, that was similar to the effects of diazepam applied at the same dose. Lower doses of dimebon (0.05–0.1 mg/kg) evoked anxiolytic-like changes in some, but not all behavioural measures in this study. In our experiments, repeated 3-day treatments with dimebon did not alter anxiety-like behaviour in the O-maze (Fig. 3C), and similar results were found after bolus treatment with dimebon at a dose of 0.5 mg/kg in 3-month-old and 7-month-old mice (Fig. 5A,C,

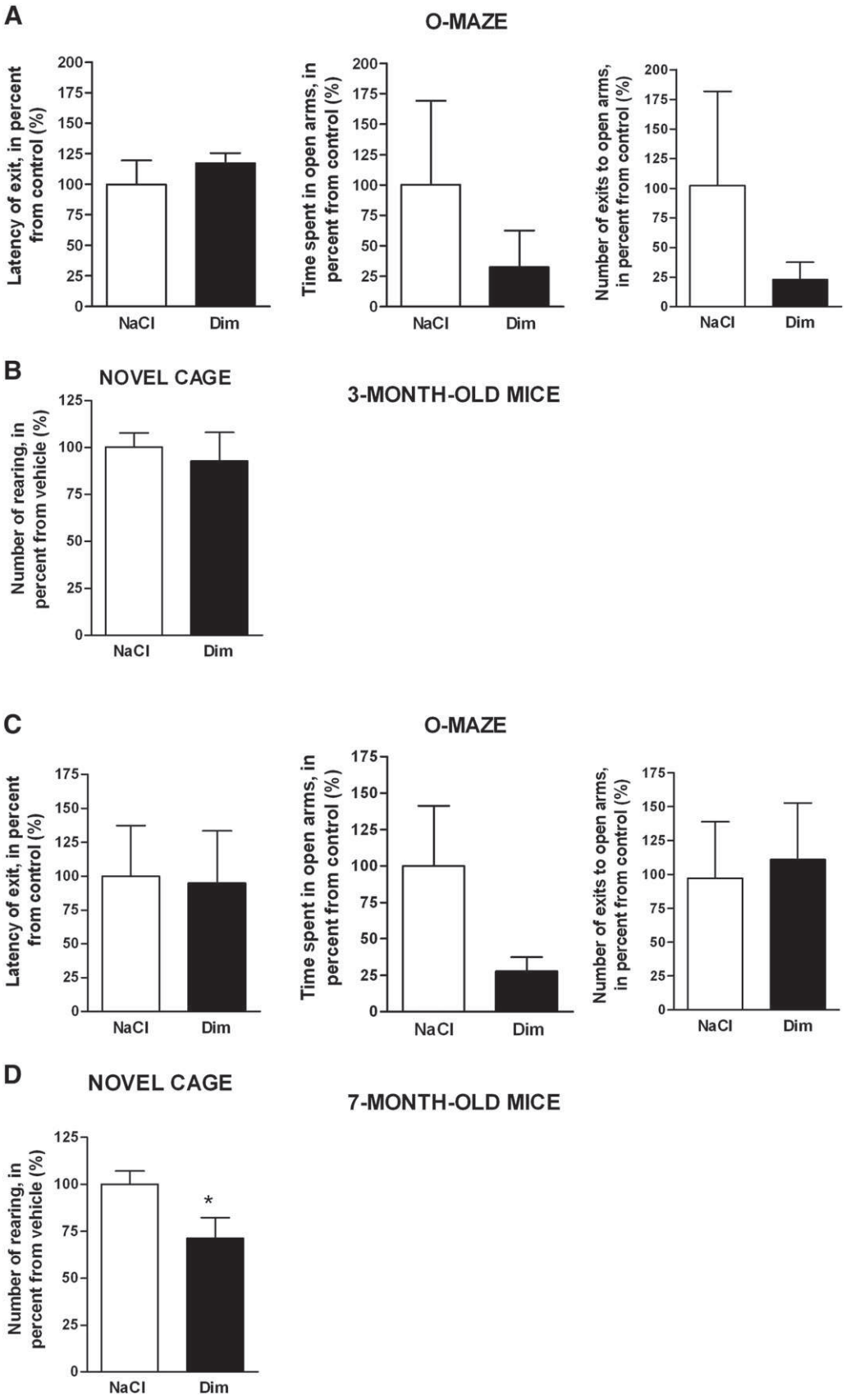

Fig. 5. Bolus injection of dimebon and parameters of anxiety, locomotion and exploration. Three-month old mice, treated with dimebon did not differ from vehicle-treated animals in (A) latency of exit to open arms and time spent therein, as well as number of exits in the O-maze ($p>0.05$, Mann–Whitney *U*-test) and (B) number of exploratory rearings in the novel cage test ($p>0.05$, unpaired *t*-test). (C) Seven-month old mice, treated with dimebon did not differ from vehicle-treated animals in the latency of exit to open arms and time spent in the open arms, as well as in the number of exits in the O-maze ($p>0.05$, Mann–Whitney *U*-test), but (B) showed reduced number of exploratory rearings in the novel cage test ($^{*}p<0.05$, unpaired *t*-test). Each column represents the mean ± SEM. NaCl: vehicle-treated group; and Dim: dimebon-treated group.

Table 1
Memory enhancing action and other behavioural effects of acute and repeated treatment with dimebon. Dimebon induced memory enhancing effect at the dose of 0.1 when injected repeatedly and at the dose of 0.5 when applied acutely. Both types of treatment induced also other behavioural effects (*see text*; n.a.: non-applicable).

			Acute treatment		Repeated treatment	
			Memory enhancing	Other effects	Memory enhancing	Other effects
Y-maze	3-m-o 0.1 mg/kg		–	+	+	+
Step down avoidance	3-m-o	0.1 mg/kg	–	–		
		0.5 mg/kg	+	–		
	7-m-o 0.5 mg/kg		+	+	n.a.	n.a.

see also below). Mice treated with dimebon demonstrated an insignificant decrease in the duration of time spent in the O-maze; statistically, this difference was far from significant, and is due to the presence of a few outliers. The latency of exit to open arms, and the number of exits had similar values in vehicle- and dimebon-treated groups, thus additionally suggesting a lack of effect by dimebon on anxiety-related behaviour under these testing conditions.

The differences between our experimental results and the above described study might be due to differences in dimebon doses, species and anxiety paradigms employed, as well as distinct animal testing times with respect to dosing. In order to rule out potential confounds in the evaluation of the cognitive effects of dimebon, mice were tested in O- and Y-mazes using consistent time schedules, 15 min after drug administration in both tests. In contrast to this testing protocol, changes in the anxiety-like behaviour of dimebon-treated rats were investigated at a time point which is believed to be closer to the peak of dimebon concentration in the brain, as other studies have revealed maximal levels of the drug in the rat brain 50–60 min after its intragastrical administration at doses of 0.05–1 mg/kg (Giorgetti et al., 2010). As these doses of dimebon were similar to those used in the present study, we assume that the concentration of dimebon in the brain peaks during a comparable time period, i.e. 30 min–1 h 30 min after dosing. Thus, it can be speculated that among other possible reasons, the anti-anxiety effects of dimebon in the O-maze experiment were not observed because of lower concentrations of the drug in the mouse brain at the moment of testing in this paradigm. In support of this explanation, we found that in the Y-maze, a significant prolongation of drinking behaviour in dimebon-treated mice was detected on Day 1, Trial 2, i.e. approximately 1 h after the first drug administration, but not during testing in Trial 1, which was carried out only 15 min after treatment (Fig. 3A,B). Together, our results in the Y- and O-maze paradigms, and previously obtained data on the inconsistent appearance of the anti-anxiety effects of dimebon in rats treated with low drug doses, led us to suggest that the proposed anxiolytic-like action of this compound does not occur in all test situations. Similar results were found for other drugs as well (Haller et al., 2000; Merali et al., 2003; Reddy and Devi, 2006).

Suggested anxiolytic-like effect of dimebon in the Y-maze might contribute to the animals' performance by the facilitation of mouse exploration in the anxiogenic situation of novelty, which generally increases the chances of finding a bottle with water and receiving a reward. In addition, the enhanced rewarding impact of the training procedure may be due to the prolongation of water intake in water-deprived mice; increased reward during training may accelerate the acquisition of the Y-maze appetitive task above and beyond the immediate effects of dimebon as a cognitive enhancer.

Supplementary tests rule out the possibility that dimebon merely increases exploratory behaviour (Fig. 3D) and elevates the metabolic need for water; the latter factor was assessed for repeated bolus administration of dimebon in both water-deprived mice and under normal drinking conditions (Fig. 3E,F). Bolus injection of dimebon did not increase 1-, 3- or 24-h water intake, regardless of previous water deprivation. Moreover, after acute treatment with dimebon, 24-h water intake was insignificantly decreased in non-water deprived animals in this study (Fig. 3F), in line with earlier reported suppressive effects of other drugs with inhibitory action on various behaviours in rodents, e.g., citalopram, on water consumption (Strekalova et al., 2006). Hence, possible changes in exploration and thirst did not seem to interfere with enhanced performance in the Y-maze and increased duration of drinking behaviour of animals treated with dimebon.

Thus, dimebon enhances spatial learning in the memory model based on positive reward. Because novelty exploration is also considered to be a rewarding stimulus, the memory enhancing effects of dimebon in the new object exploration/localization paradigms mentioned above and described by other groups (Chuhan and Taukulis, 2006; Giorgetti et al., 2010) indirectly support our findings.

Since in the Y-maze, the very first administration of dimebon induced behavioural changes (increasing the duration of drinking behaviour; Fig. 3A,B), we tested whether dimebon affected learning after a bolus injection. To achieve consistency with the Y-maze study, we selected the step-down avoidance paradigm, since this is a single trial memory test in which the animals' performance is well known to depend on intact hippocampal function (Lorenzini et al., 1996; Izquierdo et al., 2006). The object recognition test was not selected, since there are discrepancies concerning the role of the hippocampus in this task (Albasser et al., 2010).

We found that dimebon delivered at the dose of 0.5, but not 0.1 mg/kg significantly increased the latency of step down in a recall session carried out 24 h after the training session (Fig. 4A,B,D,E), as well as the number of animals classified as good learners (Fig. 4C,F) in 3-month-old mice, i.e., it evoked memory-enhancing effects, according to previously validated criteria of memory acquisition in this task (Strekalova and Steinbusch, 2009, 2010). Changes in the latency to step down under conditions of intrahippocampal administration of various active compounds and the induction of stress-induced anhedonia have been shown to correlate with other parameters of hippocampal plasticity, such as induction of the LTP in the CA1 area of the hippocampal formation (Strekalova et al., 2001, 2002; Strekalova and Steinbusch, 2010; Tokarski et al., under revision). The effective dose of dimebon also increased learning scores in the step-down avoidance test in 7-month-old mice (Fig. 4G–I). No changes in the latency to step down were found in dimebon-treated mice tested 1 h after the training session in young mice, while in 7-month-old animals it was significantly increased in the dimebon-treated group (Fig. 4E,H).

However, dimebon at the dose of 0.5 mg/kg inhibited exploration/locomotion in the novel cage test in the older, but not younger animals (Fig. 5A,D). Given the lack of memory-enhancing effects on short-term memory in young mice, these data suggest that a significant increase in the latency to step down revealed in 7-month-old mice treated with dimebon during the first recall session was due to its non-specific inhibitory effects on locomotion. Data on the suppressive effects of dimebon on exploratory vertical activity in the novel cage are in line with the well-documented sedative effects of histamine receptor blockers (Passani et al., 2007; Van Ruitenbeek et

al., 2010), which are more subtle with dimebon treatment than with other antihistamines (Iliyuchenok and Matveeva, 1989). Similarly to our study, dimebon was found to decrease exploratory behaviour in rats at a dose of 30 mg/kg (Schaffhauser et al., 2009). Interestingly, the inhibitory action of dimebon on exploratory rearing activity in the novel cage was age-dependent. Other studies have revealed differential effects of psychotropic drugs, including compounds with sedative activity, on younger vs. older rodents (Smith et al., 2002; Takase et al., 2009). The distinct locomotory effects of dimebon on 3- and 7-month-old mice in the present work might be accounted for by altered receptor sensitivity and slower drug metabolism, resulting in elevated dimebon concentrations in the brains of older animals.

Inhibitory behavioural effects of dimebon in 7 month-old-mice on exploratory rearing activity and step down avoidance behaviour measured shortly after training were detected 0.5 h and 1 h 15 min after the treatment, respectively, thus, the occurrence of these effects of dimebon corresponded a proposed time window of maximal brain concentrations of the drug in the mouse brain. No such effects of the treatment were revealed at earlier time points relative to the treatment, as no changes were observed in the latency of baseline step down behaviour and parameters reflecting animals' locomotion in the O-maze, which were assessed 15 min after injection of a dose dimebon.

The fact that the dimebon- and vehicle-treated groups in both age groups showed no difference in their baseline latencies of step down which demonstrates the absence of its effects on anxiety and locomotion under conditions of testing in the step-down avoidance apparatus. In line with these data, most of the supplementary tests in mice of both age groups revealed no effects of dimebon on the parameters of anxiety-like behaviour in the O-maze test (Fig. 5A,C) or exploratory behaviour in the novel cage test (Fig. 5B) with the above-mentioned exception of the behavioural inhibition of 7-month-old mice in the latter paradigm (Fig. 5D). Similarly to the study results on the effect of repeated dimebon administration, both 3- and 7-month-old animals acutely treated with dimebon demonstrated an insignificant decrease in the duration of time spent in the O-maze; these differences were far from the level of statistical significance. Single outliers, which might represent individual mice with increased sensitivity to the drug or/and testing procedures, could contribute to these insignificant changes. Of note, the latency of exit to open arms and the number of exits were virtually the same between the groups, again suggesting a lack of effect by dimebon on anxiety-related behaviour in the O-maze in our study. Thus, these data rule out the possibility of non-specific effects of dimebon on mouse performance in the step-down avoidance model for young animals.

All together, presented data suggest that dimebon enhances learning in both appetitive and inhibitory tasks of the hippocampus-dependent memory in mice. As it was mentioned above, dimebon administrated to a rat at the dose of 0.05–1 mg/kg was found to have a half-life in the plasma over 2 h (Giorgetti et al., 2010); similar doses used in the present work led to expect comparable pharmacokinetics of dimebon in employed here battery of memory tests. Together with the fact that dimebon was delivered 15 min prior to training, this let to propose that dimebon was present at an effective concentration in the mouse brain during phases of early and intermediate memory consolidation in both the Y-maze and step-down avoidance models (Gerlai, 2001; Cammarota et al., 2005; Da Silva Costa et al., 2009; Benchenane et al., 2010). The relatively short half-life of dimebon in rodents suggests that the non-specific effects of dimebon observed within 30 min after drug administration in the novel cage test are unlikely to underlie an increase in the latency of step down documented 24 h after training in the step-down avoidance paradigm. These changes are very likely to reflect the memory-enhancing effects of dimebon administration.

The mechanism underlying the memory enhancing effects of dimebon remains elusive. During the last few decades, the concept of multi-target drug activity has been proposed (Wong et al., 2008; Cavalli et al., 2008; Combarros et al., 2009). According to this concept, the mechanisms responsible for the beneficial actions of drugs can be realised via multiple actions of the compound on a number of receptors; in this case, effective changes in the concentrations of neurotransmitters may be much lower than if only one receptor signaling system underlies the drug's activity. On one hand, dimebon was shown to interact with a broad spectrum of neuronal receptors, which are involved in synaptic plasticity and cognitive functions (Lermontova et al., 2001; Schaffhauser et al., 2009; Grigoriev, 2009; Giorgetti et al., 2010; Okun et al., 2010). At the same time, effects from dimebon on pre-clinical and clinical measures of cognition were found at doses corresponding to brain concentrations much lower than the K1 values for many receptors which were found to be effective in models of learning and memory (Gold, 2006). Thus, it can be speculated that, in the mouse test battery employed in this study, dimebon affects a number of receptors and may act as a multi-target drug, i.e. the concentrations used in this study, which correspond to sub-threshold levels for receptor activation via a mono-target mechanism, are only able to evoke physiological effects by synergistic activation of several neurotransmitter systems (Youdim and Buccafusco, 2005; Cavalli et al., 2008). The memory-enhancing effects of dimebon may indicate simultaneous activity toward AMPA, NMDA, dopamine and serotonin receptors, all of which have been involved in inhibitory and appetitive hippocampus-dependent learning (Ungerer et al., 1998; Orsetti et al., 2001; Rogawski and Wenk, 2003; LaLumiere et al., 2003; Lynch and Gall, 2006; Balschun et al., 2006; Da Silva Costa et al., 2009; Benchenane et al., 2010). In particular, it has been hypothesised that dimebon's activity as a positive modulator of AMPA receptors and low affinity non-competitive blocker of NMDA receptors via a multi-drug mechanism, can explain the pro-cognitive action of this compound (Grigorev et al., 2003; Grigoriev 2009).

The last data suggest that it is unlikely that the effects of dimebon on memory presented here and in other reports occur via its action on neurotransmitter systems via a “one drug–one molecule” mechanism. For instance, a recent study showed that intragastrically delivered dimebon at a dose of 0.05–5.0 mg/kg is inefficient at the inhibition of AChE or blockade of the NMDA receptor; these doses, however, evoked pronounced improvement of new object recognition memory in rats. This dose range of dimebon did not affect the turnover of ACh in the hippocampus and prefrontal cortex, and is ineffective at blocking the NMDA-induced calcium influx; both effects observed at higher dimebon concentrations (Giorgetti et al., 2010; Wu et al., 2008). In another study, dimebon was found to bind to 5-HT6 receptor, where it enhanced social recognition memory; however, the weak binding affinity and the relatively low drug concentrations employed in the study cannot link the observed behavioural effects to changes in serotonin transmission (Schaffhauser et al., 2009). In addition, dimebon was shown to bind to a number of receptors, such as histamine, dopamine, norepinephrine and serotonin receptors, at concentrations which are not comparable to those used *in vivo* (Wu et al., 2008; Schaffhauser et al., 2009; Giorgetti et al., 2010). In sum, at present, a consistent view on the neurotransmitter mechanism that underlies the memory-enhancing effects of dimebon is lacking.

Interestingly, a recent study reported that dimebon had remarkable effects on hippocampal neurogenesis, at doses comparable to those used in the present study (Pieper et al., 2010). One-week treatment of rats with dimebon at a dose of 0.1 mkM/kg (0.32 mg/kg) increased the number of BrdU-positive cells in the dentate gyrus. Because activation of structural plasticity and neurogenesis in the hippocampus is well demonstrated to be implicated in the mechanisms of contextual and spatial learning (Epp et al., 2007; Yang et al., 2008; Li et al., 2010; Pieper et al., 2010), we suggest that this effect might be one of the potential mechanisms which underlie the memory enhancing action of dimebon observed in the current study. Further experiments are required to address the possible link

between the mnemonic effects of dimebon and its properties as a proneurogenic compound.

5. Conclusion

Recently, a series of new functional analogues of dimebon with predominant action on several proposed mechanisms discussed above was developed (Lermontova et al. 2003; Perlovich et al., 2009; Bachurin et al., unpublished results). A comparative analysis of these compounds in the battery of tests employed in the current study will hopefully help to elucidate its mechanism of memory-enhancing action in the future. Since the hippocampus was shown to be a primary brain structure, the function of which is compromised during early stages of Alzheimer's disease (Foerstl, 2009), this suggests the usefulness of the battery of mouse models of hippocampus-dependent memory employed in the present work for such studies as well as, in general, for fundamental and pre-clinical aspects of testing drug candidates for the treatment of this pathology.

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Anhedonic-like traits and lack of affective deficits in 18-month-old C57BL/6 mice: Implications for modeling elderly depression

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ABSTRACT

The prevalence of depression increases with aging. We hypothesized that like humans, old animals exhibit anhedonic-like behavior, along with signs of behavioral despair. In rodents, anhedonia, a reduced sensitivity to reward, which is listed as a core feature of major depression in the DSM-IVR, can be measured by a decrease in intake of and preference for sweet solutions. Here, sucrose intake, forced swimming, immobility in the modified tail suspension test, novelty exploration, grooming, anxiety and locomotor activity were compared in naïve 3- and 18-month-old male C57BL/6 mice. The absolute amounts and the ratio of consumed 1% sucrose solution to water intake was significantly smaller in 18-month-old mice than in 3-month-old mice. The consumption of 5%-sucrose solution requiring high levels of drinking effort, novelty exploration in two setups and grooming behavior in the splash test were reduced in older animals. Analysis of other behaviors suggested that the above-mentioned signs of anhedonic-like traits were unlikely to be attributable to the potential effect of aging on metabolic needs for water, taste perception, motor capabilities or the induction of essential anxiety and neophobia. A 4-week treatment with the antidepressant imipramine (7 mg/kg/day) or dimebon, a compound with suggested neuroprotective proneurogenic properties (1 mg/kg/day) restored sucrose intake and preference in 18-month-old mice. Meanwhile, young and old mice showed no differences in the parameters of behavioral despair evaluated in the forced swim and modified tail suspension tests. Thus, the behavioral profile of aged mice parallels that of humans with elderly depression, in whom the symptoms of hedonic deficits typically outweigh affective disturbances. The assessment of anhedonic-like traits with the sucrose preference test in 18-month-old mice will be useful in preclinical studies of elderly depression.

1. Introduction

The prevalence of depression is elevated in older people (Zanni and Wick, 2010; Glaesmer et al., 2011). The overall aging of the population suggests an increase in the social impact of elderly-related depressive disorders (Solhaug et al., 2011). According to the DSM-IVR, the cardinal symptom of major depression is clinically observed anhedonia, the decreased ability to experience pleasure, which is observed along with subsidiary features including affective disturbances (Hamilton, 1967; Klein, 1974). We hypothesized that old mice, like humans, exhibit anhedonic-like features and signs of behavioral despair when compared to younger animals and that these changes are sensitive to antidepressant treatment. Both features of a depressive-like state are likely to occur in parallel (Fonken et al., 2009; Snyder et al., 2011). For example, anhedonia-like behavior defined by a decrease in sucrose intake and preference in chronically stressed C57BL/6N mice accompanies an increase in floating during forced swimming and immobility behavior in the modified tail suspension test (Strekalova et al., 2004; Strekalova and Steinbusch, 2010; Kanarik et al., 2011). These changes are often associated with a reduction in novelty exploration and grooming behavior in the splash test (Strekalova et al., 2004; Skrinskaia and Nikulina, 1994; Pothion et al., 2004). Antidepressant treatment counteracts the above-mentioned depressive-like traits in rodents (Porsolt and Papp, 1998; Willner, 2005; Strekalova et al., 2011; Yacoubi et al., 2011; Surget et al., 2008).

A body of evidence suggests that older mice and rats have reduced interest in various hedonic stimuli, such as palatable food and drinking solutions, alcohol and a regular diet (Spear and Varlinskaya, 2010; Blanton et al., 1998). In the chronic stress model, older rats showed enhanced susceptibility to anhedonic-like changes in a sucrose test

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and a delayed response to the antidepressant effects of citalopram (Herrera-Pérez et al., 2008, 2010). Aging in rodents resulted in diminished place preference conditioned to a food (Rubinow et al., 2009), nicotine, cocaine, morphine and alcohol (Vastola et al., 2002; Campbell and Wood, 2000). It is also associated with lower novelty exploration (Vastola et al., 2002; Campbell and Wood, 2000) and decreased scores of grooming (Scimonelli et al., 1999; Shoji and Mizoguchi, 2011), which are considered to reflect a reduced self-rewarding activity (Pothion et al., 2004; Dubreucq et al., 2010; Gómez-Lázaro et al., 2011; Park et al., 2011). Together, these findings from animal studies parallel clinical features of elderly depression, which are predominantly related to hedonic deficits (Gallo et al., 1994, 1997).

Though affective disturbances are less typical of sentient depression (Lawton et al., 1996), translational studies report elevated scores of behavioral despair in elderly animal cohorts (Frye and Walf, 2009; Moretti et al., 2011). In the paradigm of operant extinction of escape from water, the removal of the platform in the Morris water maze induces higher scores of floating behavior in 24-month-old Wistar rats than in younger animals (Schulz et al., 2007). Similarly, the enhancement of floating behavior in the forced swim test evoked in BALB/c mice by lipopolysaccharide is two-fold higher in 20- to 24-month-old than in 3-month-old animals (Godbout et al., 2008).

The present study examined the parameters of reward sensitivity and behavioral despair in 18-month-old versus 3-month-old male mice of the C57BL/6N strain, which is commonly used in translational research on depression. The available literature suggests that aged rats and mice of various strains with behavioral signs of hedonic and affective deficits also show psychomotor disturbances when tested at certain ages (Schulz et al., 2007; Soffié et al., 1992; Bowman et al., 2006), but not at others (Frye and Walf, 2009; Moretti et al., 2011; David et al., 2001; Gould and Feiro, 2005). The efficacy of different classes of antidepressants also depends on the age of the experimental animals (Bourin et al., 1998). The use of animals within the age range at which depressive-like features emerge while general age-related behavioral changes are still minimal, and, thus, can be ignored, is important for minimizing confounding factors in rodent models of elderly depression. Our preliminary experiments demonstrated that depressive-like behaviors are accompanied by general locomotor and consumatory abnormalities in 25- but not 18-month-old C57BL6/N mice (Bolkunov et al., 2009). To determine whether 18-month-old animals display depressive features without generalized psychomotor deficits, they were compared to 3-month-old mice in an extended test battery for commonly used measures of anhedonia, behavioral despair, anxiety-like behavior, grooming, exploration and locomotion (Pothion et al., 2004; Surget et al., 2008; Strekalova, 2008; Strekalova and Steinbusch, 2009).

Clinical studies show that the symptoms of hedonic deficit typically outweigh affective disturbances in patients with elderly depression. The self-reported “lack in positive affect” is consistent in elderly patients with major depression, while the emergence of “negative affect” is variable (Lawton et al., 1996). Older primarily depressed patients were less likely to endorse crying spells, sadness, feeling fearful, being bothered, or feeling like a failure, and were less likely to have co-morbid anxiety, but tended to experience poor appetite and loss of interest in sex (Hybels et al., 2011). A study of more than 6500 participants showed that persons aged 65 years and older were less likely to acknowledge dysphoria than anhedonia (Gallo et al., 1994). A 13-year follow-up study of more than 1500 depressed patients aged 50 years and older revealed the significant prevalence of a form of disorder whose core criteria included hedonic deficit but not sadness or dysphoria (Gallo et al., 1997).

We therefore addressed whether both anhedonic and affective features change in C57BL6 mice of 18-month-old age. Anhedonic behavior defined by decreased sucrose intake and preference on one hand, and signs of behavioral despair in the forced swim and modified tail suspension tests on the other, have been extensively validated as parameters of reward sensitivity and affective traits in mice and rats (Porsolt and Papp, 1998; Willner, 2005; Strekalova et al., 2011; Yacoubi et al., 2011). In sum, using these paradigms and additional tests, the present study examined whether, in comparison with 3-month-old mice of the C57BL6N strain, 18-month-old mice (1) exhibit changes indicative of the depressive state in the sucrose test, novelty exploration, splash test, forced swim and modified tail suspension paradigms, (2) show the prevalence of one type of deficit, hedonic- or affective-like, over the other, whether (3) older animals have disturbances in daily liquid intake, anxiety-like behavior and locomotion that are non-specific to depressive-like traits and (4) parameters of depressive-like behavior in aged animals are sensitive to antidepressant treatment.

In addition to a classical antidepressant imipramine, we also assessed the effects of dimebon, an investigational drug against Alzheimer's and Huntington's diseases with proposed proneurogenic neuroprotective properties. Dimebon, a tetrahydro-g-carboline with anti-histamine effects, was reported to block the apoptosis of cortical neurons (Bachurin et al., 2001), protect the mitochondrial membrane potential (Bachurin et al., 2003; Pieper et al., 2010), promote neurite outgrowth in cultured hippocampal and cortical neurons (Protter et al., 2009; Bernales et al., 2009), dose-dependently increase neurogenesis in the rat dentate gyrus (Pieper et al., 2010), act *in vitro* as a low-affinity NMDA receptor blocker via the NR2B subunit (Perlovich et al., 2009) and enhance memory properties in middle-aged mice (Vignisse et al., 2011). The role of these factors in the neurobiology of depression was generally documented (Skolnick et al., 2009; Pittenger and Duman, 2008; Marcocci et al., 2002), however, it remains to be studied whether dimebon may exert any systemic effects via these mechanisms (Bachurin, 2003).

2. Material and methods

2.1. Animals, housing and general experimental conditions

Three- and 18-month-old C57BL/6N mice were used in this study. Following purchase from the supplier, mice were housed in individual cages for at least 2 weeks before testing. Standard laboratory conditions (22 ± 1 °C, 55% humidity, food and water *ad libitum*) and a reverse 12 h:12 h light–dark cycle (lights on: 21:00 h) were applied throughout the study. Mice were tested during the dark period of the light cycle. All experiments were carried out in accordance with the European Committees Council Directives.

2.2. Study design

2.2.1. Experiment 1: study of behavioral measures of hedonic sensitivity in old versus young mice

Experiment 1 examined the consumption of palatable solutions by old versus young mice using two paradigms of the sucrose intake test. A decrease in sucrose intake and preference over water is generally taken as a putative sign of anhedonia in rodents (Strekalova and Steinbusch, 2010; Porsolt and Papp, 1998; Willner, 2005). This experiment aimed to investigate the effects of aging on behavioral measures of hedonic status in mice. Three- and 18-month-old mice were tested in a two-bottle free-drinking paradigm with a 1% sucrose solution (see Section 2.3.1). Experimental groups comprised 15 mice, body weight was compared to young and old mice. To estimate liquid intake during aging, 24-h water consumption was compared between two groups in a separate test; each group comprised 10 animals (see Section 2.3.2).

The consumption of a 5% sucrose solution requiring a high level of drinking effort from a bottle with a narrow nipple was then assessed in young versus old animals in a one-bottle paradigm (see Section 2.3.3, five animals in each group). In order to assess the ability of sweet taste of older mice, the intake of a 5% sucrose solution from regular bottles was evaluated one week earlier in two groups of mice in a free-access one-bottle paradigm (see Section 2.3.3). In order to compare the physical ability of young and old mice to drink

in a situation that required high levels of effort, their water intake was estimated on the fourth day thereafter (between the two tests on sucrose intake; see Section 2.3.3).

2.2.2. Experiment 2: study of parameters of behavioral despair during aging

Experiment 2 scored parameters of behavioral despair in younger and older mice tested for hedonic sensitivity in the modified forced swim (Section 2.3.4) and the modified tail suspension (Section 2.3.5) tests; behavior was videotaped. First, CleverSys software (CleverSys, Reston, VA, USA) was applied for automated scoring of the floating and immobility behaviors, as well as the duration of swimming in the forced swim test and of locomotor activity in the modified tail suspension test. The duration of climbing behavior and the number of diving events were scored supplementary in the forced swim test. Automated scoring of behaviors was analyzed per 1-minute interval and per entire scoring period; for technical reasons, only the first 5 min were scored (see Sections 2.3.4 and 2.3.5).

Second, the latencies of the first episodes of floating and immobility behavior, as well as the duration of these behaviors, were scored manually off-line. Both tests were validated pharmacologically for sensitivity to antidepressant treatment. In a separate validation study, 10 mice at the age of 3 months were treated either with a single acute i.p. injection of imipramine at the dose 30 mg/kg 30 min prior to the first exposure to a forced swim or modified tail suspension tests, or with vehicle (see Section 2.4) Behavior was scored as described below (see Sections 2.3.4 and 2.3.5). This scheme of pharmacological validation was adapted from originally published studies with tricyclics in these tests (Steru et al., 1985, 1987; Porsolt et al., 1987). To enable a data comparison from two experiments, data obtained on older mice and on pharmacologically treated mice were normalized to the means of values of younger and vehicle-treated 3-month-old mice, respectively.

2.2.3. Experiment 3: study of parameters of locomotion, exploration, grooming and anxiety

Experiment 3 examined potential aging-related changes in the parameters of anxiety and locomotion that could interfere with the evaluation of depressive-like behaviors. Grooming behavior and three parameters of novelty exploration were scored in groups of both ages in the splash test, the True scan open-field and novel cage tests to further assess the hedonic/motivational state of old mice. Three- and 18-month-old mice were analyzed in the True scan open-field, novel cage, O-maze and dark/light box ($n = 12$ and $n = 11$, respectively; see Sections 2.3.6–2.3.10) and in the splash test ($n = 15$ in each group; see Section 2.3.8).

2.2.4. Experiment 4: effects of imipramine and dimebon on hedonic and affective behaviors

In Experiment 4, we investigated whether the classical antidepressant imipramine and potentially neuroprotective proneurogenic drug dimebon administered to 18-month-old mice for 4 weeks at the doses 7 mg/kg/day and 1 mg/kg/day, respectively, affected the parameters of the sucrose preference test, the latter indicating the state of anhedonia. These animals were tested for floating and immobility in visually scored modified forced swim and modified tail suspension tests (see Sections 2.3.4 and 2.3.5). The dose of imipramine was based on previous studies with CD1 mice, in which its chronic administration at 7 mg/kg/day effectively reduced the stress-induced decrease in sucrose intake and preference, floating behavior and alteration of hippocampal gene expression typical of the subgroup of mice susceptible to anhedonia (van Miegem et al., 2009). The dosage of dimebon was determined according to its efficacy in clinic (Doody et al., 2008), as well as in improving learning in the Morris water maze paradigm in aged Wistar rats treated with 1 mg/kg/day of this compound for 3 weeks (Lermontova et al., 2000) and in C57BL6N mice in a step-down avoidance and conditioned fear extinction tests (Vignisse et al., submitted for publication). Drugs were delivered in the drinking water (see Section 2.4).

Body weight and baseline behaviors, including the initial preference for a 1% sucrose solution (see Section 2.3.1), were evaluated in order to form experimental groups which are initially balanced in these parameters, as described elsewhere (Strekalova et al., 2006). Each group comprised 5 to 10 mice, the number of animals being indicated in the legend of Fig. 5.

2.3. Behavioral tests

2.3.1. Two-bottle sucrose preference test

Mice of both ages were simultaneously given a free choice between two bottles, one with 1% sucrose solution and another with tap water, for 8 h, between 09.00 and 17.00. The beginning of the test started with the onset of the dark (active) phase of the animals' cycle. No previous food or water deprivation was applied before the test. To minimize the spillage of liquids during the sucrose test, specially manufactured bottles with a glass tip (length of 6 cm, internal diameter 2 mm; they were inserted in flavorless rubber bottle-stoppers) were used. Special attention was devoted to sugar storage to avoid its contact with flavors and plastic, and to washing of bottles, where minimal amounts of detergent were used. Bottles were filled in advance (during the preceding evening) and were kept upside down for at least 12 h prior to testing. In order to balance the air temperature between the room and the drinking bottles, they were kept in the same room where the testing took place. This measure prevents the physical effect of liquid leakage resulting from growing temperature of air and pressure inside the bottles, when they are filled with liquids which are cooler than the room air. To prevent the possible effects of a side-preference in drinking behavior, the position of the bottles in the cage was switched after 4 h during the test. The test was carried out before weekly cage change. With this method proposed, the error of measurement of liquid intake does not exceed 0.1 ml.

The intake of water and 1%-sucrose solution and total intake was estimated by weighing the bottles before and after access to liquids. The preference for sucrose was calculated as the percentage of the sucrose solution consumed out of the total amount of liquid drunk:

$$\textbf{Sucrose Preference} = [\mathbf{V}(\textbf{Sucrose solution})/\mathbf{V}(\textbf{Sucrose solution}) + \mathbf{V}(\textbf{Water})] \times \mathbf{100}\%$$

Changes in sucrose preference in C57BL/6N mice evaluated using the above-described protocol correlated with molecular, electrophysiological, behavioral and biochemical alterations characteristic of a depressive-like state (Strekalova et al., 2011). Further details of methodological conditions were applied as described elsewhere (Strekalova and Steinbusch, 2010).

2.3.2. Daily water intake in one-bottle drinking test

A 24-h water drinking test was carried out simultaneously in both groups of mice in a one-bottle paradigm starting at 17.00. Specialized drinking bottles such as those employed in the above-described sucrose test (see Section 2.3.1) were used. A test procedure was carried out as described elsewhere (Strekalova and Steinbusch, 2010). The same precautions concerning possible liquid spillage as in the sucrose test were applied: bottles were filled with tap water in advance and kept in the experimental room 12 h prior to the test, being held upside down during this time. The intake of water was estimated by weighing the bottles before and after access to liquids. The test was carried out two days before weekly cage change.

2.3.3. Sucrose and water intake in high-effort and free-access one-bottle drinking tests

In a high-effort one-bottle drinking paradigm, the amount of concentrated sucrose solution ingested is taken as a measure of the

animal's motivation to receive a pleasurable stimulus. In this test, mice of both ages were simultaneously given access to a 5% sucrose solution or tap water for 8 h (between 09.00 and 17.00 h), the ingestion of which required a high level of effort due to the narrow bottle tip, the internal diameter of which was 1.0 mm instead of 2.0 mm as in a regular bottle. Preliminary studies showed that this modification resulted in a reduction in the 24-h water intake of at least 50% of normal amounts. The consumption of sucrose solutions from these bottles was high, suggesting that it can be used to measure animals' effort/motivation to receive a reward. The intake of a 5% sucrose solution was also evaluated in a single-bottle free-drinking paradigm using regular bottles for 8 h. The intake of water and sucrose solution was estimated by weighing the bottles before and after access to liquids.

2.3.4. Forced swim test

The test protocol was adapted from a previously described procedure with minor modifications (Porsolt and Papp, 1998; Strekalova et al., 2005; Ducottet et al., 2004). Mice were subjected to two 6-min swimming sessions spaced 24 h apart in a transparent cylinder (Ø 17 cm) filled with water (+23 °C, water height 13 cm, height of cylinder 20 cm, illumination intensity 25 lx). The slightly deeper water used in our protocol was shown to increase test sensitivity (Cryan et al., 2005a). First, by means of the data from the automated scoring system, the duration of floating, swimming and climbing behavior and the number of diving events during the first five minutes and during one-minute intervals within the scoring period were analyzed (see Section 2.2.2).

Second, scoring by visual observation was carried out by two independent observers who were blinded with regard to the animals' age and treatment (where applicable). The latency of the first episode of floating and the duration of floating behavior were recorded during 2-min intervals within the 6-min swimming session and for a total period on day 1 and day 2 of the test (see Section 2.2.2). Floating behavior was defined in accordance with the commonly accepted criteria of this behavior. Namely, a mouse was considered to be floating when only slight movements of its limbs and tail could be detected, without signs of searching activity; passive movements of the body while drifting on the surface of the water have no direction (Strekalova et al., 2005; Ducottet et al., 2004; Cryan et al., 2005a; Mineur et al., 2006). Latency of floating was determined as the time between the placement of a mouse in a water tank and the first bout of floating behavior.

2.3.5. Modified tail suspension test

The protocol used in this study was adapted from a previously proposed procedure (Mineur et al., 2006). Mice were subjected to the modified tail suspension by being hung by their tails with adhesive tape to a rod 50 cm above the floor for 6 min. Two animals were tested simultaneously in a dark room where only the area of the modified tail suspension construction was illuminated by a spotlight from the ceiling; the lighting intensity on the height of the mouse position was 25 lx. The trials were recorded by a video camera positioned directly in front of the mice while the experimenter observed the session from a distance in a dark area of the experimental room. This procedure was carried out twice with a 24-h interval between tests, similarly to previously reported protocols (Vaugeois et al., 1997; Gavioli et al., 2004). First, during the additional automated scoring, the duration of immobility and activity was scored during the first five minutes and during one-minute intervals within the scoring period (see Section 2.2.2).

Second, in line with generally accepted protocols, the latency of the first episode of immobility and the total duration of this behavior were scored manually as described elsewhere (Mineur et al., 2006; Vaugeois et al., 1997), and the scoring was performed during 2-min sub-intervals within a 6-min session and summarized for the entire period on day 1 and day 2 (see Section 2.2.2). Therefore, videotapes were scored by two independent observers who were blinded with regard to the animals' age and treatment by manual analysis, in accordance with the commonly accepted criteria of immobility (Cryan et al., 2005b; Ibarguen-Vargas et al., 2009). The immobility behavior was defined as the absence of any movements of the animals' head and body. The latency of immobility was determined as the time between the onset of the test and the first bout of immobility. The means of the values obtained by the two observers were analyzed.

2.3.6. TruScan open-field test

Mice were placed into True Scan activity boxes (26 cm × 26 cm × 39 cm; Coulbourn Instruments, Allentown, PA, USA) for 10 min. Boxes were evenly illuminated with white light, with an illumination intensity of 25 lx. Horizontal movements (speed), resting time, the duration of exploration during rearing and of nose poke behavior, were scored automatically by red beam cells using TruScan software (Coulbourn), as described elsewhere (Strekalova et al., 2004). Animals were placed in the experimental room at least 1 h prior to the experiment. The testing was carried out in the morning between 09.00 and 12.00. Mice of both ages were tested simultaneously; two animals were used in each run. Boxes were cleaned with water between the runs.

2.3.7. Novel cage test

The novel cage test was performed to assess exploration of a new environment, as described elsewhere (Strekalova et al., 2004). Mice were introduced into a standard plastic cage the size of their home cage filled with small amounts of fresh sawdust. The number of exploratory rearings was counted under red light during a 5-min period. The testing was carried out in a dark quiet room in morning hours. Behavior was videotaped and analyzed by trained observers blind to the animals' age.

2.3.8. Splash test

This test was performed as described elsewhere (Pothion et al., 2004; Surget et al., 2008). A 10% sucrose solution was spread on the dorsal surface of a mouse coat, which because of its high viscosity induces lasting grooming behavior in mice. As animals were single-housed, no prior isolation needed to be applied on this occasion. The parameters generally accepted for splash tests, i.e. latency of the first episode of grooming, number of grooming episodes and duration of grooming behavior, were measured in 3-and 18-month-old mice by trained observers.

2.3.9. Elevated O-maze

The apparatus (Technosmart, Rome, Italy), which consisted of a circular path (runway width 5.5 cm, diameter 46 cm), was placed 50 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 25 lx. The apparatus was placed on a dark surface in order to reduce reflection and maintain control over lighting conditions during testing. Anxiety-like behavior was assessed using previously validated parameters (Strekalova et al., 2005; Vignisse et al., 2011). Mice were placed in one of the closed-arm compartments of the apparatus. The latency of the first exit to the anxiety-related open compartments of the maze, the total duration of time spent therein and the number of exits to the open arms were scored during a 5-min observation period.

2.3.10. Dark/light box

The dark/light box (Technosmart, Rome, Italy) consisted of two plexiglass compartments, one black/dark (15 cm × 20 cm × 25 cm) and one lit (30 cm × 20 cm × 25 cm), connected by a tunnel. Anxiety-like behavior was assessed by earlier validated measures (Strekalova et al., 2005; Vignisse et al., 2011). Mice were placed into the dark compartment, from where they could visit the lit box, illuminated by light of 25 lx intensity. The latency of the first exit to the light compartment, the total duration of time spent in the lit box and the number of visits to this anxiety-related compartment were scored by visual observation over 5 min.

2.4. Drugs and drug administration

For a validation study with tests for affective behavior, imipramine (Sigma Aldrich, St. Louis, MO, USA) was dissolved in water for injection and administrated to mice via a single i.p. injection at the dose of 30 mg/kg 30 min prior to the first exposure to forced swim or modified tail suspension tests. The same treatment scheme was applied to administer a vehicle; the volume of imipramine and vehicle injections was 0.01 ml/kg.

In the study with 3- and 18-month-old mice, imipramine (from the same source as in above-mentioned study) and dimebon (obtained from the Institute of Physiologically Active Compounds, Chernogolovka, Russia) were administered for 4 weeks via drinking water at the doses of 7 and 1 mg/kg/day, respectively. Both drugs were dissolved in tap water; the solutions were changed on average every 3 and 5 days, respectively. Due to the light sensitivity of imipramine, its solution was renewed more frequently, and the bottles were covered with aluminum. Calculation of the drug concentrations in drinking water was based on the means of a 3-day evaluation of daily water consumption in both groups of mice, which were in fact similar (Fig. 1D), and on the desirable treatment dosage. This method of dosing in C57BL6N mice was previously validated in chronic stress experiments using citalopram (Strekalova et al., 2006).

2.5. Statistical analysis

Data were analyzed using the statistical software package Prism 5 (Chicago, IL, USA) unless otherwise specified. Despite normal distribution of the data from drinking tests, two-group comparisons were analyzed with non-parametric Mann–Whitney *U* test and multiple group comparisons were treated by non-parametric Kruskal–Wallis test, since few values were close to the error of measurement and they were considered as arbitrarily taken. Because of a limited accuracy of manual scoring for the forced swim and modified tail suspension behaviors, these data were also analyzed using this method. To compare variances, F test was applied. Body weight, other visually scored behaviors and the automated scores of the forced swim and modified tail suspension were analyzed using an unpaired *t*-test; the latter analysis was carried out using CleverSys software (CleverSys, Reston, VA, USA). The level of confidence was set at 95% ($p<0.05$).

3. Results

3.1. Experiment 1: study of behavioral measures of hedonic sensitivity in old versus young mice

3.1.1. Two-bottle sucrose preference test

In Experiment 1, 18-month-old animals showed significantly lower preference to 1% sucrose solution over water ($p=0.0005$, $U=33.0$; Fig. 1A), lower sucrose intake ($p=0.0016$, $U=41.0$; Fig. 1B) and a tendency to drink more water ($p=0.0704$, $U=76.5$; Fig. 1C) compared to 3-month-old mice. The 24-h water intake in a one-bottle paradigm did not differ between groups ($p=0.39$, $U=41.5$; Fig. 1D), demonstrating their similar metabolic need for water. Aged mice had a significantly higher body weight ($p=0.016$, $t=12.85$, $df=28$; Fig. 1E) than the young mice, suggesting that the above-described differences in sucrose intake and preference are unrelated to differences in body mass and caloric requirement. The reproducibility of decreases in sucrose intake and preference found in 18-month-old mice was demonstrated in independent experiments; there were no seasonal effects observed in these studies (*data not shown*).

3.1.2. Sucrose and water intake in high-effort one-bottle drinking test

In the high-effort one-bottle drinking test, the amount of 5% sucrose liquid consumed was taken as a measure of the animal's motivation to receive a pleasurable stimulus. This parameter was significantly lower in 18-month-old mice than in 3-month-old mice ($p=0.01$, $U=3.0$; Fig. 1G). While young mice showed a significant increase in 5% sucrose intake in comparison to intake of water, which was separately evaluated in a high-effort drinking paradigm ($p=0.02$, $U=21.0$; Fig. 1G), older animals showed no such difference ($p>0.05$, $U=6.0$). In this experiment, the two groups of mice showed similar levels of water intake ($p>0.05$, $U=15.5$) suggesting comparable ability to drink from bottles with narrow tips for 3- and 18-month-old animals. The intake of the 5% sucrose solution in free-drinking conditions showed no difference between groups, indirectly demonstrating the undisturbed ability of the older group to taste the sucrose solution at the concentration employed ($p>0.05$, $U=15.0$; Fig. 1G).

3.2. Experiment 2: study of parameters of behavioral despair during aging

3.2.1. Forced swim test

Automated analysis revealed no differences between young and aged groups in the total duration of floating behavior ($p=0.31$, $df=26$; *t*-test) and swimming behavior ($p=0.26$, $df=26$; data not shown). Experimental groups showed similar mean values for these variables measured during one-minute intervals ($p>0.05$; Fig. 2A). The duration of climbing and the number of diving events did not differ between 3- and 18-month-old animals ($p=0.93$, $df=28$ and $p=0.11$, $df=26$, respectively; Fig. 2B,C). Aged animals demonstrated significantly higher variability in the latter behavior than young mice ($p<0.0001$, F test).

Manual analysis of the forced swim test showed that 3- and 18-month-old mice had similar values of latencies of floating on day 1 (3-month-old mice: 92.9 ± 9.3 s, 18-month-old mice: 105.9 ± 6.6 s; $p=0.06$, $U=74.5$) and day 2 (3-month-old mice: 9.1 ± 2.3 s, 18-month-old mice: 11.3 ± 3.3 s; $p=0.39$, $U=105.0$; Fig. 3A,C). The imipramine-treated group had longer latency of floating than vehicle-treated mice (day 1: $p=0.005$, $U=29.0$, day 2: $p=0.0007$, $U=17.5$). Multiple comparison revealed significant differences in the normalized values of this parameter between three groups of 3-month-old mice (day 1: $p=0.01$, Kruskal–Wallis test $=8.49$; day 2: $p=0.004$, Kruskal–Wallis test $=10.74$). Total duration of floating was similar in the age comparison experiment on day 1 ($p=0.3$, $U=87.5$, mean values of floating in young and old mice: 154.2 ± 13.7 s and 143.9 ± 12.0 s, respectively) and day 2 ($p=0.09$, $U=71.0$, mean values of floating in young and old mice: 250.7 ± 10.6 s and 234.1 ± 7.6 s, respectively; Fig. 3B,D). This parameter was significantly lower in imipramine-treated versus vehicle-treated mice on days 1 and 2 ($p=0.004$, $U=15.0$ and $p=0.02$, $U=23.0$, respectively). Multiple comparison revealed significant differences in normalized values of total duration of floating between three groups of 3-month-old mice (day 1: $p=0.04$, Kruskal–Wallis test $=6.02$; day 2: $p=0.02$, Kruskal–Wallis test $=8.14$). A comparison of scores of floating measured over 1- or 2-min intervals did not reveal any differences between the groups of two ages (*data not shown*).

3.2.2. Modified tail suspension test

Analysis with the CleverSys software found no differences between 3- and 18-month-old mice in the total duration of immobility behavior ($p=0.18$, $df=23$) and locomotor activity ($p=0.12$, $df=23$; data not shown). Aged and young animals showed no significant differences in the duration of these behaviors scored during one-minute intervals ($p>0.05$; Fig. 2C).

Manual analysis of the modified tail suspension test indicated that 3- and 18-month-old mice had no difference in latencies of immobility on day 1 (3-month-old mice: 36.4 ± 6.5 s, 18-month-old mice: 43.3 ± 7.7 s; $p=0.35$, $U=95.5$; Fig. 3E) and day 2 (3-month-old mice: 33.3 ± 6.3 s, 18-month-old mice: 37.1 ± 5.8 s; $p=0.32$, $U=101.0$; Fig. 3G). Animals injected with imipramine demonstrated significant increase of latency of immobility as compared with vehicle-treated mice on day 1 ($p=0.01$, $U=20.0$) and day 2 ($p=0.03$, $U=30.5$). Multiple

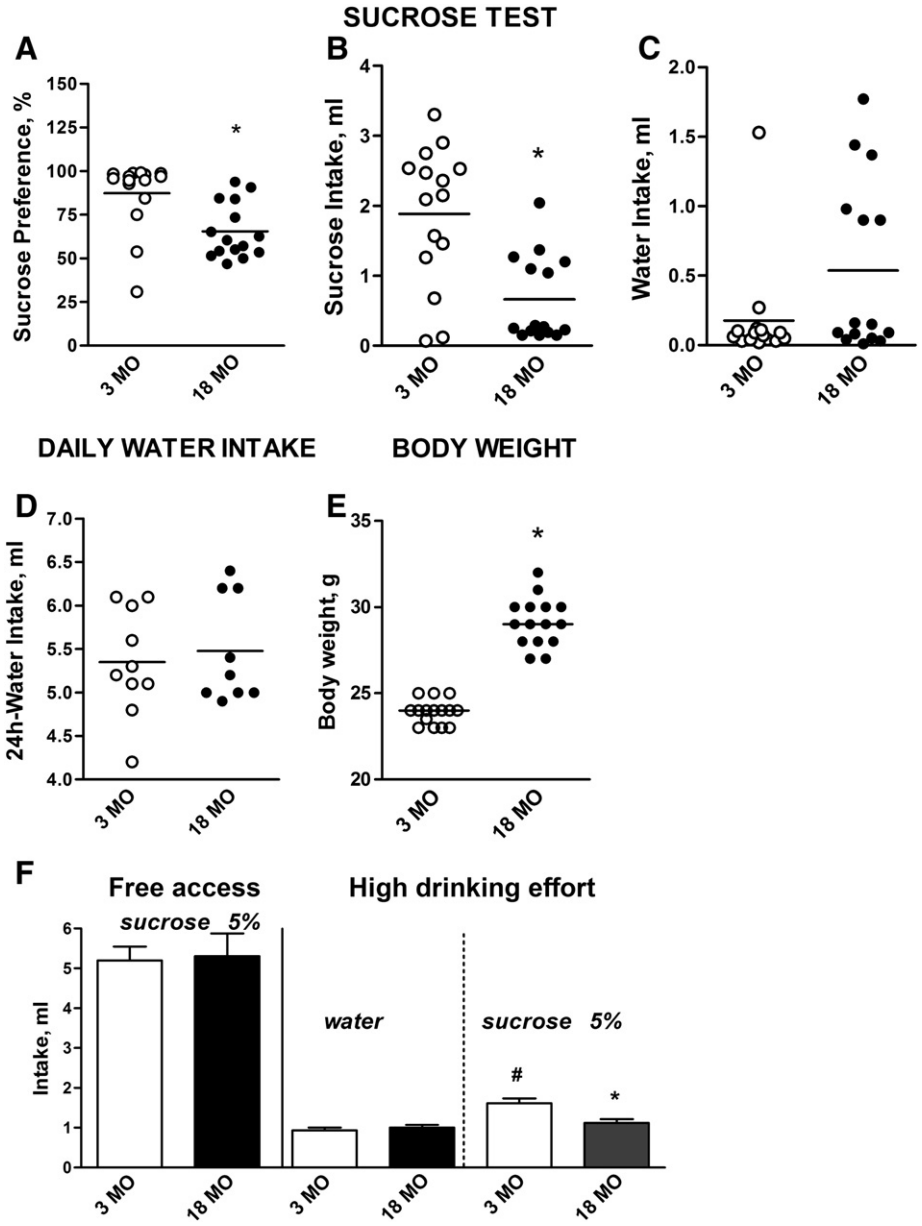

Fig. 1. Decreased sucrose intake and preference in 18- versus 3-month-old mice. In a free-access paradigm, in comparison with 3-month-old mice, 18-month-old mice exhibited significantly lower sucrose preference (A), decreased sucrose intake (B) and a tendency to increased water intake (C) (*$p<0.05$, 3- vs. 18-month-old mice; Mann–Whitney *U*-test). (D) Twenty-four-hour water consumption was similar in both groups ($p>0.05$, 3- vs. 18-month-old mice; Mann–Whitney *U*-test). (E) Body weight was significantly higher in aged mice (*$p<0.05$, 3- vs. 18-month-old mice; unpaired *t*-test). (F) In a high-effort drinking test, intake of 5% sucrose solution was significantly lower in 18-month-old mice than in 3-month-old mice (*$p<0.05$, 3- vs. 18-month-old mice; Mann–Whitney *U*-test). The young mice showed significantly higher consumption of 5% sucrose solution than intake of water (#$p<0.05$, intake of water vs. 5% sucrose solution, 3-month-old mice, Mann–Whitney *U*-test); older animals showed no such difference ($p>0.05$, intake of water vs. 5% sucrose solution, 18-month-old mice Mann–Whitney *U*-test). There was no significant difference in water intake between the groups ($p>0.05$, 3- vs. 18-month-old mice, Mann–Whitney *U*-test). The intake of 5% sucrose solution in free-drinking conditions did not differ between groups. Bars represent the means of the groups. Each column represents the mean ± SEM. 3 MO 3-month-old mice (A–C: n = 15; D–E: n = 10; F: n = 5); 18 MO: 18-month-old mice (A–C: n = 15; D–E: n = 10; F: n = 5).

comparison revealed significant differences in normalized values of latency of immobility between three groups of 3-month-old mice (day 1: $p=0.02$, Kruskal–Wallis test = 7.11; day 2: $p=0.02$, Kruskal–Wallis test = 7.96). 3- and 18-month-old mice did not differ in duration of immobility on day 1 (3-month-old mice: 160.1 ± 10.4 s, 18-month-old mice: 150.0 ± 9.6 s; $p=0.54$, $U=84.0$; Fig. 3F) and day 2 (3-month-old mice: 242.1 ± 10.7 s, 18-month-old mice: 227.4 ± 8.8 s; $p=0.16$, $U=78.0$; Fig. 3H). Imipramine-treated mice showed decreases in duration of immobility on days 1 and 2 ($p=0.04$, $U=27.0$ and $p=0.002$, $U=14.0$). Similarly to the outcome of the forced swim test, potentially more sensitive evaluation of both measured parameters at shorter intervals failed to reveal any differences between the groups (*data not shown*).

3.3. Experiment 3: study of locomotor, exploration, grooming and anxiety-like behaviors

3.3.1. True Scan open-field and novel cage tests

In the True Scan open-field test, the mean speed of movement and resting time were similar in 3- and 18-month-old mice ($p=0.80$ df = 21 and $p=0.54$, df = 21.0; respectively; Fig. 4A,B). In comparison to young mice, older mice displayed a significantly reduced duration of nose poke behavior and rearing activity in the open-field test ($p=0.007$, df = 19 and $p=0.02$, df = 18, respectively; Fig. 4C,D) and fewer rearings in the novel cage test ($p=0.04$, df = 71; Fig. 4E). These data demonstrate the lack of locomotor disturbances under the lighting conditions used in the behavioral studies of 18-month-

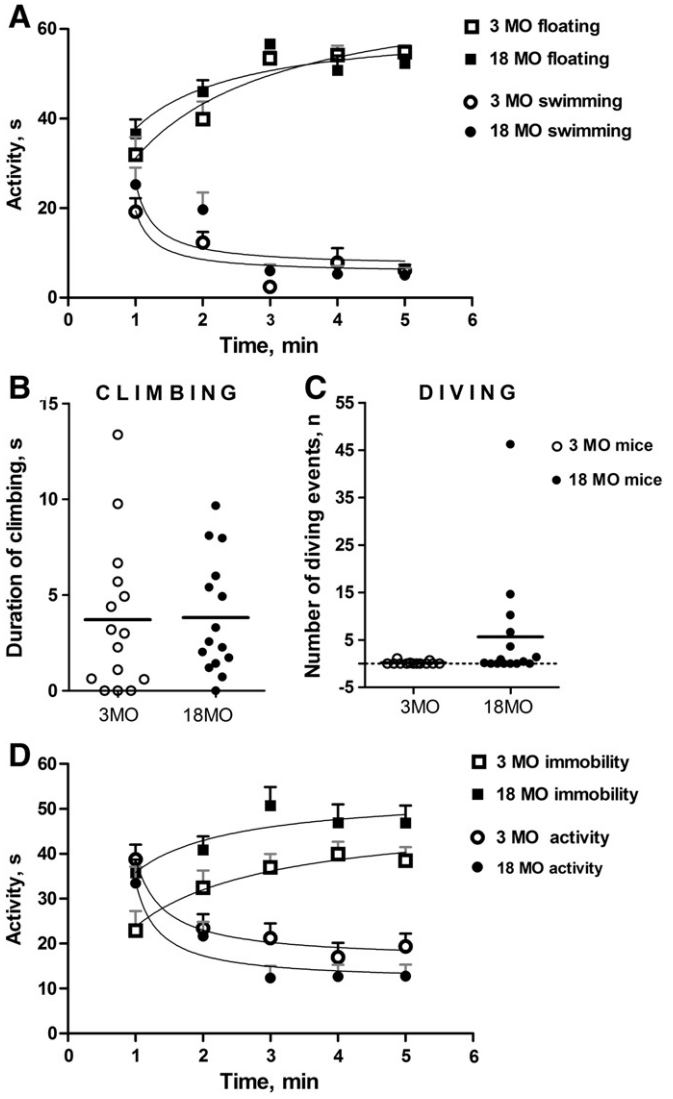

Fig. 2. Automated analysis of the forced swim and modified tail suspension tests in young and old mice. (A) In the forced swim test, 3- and 18-month-old mice showed a similar mean duration of floating and swimming behaviors scored using the CleverSys software during one-minute intervals ($p > 0.05$; unpaired *t*-test). (B) Young and aged mice showed a similar duration of climbing and (C) number of diving events ($p > 0.05$; unpaired *t*-test). (D) In the modified tail suspension test, groups of aged and young animals showed no differences in the total duration of immobility behavior and activity. Bars represent the means. 3 MO: 3-month-old mice ($n = 15$); 18 MO: 18-month-old mice ($n = 15$).

old mice and their lowered exploration of novelty, compared with 3-month-old animals.

3.3.2. Grooming behavior in splash test

Compared with 3-month-old animals, 18-month-old mice exhibited significantly longer latency of the first episode of grooming ($p = 0.03$, $U = 58.5$), lower number of grooming episodes ($p = 0.04$, $U = 63.5$) and shorter duration of grooming behavior ($p = 0.02$, $U = 57.0$; Fig. 4F).

3.3.3. Anxiety-like behavior

In the O-Maze and Dark/light box paradigms, 18-month-old mice showed non-significantly longer latencies of exit to anxiety-related areas than 3-month-old mice ($p = 0.07$, $df = 20$ and $p = 0.69$, $df = 20$, respectively; Fig. 4G,J). Both groups had similar time spent in open arms ($p = 0.30$, $df = 20.0$; Fig. 4H) and lit compartment ($p = 0.40$, $df = 20$; Fig. 4K), as well as number of exits to the open arms ($p = 0.43$, $df = 20$; Fig. 4I) and to the lit box ($p = 0.83$, $df = 20$; Fig. 4L). These data suggest overall similar scores of anxiety-like behavior in 3- and 18-month old mice in employed here testing conditions.

3.4. Experiment 4: effects of imipramine and dimebon in tests for depressive-like behavior

3.4.1. Sucrose preference test

Baseline testing in the sucrose test (before the onset of chronic drug administration) revealed significantly lower sucrose intake and preference in 18-month-old than in 3-month-old mice ($p < 0.0001$, $U = 2.0$ and $p = 0.0007$, $U = 6.5$, respectively; *data not shown*); older animals consumed significantly more water ($p = 0.0007$, $U = 6.5$), but their total liquid intake did not differ from that of younger animals ($p = 0.10$, $U = 25.0$). After chronic dosing, sucrose intake and preference were significantly changed ($p = 0.003$, Kruskal–Wallis statistic = 16.21 and $p < 0.0001$, Kruskal–Wallis statistic = 25.21, respectively). In imipramine-treated 18-month-old mice, both parameters were significantly higher than in non-treated mice of this age ($p = 0.003$, $U = 2.00$; and $p = 0.002$, $U = 0.00$, respectively; Fig. 5A,B). While these parameters were significantly decreased in non-treated 18- versus 3-month-old mice ($p = 0.0007$, $U = 6.5$ and $p < 0.0001$, $U = 2.0$, respectively), such differences were not found in aged imipramine-treated groups ($p = 0.95$, $U = 24.5$ and $p = 0.59$, $U = 20.0$). Treatment with imipramine did not affect either of these variables in young mice ($p = 0.13$, $U = 19.50$ and $p = 0.36$; $U = 25.0$, respectively).

Similarly to imipramine-treated aged mice, 18-month-old mice dosed with dimebon showed a significantly higher sucrose intake and preference than non-treated mice of this age ($p = 0.002$, $U = 7.0$ and $p = 0.04$, $U = 17.0$; Fig. 5A,B). Sucrose intake in aged dimebon-treated mice did not differ from that in young mice ($p = 0.63$, $U = 43.0$), while sucrose preference was significantly higher in the 3-month-old group ($p = 0.002$, $U = 11.0$). The latter effect was due to increased water intake in the dimebon-treated 18-month-old group versus the 18-month-old non-treated group ($p = 0.003$, $U = 8.0$). Sucrose preference was significantly higher in imipramine-treated than in dimebon-treated 18-month-old mice ($p = 0.001$, $U = 1.0$), while sucrose intake did not differ ($p = 0.85$, $U = 23.0$).

Multiple group comparison indicated that water intake was significantly altered ($p = 0.0004$, Kruskal–Wallis statistic = 20.64, Fig. 5C). Treatment with imipramine did not affect water intake in young animals ($p = 0.31$, $U = 24.0$) and decreased this variable in old group ($p = 0.006$, $U = 2.0$); as it is mentioned above, dimebon reduced this measure in 18-month-old mice ($p = 0.003$, $U = 8.0$). Multiple group comparison revealed significant changes in total intake of liquids ($p = 0.01$ Kruskal–Wallis statistic = 12.55, Fig. 5D); this parameter was augmented in 18-month-old mice treated with dimebon versus non-treated aged animals ($p = 0.003$, $U = 8.0$) and was not changed in imipramine-treated 3-month-old mice ($p = 0.31$, $U = 24.0$) nor in 18-month-old mice ($p = 0.35$, $U = 13.0$, vs. young mice).

3.4.2. Effects of imipramine and dimebon on measures of affective behavior of young and old mice

Manual scoring of the forced swim test showed that 3- and 18-month-old imipramine-treated mice had higher latency of floating than non-treated animals (day 1: $p = 0.005$, $U = 8.5$ and $p = 0.08$, $U = 10.0$, day 2: $p = 0.008$, $U = 8$ and $p = 0.007$, $U = 3$, respectively; Fig. 5E,F). No difference between dimebon-treated and non-treated groups of old mice in this parameter was revealed (day 1: $p = 0.93$, $U = 38.5$, day 2: $p = 0.79$, $U = 36.5$). Experimental groups 3- and 18-month-old mice treated with imipramine showed significantly decreased duration of floating than non-treated control animals (day 1: $p = 0.03$, $U = 16.0$ and $p = 0.04$, $U = 12.50$; day 2 ($p = 0.004$, $U = 6.0$ and $p = 0.005$, $U = 3.0$, respectively). A comparison of scores of floating measured in dimebon-treated and non-treated 18-month-old mice did

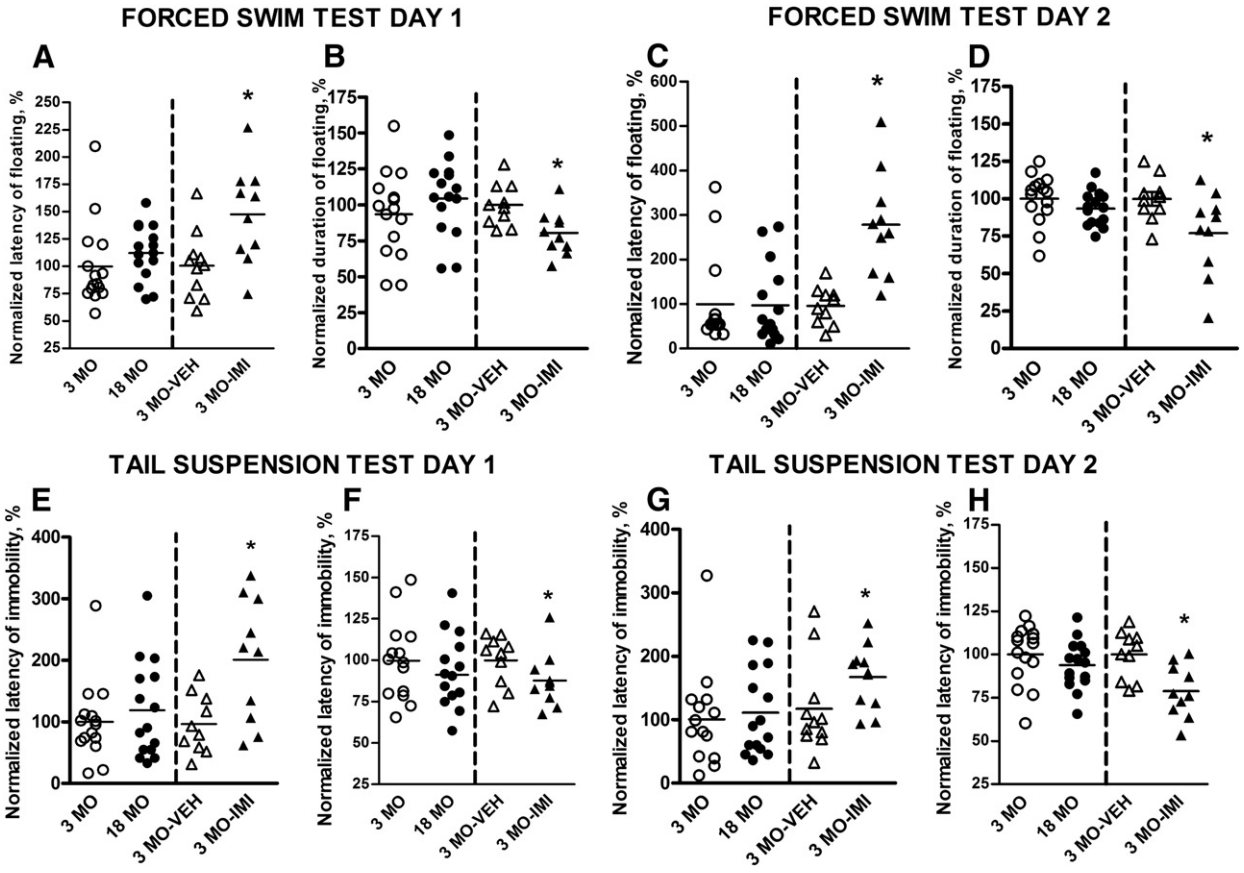

Fig. 3. Similar floating and immobility behaviors of visually scored 3- and 18-month-old mice. On both day 1 (A) and day 2 (C) of the forced swim test, imipramine-treated group had elevated latency of floating (*$p<0.05$, vs. vehicle-treated and #$p<0.05$, vs. non-treated 3-month-old mice); 3- and 18-month-old mice had a similar latency of floating ($p>0.05$; Mann–Whitney *U*-test). On day 1 (B) and day 2 (D), duration of floating was lower in imipramine-treated group (*$p<0.05$, vs. vehicle-treated and #$p<0.05$, vs. non-treated 3-month-old mice); and did not differ between 3- and 18-month-old animals ($p>0.05$; Mann–Whitney *U*-test). In the modified tail suspension test, on both day 1 (E) and day 2 (G), imipramine-treated group had elevated latency of immobility (*$p<0.05$, vs. vehicle-treated and #$p<0.05$, vs. non-treated 3-month-old mice); 3- and 18-month-old mice had a similar latency of immobility ($p>0.05$; Mann–Whitney *U*-test). On day 1 (F) and day 2 (H), duration of immobility was lower in imipramine-treated group (*$p<0.05$, vs. vehicle-treated and #$p<0.05$, vs. non-treated 3-month-old mice); and did not differ between 3- and 18-month-old animals ($p>0.05$; Mann–Whitney *U*-test). Bars represent the means. 3 MO: 3-month-old mice ($n=15$); 18 MO: 18-month-old mice ($n=15$); 3 MO-VEH: vehicle-treated 3-month-old mice ($n=10$); 3 MO-IMI: imipramine-treated 3-month-old mice ($n=10$).

not reveal any differences between the groups (day 1: $p=0.73$, $U=31.0$; day 2: $p=0.89$, $U=38.0$).

Similarly to the outcome of the forced swim test, manual analysis of the modified tail suspension test revealed the differences between the same groups. Young and aged imipramine-treated mice showed significantly longer latencies of the immobility as compared with non-treated control groups (on day 1: $p=0.04$, $U=17.5$ and $p=0.02$, $U=7.0$ and day 2: $p=0.04$, $U=17.5$ and $p=0.02$, $U=4.0$ respectively; Fig. 5G,H). No difference between dimebon-treated and non-treated groups of old mice in this parameter was found (day 1: $p=1.0$, $U=31.5$, day 2: $p=0.86$, $U=37.5$). Imipramine-treated 3- and 18-month-old mice showed significantly decreased duration of immobility as compared with non-treated control animals (day 1: $p=0.01$, $U=12.0$ and $p=0.04$, $U=8.0$, and day 2: $p=0.008$, $U=8.0$ and $p=0.003$, $U=2.0$ respectively). This parameter was not changed in old mice treated with dimebon as compared with non-treated animals of that age (day 1: $p=0.89$, $U=38.0$; day 2: $p=0.45$, $U=31.0$).

4. Discussion

4.1. Anhedonic-like traits of older mice and their reversal by antidepressant treatment

In a 1% sucrose preference test, 18-month-old mice showed significantly reduced sucrose intake and preference (Fig. 1A,F) that were previously shown to correlate with features of depression in stressed C57BL/6N mice (Strekalova and Steinbusch, 2009, 2010; Strekalova et al., 2008, 2011; Strekalova, 2008; Tokarski et al., 2009). Such abnormalities were not observed in a subset of stressed resilient mice whose sucrose intake and preference was preserved during stress, nor in stressed animals chronically treated with antidepressants imipramine or citalopram. From that, the data obtained here suggest hedonic deficit in 18-month-old mice. Restoration of normal sucrose intake and preference in these animals by a 4-week treatment with the classical antidepressant imipramine (Fig. 5A,B) supports this suggestion. Importantly, the decrease in sucrose preference in 18-month-old mice occurred at the expense of both a reduction in sucrose intake (Fig. 1B) and an increase in water consumption (Fig. 1C); the latter effect was compensatory, as the total liquid intake and 24-h water consumption was unaltered. The concomitance of a decrease in sucrose intake and preference additionally points to the occurrence of anhedonia in older mice.

The amount of highly palatable 5% sucrose solution consumed in a high-effort drinking paradigm was significantly lower in 18-month-old mice (Fig. 1G); such effects in analogous paradigms are regarded as a sign of decreased hedonic sensitivity (Hsiao and Chen, 1995). The similar intake of water in the two groups rules out a potential physical deficit as a possible confound in the assay. Comparable intake values of a 5%- sucrose solution in non-restricted drinking conditions in two groups suggest them to be equally capable of tasting a sucrose solution. Thus, diminished intake of sucrose solution by aged animals

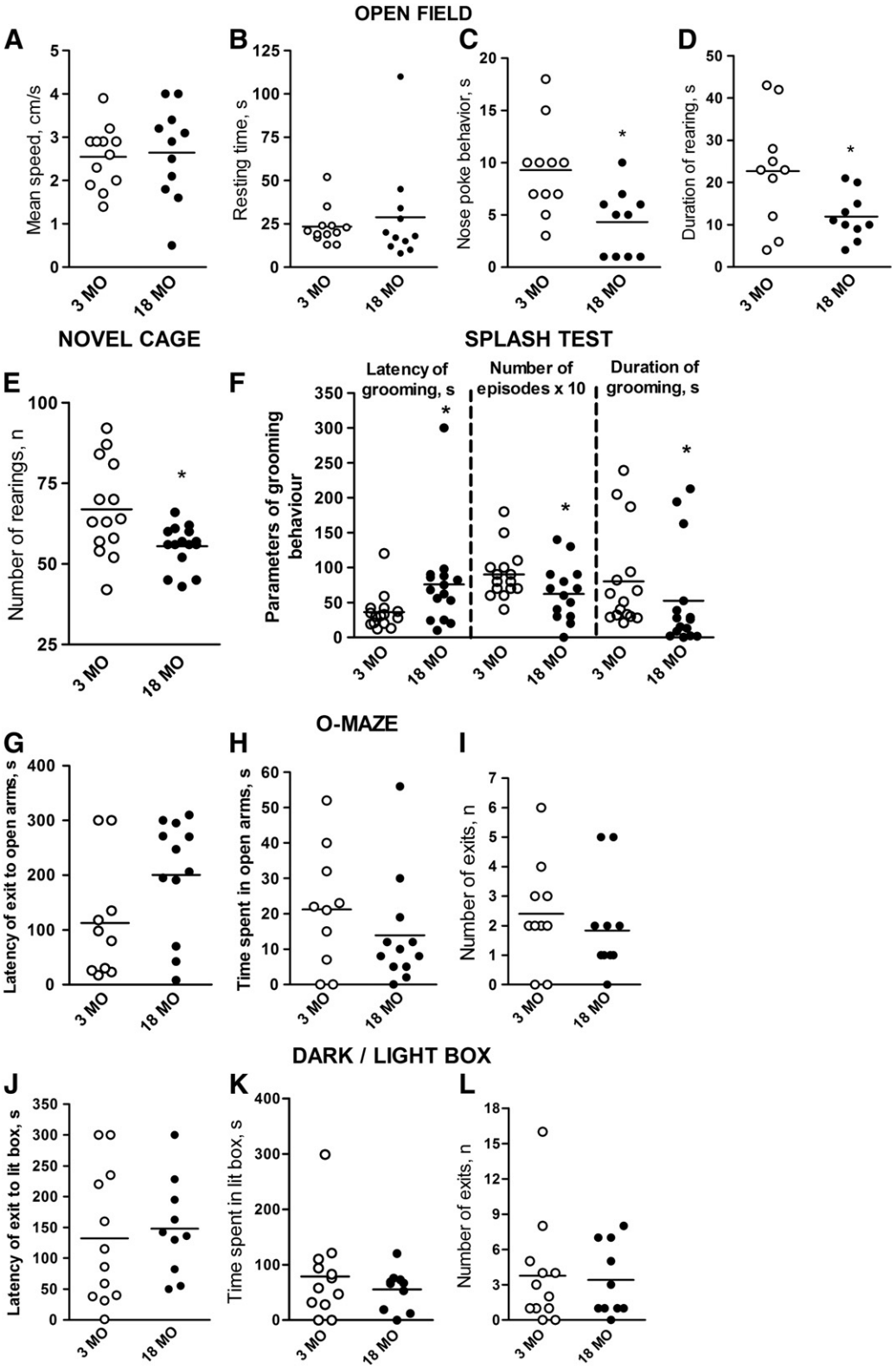

Fig. 4. Parameters of locomotion, exploration, grooming and anxiety-like behavior in 3- versus 18-month-old mice. Mean speed (A) and resting time (B) were similar in old and young mice ($p > 0.05$; unpaired *t*-test). In comparison to young mice, older mice displayed a significantly shorter duration of nose poke behavior (C) and rearing activity (D) in the open-field test (*$p < 0.05$; unpaired *t*-test), and fewer rearings in the novel cage test (E) (*$p < 0.05$; unpaired *t*-test). (F) 18-month-old mice showed higher latency of the first episode of grooming, lower number and duration of grooming episodes than 3-month-old mice (*$p < 0.05$; Mann–Whitney *U*-test). In the O-Maze test, in comparison with 3-month-old mice, 18-month-old animals showed non-significantly longer latencies of exit to the open arms (G) and similar values of time spent therein (H) and number of exits to this area (I) ($p > 0.05$; unpaired *t*-test). In the dark/light box, there were no differences between the groups in latency of exit to the lit compartment (J) time spent in this zone (K) or the number of exits to the illuminated box (L) ($p > 0.05$; unpaired *t*-test). Bars represent the means. 3 MO: 3-month-old mice (A–E and G–L: n = 12; F: n = 15); 18 MO: 18-month-old mice (A–E and G–L: n = 11; F: n = 15).

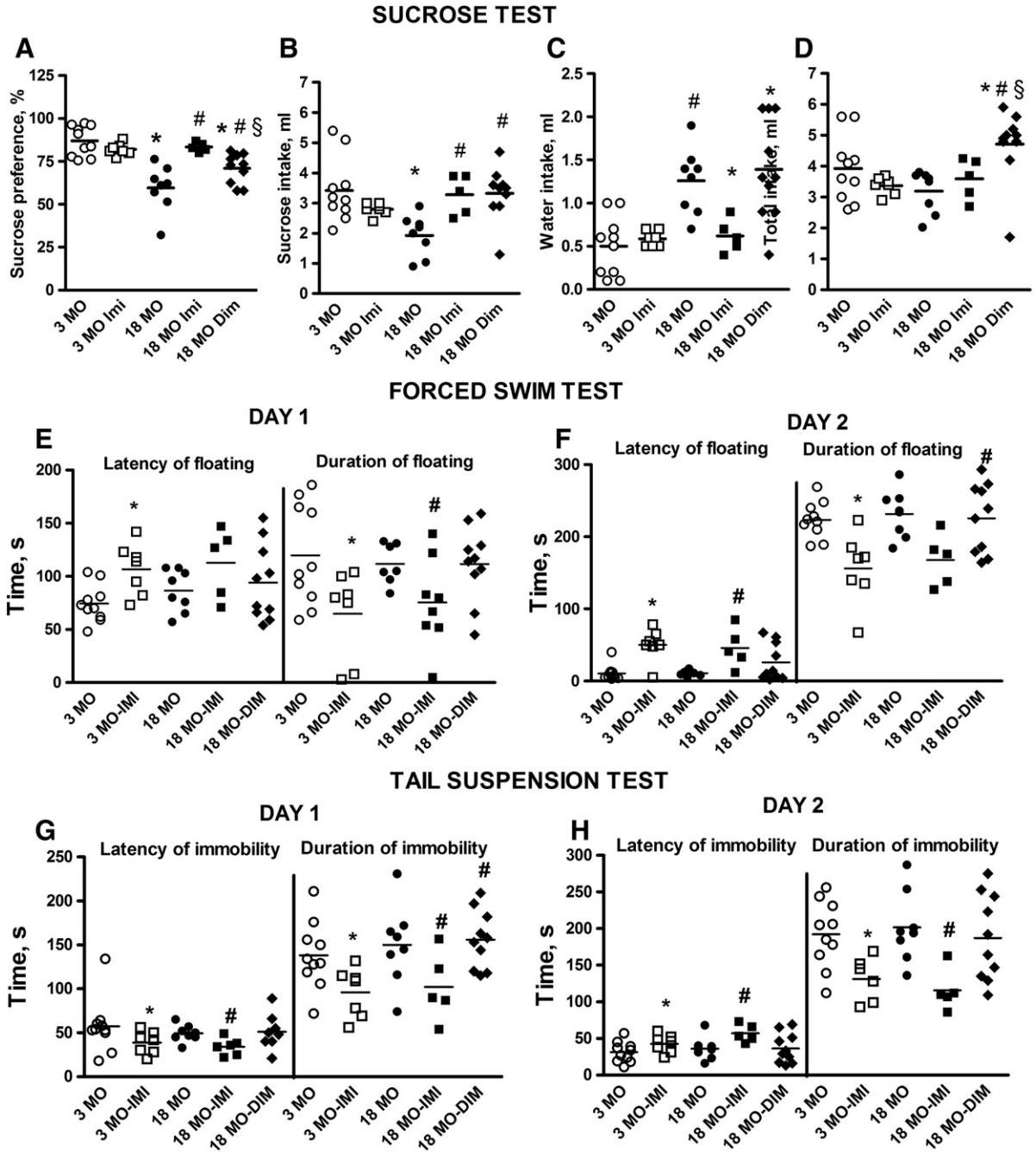

Fig. 5. (A) Sucrose preference was decreased in non-treated older animals (*$p<0.05$, old vs. young mice; Mann-Whitney U-test), but not in 18-month-old mice treated with imipramine ($p>0.05$); it was significantly elevated in both treated groups (#$p<0.05$, vs. 18-month-old non-treated group; Mann-Whitney U-test). Dimebon-treated 18-month-old group had significantly lower sucrose preference than young mice (*$p<0.05$; Mann-Whitney U-test) and mice of this age that received imipramine (§$p<0.05$; Mann-Whitney U-test). (B) Sucrose intake was significantly reduced in older group (*$p<$;0.05, Mann-Whitney U-test), while aged imipramine- and dimebon-treated animals did not differ from young mice ($p>0.05$, vs. 3-month-old mice); sucrose intake was similar in the latter two groups ($p>0.05$, Mann-Whitney) and significantly higher in these groups compared to non-treated aged mice (#$p<0.05$, vs. 18-month-old non-treated group; Mann-Whitney U-test). (C) Aged non-treated and dimebon-treated mice showed enhanced water intake in comparison with young mice (*$p<0.05$, Mann-Whitney U-test); this was not found in imipramine-treated animals treated with imipramine, which showed a significant decrease in this measure in comparison to the two other 18-month-old groups (#$p<0.05$; Mann-Whitney U-test). Young imipramine-treated mice had no changes in water consumption compared to the non-treated animals (p>0.05, Mann-Whitney). (D) Total liquid intake was elevated in all groups of old mice in comparison to young mice (*$p<0.05$ for dimebon-treated mice, #$p<0.05$ for non-treated and §$p<0.05$ for imipramine-treated mice). Young imipramine-treated mice showed no differences in this parameter. (E) Imipramine-treated mice of both ages had longer latency of floating and (F) decreased duration of floating on days 1 and 2 than non-treated groups (*$p<0.05$, Mann-Whitney U-test). There were no changes in floating in dimebon-treated old mice ($p>0.05$, Mann-Whitney). In the modified tail suspension test, the latency of immobility (G) was significantly longer and (H) the duration of immobility was significantly shorter in imipramine-treated mice of both ages (*$p<0.05$, Mann-Whitney U-test). Older animals treated with dimebon had no changes in parameters of immobility ($p>0.05$, Mann-Whitney). Bars represent the means. 3 MO: non-treated 3-month-old mice (n = 10); 3 MO-IMI: 3-month-old mice treated with imipramine (n = 7); 18 MO: 18-month-old mice (n = 8); 18 MO-IMI: 18-month-old mice treated with imipramine (n = 5); 18 MO-DIM: 18-month-old mice treated with dimebon (n = 10).

is likely to be due to their lower motivation for pleasurable stimulation rather than impaired ability to drink from narrow tips or taste a sweet.

The lack of change in total liquid intake in the sucrose test and 24-h water consumption in this group (Figs. 1D, 5D) indicates that its physiological need for water is not altered and cannot account for the observed changes in the sucrose test. The significantly higher body mass in aged mice (Fig. 1E) suggests that the decrease in sucrose intake is not related to changes in caloric needs; besides, the sucrose test parameters were shown to be unrelated to individual body weights (Strekalova et al., 2011). Moreover, 3- and 18-month-old mice displayed similar scores for locomotion and anxiety (Fig. 4A,C–G). This is consistent with the unchanged drinking behavior in novel testing conditions of a high-effort drinking paradigm, showing no indications for neophobia and psychomotor deficits in aged mice (Fig. 1F). These measurements rule out any essential interference of metabolic, motor and emotional changes with the evaluation of proposed anhedonic-like behavior of 18-month-old mice.

Remarkably, 18-month-old mice displayed less nose-poking behavior and a shorter duration of exploratory rearing activity in the open-field test, as well as fewer rearings in the novel cage test (Fig. 4B–D). Low interest in novelty is regarded as reflecting diminished sensitivity to reward/motivation for hedonic stimulation; it parallels elderly depression (Gallo et al., 1997; Bourin et al., 1998) and can be reversed by antidepressant therapy (Willner, 2005; van Miegem et al., 2009; American Psychiatric Association, 2000). This feature generally correlates with aging in rodents (Skrinskaia and Nikulina, 1994; Schulz et al., 2007; Meier et al., 2010; Soffié et al., 1992) and particularly with the occurrence of stress-induced anhedonic behavior in C57BL6N mice (Strekalova et al., 2004; Porsolt and Papp, 1998). Thus, data on superficial exploration of 18-month-old mice indirectly support the outcome of the sucrose test.

4.2. Reduced grooming of 18-month-old mice in a splash test

Similarly to the effects of chronic mild stress (Pothion et al., 2004; Surget et al., 2008), aging was found to result in suppressed grooming behavior in a classical splash test model: 18-month-old mice exhibited significantly shorter duration of grooming behavior than 3-month-old animals (Fig. 4F). This feature has been reported to correlate with key manifestations of a depressive-like state in mice, such as physical appearance, the choice test, deterioration of the fur state, vanilla pasta test, reward maze test and others that extensively validate this effect as an important trait of depressive-like syndrome in mice (Willner, 2005). Similar findings were reported by other groups (Scimonelli et al., 1999; Shoji and Mizoguchi, 2011). Together, these results are consistent with the above manifestations of depressive-like features of older mice in sucrose and exploration tests.

4.3. Eighteen-month-old mice display no behavioral signs of affective disturbances

Both manual and automated scoring of the forced swim and modified tail suspension tests, whose protocols were validated in the present study and in other experiments with various antidepressants under a number of conditions (van Miegem et al., 2009; Strekalova et al., 2005, 2006, 2008; Ducottet et al., 2004; Cryan et al., 2005a; Mineur et al., 2006; Vaugeois et al., 1997; Gavioli et al., 2004; Cryan et al., 2005b; Ibarguen-Vargas et al., 2009), revealed the lack of differences between 3- and 18-month-old animals in terms of floating and immobility measured either during the entire 6-min period (Figs. 2, 3, 5) or over 1- or 2-min intervals (*data not shown*). Other behaviors, such as the duration of swimming behavior/activity (Fig. 2A,D) and the latency of the first episode of floating/immobility (Fig. 3A,E) also showed no difference between groups. Furthermore, the duration of climbing behavior and diving events during forced swimming, which were shown to be sensitive to some types of antidepressant treatment (Hayashi et al., 2011), did not differ between the two groups (Fig. 3B,C). The latter parameter, however, was significantly more variable in 18-month-old mice, with a non-significant increase (Fig. 3C) that suggests a trend towards psychomotor disturbances in animals of this age. In summary, 18-month-old mice of the C57BL6/N strain did not demonstrate changes in measures for affective behaviors in the forced swim and modified tail suspension test.

Other reports are consistent with our data. Aging did not change the floating behavior of CC57Br, A/He and C3H/He, which were tested at the age of about 15 months (Skrinskaia and Nikulina, 1994). A lack of differences in mobility scores in the modified tail suspension and forced swim tests was reported in 10- to 15-month-old C57BL6 mice (David et al., 2001; Bourin et al., 1998; Ohashi et al., 2006). Studies that were predominantly performed on rodents of older age, however, reported increased floating and immobility behaviors; the various outcomes in these tests can be accounted for by the different age categories. Since most of them evidence higher scores of behavioral despair accompanied by psychomotor deficits, the validity of these findings may be limited (Skrinskaia and Nikulina, 1994; Schulz et al., 2007; Soffié et al., 1992; Bowman et al., 2006).

4.4. Comparison of 3- and 18-month-old mice in additional tests

Similar values for the speed of movement and resting time in the open-field test suggest a lack of motor disturbances in 18-month-old mice (Fig. 4A,B). The onset, frequency and duration of grooming, as well as nose-poking behavior, exploratory rearing, and vertical activity in the novel cage test were inhibited in 18-month-old mice, in comparison to a younger group (Fig. 4C–F). As discussed above, decreases in these behaviors parallel reduced hedonic sensitivity and other depressive-like features in rodents. Elevated anxiety is a well-known feature of aging and depressive disorder (American Psychiatric Association, 2000), but not always accompanies anhedonia/depressive syndrome in clinical and animal studies (Hybels et al., 2011; Pachana et al., 2011; Sobrian et al., 2003). Our experiments found no evidence of such changes in 18-month-old mice (Fig. 4G–L). This may be due to the applied here "mild" protocols of the O-Maze and dark/light box anxiety paradigms (Strekalova et al., 2005, 2006), while most of studies which reported increased anxiety-like behavior during aging, employ highly anxiogenic testing (Skrinskaia and Nikulina, 1994; Schulz et al., 2007; Meier et al., 2010; Lawton et al., 1996). In summary, in the protocols used here for anxiety-like behaviors and other behavioral analysis, aged mice displayed no alterations in measures of anxiety-like behavior and locomotion, excluding possible confounds with evaluation of depressive-like traits.

4.5. Effects of imipramine and dimebon in old mice

Both drugs elevated sucrose intake and preference in 18-month-old mice (Fig. 5B) that is characteristic of the effects of antidepressants (Porsolt and Papp, 1998; Strekalova et al., 2011; van Miegem et al., 2009). Thus, elderly-related manifestations of anhedonia are reversible by antidepressant treatment with imipramine that validates the proposed model pharmacologically. In contrast to imipramine, dimebon-treated mice showed a significant increase in the total intake of liquid (Fig. 5D) that may suggest the emergence of the characteristic antihistamine effect of increasing water intake, reported in patients after a four-week course of dimebon, and in mice after five-day treatment, at comparable doses (Vignisse et al., 2011; Bachurin and Grigoriev, 2008; Magrani et al., 2006). Imipramine reduced water intake that was obviously owed to the compensation of increased consumption of a sucrose solution (Fig. 5A–C). Interestingly, dimebon exhibited anti-anxiety effects in mice and rats (Vignisse et al., 2011; Magrani et al., 2006), which similarly to the situation with many other drugs, overlap with its antidepressant action (Porsolt and Papp, 1998; Willner, 2005).

Further experiments are required to see whether one of the above-discussed mechanisms (Bolkunov et al., 2009; Strekalova, 2008; Strekalova and Steinbusch, 2009; Hybels et al., 2011; Bachurin et al., 2001, 2003) or other effects of dimebon underlie its activity in sucrose test.

In the modified protocols of the forced swim and modified tail suspension tests, imipramine but not dimebon decreased duration of floating and immobilization during testing on both days (Fig. 5D,E). These effects were found in both young and old mice. The latency of the first episode of floating was also decreased by imipramine in mice of both ages; no such effect was found in dimebon-treated mice. Thus, the effects of antidepressant treatment in the tests for animal parallels behavioral despair where originally 3- and 18-month-old mice display similar behavior did not differ between the groups.

5. Conclusions

The present study demonstrates the occurrence of anhedonic-like traits in 18-month-old mice which are not accompanied by manifestations of behavioral despair and mimic the clinical profile of primary elderly depression where hedonic deficits outweigh affective symptoms. Anhedonic-like changes in older mice found in a sucrose preference test were sensitive to a chronic antidepressant treatment and were accompanied by decreases in sucrose intake in a high-effort drinking paradigm, grooming in a splash test and novelty exploration. Additional studies ruled out potential confounds in the analysis of behavioral features that are specific to depressive-like syndrome, in pre-clinical modeling of elderly depression in 18-months-old mice of this strain.

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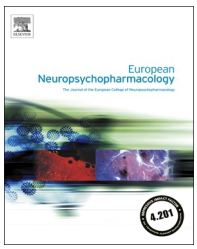

Concomitant manipulation of murine NMDA- and AMPA-receptors to produce pro-cognitive drug effects in mice

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Abstract
Bifunctional drug therapy targeting distinct receptor signalling systems can generate increased efficacy at lower concentrations compared to monofunctional therapy. Non-competitive blockade of the NMDA receptors or the potentiation of AMPA receptors is well documented to result in memory enhancement. Here, we compared the efficacy of the low-affinity NMDA receptor blocker memantine or the positive modulator of AMPA receptor QXX (in C57BL/6 J at 1 or 5 mg/kg, ip) with new derivatives of isothiourea (0.5–1 mg/kg, ip) that have bifunctional efficacy. Low-affinity NMDA blockade by these derivatives was achieved by introducing greater flexibility into the molecule, and AMPA receptor stimulation was produced by a sulfamide-containing derivative of isothiourea. Contextual learning was examined in a step-down avoidance task and extinction of contextual memory was studied in a fear-conditioning paradigm. Memantine enhanced contextual learning while QXX facilitated memory extinction; both drugs were effective at 5 mg/kg. The new derivative IPAC-5 elevated memory scores in both tasks at the dose 0.5 mg/kg and exhibited the lowest IC_{50} values of NMDA receptor blockade and highest potency of AMPA receptor stimulation. Thus, among the new drugs tested, IPAC-5 replicated the properties of memantine and QXX in one administration with increased potency. Our data suggest that a concomitant manipulation of NMDA- and AMPA-receptors results in pro-cognitive effects and supports the concept bifunctional drug therapy as a promising strategy to replace monofunctional therapies with greater efficacy and improved compliance.

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1. Introduction

The development of novel memory enhancers for treating cognitive deficits associated with neurological and psychiatric disorders remains an important focus of study in central nervous system research. Recent advances in preclinical and clinical development in this field identify the glutamatergic system as a central element (Francis, 2008; Lynch et al., 2011). Many promising new drug candidates that target NMDA or AMPA receptor function are progressing through preclinical and clinical development as potential memory enhancers to be applied in patients with Alzheimer's disease, vascular cognitive impairment, schizophrenia, attention deficit hyperactivity disorder, post-traumatic stress disorder and other neuropsychiatric disorders (O'Neill et al., 2004; Olivares et al., 2012).

Compounds that block the N-methyl-D-aspartate (NMDA) receptor via a low-affinity mechanism (partial NMDA receptor antagonists), or those that stimulate glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (positive modulators of AMPA receptor), have been reported to induce profound effects on neurotransmission and improve cognitive function in clinical and preclinical experiments with animals (Francis, 2008; Lynch et al., 2011; Gonda, 2012). Memantine, a low-affinity NMDA receptor blocker is a drug used worldwide for the treatment of cognitive impairments in patients with Alzheimer's disease, vascular dementia, and in Parkinson's disease (Parsons et al., 2007; Olivares et al., 2012). A number of promising compounds with a similar mode of action are currently at advanced stages of preclinical testing (Olivares et al., 2012; Ihalainen et al., 2011; Smith et al., 2011; Kaplan and Moore, 2011). Positive modulators of AMPA receptors are also reported to be effective in patients suffering from neurological and psychiatric disorders that include post-traumatic stress disorder and vascular dementia (O'Neill et al., 2004; Pirotte et al., 2010; Lynch et al., 2011). Glutamatergic receptors offer multiple binding sites that can be targeted, but the administration of many present problems as a consequence of their side-effects (Jones, 2010; Emre et al., 2010; Gonda, 2012). Therefore, the development of interventions that decrease the adverse effect profiles of low-affinity NMDA receptor blockers/AMPA receptor positive modulators, while preserving their valuable effects on cognitive functions, is an important priority in the field of memory enhancer research and development.

The concept of multi-target drug activity has been proposed (Cavalli et al., 2008; Combarros et al., 2009). According to this concept, the mechanisms responsible for the beneficial actions of drugs can be realized via multiple actions of the compound on a number of receptors; in this case, effective changes in the concentrations of neurotransmitters may be much lower than in a situation in which only one receptor signalling system underlies the drug's activity. The theoretical concept of multi-target therapy has found increasing practical application over the last few years. For example, the memory enhancer JWSUSC75IX was developed as a potent AChE inhibitor and high-affinity antagonist of muscarinic M2 receptors (Terry et al., 1999) and ladostigil, a compound with memory-enhancing and antidepressant activities, was designed to combine the properties of inhibitors of AChE with central MAO-A and MAO-B inhibitors (Weinstock et al., 2003); both drugs have shown higher efficacy and reduced side-effects in comparison with the monotherapies or therapeutic regimen combined with original compounds, from which these bifunctional molecules were derived. Other drugs that combine useful pharmacophores in a single molecule, but do not lose their activities were recently introduced (Youdim and Buccafusco, 2005). Similarly, the discovery of several types of molecule that combine pro-cognitive effects exerted via the mechanisms of low-affinity blockade of NMDA receptors and stimulation of AMPA receptors, whose importance is already supported by clinical studies, may offer valuable therapeutic possibilities for several disorders, with improved clinical efficacy and decreased side-effects.

The present study sought to investigate the utility of compounds exerting a concomitant NMDA- and AMPA-receptors activity in a memory-enhancing role and assess a role for the magnitudes of their electrophysiologically measured effects in mnemotropic activities. Therefore, we used a series of new compounds that combine the two above-mentioned effects: four derivatives of isothiurea for immediate comparison because of their identical chemotype and one thiadiazole that was used as additional reference (for chemical structures, see Supplementary data). The chemical design of these drugs was guided by the need to reduce the affinity of the molecule for the NMDA receptor intrachannel binding site. This was achieved by enhancing molecular flexibility; compounds with lower affinities for this site are known to enhance memory with fewer adverse effects than competitive NMDA receptor blockers (Blanpied et al., 1997; Parsons et al., 2007; Chen et al., 2012; see Supplementary data). The derivatives whose essential neuroprotective and cognitive-enhancing activities, as well as lack of neurotoxic effects in a number of assays had been previously demonstrated (Bachurin et al. 2001; Grigoriev et al., 2003; Tkachenko et al., 2004, 2005) were selected for the next step, where new ampakine-like pharmacophore groups containing, similarly to ampakines (Arai and Kessler, 2007), sulphamide fragments, were introduced in their structures. The estimation of potential interactions between pharmacophores was made as described elsewhere (Sheridan, 2002, see also Supplementary data).

New compounds were described as 'high' or 'moderate' relative to the potency of model molecules with mono-effects on glutamate receptors, a low-affinity NMDA receptor blocker memantine and new positive stimulator of AMPA receptor QXX (N,N′-substituted 3,7-diazabicyclo [3,3,1] nonane. QXX was shown to be the effective positive modulator of the AMPA receptor (Lavrov et al., 2007; Bachurin et al., 2008; Anokhin et al., 2008; Palyulin et al., 2009; see also Supplementary data). The bifunctional drugs were compared against each other and to the effects of the monofunctional compounds in two mouse tasks whose neurobiology is well known to be dependent on AMPA and NMDA receptor-dependent synaptic plasticity: the step-down passive avoidance paradigm and a model of extinction of contextual freezing (Costa et al., 2008; Clem and Huganir, 2010; Yamada et al., 2011).

2. Experimental procedures

2.1. Study design

A series of new compounds, IPAC 1–5, were designed and synthesized (see Supplementary data) and tested for their electrophysiological properties (*see below*). Their EC_{50} values for non-competitive blockade of NMDA receptors and the magnitude of AMPA receptor stimulation are expressed as per cent of control and were compared to those model molecules with mono-functional properties and classified as 'high' (effects that are equal or exceeding those of model drug) or 'moderate' (with lower than in model drugs effects). Among the newly synthesized drugs for selected behavioural analysis, one compound was from the class of thiadiazoles and served as additional reference treatment. Four drugs belonged to isothiourea derivatives and were used for direct drug comparison. These drugs had the following combinations of effects: (1) high effects on NMDA and AMPA receptors, (2) moderate effects on NMDA and AMPA receptors, (3) high effects on NMDA and moderate effects on AMPA receptors, and (4) moderate effects on NMDA and high effects on AMPA receptors.

The effects of five new compounds IPAC 1–5, memantine and QXX were investigated in the battery of behavioural tests (Figure 2). In the step-down passive avoidance task (*see below*), mice received an intraperitoneal injection of drug or vehicle (*see below*) and, 15 min afterwards, were subjected to a training session. The test for recall was carried out 1 and 24 h later. A second cohort of mice was trained in the contextual fear-conditioning paradigm and 24 h later were tested for memory recall (*see below*). The animals were injected with drug or vehicle and were subjected to a memory extinguishing session. 24 h later, animals were scored for recall of memory extinction. To control for anxiety-like behaviour after the drug administration, a group of mice were injected with the compounds, followed 15 min later, by the dark–light box test (*see below*). The number of animals per group is indicated in the legends of Figures 3–5. The combinations of electrophysiological parameters of the four isothiourea derivatives on glutamate receptors were related to their behavioural effects in mouse memory models and compared to the effects of reference drugs.

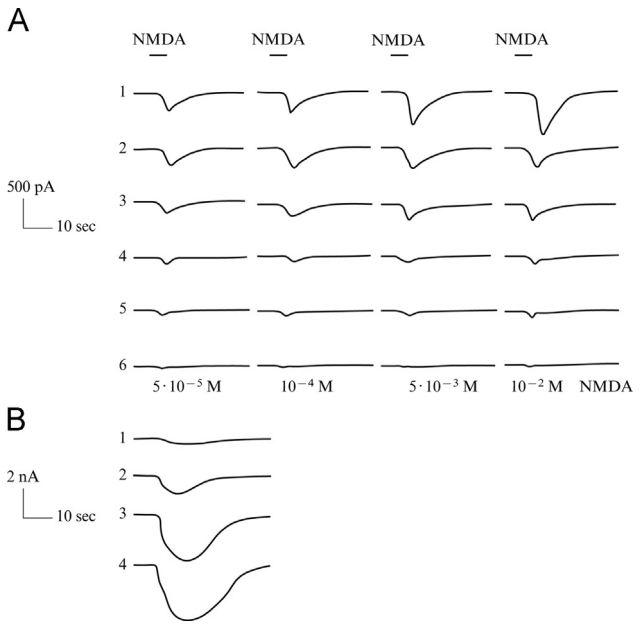

Figure 1 Electrophysiological recordings of NMDA- and AMPA-mediated currents after drug application: an example with IPAC-5. (A) The application of IPAC-5 has evoked dose-dependent inhibition of NMDA-induced currents in rat cortical neurons. **1**—control (drug-free); **2**—0.4 μM IPAC-5; **3**—0.7 μM IPAC-5; **4**—1.0 μM IPAC-5; **5**—5.0 μM IPAC-5; **6**—10.0 μM IPAC-5. (B) Transmembrane currents induced in Purkinje neurons by kainic acid (20 μM) were dose-dependently increased, as compared to control (application of kainite only: **1**), by IPAC-5: **2**—2.0 μM, **3**—10.0 μM; **4**—30.0 μM.

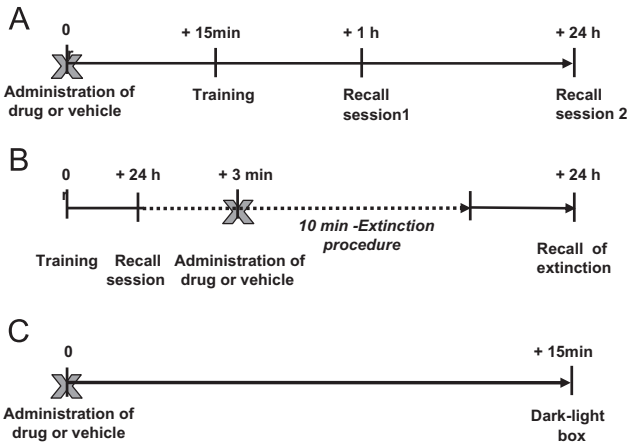

Figure 2 Schemes of treatment and behavioural testing. (A) In the step-down avoidance test, a single training session was carried out 15 min after the bolus i.p. administration of drug or a vehicle; mice were tested for memory recall 1 h and 24 h thereafter. (B) In the paradigm of extinction of contextual freezing, mice were subjected to a single training session and 24 h later tested for a memory recall. At the end of this session, they were injected with drug or a vehicle and instantly placed back for a 10-min extinction procedure. A test for recall of memory extinction was carried out 24 h thereafter. (C) Total time spent in the lit compartment of the dark/light box was estimated 15 min after a bolus administration of drugs or a vehicle.

2.2. Electrophysiological experiments

Freshly isolated neurons from 9-to-16-day-old rat pups were used for the patch-clamp technique; NMDA-receptor-mediated currents were studied in cortical neurons and AMPA-receptor-mediated currents were studied in Purkinje neurons of the cerebellum, as described elsewhere (Grigoriev et al., 2003; Perlovich et al., 2009). Briefly, for cell isolation, a selected region of the brain was cut into slices 0.4–0.6 mm wide followed by incubation in buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4) for 1 h. The slices were transferred to fresh buffer solution with 2 mg/ml of Pronase (Serva, Heidelberg, Germany) and 1 mg/ml of Collagenase (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30–60 min. Then, the slices were transferred to the fresh buffer solution and incubated about 20 min. Slices were incubated at 34 °C and pre-gassed with 100% O_2. Finally, the slices were mechanically dissociated into individual cells by means of Pasteur pipettes. For cortical neurons, the composition of extracellular saline was 150 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.32, and for Purkinje neurons it was 150 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 2.0 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.32. The composition of the intracellular saline was 140 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM ATP. The transmembrane currents were registered in the configuration of

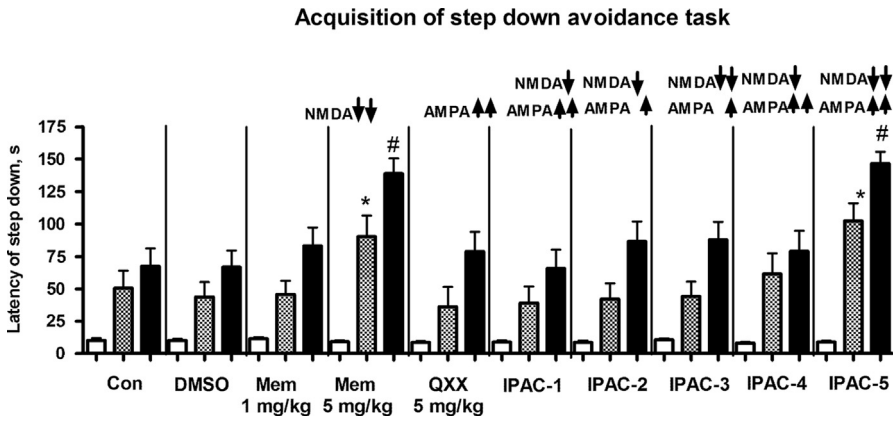

Figure 3 Effects of IPAC 1-5 drugs on contextual learning in the step-avoidance model. (A) In the step down passive avoidance test, there was an increase of the latencies of step down measured during two recall sessions (+1 h and +24 h) in comparison to the training session (baseline) in all experimental groups demonstrating that mice acquired the task ($p<0.05$ vs. training values, Wilcoxon test). Mice treated with memantine at a dose of 5 mg/kg (n=22) or IPAC-5 at a dose of 0.5 mg/kg ($n=22$) revealed significant increase in the latency of step down evaluated 1 h after training ($^{*}p<0.05$, Mann-Whitney *U*-test) and 24 h after training ($^{\#}p<0.05$, Mann-Whitney *U*-test) as compared with respective saline-treated ($n=20$) or DMSO-treated ($n=22$) control groups, respectively. No such effects were observed in animals treated by memantine at a dose of 1 mg/kg ($n=20$), QXX at a dose of 5 mg/kg ($n=15$), IPAC-1 ($n=22$), IPAC-2 ($n=22$), IPAC-3 ($n=22$) or IPAC-4; ($n=20$; $p>0.05$, Mann-Whitney *U*-test). Each column represents the mean ± SEM. Arrows indicate the effects of drugs on NMDA- and AMPA-receptors: 'high' (↓↓) or 'moderate' (↓) blockade and 'high' (↑↑) or 'moderate' (↑) positive modulation (see Table 1). Experimental groups: **Con**: saline-treated control; **DMSO**: DMSO-treated control; **Mem**: memantine-treated.

the 'whole cell' using the electrophysiological EPC-9 set-up (HEKA, Lambrecht, Germany); data were processed with HEKA software (Pulsefit/HEKA, Lambrecht, Germany). Compounds were exposed to neurons by the fast perfusion method (Grigoriev et al., 2003; Perlovich et al., 2009).

As mentioned above, the effects on NMDA receptors were studied on isolated cortical neurons that contained a population of NMDA receptors; NMDA solutions of 1-100 μM containing 7 μM of glycine were applied to activate the receptor. A representative picture of NMDA-mediated currents is shown in Figure 1A. Before the first drug application, recordings of NMDA-receptor mediated transmembrane currents were carried out three times after the application of a solution with NMDA and glycine and were separated by a minimum of 2 min. The physiological solution in a recording chamber was replaced with the test solutions containing specific concentrations of compounds that were increased throughout the testing period. The application of each test concentration was accompanied by the concomitant triple application of a solution of NMDA and glycine. After each drug application, a 3-min wash-out with physiological solution was carried out and responses to three applications of a solution of NMDA and glycine were recorded for a control. The next drug concentration was applied thereafter and was again followed by a wash-out session and triple application of NMDA and glycine. The mean of amplitudes of the NMDA-mediated currents measured during all applications of NMDA and glycine was taken as a control value, and means of the measurements following the drug applications were normalized to the control values and expressed as a percentage. Normalized currents for control and treatment recordings were obtained for 4-6 cortical neurons, and were analyzed with the specialized software as mentioned above. An IC_{50} for each compound was calculated using the Master PlexReaderFit (Hitachi Solutions America, Ltd., San Francisco, CA, USA). Using this programme, the standard curves were generated; each curve set contained 5-8 data points. For compounds where blocking was evident at1 μM, 5-8 concentrations from a concentration of 0.1 μM were investigated with incremental steps of 0.2 μM. For those compounds that were not active at 1 μM, concentrations of 10 μM or 30 μM were tested and, once an effect was observed, 5-8 concentrations with incremental steps of 5 μM were applied. As asymmetry at the inflection point of the dose/response curve could be not excluded for the new compounds tested in our studies, the Parameter Logistic (5-PL) nonlinear regression model equation was used to determine the IC_{50} value. This model incorporates an asymmetry factor in the analysis.

The effects of tested compounds on the stimulation of AMPA receptors were investigated on isolated Purkinje neurons using partial receptor agonist kainic acid (KA), which induces AMPA-receptor mediated currents while evoking relatively low receptor desensitization. A representative picture of AMPA-mediated currents is shown in Figure 1B. Baseline recordings of AMPA-receptor mediated transmembrane currents were carried out three times after each application of KA (20 μM) that were spaced from each other in this and any other applications during recordings by 2 min. Thereafter, the physiological solution in the recording chamber was replaced with increasing concentrations of test compounds. The application of each tested concentration was accompanied by a concomitant triple application of KA at above-indicated concentration. After each drug application, a 3-min wash-out with physiological solution was carried out and responses to three applications of KA were recorded for a control. The next drug concentration was then applied followed by a wash-out session and triple application of KA. The mean of amplitude of the AMPA-mediated currents measured during all applications of KA was taken as a control value (100%), means of measurements of this parameter during drug applications were normalized to control for each drug concentration and expressed as a percentage. Each drug concentration was tested on 4-6 Purkinje neurons; 5-8 concentrations in a range from 0.001 μM to 30 μM of each drug were tested.

2.3. Animals and behavioural testing

Male C57BL/6J mice aged 3.5 months were used. Animals were housed in standard laboratory conditions as described elsewhere

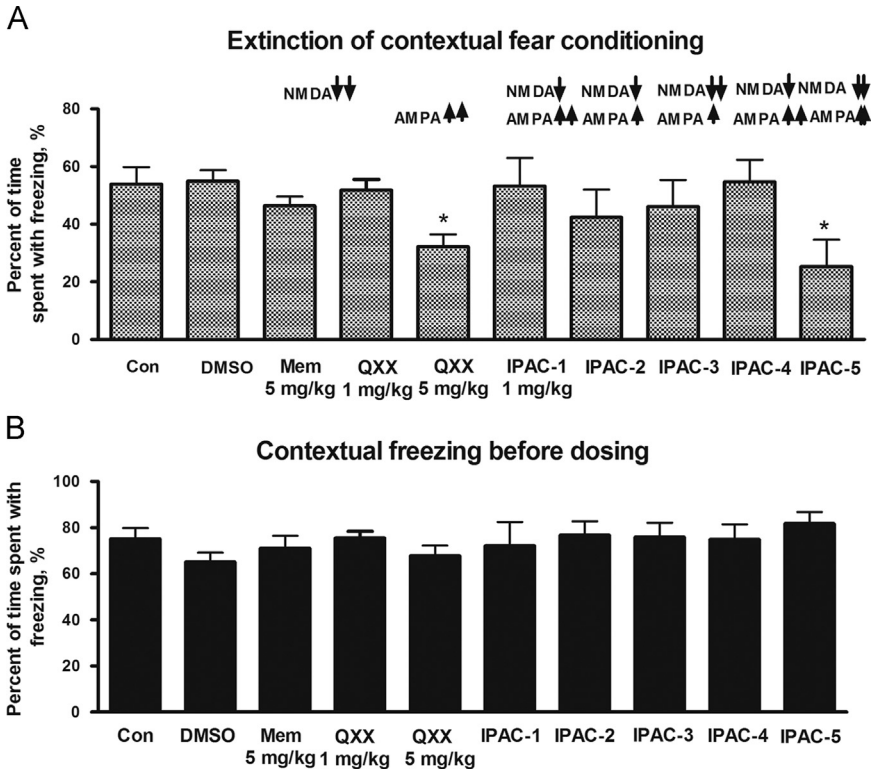

Figure 4 Effects of new compounds on extinction of contextual freezing in a fear-conditioning paradigm. (A) In the paradigm of extinction of conditioned freezing, during recall of memory extinction trials, a significant decrease in the percentage of time spent in freezing vs. the DMSO-treated group ($n=10$) was found in mice treated with QXX at a dose of 5 mg/kg ($n=15$) and IPAC-5 ($n=11$) ($^*p<0.05$, Mann–Whitney *U*-test). Experimental groups treated with memantine at a dose of 5 mg/kg ($n=15$), QXX at a dose of 1 mg/kg ($n=15$), IPAC-1 ($n=12$), IPAC-2 ($n=11$), IPAC-3 ($n=12$) and IPAC-4 ($n=13$) showed no changes in freezing behaviour during a recall of extinction session, as compared with the respective control groups ($p>0.05$, Mann–Whitney *U* test). (B) During a recall session of fear conditioning, before dosing, all groups showed similar freezing rates that ruled out pre-existing differences in the level of learning between the experimental groups ($p>0.05$, Kruskall–Wallis and Mann–Whitney *U* tests). Each column represents the mean ± SEM. Symbols are as in Figure 3.

(Vignisse et al., 2011; see also Supplementary data). All experiments were carried out in accordance with the European Communities Council Directive for the care and approved by governmental bodies for animal care and welfare.

2.3.1. Step-down passive avoidance model

Fifteen minutes after the i.p. administration of the drug or vehicle, mice were trained in a step-down avoidance paradigm as described elsewhere (Strekalova et al., 2001; Vignisse et al., 2011; see also Supplementary data). Briefly, during the training session, mice were placed on the platform inside a transparent cylinder. After removal of the cylinder, the time until the animal left the platform with all four paws was measured as baseline latency of step-down. Immediately after step-down, mice received a single electric foot shock (0.5 mA, 2 s) and were returned to their home cages. One hour and 24 h later, during the recall trial session, animals were exposed to the apparatus again by being handled in the same way as in the training session; no foot shock was delivered. Latency of step-down with all four paws was measured until 180 s had elapsed.

2.3.2. Paradigm of extinction of contextual freezing

The test procedure was adapted from a previously described protocol (Strekalova et al., 2003). The apparatus (Evolocus LLC Tarrytown, NY, USA and Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 cm × 25 cm × 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter). An alternating single electric current (AC, 50 Hz; 0.7 mA) was delivered after a 2-min acclimatization of a mouse to a chamber. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behaviour was scored by visual observation during a test of memory recall that was carried out 24 h later. The occurrence of freezing behaviour was assessed every 10 s for 180 s; each 10-s score was assigned to a freezing or non-freezing period, and the percentage of time spent in freezing was calculated. At the end of a recall session, mice were retrieved from the apparatus and injected with drugs or vehicles and immediately placed back for 10-min memory extinction. During a memory extinction session, no foot shock was applied, and animals were free to explore the apparatus. Twenty-four hours later, freezing behaviour was scored in a 180-s recall of extinction session as in the previous trial and percentage of time spent in freezing was calculated.

2.3.3. Dark-light test

The test protocol was applied as described elsewhere (Malatynska et al., 2012; see also Supplementary data). Briefly, mice were placed into the black compartment from where they could visit the lit box. The total duration spent in the lit compartment was scored by visual observation over 5 min and was taken as a measure of anxiety-like behaviour; a latency of the first exit to this area and a total number of exits were scored as well.

2.4. Drug administration

Selection of doses of memantine (1 and 5 mg/kg) and the timing of dosing (single injection 15 min prior to training) were based on previously reported studies, in which memantine induced effects in similar paradigms as employed here (Bachurin et al., 2001; Zoladz et al., 2006; Saab et al., 2011). Preliminary data suggested comparable characteristics of pharmacokinetic between memantine and QXX, which enabled the application of the two drugs with the same dosing regimen (Bachurin et al., 2008). In previous studies, the newly synthesized compounds IPAC 1-5 and QXX revealed effects on exploratory and locomotor behaviours 15–30 min after their intragastric administration to mice (Lavrov et al., 2007; Palyulin et al., 2009) that defined the timing of the dosing for the current study and the limits of the concentrations used (see Table 1). These data have revealed the ability of the new compounds, at the range of doses indicated in Table 1, to evoke central activity following systemic bolus administration (Lavrov et al., 2007; Palyulin et al., 2009) and chronic administration (Bachurin et al., 2001). Because of the above-mentioned behavioural effects, IPAC-1 and -3 were used at a dose of 1 mg/kg and IPAC-2, IPAC-4 and IPAC-5 were applied at a dose of 0.5 mg/kg. All drugs, except memantine, which was dissolved in saline, were first dissolved in 100% DMSO (Sigma-Aldrich, St. Louis, MO, USA) and then the injection solution was diluted so the final concentration of DMSO was 0.2%. A control group of mice was injected with saline; a separate group of mice was treated with a vehicle 0.2% DMSO solution. All solutions were delivered in a volume of 0.01 ml/kg of body weight by intraperitoneal injection.

2.5. Statistical analysis

Behavioural data were analyzed with a statistical software package (Prism 5, Chicago, IL, USA). The Friedman test and Dunn's multiple comparison test was applied for repeated measurements; the Kruskal–Wallis test followed by the Mann–Whitney *U*-test was used for a group comparison (see also Supplementary data). Independent data sets obtained in the dark-light box were treated with one-way ANOVA analysis followed by the unpaired *t*-test. Electrophysiological data were treated by *t*-test with software from HEKA (Lambrecht, Germany). The level of confidence was set at 95% ($p<0.05$).

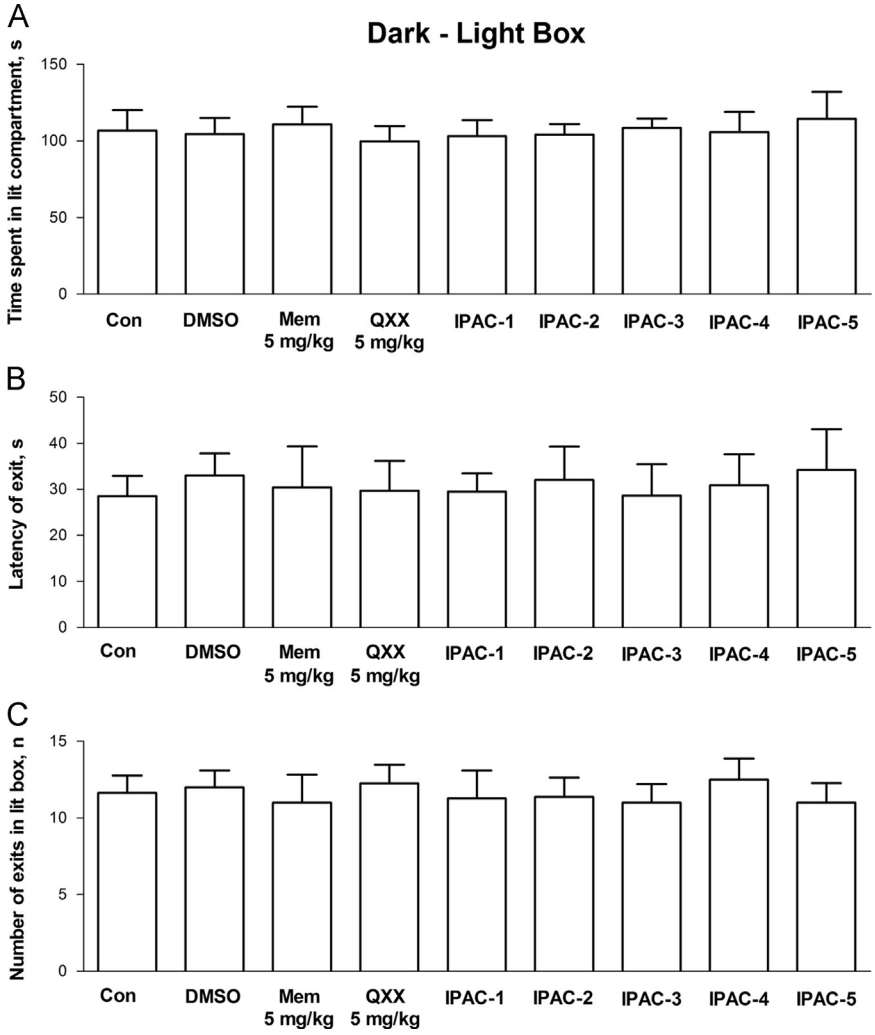

Figure 5 Effects of IPAC 1-5 on anxiety-like behaviour in the dark-light box. In the dark-light box, mice treated with memantine at a dose of 5 mg/kg ($n=8$), QXX ($n=9$), IPAC-1 ($n=8$), IPAC-2 ($n=8$), IPAC-3 ($n=8$), IPAC-4 ($n=9$), and IPAC-5 ($n=10$) did not differ in terms of (A) the time spent in the lit compartment, (B) latency of exit in the lit area and (C) number of exits to the lit box from respective non-treated ($n=8$) and vehicle-treated control mice ($n=8$). Each column represents the mean ± SEM. Symbols are as in Figure 3.

3. Results

3.1. Electrophysiological properties of new compounds IPAC 1-5 and model drugs

The newly synthesized drugs IPAC 1-5 selected for this study were compared with the actions of memantine or the AMPA receptor potentiator QXX. They exhibited similar effects on NMDA- and AMPA-receptor-mediated currents over a range of effective concentrations (Table 1). The IC_{50} of the novel drugs acting on NMDA receptor was in a range from 0.8 to 21 μM, and was comparable to memantine in our experiments, which had an IC_{50} of 1.4 μM. The maximal magnitude of the potentiaton of AMPA-receptor-mediated current by new drugs was 148-1050% of the control values and the effective doses for inducing positive modulation of AMPA-receptors varied between 0.1 μM and 30.0 μM; for the QXX for the QXX, these parameters were 210% and 0.001 μM, respectively. Based on these results, IPAC-1 and IPAC-4 were defined as drugs that have a moderate NMDA blockade effect on NMDA, and induce high potentiation of AMPA receptors. IPAC-2 was classified as a compound with moderate effect in blocking NMDA receptors and moderate potentiation of AMPA receptors. IPAC-3 had high effect in blocking NMDA receptors and moderate potentiation of AMPA receptors. IPAC-5was found to have high effect as NMDA receptor blocker and high effects of potentiation of AMPA receptors.

3.2. Step-down avoidance task

In the step-down experiment, there was no statistical difference in baseline measurements of the latencies of step down ($p=0.45$, Kruskal-Wallis test), suggesting that the

Table 1 Electrophysiological properties of new compounds IPAC 1-5 and model drugs[a]. The EC_{50} values for non-competitive blockade of NMDA receptors and the magnitude of AMPA receptor stimulation of IPAC 1-5 drugs were expressed as per cent of control and were compared to those model molecules with mono-functional properties (*see the text*). These effects were classified as 'high': effects that are equal or exceeding those of model drug (↓↓—for blockade, ↑↑—for stimulation) or 'moderate': with lower than in model drugs effects (↓—for blockade, ↑—for stimulation). IPAC 1-5 drugs had following combinations of effects: (1) high effects on NMDA and AMPA receptors, (2) moderate effects on NMDA and AMPA receptors, (3) high effects on NMDA and moderate effects on AMPA receptors, and (4) moderate effects on NMDA and high effects on AMPA receptors.

Potency to affect NMDA-R[b] or/and AMPA-R[c]	Drug	Blockade of NMDA-R IC_{50}	Potentiation of AMPA-R in % from control, dose of maximal effects and range of effective doses	Minimal doses at which IPAC 1-5 affect exploration and/or locomotion
Non-competitive blockade of **NMDA-R**—high ↓↓ **AMPA-R** potentiation—high ↑↑	**Memantine**	1.4±0.15 μM	n.a.	n.a.
	QXX	n. a.	210% (0.001 μM) 0.00001-0.01 μM	n.a.
Non-competitive blockade of **NMDA-R**—moderate↓**AMPA-R** potentiation—high ↑↑	**IPAC-1**	21.4±1.58 μM	230% (0.01-0.1 μM) 0.001-1.0 μM	>1 mg/kg
Non-competitive blockade of **NMDA-R**—moderate↓**AMPA-R** potentiation—moderate↑	**IPAC-2**	3.2±0.47 μM	150% (0.5 μM) 0.5-1.0 μM	>0.5 mg/kg
Non-competitive blockade of **NMDA-R**—high ↓↓ **AMPA-R** potentiation—moderate ↑	**IPAC-3**	0.8±0.11 μM	148% (0.5 μM) (0.02-1.0 μM)	>1 mg/kg
Non-competitive blockade of **NMDA-R**—moderate↓**AMPA-R** potentiation—high ↑↑	**IPAC-4**	7.0±0.8 μM	360% (30 μM) (0.1-30 μM)	>0.5 mg/kg
Non-competitive blockade of **NMDA-R**—high ↓↓ **AMPA-R** potentiation—high ↑↑	**IPAC-5**	0.4±0.07 μM and 21.2±2.15 μM	1050% (30 μM) 1.0-30 μM	>0.5 mg/kg

[a]See the text.
[b]NMDA-receptor.
[c]AMPA-receptor.

administration of drugs did not induce any behavioural changes in the animals before training and had no toxic effect (Figure 3). In comparison with baseline values, the latencies of step down were significantly increased in vehicle- and drug-treated groups during the first recall session ($p<0.05$, Fridman test). The latencies of step down were significantly elevated in all groups of animals during the second recall session ($p<0.05$, Wilcoxon test), indicating that all mice acquired the task. A multiple group comparison performed with the Kruskal–Wallis test revealed a statistical difference between the groups in the latency of step down evaluated 1 h after training ($p=0.0012$, Kruskal–Wallis test; Figure 3). Group comparison performed with the Mann–Whitney *U* test showed that the latencies of step-down increased during the first recall trial in mice treated with memantine at a dose of 5 mg/kg, and with IPAC-5, as compared to saline or DMSO-treated groups, respectively (memantine: $p=0.041$, $U=151.0$; IPAC 5: $p=0.016$, $U=120.0$). No such difference was found between other drug-treated and vehicle-treated control groups ($p>0.05$, Mann–Whitney *U* test). DMSO-treated and non-treated control groups had similar values for this parameter ($p=0.910$, $U=215.0$; Mann–Whitney *U* test). These results provide evidence for a lack of effect of the vehicle on measured parameters of learning and suggest improved scores of short-term memory of the three groups of animals subjected to the above treatment.

A multiple group comparison performed with the Kruskal–Wallis test revealed a statistical difference between the groups in latencies of step down measured during the second recall session ($p=0.01$, Kruskal–Wallis test). This parameter was significantly elevated in mice treated with memantine at a dose 5 mg/kg, and with IPAC-5, as compared with saline- or DMSO-treated control groups ($p=0.006$, $U=104.5$and $p=0.004$, $U=91.0$ respectively; Mann–Whitney *U* test; Figure 3). Such differences were not found between other drug-treated groups compared with control groups ($p>0.05$, Mann–Whitney *U* test). DMSO-treated and non-treated control groups showed no difference in this parameter ($p=0.48$, $U=218.5$, Mann–Whitney *U* test). These data show that vehicle administration did not interfere with long-term learning of the step-down avoidance task and demonstrate enhanced scores of this type of memory in animals treated with memantine at a dose of 5 mg/kg and IPAC-5.

3.3. Extinction of contextual fear conditioning

Overall group comparison revealed significant changes in the percentage of time spent in freezing during a recall of extinction ($p=0.023$, Kruskall–Wallis test). Significant decrease in this parameter vs. respective control groups was found in mice treated with QXX at a dose of 5 mg/kg and IPAC-5 ($p=0.0013$, $U=20.5$ and $p=0.0062$, $U=31.0$, Mann–Whitney *U* test, respectively; Figure 4A). Other drug-treated groups showed no changes in freezing behaviour during a recall of extinction session, as compared with the respective control groups ($p>0.05$, Mann–Whitney *U* test). DMSO-treated and non-treated control groups showed no difference in this parameter either ($p=0.32$, $U=57.0$, Mann–Whitney *U* test; Figure 4A). Hence, the obtained results demonstrate that vehicle administration did not interfere with memory extinction in the current experiment. They show that the QXX employed at a dose of 5 mg/kg and IPAC-5 facilitates extinction of contextual memory. Multiple comparison of the duration of freezing behaviour during a recall session, before dosing of animals, evidenced the lack of pre-existing differences between experimental groups, thus excluding possible confounds in evaluation of changes of extinction that might result from different initial levels of learning ($p>0.05$, Kruskall–Wallis test; Figure 4B).

3.4. Dark-light box

One-way ANOVA followed by a post-hoc Dunnett's multiple comparison test revealed no differences between the dosed animals and respective control groups in the time spent in the lit compartment ($p=0.9935$, $F=0.1756$, $R^2=0.02115$; Figure 5A). This suggests that all groups exhibited similar levels of anxiety-like behaviour. Likewise, there was no statistical difference between the groups in the latencies of exit to lit area and number of exits ($p=0.9890$, $F=0.1842$, $R^2=0.02048$ and $p=0.9764$, $F=0.1697$, $R^2=0.02239$; Figure 5B and C, respectively; ANOVA and Dunnett's multiple comparison test), suggesting that the administration of drugs did not change locomotion and did not induce any toxic effects.

4. Discussion

Here we found that, among the new drugs tested, IPAC-5 replicated the properties of memantine and QXX in one administration with increased potency. IPAC-5 elevated memory scores in both the step-down passive avoidance task and a paradigm of extinction of conditioned freezing (Figures 3 and 4A). In the former model, it significantly increased the latencies of step down evaluated in mice 1 and 24 h after training that suggests enhanced short-term and long-term memories (Strekalova and Steinbusch, 2010; Vignisse et al., 2011). Importantly, baseline latencies, measured 15 min after drug administration and prior to training, were unaffected. This indicated a lack of change in locomotion and anxiety and makes the occurrence of neurotoxic effects, which can be observed with competitive NMDA receptor blockers at moderate doses (Olney et al., 1989) and non-competitive NMDA receptor blockers at high doses (Creeley et al., 2008; Chen et al., 2012), very unlikely in a present experiment. Administration of memantine has evoked similar effects in both recall trials that validated applied here the step-down avoidance learning protocol in mice as a memory paradigm that is sensitive to a low-affinity blockade of NMDA receptors. Whilst some labs report memantine-induced cognitive dysfunctions, our data are in line with results obtained in intact mice and rats, which showed beneficial effects of memantine administration at the dose below 10 mg/kg in the active avoidance, water maze, delayed non-match-to-sample task and object recognition tasks without affecting sensomotor functions (Zoladz et al., 2006; Creeley et al., 2008; Minkeviciene et al., 2008; Loskutova and Kostjunina, 2009; Smith et al., 2011; Saab et al., 2011; Ihalainen et al., 2011). The differences in terms of chronicity of training/dosing and

motivational impact between the paradigms employed in the studies that showed differential effects of memantine on memory might explain distinct outcomes. While AMPA receptors are reported to be implicated in the acquisition of various forms of fear-related memories, as it is discussed above, in the present study, the AMPA-receptor potentiator QXX used at the same dose as memantine (5 mg/kg) was ineffective in the step-down avoidance test. This does not exclude the possibility that potentiation of AMPA receptors under different conditions could have such effect, however, our data suggest higher sensitivity of contextual learning in applied test of inhibitory learning to low-affinity NMDA receptor blockade mechanism. The IC_{50} of IPAC-5 on NMDA receptor blockade was lower than that of memantine (0.4 μM vs. 1.4 μM) that alone could explain the memory enhancing effects of this new drug in the step-down avoidance model. At the same time, IPAC-3 had low IC_{50} as well (see Table 1), but did not enhance contextual memory. This drug had moderate action on the AMPA receptor.

In the paradigm of extinction of contextual fear conditioning, the duration of freezing scored during a recall of extinction trial was significantly reduced by the administration of IPAC-5 and QXX at the dose 5 mg/kg (Figure 4A). No such effects were found after the dosing with memantine. Thus, while NMDA receptors were shown to be implicated in the extinction of fear-related learning (Falls et al., 1992) and NMDA low-affinity receptor blockers were found to interfere with some of its forms at higher doses (Yamada et al., 2011), no evidence that the extinguishing of contextual freezing is dependent on NMDA low-affinity blockade was obtained in the present studies. Our results suggest that extinction of contextual freezing here is predominantly sensitive to the AMPA receptor positive modulation; a role of AMPA receptors is well established in a literature (Clem and Huganir, 2010; Kaplan and Moore, 2011). In line with this finding, in a similar mouse model of extinction of conditioned fear, another AMPA receptor potentiator, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA) was reported to decrease freezing scores at doses that are comparable to those used in a current study (3, 10, 30 mg/kg; Yamada et al., 2011). Similar values of freezing behaviour measured during a recall session evidenced a lack of pre-existing differences between experimental groups and ruled out a possibility for confounds resulting from this factor (Figure 4B).

As it is mentioned above, IPAC-5 is characterized by remarkably high AMPA receptor potentiation (Table 1) that could alone account for its facilitating effect on memory extinction, taking into account evidences for the predominant sensitivity of the performance of the animals to the application of the stimulator of AMPA receptors in this model. However, another drug, IPAC-4, was highly effective as a positive modulator in comparison to QXX (360% vs. 210% for QXX); this compound had a modest effect on the NMDA receptor (with IC_{50} 21 μM vs. 1.4 μM for memantine) and did not facilitate extinction of contextual freezing. Similarly, IPAC-1, a highly positive modulator of AMPA receptors (230% from control), though a distinct chemical class, also produced no change in memory extinction in mice (Figure 4A). Hence, three compounds of different chemical classes that displayed similarly significant potentiating effects on AMPA receptors were not accompanied by 'high' or any efficacy towards NMDA receptors: QXX at low dose, IPAC-1 and IPAC-4, were equally ineffective in the fear conditioning model.

A separate study employing the dark-light box was carried out to assess the possible side effects of the new drugs. All groups spent similar time spent in the lit box (Figure 5A); the number of exits and the latency of the first exit to the lit area (Figure. 5B and C) were similar in all groups, which indicated that acute neurotoxic effects were unlikely. Locomotion and anxiety scores were not changed by the treatments. The former finding rules out possible effects on anxiety by these glutamatergic drugs (Kapus et al., 2008; Takahashi et al., 2009). Memantine, at the higher than those used in this study doses, evokes an anti-anxiety effect in the elevated plus-maze test (Minkeviciene et al., 2008). The neurotoxic effects, which are typical for MK-801 and competitive NMDA receptor blockers with high affinity to this receptor, were not expected here as these compounds were designed as low affinity NMDA receptor blockers. This was confirmed in independent experiments (a constant of binding of IPAC 1-5 was 0.8-29.3 μM, while this constant for MK-801 was 4.9 nM; Grigoriev and Bachurin, unpublished results). As expected, in the data presented here, and in previous rodent studies using higher doses, the bolus and chronic injections of memantine and IPAC-5 suggest that any neurotoxic effects are highly unlikely (Bachurin et al., 2001; Willmore et al., 2001).

Thus, in comparison to the other newly synthesized drugs, IPAC-5 was characterized by the lowest IC_{50} values of NMDA receptor blockade and highest potency of AMPA receptor stimulation and was the only compound with true bifunctional activities that enhanced learning in NMDA- and AMPA-receptor manipulation sensitive paradigms. Moreover, the new compound was effective at a dose of 0.5 mg/kg, which is ten times lower than the doses at which both memantine and QXX induced their effects on mouse learning; doses of memantine and QXX at 1 mg/kg were ineffective. Meanwhile, as mentioned above, the magnitude of the electrophysiological effects on glutamatergic receptors lay in the same range as the tested drugs with mono- and bifunctional properties with respect to NMDA and AMPA receptors.

While the complete mechanism of action of the newly synthesized compounds remain to be elucidated in order to discover whether there are other off-target effects that might contribute to the superior efficiency of memory-enhancing effects of IPAC-5, it is clear that this compound has 'high' effects on the two glutamate receptors that suggests that the concomitant manipulation of NMDA- and AMPA-receptors has pro-cognitive action. Importantly, the memory-improving activity of IPAC-5 was further supported in studies (*data not shown*) that used learning paradigms based on positive reinforcement: new object recognition model and new object localization test, as well as Morris water maze and instrumental learning of active avoidance task (Bachurin et al., 2001; Beznosko and Bachurin, *unpublished data*).

It is of interest that a [3H] Ifenprodil binding study has revealed that IPAC-5 that blocks ifenprodil binding site at the NR2B subunit of the NMDA receptor at a very low concentration ($IC_{50}=0.4\pm0.1$ mcM) suggesting that this effect may mediate the possible molecular mechanisms of action of this compound. While the role of NR2B subunit in

the mechanisms of learning is rather complex (Cull-Candy et al., 2001), its pharmacological blockade produced memory-enhancing effects (Higgins et al., 2005). Further electrophysiological experiments (LTP/LTD) would be useful in order to address the knowledge gap between the patch clamp data with IPAC-5 and its pro-cognitive effects from the behavioural experiments, and these are the subject of our ongoing studies.

A number of other factors, apart from those suggested here, could potentially contribute observed differences in mnemotropic effects between the drugs. However, IPAC-5 was reported to affect glutamate-induced Ca^{2+} uptake and prevent NMDA-induced convulsions (Bachurin et al., 2001) that evidences its pronounced effects on glutamatergic system and additionally suggests rather glutamatergic mechanisms to underlie the mnemotropic action of this drug. Further, because IPAC-2–5 belongs to the same chemotype it is not likely that differential pharmacokinetics, their penetration across blood–brain barrier, or bioavailability are so distinct to account for the altered behavioural effects. IPAC-1 is of a different chemical class but similar group of applied here drugs IPAC 2–5 and was used as an external reference in this study; a comparison of all five drugs at higher doses in tests for locomotion suggests similar pharmacokinetics of these drugs (Grigoriev, unpublished data). As it is mentioned above, preliminary data suggest similar pharmacokinetic properties of QXX to those of memantine (Bachurin et al., 2008). Similar doses of all new drugs tested in this work affected locomotion and anxiety in a battery of tests (see Table 1) and together suggest that they all cross the brain-blood barrier and have similar pharmacodynamics. Thus, while currently there are no immediate data that can be presented to exclude all potential differences between the drugs that are unrelated to their effects on receptors, it is quite unlikely that such differences fully determine reported here functional effects. Further experiments that address these questions are under way.

The data presented here support the argument that simultaneous, low-affinity blockade of the NMDA receptor and stimulation of AMPA receptor is a promising strategy to decrease the minimal effective dose and possible adverse events that may be observed in the clinic. Our results argue for the development of multi-target drug mechanism in the modulation of cognitive functions and suggest further its potential for clinical implications.

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ISAO, FCT and KNAW had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

Author **S.B.** designed the study with drug synthesis and screening in vitro. Author **A.P.** carried out drug design and synthesis and structure verification. Author **V.G.** managed the electrophysiological testing of drugs and statistical analysis of the outcome from this part of the study. Authors **J.V.** and **A.B.** undertook the behavioural experiments and statistical analysis of behavioural data. Authors **H.S.** and **L.B.** managed the literature searches and helped to design the study. Author **T.S.** wrote the protocol of behavioural studies and the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflicts of interest

All other authors declare that they have no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2013.06.010.

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ORIGINAL RESEARCH REPORT

Altered emotionality, hippocampus-dependent performance and expression of NMDA receptor subunit mRNAs in chronically stressed mice†

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Abstract

N-Methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission in the hippocampus is implicated in cognitive and emotional disturbances during stress-related disorders. Here, using quantitative RT-PCR, we investigated the hippocampal expression of NR2A, NR2B and NR1 subunit mRNAs in a mouse stress paradigm that mimics clinically relevant conditions of simultaneously affected emotionality and hippocampus-dependent functions. A 2-week stress procedure, which comprised ethologically valid stressors, exposure to a rat and social defeat, was applied to male C57BL/6J mice. For predation stress, mice were introduced into transparent containers that were placed in a rat home cage during the night; social defeat was applied during the daytime using aggressive CD1 mice. This treatment impaired hippocampus-dependent performance during contextual fear conditioning. A correlation between this behavior and food displacement performance was demonstrated, suggesting that burrowing behavior is affected by the stress procedure and is hippocampus-dependent. Stressed mice ($n = 22$) showed behavioral invigoration and anomalous anxiolytic-like profiles in the O-maze and brightly illuminated open field, unaltered short-term memory in the step-down avoidance task and enhanced aggressive traits, as compared to non-stressed mice ($n = 10$). Stressed mice showed increased basal serum corticosterone concentrations, hippocampal mRNA expression for the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio; mRNA expression of NR2B and NR1 was unchanged. Thus, stress-induced aberrations in both hippocampal-dependent performance and emotional abnormalities are associated with alterations in hippocampal mRNA NR2A levels and the NR2A/NR2B ratio and not with mRNA expression of NR2B or NR1.

Keywords

Burrowing behavior, contextual fear memory, hippocampus, *N*-methyl-D-aspartate receptor, predator stress, social defeat

History

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Introduction

N-Methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission is involved in the etiology of stress-related cognitive deficits and behavioral abnormalities (Cull-Candy et al., 2001). This particularly implicates altered expression of the two NR1 (GluN1) and two NR2 (GluN2) subunits (NR2A and NR2B) of NMDAR tetramers, which are the most abundant in the hippocampal formation, a brain structure that mediates stress-induced aberrations in both learning and emotional behavior (Li & Ju, 2012). Both NR2A and NR2B were shown to have distinct pharmacology and a role in the regulation of NMDAR, and have been suggested to be differentially involved in the mechanisms of learning and emotionality (Fleischmann et al., 2003; Li & Tsien, 2009). A body of evidence has demonstrated the involvement of NR2A, NR2B and NR1 subunits in the neurobiology of neuropsychiatric conditions such as anxiety, psychosis, impulsivity, Alzheimer's disease and major depression (Davies et al., 2012; Geissler & Lesch, 2011; Tsang et al., 2008).

Most experiments investigating the roles of NMDAR subunits in neuropsychiatric symptoms target selective subunits of this receptor using pharmacological and genetic manipulations that are frequently applied *in vitro* (Boyce-Rustay & Holmes, 2006; Cui et al., 2013; Longordo et al., 2009). However, fewer studies have addressed the changes in NMDAR subunit expression in

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disease-modeling conditions. Understanding changes in relative-fold mRNA expression of NMDAR subunits in animal models of cognitive and emotional disturbances related to chronic stress is important for the identification of new pharmacological interventions and therapies that effectively alter glutamatergic transmission (Vignisse et al., 2013). Previous work on gene expression (Strekalova et al., 2011) has highlighted abnormal hippocampal expression of NMDAR subunits in mice subjected to repeated stressors. We have hypothesized that in male C57BL/6J mice, a novel 2-week stress procedure, comprising ethologically valid stressors, exposure to a rat and social defeat, results in concomitant cognitive, emotional and NMDA receptor expression abnormalities in the hippocampus. Thus, the current study specifically investigated the hippocampal relative-fold mRNA expression of NR2A, NR2B and NR1 subunits in the above-mentioned chronic stress procedure in mice that mimics clinically relevant situations of stress-related disturbances in learning and emotionality (Cline et al., 2012; Strekalova & Steinbusch, 2010).

Materials and methods

Animals and housing

Male C57BL/6J and CD1 mice were 3 months old, Wistar rats of 3–5 months age were used for applying predator stress; mice and rats were provided by Harlan, the Netherlands and Charles River, France, respectively. Fourteen days before the behavioral experiments, mice were single housed under a reverse 12 h:12 h light–dark cycle (lights on: 21:00 h) in standard laboratory conditions (22 ± 1 °C, 55% humidity, food and water *ad libitum).* All experiments were carried out in accordance with the European Committees Council Directives and had been approved by the ethics committee of Maastricht University for animal research (CPV, DEC-UM 2009-109).

Experimental conditions and study outline

This study applied only ethological stressors to male C57BL/6J mice, for 14 d, based on previous work (adapted from Cline et al., 2012; Couch et al., 2013). The stress procedure consisted of dark-cycle rat exposure between the hours of 09:00 h and 18:00 h and light-cycle application of a social defeat paradigm, combined with exposure to an aggressive CD1 mouse. Body weight and parameters of social behavior were determined 1 week before the chronic stress procedure in a social interaction test as described elsewhere (Strekalova et al., 2004). The experimental and control groups were balanced for these parameters. Ethological stressors were applied to a stress group for 14 d, as described below. The control group received daily handling only. At the end of the stress experiment, 12 h after the application of the last stressor, short-term memory in the step-down avoidance task (Vignisse et al., 2011) and aggressive behavior in a resident-intruder test (Strekalova et al., 2004) were investigated in stressed and control mice. At this time, body weight was also assessed. The next day (day 1), mice were tested in the elevated O-maze and food displacement tube tests (Strekalova & Steinbusch, 2010). On days 2 and 3, respectively, contextual fear conditioning training and testing for recall (Vignisse et al., 2013) were performed. The open-field testing was carried out on day 4 (under red light) and on day 5 (under white light). All behavioral tests were recorded on video.

We used a battery of behavioral tests based on previous literature (Calabrese et al., 2012). Although it is possible that the different behavioral tests may affect subsequent tests, previously published test batteries with similarly employed paradigms have revealed an absence of any testing effects in C57Bl/6J mice (Malatynska et al., 2012; Strekalova & Steinbusch, 2009, 2010; Vignisse et al., 2011, 2013). Hence, we considered possible interfering effects of multiple behavioral test to be minimal in the current study.

A separate cohort of mice from both groups was pre-exposed to a mixture of CO_2 and O_2 and euthanized via cervical dislocation (according to Dutch law), for gene expression analysis 24 h after the termination of the stress procedure.

Chronic stress procedure

Rat exposure while in a small container

Mice were introduced into cylindrical containers, which were placed into a rat home cage for 15 h (overnight, from 18:00 h to 9:00 h). Containers (15 cm × Ø 8 cm) were made from customized transparent plastic with holes in the covers (Ø < 0.5 cm). This ensured protection of the mouse from the rat, but allowed visual and odor contact. During the weekends, mice were kept in their home cages, situated on top of the rat cages.

Social defeat stress

Social defeat procedures took place during the dark phase of the light cycle (between 12:00 h and 16:00 h). To enable visual control by the experimenter over the resident-intruder confrontation, the test was carried out under red light. In a preliminary test, aggressive CD1 mice that were able to attack the counter-partners in less than 60 s, without injuring them, were selected for this procedure; these mice were introduced into the home cages of mice from the stress group during social defeat sessions for 5 min. During social defeat stress, test mice typically showed flight responses, submissive postures and vocalizations. Pairs of mice were carefully observed in order to prevent physical harm. In rare cases, aggressive mice were immediately removed from the cage of resident mice. After a 5-min period of social defeat, C57BL/6J mice were placed into small containers and put inside a CD1 mouse cage, where they stayed for a 3 h-period. After the 3 h period, the 5-min social defeat procedure was repeated. In order to randomize the procedure, the same pairs of C57BL/6J and CD1 mice were never put together.

Behavioral procedures

Step-down passive avoidance model

The step-down passive avoidance test was used as described elsewhere (Strekalova et al., 2001; Vignisse et al., 2011, 2013). The step-down apparatus (Evolocus LLC Tarrytown, NY and Technosmart, Rome, Italy) was a transparent plastic cubicle (25 cm × 25 cm × 48 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden

platform (7 cm × 7 cm × 1.5 cm) was placed. The illumination strength was 25 lux. A shocker was used to deliver an alternating electric current (AC, 50 Hz). In this paradigm, mice are trained to avoid an electric shock by staying on the platform above the grid floor. During the training session, mice were placed on the platform inside a transparent cylinder for 30 s to prevent them from stepping down immediately. After removal of the cylinder, the time until the mouse left the platform, with all four paws, was measured as baseline latency of step-down. Immediately after step-down, mice received a single electric foot shock (0.5 mA, 2 s) and were returned to their home cages. One hour later, during the recall trial session, mice were exposed to the apparatus again by being handled in the same way as in the training session; no foot shock was delivered. Latency of step-down with all four paws was measured until 180 s had elapsed.

Elevated O-maze

Testing on the elevated O-maze was carried out as described elsewhere (Strekalova et al., 2005). The O-maze consisted of a black circular path (runway width 5.5 cm, Ø = 46 cm) with two opposing compartments protected by walls made of polyvinyl-chloride (height = 10 cm) and two open sectors of equal size. The maze was elevated 20 cm above the ground and illuminated from the top with red light. At the start of the testing session, mice were placed inside one of the two closed compartments. The test was recorded with a web camera. The latency to the first entry into the anxiety-related open arms of the maze, total number of entries into the open arms and total duration of time spent in open arms were scored for 5 min.

Food pellet displacement (burrowing) behavior in a tube test

In order to further assess hippocampal function, all experimental groups were tested for burrowing behavior. Burrowing behavior, a tendency to displace small objects, e.g. small stones or food pellets, from a tube inside the home cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation. Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the middle of a mouse home cage, the latency to displacement of the first food pellet, time required to empty the tube, number of pellets removed after 1 h and 1 h 30 min were assessed in stressed and control mice during the dark phase (Strekalova & Steinbusch, 2009, 2010). Time elapsed was 90 min.

Contextual fear-conditioning paradigm

The contextual fear-conditioning test procedure was adapted from previously described protocols (Strekalova et al., 2003; Vignisse et al., 2013). The apparatus consisted of a transparent plastic cubicle (25 cm × 25 cm × 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter). A shocker was used to deliver an alternating electric current (AC, 50 Hz; 0.7 mA, 2 s) after a 2-min acclimatization of a mouse to the chamber. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behavior was scored by visual observation during a test of memory recall that was carried out 24 h later. The occurrence of freezing behavior in the chamber was assessed every 10 s for 180 s; each 10-s score was assigned to a freezing or non-freezing period, and the percentage of time spent in freezing was calculated.

Open field

The open-field apparatus consisted of four square arenas (25 cm × 25 cm × 40 cm), made of wood covered with white resopal. Mice were put in the center of one of the four square open field arenas, and their behavior was video recorded for 10 min. The open field was illuminated with white light (25 lux) or red light. Behavior was analyzed off-line using the Any-maze software (Stoelting Co, Wood Dale, IL). Among other parameters, time spent immobile was analyzed in the central (area 20 × 20 cm) and peripheral (remaining part of the apparatus) zones.

Resident-intruder test

The resident-intruder test procedure was performed as described elsewhere (Strekalova et al., 2004). In this paradigm, the C57BL/6J mice were placed individually in an observation cage (30 cm × 60 cm × 30 cm) for 30 min. Thereafter, a male CD1 mouse, which was group housed before the test, was introduced as an intruder to the same cage and left with the resident mouse for 8 min. During the observation period, resident and intruder mice were scored for aggressive social behaviors. Latency of the first attack and number of attacks were scored.

Brain dissection and blood collection

On the day following the termination of the stress procedure, a cohort of mice from both groups was euthanized as described above, their hippocampi were dissected and trunk blood was collected for corticosterone evaluation.

Corticosterone concentration

To assay serum corticosterone, trunk blood was stored at 4 °C overnight and centrifuged at 10 × *g* for 10 min; the assay was performed as previously described (Pawluski et al., 2012). Serum was collected and stored a −80 °C until use. All samples were run in duplicate. For total serum corticosterone concentrations, a commercially available radioimmunoassay (RIA) kit for rat corticosterone from MP Biomedicals (corticosterone I25 for rats and mice, MP Biomedicals, LLC, Orangeburg, NY) was used. Average intra- and inter-assay coefficients of variation for all corticosterone assays were below 10%. Assay sensitivity was 7.7 ng/mL.

RNA isolation and RT PCR

RNA was extracted as previously described (Couch et al., 2013) using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) and first strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 μg total RNA was converted into cDNA. Quantitative RT-PCR (qPCR) for *NR2A*, *NR2B*, *NR1* genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using TaqMan probes and the CFX96 Real-time System (BioRad, Hercules, CA). Cycling conditions and

sequences of primers used are indicated in Table 1 of supplementary data. Results were normalized to GAPDH mRNA expression and calculated as relative-fold changes compared to control mice as described elsewhere (Couch et al., 2013). Results of the qPCR measurements were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH expression in each cDNA sample ($2-\Delta\Delta$ Ct method). Results are given as expression-folds compared to the mean expression values in non-stressed control mice (Couch et al., 2013, adapted from Livak & Schmittgen, 2001).

Statistical analysis

GraphPad Prism 5.00 (San Diego, CA) was used for analyses. A comparison of normally distributed independent variables was carried out using unpaired two-tailed t tests. Independent measurements that were not normally distributed were analyzed via the non-parametric Mann–Whitney test, and repeated measurements were compared by the Wilcoxon non-parametric test. The Pearson test was applied for correlation analysis. The level of confidence was set at 95% ($p<0.05$).

Results

Stressed mice showed a significant loss of body weight compared to the control mice ($p<0.0001$, $t=6.801$, df = 21; unpaired t test; Figure 1A) and a significant elevation of circulating corticosterone concentration ($p=0.030$, $U=39$; Mann–Whitney test, Figure 1B), indicating a profound impact of the 2-week stress procedure. In comparison to control mice, the open-field activity of the stressed mice was significantly higher both in the peripheral ($p=0.040$, $U=61.00$, Mann–Whitney test) and even more notably, in the central zones ($p=0.011$; $U=73.50$) of the apparatus, when white lighting was employed (Figure 1C), but not when activity was scored under red light ($p=0.15$, $U=68.00$ and $p=0.64$, $U=97.50$, respectively). There was a significantly lower latency to the first exit in the elevated O-maze ($p=0.010$, $U=49.50$, Mann–Whitney test), increased time spent in the open arms ($p=0.020$, $U=55.50$) and number of entries into the open arms ($p=0.028$, $U=58.50$; Figure 1D) in stressed versus control mice. Together, these data indicate differences in the brightly lit open field induced by stress: behavioral invigoration and an anomalous ''anxiolytic-like'' profile. In addition, chronically stressed mice displayed a significant increase in the number of attacks and no change in the latency to the first attack in the resident-intruder test ($p=0.049$, $t=1.698$, df = 30 and $p=0.12$, $t=1.78$, df = 23; unpaired t test; Figure 1E).

Both stressed and control mice showed a significant increase in the latency of step down 1 h after training, in comparison to baseline latencies ($p=0.033$, $W=-37.00$ and $p=0.05$, $W=-51.00$, respectively, Wilcoxon test). There was no significant difference between stressed and control mice in the latencies for step-down evaluated at baseline conditions ($p=1.0$, $U=71.50$) or 1 h after training ($p=0.98$, $U=71.00$; Figure 2A), indicating similar scores of anxiety-like behavior and short-term memory in these groups. In the contextual fear-conditioning paradigm, stressed mice spent a significantly shorter time freezing during a recall session than the control group ($p=0.021$, $U=50.00$, Mann–Whitney test; Figure 2B), which demonstrates impaired hippocampus-dependent contextual memory in this group. Stressed mice showed no significant inhibition of burrowing behavior in comparison to the control group, as assessed by latency for the first pellet displacement ($p=0.18$, $U=73.00$; Figure 2C) and number of pellets displaced over time intervals of 0–60 min ($p=0.14$, $U=53.50$) and 60–90 min ($p=0.34$, $U=47.50$, Mann–Whitney test). Nonetheless, there was a significant correlation between the two latter measures and contextual freezing ($r=0.36$, $p=0.046$ and $r=0.36$, $p=0.048$, respectively). There was no significant correlation between the duration of freezing and the latency for pellet displacement ($r=-0.16$, $p=0.39$, Pearson correlation).

Concerning mRNA levels of NMDA receptor subunits in the hippocampus, in comparison to control mice, the stressed group had a significant increase in expression of NR2A ($p=0.029$, $t=2.050$, df = 15, unpaired t test; Figure 3A) and no significant change in the expression of NR2B ($p=0.27$, $t=0.6431$, df = 15, Figure 3B). The ratio of NR2A/NR2B was significantly increased ($p=0.015$, $t=2.452$, df = 13; Figure 3C) but the expression of the NR1 subunit was unaltered ($p=0.64$, $t=0.4665$, df = 14; Figure 3D).

Discussion

In accordance with our hypothesis and in line with the literature (Calabrese et al., 2012; Cull-Candy et al., 2001; Fleischmann et al., 2003; Li & Ju, 2012), the current study implicates altered expression of NMDAR subunits of the hippocampus in stress-induced deficits in both cognitive and emotional traits. The present study showed that in C57BL/6J mice, a 2-week ethological stress paradigm, comprised of exposure to a rat and social defeat, resulted in the impairment of contextual fear conditioning, as well as behavioral disinhibition in the open field test and increased aggressive behavior. These cognitive and emotional abnormalities were accompanied by increases in the hippocampal mRNA expression of the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio, while the mRNA expression of NR2B and NR1 was unchanged.

A significant reduction in body weight and increase in basal serum corticosterone concentration of stressed mice demonstrated the impact of the 2-week stress procedure (Figure 1A, B). Stressed mice displayed behavioral hyperarousal under stressful testing conditions, as shown by a significant decrease in the time spent immobile in the central and peripheral parts of the brightly illuminated open field, but a lack of these changes during stress-free open field testing under red light (Figure 1C). Behavioral alterations of the stressed group in the open field illuminated with white light are in agreement with behavioral changes in these mice in the elevated O-maze. In the O-maze test, stressed mice showed a significant shortening of the latency to enter the open arms, an increase in the time spent and the number of entries into the open arms (Figure 1D). Together, these findings indicate that the stress procedure evoked anomalous ''anxiolytic-like''

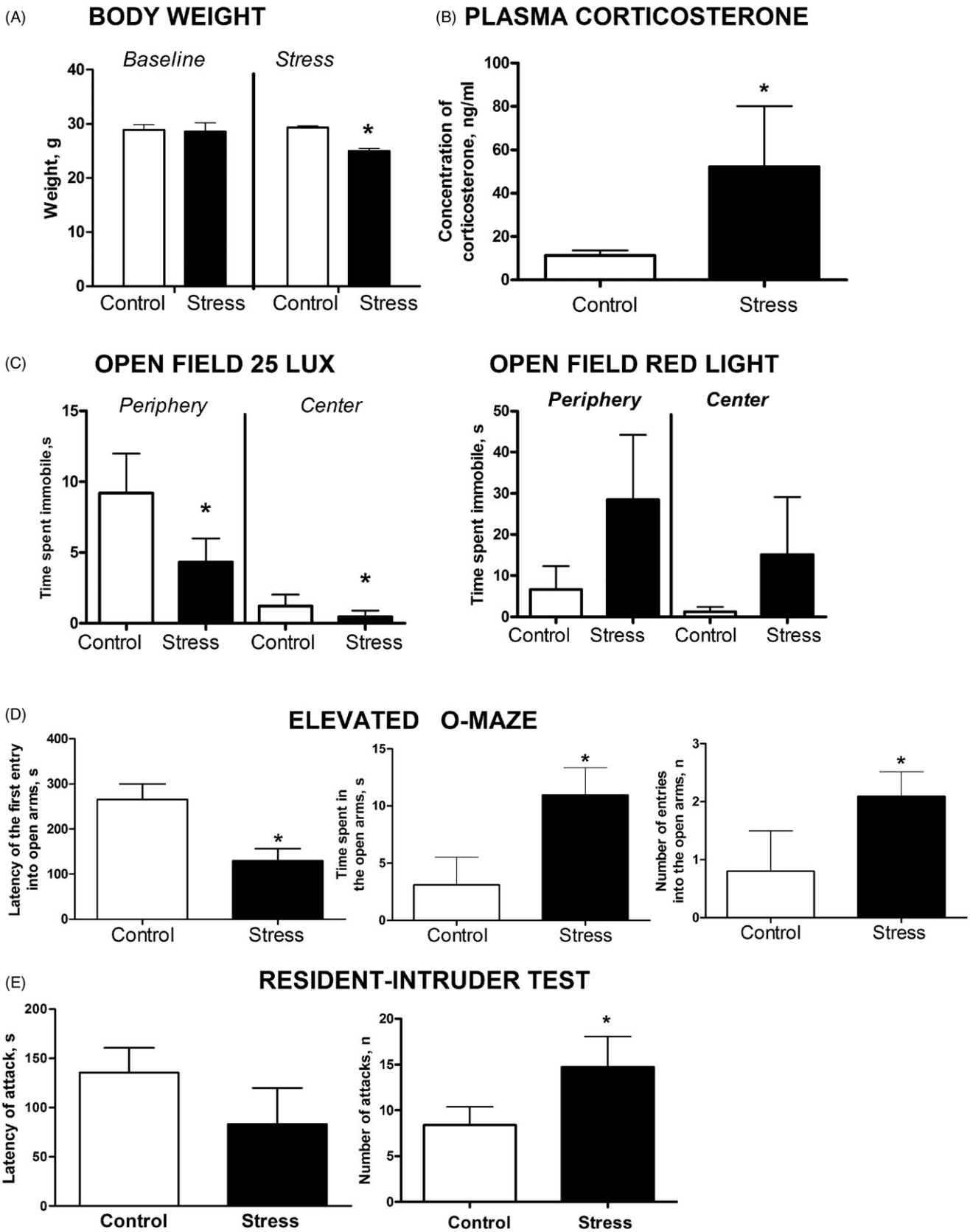

Figure 1. Exposure of mice to stressors for 2 weeks affects body weight, serum corticosterone and parameters of emotionality. (A) Weight loss and (B) increased serum corticosterone concentration in the stress group. (C) Stressed mice showed a reduced total time spent immobile at the periphery and in the center of the open field lit with white light. There were no significant differences in locomotor behavior between groups tested under red light. (D) Stressed mice displayed decreased latency of entries into the open arms, an increased time spent therein and increased number of entries. (E) Elevated aggressive behavior (number of attacks) in stressed mice. *$p < 0.05$ versus control (A, E: unpaired t test, B–D: Mann–Whitney test). Control group, $n = 10$; stress group, $n = 22$. All data are means ± standard error of the mean (SEM).

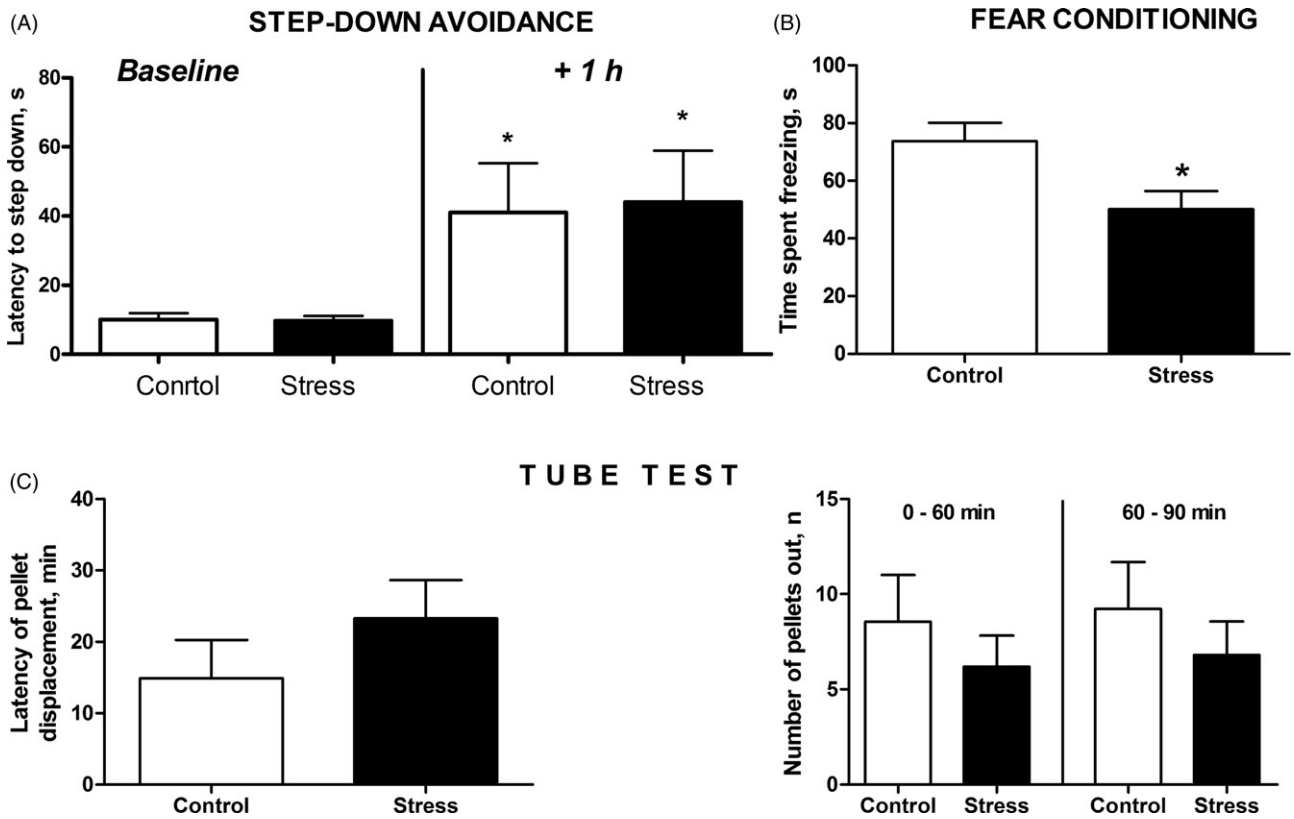

Figure 2. Hippocampus-dependent performance in stressed mice. (A) Stressed and control mice showed a significant increase in the latency for step down 1 h after training, in comparison to baseline latencies; $*p > 0.05$ versus baseline, Wilcoxon test; there were no differences between groups. (B) Stressed mice spent a significantly shorter time freezing, during a recall session in the fear-conditioning paradigm; (C) there were no significant differences for latency to food displacement or number of pellets displaced at 0–60 min and 60–90 min in the tube test. $*p < 0.05$ versus control; Mann–Whitney test. Control group, $n = 10$; stress group, $n = 22$. All data are means ± the standard error of the mean (SEM).

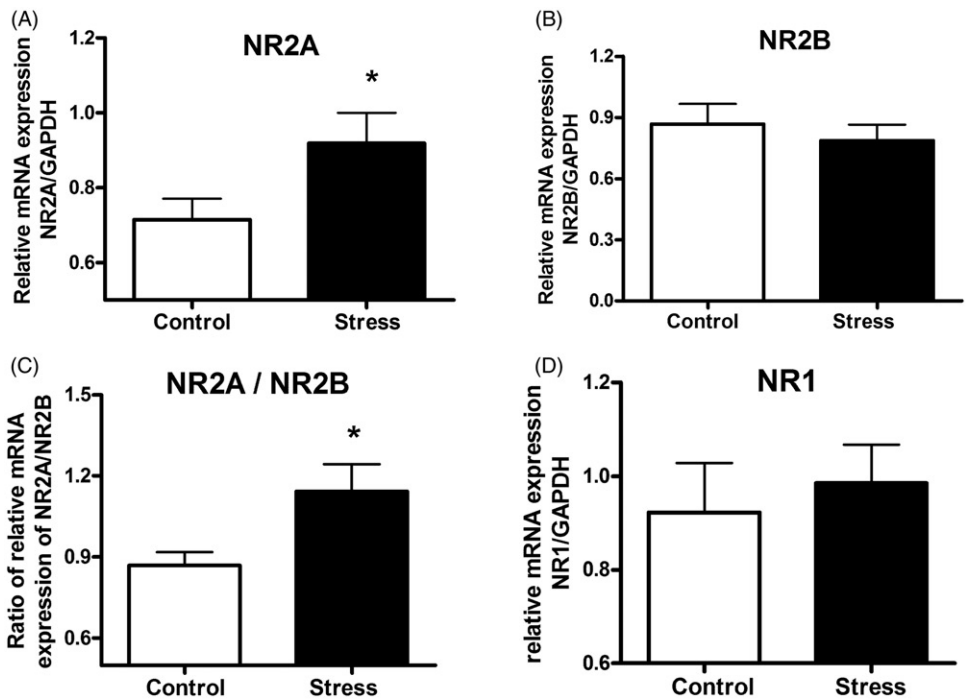

Figure 3. Hippocampal expression of mRNAs for *N*-methyl-D-aspartate receptor (NMDAR) subunits in stressed mice. In the stressed group, relative to controls: (A) mRNA expression of the NR2A subunit of the NMDAR was significantly greater; (B) mRNA expression of NR2B was not altered; (C) the ratio of mRNAs for NR2A/NR2B was significantly increased; (D) mRNA expression of the NR1 subunit of NMDAR was not altered; $*p < 0.05$ versus control; unpaired *t* test. Control group, $n = 9$; stress group, $n = 12$. Data are means ± the standard error of the mean (SEM).

traits and behavioral invigoration in mice. In our experiments, these changes were accompanied by a significant increase in the number of attacks by stressed mice in the resident-intruder paradigm, which is an indicator of enhanced aggressive traits (Figure 1E). This is in agreement with previous work showing that increased anxiety levels typically correlate with increased scores of aggressive behavior in C57BL/6J male mice (Willner, 2005).

In line with our findings, different chronic stress procedures have been reported to cause an increase in time spent in anxiety-related compartments of the elevated plus maze and the dark/light box in rodents, which was interpreted as a sign of ''anxiolytic-like'' effects (Cancela et al., 1995; D'Aquila et al., 1994; Hata et al., 2001; Sanchez, 1997). Such phenomena, also considered as manifestations of impulsivity and disinhibition, are well-known consequences of chronic stress in rodents (Belujon & Grace, 2011; Strekalova et al., 2005; Willner, 2005). It is commonly accepted that the limbic system, in general, and the hippocampus, in particular, have a central role in the behavioral inhibition system (Abela & Chudasama, 2013; Geissler & Lesch, 2011; Gray & McNaughton, 1983; McNaughton et al., 1997; McNaughton & Gray, 2000; Rawlins et al., 1985), while the crucial role of the hippocampus for memory was established much earlier (Squire, 1992). Behavioral invigoration, such as during stress, is likely to be associated with alterations of other hippocampal functions, which may include learning processes and molecular changes related to gene expression regulating glutamatergic neurotransmission (Belujon & Grace, 2011; Calabrese et al., 2012; Geissler & Lesch, 2011). Thus, these potential changes were investigated in the present study.

Stressed mice had a normal acquisition of the short-term step-down avoidance task, a form of cortex-dependent learning, which was shown by their unaltered latencies to step-down (Figure 2A). However, a lower percentage of freezing during the recall session of the fear-conditioning test in stressed mice indicates a deficiency in their long-term hippocampus-dependent memory (Figure 2B). The possibility that a hyperactivity of chronically stressed mice in our study interferes with scoring of freezing was largely ruled out by a lack of differences in locomotor activity during a 2-min acclimatization pre-training period between stressed and control mice (Strekalova et al., 2003). Also, both groups had similar values for the baseline latencies of step-down behavior (Figure 2A), indicating similar anxiety-like traits when assessed under the testing conditions that were subsequently used to assess contextual learning. Baseline step-down behavior was previously reported as a highly sensitive measure of subtle changes in anxiety and locomotion in C57BL/6J mice (Strekalova & Steinbusch, 2009, 2010; Vignisse et al., 2011, 2013).

The changes in contextual freezing significantly correlated with decreased burrowing behavior, although burrowing parameters were not altered significantly (Figure 2C). A tendency to displace food pellets is often regarded as not a fully specific indicator of hippocampal dysfunction in rodents (Hart et al., 2012; Kaczmarczyk et al., 2013; Tarr et al., 2012) and its neurobiology is debatable. Hence the finding of a correlation between this behavior and hippocampus-dependent learning in the present study might be potentially important in relating the burrowing behavior to the dorsal hippocampus, as originally proposed (Deacon et al., 2002).

This study revealed stress-induced increases in the hippocampal expression of NR2A and the NR2A/NR2B ratio (Figure 3A, C), which were previously shown to accompany elevated anxiety (Boyce-Rustay & Holmes, 2006; Calabrese et al., 2012; Gao et al., 2010), impulsivity and aggression (Bortolato et al., 2012; Meyer et al., 2004), home cage hyperactivity and a stress-induced increase in peripheral concentrations of corticosterone (Huang et al., 2010; Longordo et al., 2009) in various conditions. A limitation of our study, however, is the need for confirmation that the mRNA changes result in corresponding changes in subunit protein levels and in altered synaptic function in the hippocampus. In agreement with our data, separate studies have reported that molecular changes such as we found are associated with a disruption of long-term memory, but not short-term learning (Calabrese et al., 2012; Cui et al., 2013; Huang et al., 2010). In the present experiment, mRNA expression of NR2B and the NR1 in the hippocampus was not significantly changed by stress (Figure 3). However, previous work has reported a significant decrease in NR2B expression in several brain areas during stress (Cull-Candy et al., 2001; Huang et al., 2010), aging (Dere et al., 2003) and compromised plasticity (Bortolato et al., 2012). Previous work has also reported a decrease in NR1 mRNA after stress (Cull-Candy et al., 2001; Schenberg et al., 2006). Discrepancies between our findings and others may be due to the different stress paradigms employed. However, previous work we have done in a gene expression profiling Illumina study (Integragen, Evry, France and Northwestern Chicago University, USA and Ingenuity Systems, Redwood city, CA), using hippocampi obtained in a similar chronic stress model (Strekalova et al., 2011), suggests diminished expression of NR1 receptor subunit mRNA in stressed mice in particular, and speaks for systemic differences in the hippocampal glutamatergic receptors expression in these mice in general. In addition to NR1 expression results, these microarray data pointed to statistically significant changes in several elements of the glutamatergic system in the hippocampal formation of chronically stressed mice: NR2B receptor, AMPA receptor, glutamate metabotropic receptor 5 and in NR2A/NR2B ratio, which changes are overall in line with the outcome from mRNA evaluation in the current study. Remarkably, a segregation of stress-susceptible and stress-resilient individuals in the microarray study revealed differential expression of the above genes between the sub-groups that additionally supports functional importance of molecular changes in response to stress, obtained both in the microarray and mRNA experiments

In summary, this study demonstrates that elevated hippocampal expression in stressed mice of mRNAs for NR2A and of the NR2A/NR2B ratio, but not for NR2B and NR1, is associated with concomitant abnormalities in both cognitive and emotional elements that mimic one of the most characteristic consequences of experiencing chronic stress in humans. These data suggest that a paradigm comprising ethological stressors evokes behavioral disinhibition and molecular changes that likely mimic epidemiologically

spread human syndromes associated with stress-related emotional and cognitive deficits. Thus, the stress paradigm employed in the present study can be useful for translational studies in the search for pharmacological compensation of these combined symptoms of stress-related pathologies.

Declaration of interest

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Research report

Deuterium content of water increases depression susceptibility: The potential role of a serotonin-related mechanism

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HIGHLIGHTS

- Geographical distribution of deuterium correlates with depression rate.
- Deuterium depleted water reduces stress-induced depressive-like signs in mice.
- Hippocampal proliferation after stress is rescued by deuterium depleted water.
- Deuterium depleted water induces SSRI-like changes in EEG parameters of sleep.
- Above-indicated effects may be due to normalization of hippocampal 5-HTT level.

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ABSTRACT

Environmental factors can significantly affect disease prevalence, including neuropsychiatric disorders such as depression. The ratio of deuterium to protium in water shows substantial geographical variation, which could affect disease susceptibility. Thus the link between deuterium content of water and depression was investigated, both epidemiologically, and in a mouse model of chronic mild stress. We performed a correlation analysis between deuterium content of tap water and rates of depression in regions of the USA. Next, we used a 10-day chronic stress paradigm to test whether 2-week deuterium-depleted water treatment (91 ppm) affects depressive-like behavior and hippocampal SERT. The effect of deuterium-depletion on sleep electrophysiology was also evaluated in naïve mice. There was a geographic correlation between a content of deuterium and the prevalence of depression across the USA. In the chronic stress model, depressive-like features were reduced in mice fed with deuterium-depleted water, and SERT expression was decreased in mice treated with deuterium-treated water compared with regular water. Five days of predator stress also suppressed proliferation in the dentate gyrus; this effect was attenuated in mice fed with deuterium-depleted water. Finally, in naïve mice, deuterium-depleted water treatment increased EEG indices of wakefulness, and decreased duration of REM sleep, phenomena that have been shown to result from the administration of selective serotonin reuptake inhibitors (SSRI). Our data suggest that the deuterium content of water may influence the incidence of affective disorder-related pathophysiology and major depression, which might be mediated by the serotoninergic mechanisms.

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1. Introduction

Major depressive disorder, a common and recurrent disorder, has been projected to become the second leading cause of disability worldwide by 2020. It is associated with considerable morbidity and increased mortality and is challenging to treat effectively. Indeed, environmental factors, such as insolation and amount of day light [1,2], annual fluctuations of air temperature [3], a content of certain minerals in soil and water [4,5] were shown to be important interacting factors for neuropsychiatric disorders including depression. Isotope content of natural waters is one of the factors that greatly varies across different geographical areas [6,7] and can profoundly affect basic physiological processes [8]. However, it is unclear whether isotope content has an impact in the prevalence of affective disorders, or whether it might alter the phenotype in animal models of depression.

Natural water is a mixture of nine water isotopologues formed by stable isotopes of hydrogen [^{1}H, protium (H) and ^{2}H, deuterium (D)] and oxygen (^{16}O, ^{17}O, ^{18}O). The term 'isotopologue' refers to a molecular entity that differs only in isotopic composition [9]. The abundance of nine water isotopologues in environmental water, expressed as the deviation (δ) relative to the international standard 'Vienna Standard Mean Ocean Water 2′ (VSMOW2) standard, varies systemically by location and climatic conditions due to isotopic fractionation accompanying evaporation-condensation process, when the air mass move inland over topographic features. The content of heavy isotopologues decreases proportionally with the distance from the ocean and altitude. It is also influenced by other factors, such as latitude, humidity, and a seasonal temperature [7,10–12]. The spatial isotopic distribution in tap waters reflects the regional variation of isotopes in the local environment [13], which shows considerable variation across the United States (US). However, the lowest levels of heavy isotopologues are detected in the plateau of Antarctica whose water is used as another international standard, Standard Light Antarctic Precipitation 2 (SLAP2) (International Atomic Energy Agency, 2009). This water is characterized by 43% reduction in deuterium content (D/H ratio 89.1 vs. 155.8 ppm) and by 5% reduction in oxygen-18 content (^{18}O/^{16}O ratio 1894.9 ppm vs. 2005.2 ppm), as compared to VSMOW2.

2. Objectives

Here, we performed an epidemiological analysis of the relationship between deuterium content of drinking water in the US, the most abundant isotope after protium and isotopes of oxygen, and prevalence of depression. We then assessed the antidepressant-like properties of deuterium depleted water (D91 = 91.7 ppm) against the pharmacological reference citalopram using three well established paradigms of depression-like state and stress in mice: a chronic stress depression model [14], stress-induced suppression of hippocampal neural proliferation [15] and EEG analysis of sleep [16].

3. Methods

3.1. Epidemiological analysis

Depression rates were obtained for each state in the US from the morbidity and mortality weekly report (MMWR) on depression in adults in the US in the period 2006–2008 [17]. Multiple samples of tap water were collected over the period 2004–2013, and the proportion of deuterium was averaged to give a value for each state in the continental USA. We then performed a correlation analysis (Pearson's r) between content of deuterium in tap water and prevalence of depression across the USA.

3.2. Animals

Studies were performed using 3.5-month-old male C57BL/6J mice. 3.5-month-old male CD1 mice were used as resident intruders for social stress and 2–5-month-old Wistar rats were used for predator stress. Animals were kept in standard laboratory conditions as previously described [14]. Details on the animals' housing can be found in Supplementary methods. Experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals, and were approved by respective local governmental bodies.

3.3. Water isotopologues and drug therapy

Experimental solutions replaced normal drinking water. Control water (D140 = 140.3 ppm deuterium) and deuterium-depleted water (D91 = 91.7 ppm deuterium) were obtained from Almaz Kotovsk Tambov Reg/Timantti AB, Stockholm, Sweden (for details, see Supplementary tables). Citalopram was dissolved in tap water; the solutions were changed every 3 days. The citalopram dose (15 mg/kg/day), in D140 drinking water, was based on the previously validated dosing method [18]. We measured water intake between D91 and D140 groups; no significant difference between these groups was found ($p > 0.05$) (Supplementary Fig. 1).

3.4. Chronic stress study

Mice were either assigned to control ($n = 30$) or stress ($n = 63$) groups. The group means were matched for mouse social behavior, body weight and sucrose preference, after Strekalova et al. [19]. 25 of the stressed mice were treated with D140, 19 with D91, and 19 were treated with the antidepressant, citalopram (15 mg/kg/day, via drinking water). Control mice were treated either with D91, D140 or citalopram ($N = 10$ in each group). D91, D140 and citalopram were administrated starting 7 days prior the onset of stress and lasted for the entire duration of the stress procedure (*see below*).

We employed a 10-day stress protocol [14] comprising a dark cycle rat exposure stress (predator stress) and light cycle semi-random application of two of three stressors: social defeat stress, restraint stress and tail suspension stress. Briefly, between the hours of 09:00 and 18:00 two stressors per day were employed in the following sequence: social defeat for 30 min, restraint stress for 2 h and tail suspension for 40 min with an inter-session interval of at least 4 h. All mice were weighed on the 7th and 10th day of stress, and scored for a coat state 15 h after the termination of the last stressor. 12 h after the last stressor, after being weighed and scored for coat disintegration, all mice were tested in 8-h sucrose test, to assess hedonic traits. Immediately thereafter their forced swimming was assessed to evaluate changes in affective behavior. Chronically stressed mice were killed ~30 h after the last stressor, and their hippocampi were isolated, quantitative RT-PCR of SERT expression was performed as described elsewhere [14]. For details on the behavioral and molecular methods, see Supplementary methods.

3.5. Hippocampal neural cell proliferation after a 5-day predation stress

Mice were randomized to two groups; one received D91 for 2 weeks and the other D140. Both groups underwent predator (rat exposure) stress for 5 consecutive nights; treatment with D91 or D140 continued throughout the stress exposure (see Supplementary methods). Before the first stress session, mice received four intraperitoneal injections of Bromodeoxyuridine

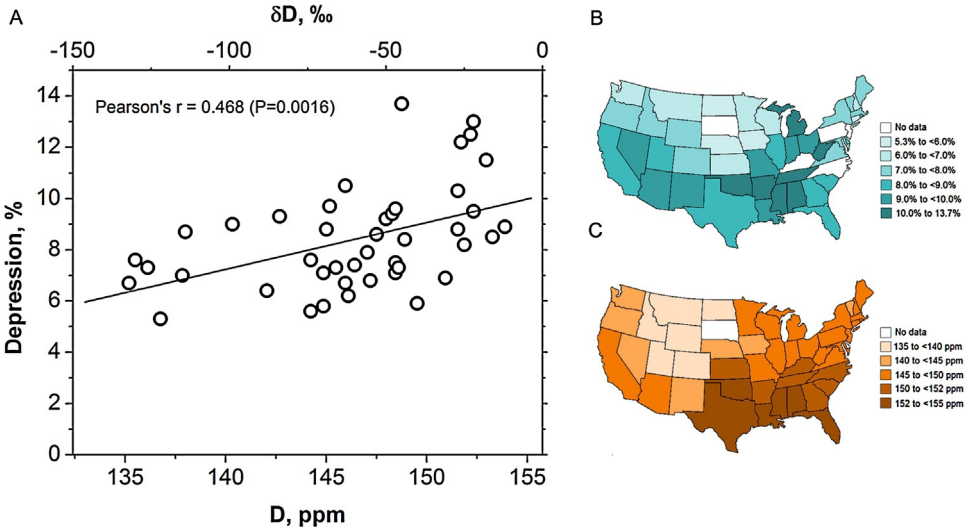

Fig. 1. Depression rates and deuterium content of tap water. (A) Correlation between reported rates of major depressive disorder in US states and content of deuterium in tap water in the USA. (B) The reported prevalence of individuals with depression in among adults aged ≥18 years in each US state [Centers for Disease Control and Prevention. Mental illness surveillance among adults in the United States. MMWR 2011;60 (Suppl):1–30]. (C) Geographical distribution of average deuterium content in the tap water in the continental USA from samples collected from each state for 2004–2013.

(Sigma–Aldrich, St. Louis, MO, USA; 200 mg/kg/kg) dissolved in 0.9% NaCl and 0.007 M NaOH, spaced one from the other by 2 h. They were killed 24 h after the last stress session, perfused with 4%-paraformaldehyde, and brains were removed and treated as described elsewhere [20]; see Supplementary methods for more detail. Immunofluorescence, confocal microscopy and quantification for detection of BrdU and Ki67 were performed as described in Supplementary methods.

3.6. EEG sleep study

12 naïve C57BL6J mice were implanted with electrodes for polygraphic recordings as described elsewhere [16]. The citalopram-treated group was not included in the EEG experiment with sleep analysis, since the effects of SSRIs on sleep were intensely investigated and are well described in a literature of the last decades (for a review, see Ursin [21]). 2 weeks after surgery and recovery period, animals were connected to recording cables and allowed to habituate to the recording chambers for 1 week. Lighting schedule in the chambers was kept reversed (12 h:12 h, light-off at 9:00), food and water were available at libitum. Thereafter, D91 or D140 delivery was started simultaneously with the polygraphic recordings that lasted 14 days. Data were recorded and analyzed as described elsewhere [22].

3.7. Statistics

Data were analyzed with GraphPad Prism version 5.0 for Windows (San Diego, CA). One- and two-way ANOVA was used followed by a Tukey's post-hoc comparisons (or more strictly, Tukey–Kramer, owing to unequal group sizes). *t*-tests were applied for two-group, two-tailed comparisons. A type I error rate of $p < 0.05$ was adopted, and data are shown as mean ± SEM.

4. Results

4.1. Depression prevalence and deuterium content in tap water

We performed correlation analysis using Pearson's *r*, for the relationship between incidence of depression and deuterium content of tap water (Fig. 1). There was a significant correlation ($r = 0.468$; $p = 0.0016$; $F = 11.49$) between deuterium content of tap water and rates of depression. From the linear equation we estimate that prevalence of depression is increased by 1.8% (95% confidence intervals 0.7–2.9%; $R^2 = 0.219$; $p = 0.0016$; $F = 11.49$) for a 10 ppm increase in deuterium level in tap water (see Supplementary Table 3 state-by-state tap water deuterium content.) In order to allow more stringent control of experimental variables, we pursued further work in animal models.

4.2. Assessment of anhedonia induction

Two-way ANOVA revealed a significant effect of stress on sucrose preference ($F_{1,120} = 37.83$, $p < 0.001$), and an interaction between stress and treatment ($F_{2,120} = 4.84$, $p = 0.009$). *Post-hoc* Tukey-corrected *t*-tests showed a significant reduction in sucrose preference from baseline for the non-treated stressed group ($p < 0.05$), but when treated with citalopram and D91 there was no difference from baseline in terms of sucrose preference. This suggests that D91, like citalopram, prevents a reduction in sucrose preference for the stressed cohort (Fig. 2A).

4.3. Effects of treatment on floating behavior

'Behavioral despair' measured in the modified Porsolt forced swim test (FST) by occasion of floating behavior is another sign of depressive-like state in rodents [23]. There was a significant main effect of stress for latency to floating in the FST as revealed by two-way ANOVA ($F_{1,87} = 14.19$, $p < 0.001$), but there was no significant main effect of treatment ($F_{2,87} = 0.82$, $p = 0.44$) and no significant interaction ($F_{2,87} = 0.92$, $p = 0.40$). Post-hoc tests showed a significant reduction in latency to floating for stressed compared with control mice treated with D140 ($p < 0.01$), but there was no significant difference between stressed and control mice that received citalopram or D91 ($p > 0.05$; Fig. 2B). There was also a significant main effect of stress for time spent floating ($F_{1,81} = 9.11$, $p = 0.0033$), with stressed mice floating for longer than controls, and a significant main effect of treatment ($F_{2,87} = 3.14$ $p < 0.05$), but no significant interaction between treatment and stress ($F_{2,87} = 1.28$, $p = 0.284$) (Fig. 2C). Post-hoc tests showed significant differences

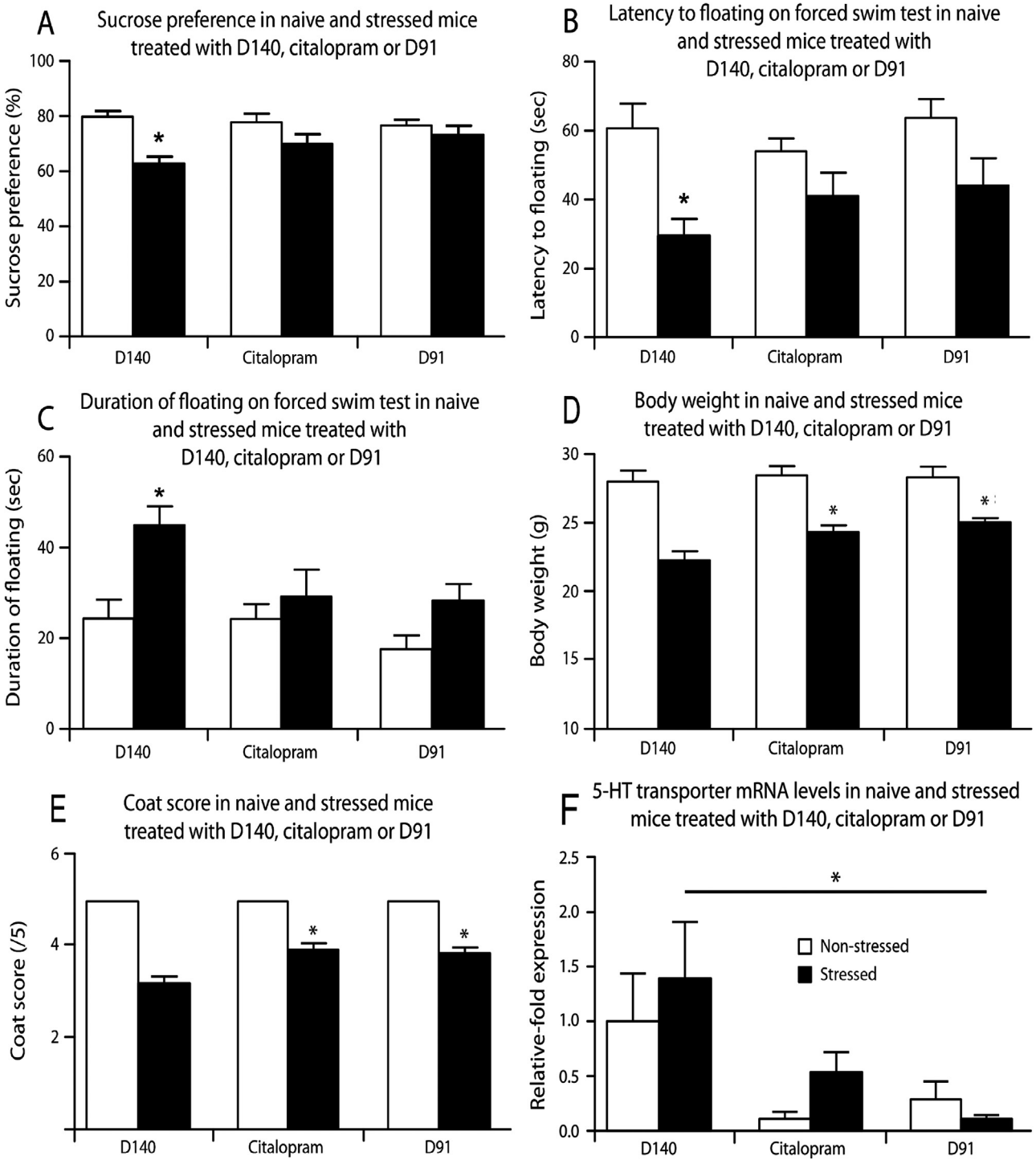

Fig. 2. Citalopram or D91 ameliorates stress-induced depressive-like changes in mice. (A) D140 treated stress mice showed a significant reduction in sucrose preference at day 10 ($p < 0.01$, Tukey's post hoc test). However, citalopram-treated and D91-treated stressed mice displayed no alteration in sucrose preference ($p > 0.05$, Tukey's post hoc test). (B) In the forced swim test, the latency to float was significantly increased by stress in the D140-treated mice ($p < 0.01$), but this was not the case in the citalopram or D91-treated groups ($p > 0.05$; Tukey's post hoc test). (C) The duration of floating was significantly elevated in stressed mice that received D140 ($p < 0.001$), but it was unaltered in D91 and citalopram treated animals ($p > 0.05$; Tukey's post hoc test). (D) All stressed groups showed a significant decrease in body weight compared with controls ($p < 0.001$ for D140 and citalopram treated animals, $p < 0.01$ for D91 treated mice). Post-hoc testing revealed that body weight was significantly higher in stressed groups treated with citalopram or D91 compared to D140 ($p < 0.01$ and $p < 0.0001$ respectively). (E) All stressed groups had significantly lower coat scores than controls while stressed citalopram or D91-treated animals had significantly higher scores than the D140-treated group ($p < 0.001$, Tukey's test). (F) The relative-fold mRNA expression level of the 5-HT transporter (SERT) was significantly lower in D91-treated stressed mice. Data are presented as relative-fold compared to control animals with all expression being normalized to the housekeeping gene GAPDH. Data are mean ± SEM (*<0.05, Tukey's post hoc test). Data are mean ± SEM.

between control and stressed D140-treated groups ($p < 0.01$) but not between other groups ($p > 0.05$).

4.4. Changes in body weight

In terms of body weight, at the end of the chronic stress paradigm there was a significant main effect of stress ($F_{1,87} = 64.09$, $p < 0.0001$), a near-significant main effect of treatment ($F_{2,87} = 2.94$, $p = 0.058$), but no significant interaction between stress and treatment ($F_{2,87} = 1.71$, $p = 0.0.187$). However, stressed mice treated with citalopram or D91 were heavier than those treated with D140 ($p < 0.01$ and $p < 0.0001$, respectively, Fig. 2D).

4.5. Evaluation of coat scores

Before the onset of the chronic stress procedure, all mice had good coat quality, with no significant difference between the groups (*data not shown*). After completion of the chronic stress procedure, a two-way ANOVA comparison showed significant main effects of both stress ($F_{1,87} = 3.59$, $p < 0.0001$); and treatment ($p = 0.0317$; Fig. 2E), and there was also a significant interaction between stress and treatment ($F_{2,87} = 3.59$, $p < 0.05$). All stressed mice showed significantly lower scores of coat state than control treated animals ($p < 0.001$). However, citalopram and D91 administration groups showed higher scores of coat state compared with the D140-treated group ($p < 0.001$).

4.6. Hippocampal gene expression of serotonin transporter (SERT)

Given the marked behavioral differences between the groups, and previous findings showing upregulation of the serotonin transporter (SERT) during anhedonia [14], we sought to elucidate whether treatment with citalopram or D91 led to changes in SERT mRNA expression. A 2-way ANOVA revealed a significant difference between treatment groups in hippocampal expression of SERT mRNA ($F_{2,47} = 4.11$, $p = 0.022$ Fig. 2F). There was no significant main effect of stress ($p = 0.542$) and no significant interaction ($p = 0.705$). There was a trend to increased SERT expression for stressed compared with non-stressed mice in the D140 and citalopram-treated group, but these were not significant on post-hoc analysis ($p > 0.05$). However, both citalopram and D91 decreased the expression of SERT, which was significantly lower in stressed D91-treated compared with stressed D140-treated mice ($p < 0.05$), but the comparison of citalopram-treated versus D140 did not survive correction for multiple comparisons ($p = 0.26$). Therefore, in addition to D91 treatment having a significant effect on behavior, it also had a significant effect on the expression level of SERT mRNA in the hippocampus.

4.7. Neural cell proliferation after a five-day stress

The number of BrdU-positive cells per mm^3 of in the dentate gyrus was significantly reduced in the stressed D140- and D91-treated groups (mean: 613.6 ± 42.59 and 838.8 ± 99.18), as compared to the non-stressed control group (mean: 1578 ± 147.1; $p = 0.0003$, $F = 18.06$, $R^2 = 0.766$, $q = 5.58$ and $q = 4.30$, respectively, one-way ANOVA). There was a significant difference in BrdU-positive cells normalized to control between stressed D91- and D140-treated mice ($p = 0.041$, $t = 2.086$, Fig. 3A). The number of Ki67-positive cells per mm^3 in the dentate gyrus was lower in the stressed D140-treated group, compared to the non-stressed control group (mean: 1405 ± 104.05, $p = 0.0008$, $t = 5.201$), but the difference between stressed D140-treated group (mean: 648.3 ± 80.1) and D91-treated mice (mean: 1371.0 ± 558.5) did not reach statistical significance in this measure normalized to control ($p = 0.102$, $t = 1.28$, Fig. 3B).

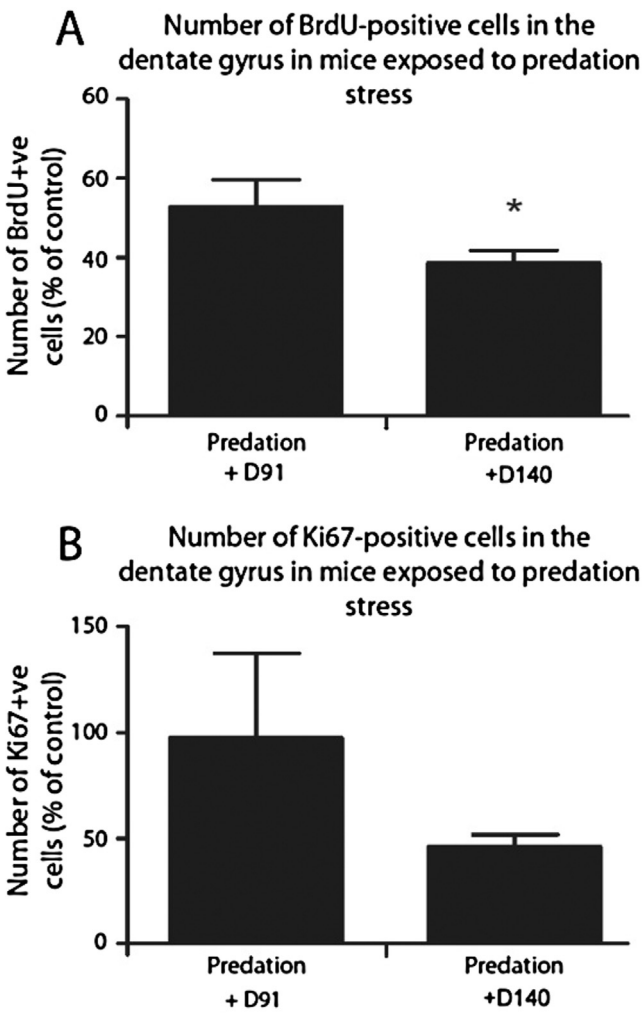

Fig. 3. Effects of D91 on hippocampal SERT and neurogenesis. In stressed animals, normalized to control, (A) the number of BrdU-positive cells per mm^3 in the dentate gyrus was significantly higher in the D91-treated than in D140-treated mice ($^*p < 0.05$, unpaired two-tailed *t*-test). (B) The number of Ki67-positive cells per mm^3 in the dentate gyrus was not significantly different between D91- treated and D140-treated mice.

4.8. EEG parameters of sleep

EEG analysis was performed two weeks after surgery and recovery/adaptation period (Fig. 4A). On day 3 following D91 administration, we noted significant changes in the duration of wakefulness (W) (5–7%, $p = 0.044$, $t = 2.30$, unpaired two-tailed *t*-test, Fig. 4B) and slow wave sleep (SWS) (5-7%, $p = 0.040$, $t = 2.35$, unpaired two-tailed *t*-test, Fig. 4C) compared to D140-treated animals, and this phenomenon remained constant throughout the recording period till day 14 ($p = 0.031$, $t = 2.50$, unpaired two-tailed *t*-test, Fig. 4A and B). D91 administration significantly suppressed REM sleep after day 4 ($p = 0.046$, $t = 2.27$, unpaired two-tailed *t*-test, Fig. 4D); this was more marked than the effect on the other parameters, and was maximal on day 11 of the recording period (40%, $p = 0.0086$, $t = 3.26$, unpaired two-tailed *t*-test). No differences between the groups were observed in the power spectra at any time period of the light cycle, or on any day ($p > 0.05$, unpaired two-tailed *t*-test), including the day 11, when maximal changes in sleep parameters were detected (Supplementary Fig. 2). The spectral

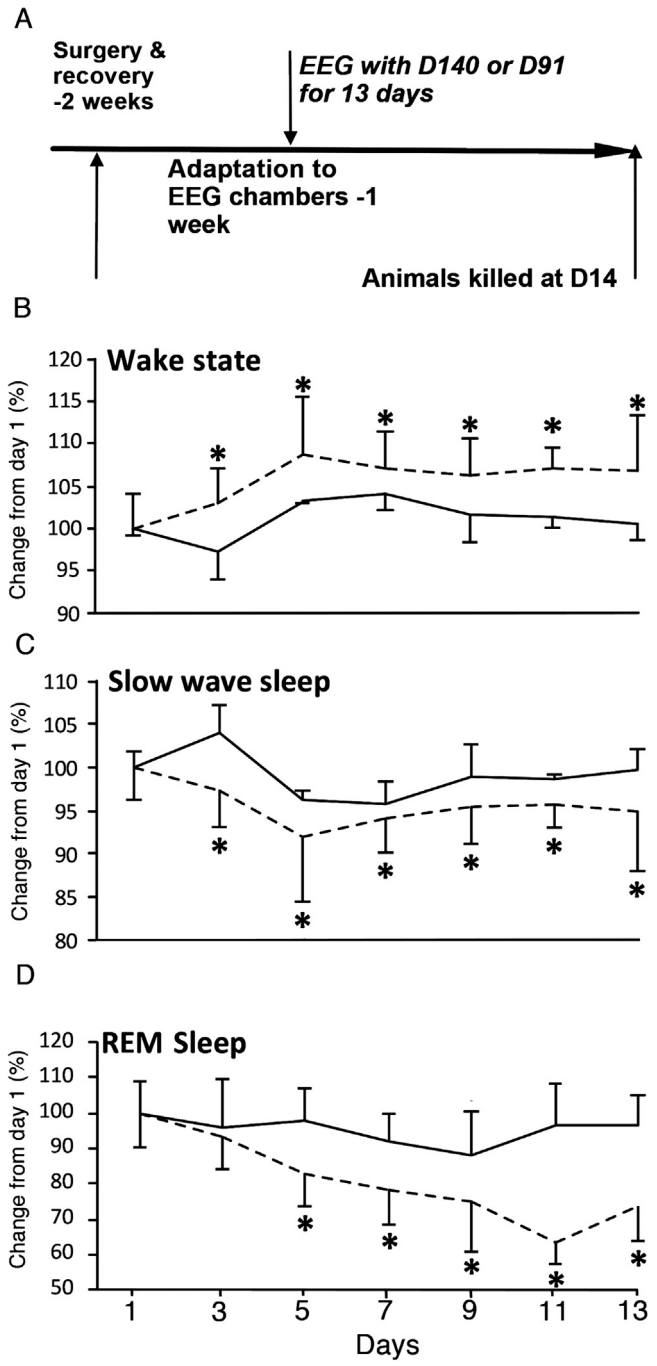

Fig. 4. Effects of D140 and D91 on EEG parameters of sleep of naïve mice. (A) Schematic timeline of the EEG experiment. After electrode implantation, habituation to the recording chambers and baseline EEG registration on the day 1, animals were continuously treated with D140 or D91 during 14-day EEG recordings. (B) Effects of D140 and D91 on waking state. There was a significant increase of the duration of the waking state in D91-treated mice in comparison to D140-treated animals throughout the treatment period. (C) Effects of D140 and D91 on the slow wave sleep. In comparison to D140-treated animals, D91-treated mice had significantly shorter duration of the slow wave sleep on days 3–13. (D) Effects of D140 and D91 on the REM sleep. A significant decrease of the duration of the REM sleep in D91-treated mice in comparison to D140-treated animals was detected throughout the treatment period. $^{*}p < 0.05$; unpaired two-tailed *t*-test. Data are mean ± SEM, expressed as percentage from the means obtained on the day 1.

bands of the EEG considered were: delta, 0.5–4 Hz; theta, 4–8 Hz, alpha, 8–11.5 Hz, sigma, 11.5–14.5 Hz; beta-1, 14.5–18.6 Hz, and beta-2, 18.6–30 Hz.

5. Discussion

We show a positive correlation between rates of affective disorder-related behavior and pathophysiology defined by the MMWR [17] and deuterium content of water, on a state-by-state basis in the USA. Given that the average daily water intake in adults ranges between 2.7 and 3.7 L per individual per day [24], the absolute consumption of the isotopologue hydrogen–oxygen–deuterium (HOD) in deuterium-containing water is around 0.7–1.2 mL for water close to the VSMOW2 standard. As such, even relatively small changes in the deuterium content of consumed water could result in substantial variations of intake. However, it should be pointed out that social, economic and other demographic factors show state-to-state variation, which could be a confounding factor, and it is not possible to highlight a causal relationship from correlational statistics. However, the strength of the association prompted further investigations in mice, which revealed that reduced deuterium intake has the potential to reverse the negative affect, and alter sleep patterns and gene expression associated with serotonergic neurotransmission.

We used a paradigm of stress-induced anhedonia in mice [14,19], using the established sucrose-preference test [25,26] which is a core feature of depression [27]. Citalopram is well documented to counteract the stress-induced decrease in sucrose preference in similar behavioral paradigms [28,29]. Chronic stress reduced sucrose preference in D140-treated mice in agreement with other reports [30,31]; however, after treatment with citalopram and D91 there was no longer a significant difference between stress groups. Importantly, neither D91 nor citalopram had an effect on sucrose test parameters in control animals ruling out a more general effect on sucrose preference. Our data using the Porsolt forced swim test showed that whilst chronic stress decreased the latency to floating, and increased the total time spent floating in mice receiving D140, both citalopram and D91 prevented pathological changes in these parameters, there was no significant difference between stressed and control mice that received citalopram or D91 ($p > 0.05$; Fig. 2B and C). Previous publications from our group showed a similar effect following chronic administration of imipramine and citalopram [18,32,33].

All stressed animals showed a decrease in body weight (Fig. 2D), but this effect was diminished in D91- and citalopram-treated animals. All treated mice showed a significant increase in body weight compared with controls and stressed mice treated with citalopram or D91 were heavier than those treated with D140, while no significant interaction between stress and treatment was revealed. A near-significant main effect of treatment was found. This was consistent with previous reports of restoration of body weight after antidepressant treatment [31,34,35]. Stressed mice also displayed a significant deterioration of coat state, which was ameliorated by both D91 and citalopram treatment (Fig. 2E). Coat disintegration is an important feature of a depressive-like (anhedonic) state in preclinical models [36,37], and improvements in coat state have been associated with antidepressant treatment [37,38].

We next wished to ascertain whether D91 treatment resulted in a corresponding change in SERT expression in the hippocampus. Whilst there was a trend to increased SERT expression in stressed compared with non-stressed D140-treated mice, in line with our previous findings [14], this comparison was not statistically significant here (Fig. 2F). Compared with stressed D140-treated mice, expression of SERT was significantly decreased in stressed mice treated with D91. Interestingly this seemed to have a greater effect size than the antidepressant citalopram, which whilst lower, did

not show a significant decrease in SERT expression. Both stressed and non-stressed animals showed a trend to decreased SERT expression with citalopram/D91 treatment compared with D140, so it is not clear that this effect is limited to stressed animals in this case. Change in SERT expression within the CNS in chronic stress and depression is supported by a number of studies [39–41], and have been associated with alterations in neuroinflammatory-inked pathways [14]. Thus, molecular changes related to the reduction of SERT expression in the CNS may underlie antidepressant-like action of both citalopram and D91 presented here.

We also found that the number of BrdU-positive cells in the dentate gyrus was reduced by stress, and this was ameliorated by treatment with D91 compared with D140 (Fig. 3A), indicating a stimulatory effect of deuterium-depleted water on hippocampal cell proliferation. The effects of stress and antidepressant/anxiolytic therapy on hippocampal neurogenesis are well established in the literature [42,43], and suggests that deuterium depletion could fulfil a similar role. Recent findings have shown that antidepressants, including SSRIs, rescue ongoing neurogenesis during stress [44] and suggested a link between suppression of SERT and the activation of the hippocampal neurogenesis [45] with a key role of BDNF-mediated processes in this effect [46].

The rapid onset in the increase of wakefulness, accompanied by a rapid decrease in slow wave sleep (SWS) remained constant over the treatment period in D91-treated animals (Fig. 4). Remarkably, the changes in REM sleep appeared progressively throughout the treatment period and reached a maximum on the 11th day when this state of sleep was decreased by about 40% in comparison to a baseline value. Analogous effects on SWS and REM sleep are reported for antidepressants inhibiting serotonin and noradrenaline reuptake [16,47]. Both acute [48,49] and 1–3 week long [49,50] dosing with citalopram or other SSRIs reduced the duration of REM and SWS and increased wakefulness. In the clinic, the REM sleep reduction observed with SSRI treatment is used as a biomarker of their therapeutic efficacy [51].

In clinical depression there is decreased latency to the first REM episode, together with an enhancement of REM during the first part of the night. Such an effect is counterbalanced by a decline in REM sleep during the second part of the night, with a decrease in total REM-sleep in a night. Conversely, SWS is decreased in the first part of the night and intermittent awakenings increased. It is possible that the changes in SERT expression may play a role in the changes to sleep EEG parameters. In contrast to the most of standard antidepressants [52,53] the power spectra of D91-treated animals was not altered throughout the treatment period regardless the light cycle. This was surprising given the reduction in REM sleep that might have been expected to alter the proportion of alpha waves.

Previous research has shown a kinetic isotope effect of water as a solvent, with 100% D_2O slowing the rate of ubiquinol derivative oxidation around 400-fold [54]. However, whilst deuterium within natural water range (89–155 ppm) is unlikely to have a significant effect on most chemical reactions, respiration in mitochondria may be a special case, as it involves a connected sequence of proton-coupled electron transfers. Very low levels of deuterium have been shown to have an impact on this process [55] and may account for some of the biological effects we have observed in our mouse paradigms.

A mechanism, by which water with reduced deuterium content exhibits antidepressant-like properties, remains to be elucidated. Based on currently available literature and own preliminary results, various speculations concerning this matter can be proposed. First, replacement of normal water with water of lower viscosity could exert physicochemical effects, leading to increased fluidity of the cell membranes and less rigid organization of phospholipid bilayers [56–58] which can, in turn alter the dispersion of neurotransmitter receptors and increase receptor affinity [59,60], affect passive blood brain barrier permeability [61] and metabolism of arachidonic acid and calcium-dependent receptor binding [62]. Currently unpublished work from our group suggests that epigenetic and post-translational regulation mechanisms may underlie the effect of D91 treatment, based on limited gene expression changes, and there may also be an involvement of factors of synaptic plasticity and BDNF/TrkB signaling.

6. Conclusions

Taken together, our study demonstrates that rates of depression correlate with geographical distribution of deuterium in the natural water in the US population. Substitution of normal drinking water with deuterium-depleted water in mice counteracts the behavioral, transcriptional and proliferative changes typical of the depressive-like state, which was comparable to the effects of the SSRI citalopram. In naïve mice, consumption of deuterium depleted water results in changes of EEG parameters of sleep that are reminiscent of the effects of noradrenaline and serotonin reuptake inhibitors. Thus, deuterium-depleted drinking water could present a novel prophylactic strategy for depression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.07.039.

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Thiamine and benfotiamine prevent stress-induced suppression of hippocampal neurogenesis in mice exposed to a predation without affecting brain thiamine diphosphate levels

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Journal of Neurochemistry (Abstract – 247 words, text – 2973 words, 4 figures)

Thiamine and benfotiamine prevent stress-induced suppression of hippocampal neurogenesis in mice exposed to a predation without affecting brain thiamine diphosphate levels

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Running title: **Anti-stress effects of thiamine and benfotiamine**

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Abbreviations

AD, antidepressant; AHN, adult hippocampal neurogenesis; BrdU, bromodeoxyuridine; S-BFT, stressed-treated with benfotiamine; S-NT, stressed-not treated; S-Thia, stressed-treated with thiamine; TD, thiamine deficiency; ThDP, thiamine diphosphate; ThMP, thiamine monophosphate.

Abstract (247 words)

Thiamine is very important for brain functioning and its deficiency causes specific lesions, oxidative damage and impairment of adult hippocampal neurogenesis (AHN). Thiamine precursors with higher bioavailability, especially benfotiamine, exert neuroprotective effects not only in thiamine deficiency (TD) but also in mouse models of neurodegeneration. As it is known that AHN is impaired by stress in rodents, we exposed C57BL mice to predator stress for 5 consecutive nights and studied the proliferation (number of Ki67-positive cells) and short survival (number of BrdU-positive cells) of newborn immature neurons in the subgranular zone of the dentate gyrus. In stressed mice, the number of Ki67- and BrdU-positive cells was reduced compared to non-stressed animals. This reduction was prevented when the mice were concomitantly treated (200 mg/kg per day in the drinking water, 20 days) with thiamine or benfotiamine. Moreover, we show that thiamine and benfotiamine counteract stress-induced bodyweight loss and increase of anxiety-like behavior, but have no effect on plasma corticosterone concentrations. Both treatments elevated brain levels of thiamine, but not of the coenzyme thiamine diphosphate (ThDP), a mediator of many thiamine-regulated processes, suggesting that the beneficial effects observed are not linked to the cofactor role of ThDP. Our study demonstrates for the first time that thiamine and benfotiamine prevents stress-induced inhibition of hippocampal neurogenesis and accompanying physiological changes, probably by non-cofactor-dependent mechanisms and not mediated by the normalization of hormonal stress response. The use of thiamine precursors might thus be considered as a complementary therapy in several neuropsychiatric disorders.

Keywords

Thiamine, benfotiamine, predator stress, proliferation, survival, immature neurons, hippocampus

Introduction (646 words)

Increasing evidence suggests that thiamine (vitamin B1) and precursors with higher bioavailability can exert prominent neuroprotective effects in the mammalian brain. It is well known that the main phosphorylated derivative of thiamine, ThDP, is an essential cofactor for glucose metabolism, and required for the activity of transketolase and the mitochondrial pyruvate and oxoglutarate dehydrogenase complexes. It is therefore not surprising that thiamine deficiency has deleterious effects on brain activity, which heavily relies on glucose oxidative metabolism (Gibson and Blass 2007). However, it has long been thought that a general impairment of brain energy metabolism does not adequately account for the selective vulnerability of diencephalic structures in TD. This has led to the idea hypothesis that thiamine may exert neuromodulatory or neuroprotective actions through mechanisms unrelated to its coenzyme role (Nghiem *et al.* 2000; Bettendorff 2013; Bettendorff *et al.* 2007; Mkrtchyan *et al.* 2015).

The high sensitivity of the brain to TD is due to the slow absorption of thiamine through the intestinal epithelium and through the blood-brain barrier (Greenwood *et al.* 1982). Therefore, lipophilic precursors with higher bioavailability have been developed in order to increase the absorption of the vitamin. A widely used precursor, benfotiamine (S-benzoylthiamine-O-monophosphate) is not lipophilic but, after oral administration, it is dephosphorylated by intestinal ecto-alkaline phosphatases to the liposoluble S-benzoylthiamine, which in turn is converted to thiamine in liver and blood (Volvert *et al.* 2008). Numerous studies on various pathological conditions, including neurological abnormalities, reported ameliorative effects of benfotiamine that was therefore investigated in our work Benfotiamine was first used as a possible treatment for microvascular complications of diabetes (Hammes *et al.* 2003; Beltramo *et al.* 2008; Pan *et al.* 2010).

As TD is associated with memory loss even before the appearance of diencephalic lesions (Vetreno *et al.* 2011) and since cognitive impairment is generally associated with hippocampal dysfunction, such possible alterations were investigated in mice undergoing TD at a pre-pathological lesion stage (Zhao *et al.* 2008). In these deficient mice, learning abilities were markedly decreased and this concomitant with an impairment of progenitor cell proliferation and neurogenesis in the dentate gyrus.

Many studies have shown that, in rodents and other mammals exposure to stress causes a marked impairment of AHN (Gould *et al.* 1998; Malberg and Duman 2003). The discovery that concomitant treatment with antidepressants protected AHN against harmful effects of

stress has raised much interest (Warner-Schmidt and Duman 2006; Miller and Hen 2015). Thus it appears that AHN is controlled by a variety of different factors and it can be anticipated that several kinds of drugs (in addition to antidepressants) could be used to protect and boost neurogenesis when it has been impaired by stressful events.

We considered the possibility that thiamine and/or benfotiamine might exert protective effects of AHN when mice are exposed to stressful events. Recently, an involvement of thiamine-dependent mechanisms in stress response was shown in the forced swim and immobilization stress models (Dief *et al.* 2015). However, the effects of increased thiamine levels on neuroplasticity during stress response were not yet addressed and no data on the effects of thiamine or benfotiamine in brain neurogenesis are available.

As stress, including predation stress, is known to inhibit hippocampal neurogenesis (Tanapat *et al.* 2001; Hanson *et al.* 2011a; Hanson *et al.* 2011b), we used a recently validated 5-day rat exposure, where mice displayed reduced proliferation of progenitor cells and survival of newborn neurons in the hippocampus, alone with lowered body weight, elevated signs of anxiety behavior and plasma corticosterone (Strekalova *et al.* 2015). These parameters, as well as brain and liver content of the coenzyme ThDP, a mediator of many thiamine-regulated processes were investigated in mice treated with thiamine or benfotiamine. While no changes in plasma CORT levels and ThDP were found, our study demonstrated, for the first time, that thiamine and benfotiamine prevent stress-induced suppression of hippocampal neurogenesis and physiological changes in mice exposed to a predation.

Methods (895 words)

Animals

Three-month-old male C57BL/6J mice were supplied by Instituto Gulbenkian de Ciência, Oeiras, Portugal). Mice were single housed under a reversed 12-hour light–dark cycle (lights on: 20h00), under controllable laboratory conditions (22±1°C, 55% humidity). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals 2010/63/EU.

Reagents

Thiamine and benfotiamine were from Sigma-Aldrich (Diegem, Belgium). Thiamine (1.7 g/L or 5 mM) was dissolved in tap water and pH was adjusted to 7 with NaOH. Benfotiamine (1.7 g/L or 3.7 mM) was dissolved in alkalinized tap water and pH was adjusted to 7 with HCl.

Bromodeoxyuridine (BrdU, Sigma-Aldrich) was dissolved in 0.9% NaCl and 0.007M NaOH. Antibodies: primary antibody rat anti-BrdU (1:500, AbD Serotec, Raleigh, NC, USA), anti-rat and anti-mouse secondary antibodies (1:500, Jackson ImmunoResearch, Europe Ltd, Suffolk, U.K.).

Experimental design

Two runs of a 5-day predation stress experiment were carried out. In each of them mice were either not stressed and not treated with drugs (NS-NT, control group), stressed not treated (S-NT), or stressed and treated with thiamine (S-Thia) or benfotiamine (S-BFT). Both of compounds were administrated with drinking water at the daily-adjusted dose of 200 mg/kg/day (and averaged 24h-liquid intake of 2.5 ml, Strekalova et al. 2006) during 20 days, a pH of their solutions was adjusted to 7.0 using NaOH or HCl.

In the first study (Fig. 1A), mice received four injections of BrdU (dissolved in 0.9% NaCl and 0.007M NaOH) at the dose 50 mg/kg spaced by 2h prior a 5-day rat exposure stress, as described previously (Strekalova *et al.* 2015) and 24h after the last stress session they were sacrificed for immunohistochemical staining with Ki67 and BrdU antibody, markers of neuronal cell proliferation and survival of immature neurons.

In the second experiment (Fig.1B), a cohort of animals was weighted across the study and tested in a step-down anxiety test prior stress and 3h post-stress and subsequently sacrificed 6h post-stress for plasma corticosterone evaluation. A separate group was subjected to stress and treatment and sacrificed 24h post-stress without behavioral testing for evaluation of the levels of corticosterone and thiamine derivatives.

Rat exposure stress

Mice were introduced into a transparent glass cylinder (15 cm high x Ø 8 cm) and placed into the rat cage (10h exposures were performed between 08:00 – 18:00) for 5 consecutive days; that was previously shown to suppress hippocampal cell proliferation (Strekalova *et al.* 2015).

Step-down anxiety test

The step-down apparatus (Evolocus LLC Tarrytown, NY, USA and Open Science, Moscow, Russia) consisted of a transparent plastic cubicle (25cm x 25cm x 50cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden platform (7cm x 7cm x 1.5cm) was placed. Mice were placed onto the platform inside a transparent cylinder and after

removal of the cylinder, the time until the animal left the platform with all four paws was taken as a measure of anxiety (Strekalova and Steinbusch 2010).

Brain collection, sectioning and immunohistochemistry

All steps were performed as described previously (Beukelaers *et al.* 2011; Strekalova *et al.* 2015). Mice were anaesthetized with nembutal (CEVA, Santé Animale, Brussels, Belgium, 0.01ml/g) and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA, Fluka Chemika, Breches, Switzerland). Brains were removed and post-fixed in 4% PFA for 12h at 4°C, followed by previously described protocols of storage, freezing and sectioning for 40-µm-thick free-floating sagittal sections using a MSE (UK) microtome. Every 4th section located 3.5 mm lateral and 0.3 mm ahead of bregma (Paxinos and Franklin 2001) was collected and processed according to previously described protocols of staining with anti- BrdU and anti-Ki67 antibodies, which were from AbD Serotec, Raleigh, NC, USA. Immunostaining with secondary antibodies (Jackson ImmunoResearch, Suffolk, U.K) was as previously described (Strekalova *et al.* 2015). Fluorescence images were acquired using the NIS-Element confocal system equipped with the Nikon A1R hybrid resonant confocal inverted microscope (Nikon Belux, Brussels, Belgium). Fields were acquired using Z-scan with a step of 1.5 µm between each confocal plane. Quantification of BrdU- and Ki67-positive cells per mm^3 of granule cell layer was carried out by an experimenter blind for experimental conditions in the granule cell layer and the subgranular zone from the beginning of the dentate gyrus to its end (0.36 mm to 2.52 mm from lateral bregma).

HPLC of thiamine derivatives

Mice were sacrificed by cervical dislocation and the hippocampus, sensor-motor cortex, liver and blood were isolated for determination of thiamine derivatives by HPLC (Bettendorff *et al.* 1991; Gangolf *et al.* 2010).

Plasma CORT assay

Trunk blood was collected in EDTA blood at sacrifice, stored at 4°C overnight and centrifuged at 10 x g for 10 minutes. Serum was collected and stored a -80°C until use. All samples were run in duplicate. For serum corticosterone levels, a commercially available ELISA kit (Sigma-Aldrich, MO, USA) was used according manufacturer's instructions. The average intra- and inter-assay coefficients of variation for all corticosterone assays below 10%. The assay had a sensitivity of 3.7 ng/mL.

Statistical analysis

Analysis was performed using GraphPad Prism software version 5.03 for Windows (San Diego CA, USA). One-way ANOVA followed by Tukey's post-hoc test was applied to compare three or more groups, except CORT data, which were treated by Kruskal Wallis test due to non-normal distribution. The level of confidence was set at 95% ($p < 0.05$) and all data are expressed as mean ± SD.

Results and Discussion (1.058 words)

First, we tested the effects of thiamine and benfotiamine on hippocampal progenitor cell proliferation (Fig. 2). The density of Ki67-positive cells was not significantly lower in the stressed non-treated mice versus the non-stressed control group (mean: 609 ± 170, and 1357 ± 254, $p>0.05$), but both thiamine and benfotiamine treatment significantly increased the density of Ki67-positive compared to stressed controls. The density of BrdU-positive cells (a marker of short-term cell survival) was significantly reduced in the stressed non-treated group, as compared to the non-stressed control group, but not in thiamine- and benfotiamine-treated groups.

By the end of the experiment, stressed pharmacologically-naive mice, but not thiamine- or benfotiamine-treated animals had a significant reduction in bodyweight compared to the controls (Fig. 3A), suggesting anti-stress properties of these compounds. Also, we evaluated a latency of step-down from a platform model, as a measure of anxiety-like behavior. In comparison to control animals, there was a significant increase in this parameter in stressed non-treated mice, suggesting higher levels of anxiety, but not in thiamine- or benfotiamine-treated groups (Fig. 3B). Experimental groups showed significant changes in plasma corticosterone levels 6h post-stress (Fig. 3C) and no such changes 24h post-stress (Fig. 3D). In the present study, however, stress-induced corticosterone levels were not altered by thiamine or benfotiamine (Figs C, D). This suggest that anti-stress-effects of applied drugs are not mediated by their effects on the hypothalamic–pituitary–adrenal axis.

Our results are consistent with recent data (Strekalova *et al.* 2015) showing that rat exposure for 5 consecutive nights can suppress proliferation and short survival in the hippocampal pool of newborn neuronal cells. Indeed, it has been reported that exposure of rats to fox odor inhibits hippocampal cell proliferation (Tanapat *et al.* 2001). Various forms of stress that were shown to precipitate or worsen depressive symptoms, were found to reduce hippocampal proliferation of newborn neurons as well (Duman 2004). In addition, many studies have

shown that antidepressant treatments reverse the effects of stress and boost AHN (Warner-Schmidt and Duman 2006; Miller and Hen 2015). Thus the effects of thiamine and benfotiamine reported here could be very similar to those of antidepressant drugs and other treatments such as electroconvulsive therapy, physical exercise and environmental enrichment.

It has long been considered (Gibson and Blass 2007) that the beneficial effects of increasing blood thiamine for brain activity were mainly due to the resulting increase in brain content of the coenzyme ThDP, which is essential for neuronal energy metabolism. If this view is correct, we should observe an increase in ThDP content in the hippocampus and cortex, especially after treatment of stressed mice with benfotiamine. There were no significant group differences in blood levels of thiamine and ThDP, though ThMP was significantly increased in benfotiamine-treated mice (Fig. 4A). No significant differences were found in thiamine and its metabolites in liver (Fig 4B). However, in the hippocampus and cortex, a significant increase the levels of thiamine, but not of ThDP were observed in thiamine- and benfotiamine-treated groups (Fig. 4C & D).

Notably, while blood and liver thiamine were strongly increased one hour after benfotiamine and thiamine treatment of WT naive mice (Volvert *et al.* 2008), no increase was observed in contents of thiamine derivatives in our conditions, *i.e.* one day after the replacement of treatment solutions by water. Thus, this delay used in the present study appears sufficient to eliminate most of the excess thiamine from blood and liver. This is presumably not the case for brain thiamine, as the latter only slowly crosses the blood-brain-barrier; this is the reason why it takes more than 10 days before thiamine deficiency can cause brain lesions in rodents (Hakim and Pappius 1983; Hazell and Butterworth 2009).

Oral administration of benfotiamine causes a rapid increase in blood thiamine, which may approach 50 µM one hour after ingestion of a large dose of benfotiamine (Volvert *et al.* 2008; Xie *et al.* 2014). However, even such high amounts of blood thiamine fail to increase the levels of the coenzyme ThDP in mouse brains, even on the long term (Volvert *et al.* 2008; Pan *et al.* 2010; this study). This suggests that the beneficial effects of benfotiamine treatment might be ascribed to increased free thiamine concentrations, either in the brain parenchyma (Fig. 4C, D) or in endothelial cells of brain capillaries. Alternatively, unidentified metabolites of thiamine or benfotiamine might exert protective effects on neurons or endothelial cells. In any event, the beneficial effects of thiamine or benfotiamine described in this study appear to be independent of the cofactor role of ThDP.

As our results suggest that benfotiamine treatment does not act by boosting energy metabolism in the hippocampus, other possible mechanisms should be considered. Previous studies have shown that stressful events, such as acute restraint stress in rats, have harmful effects on the hippocampus and theses effects are related, at least partially, to oxidative damage and inflammation (Chen *et al.* 2016). It is thus plausible that the inhibition of cell proliferation and survival that we observe after predator exposure may be linked to oxidative stress and inflammation. It is well documented that benfotiamine treatment has antioxidant and anti-inflammatory actions, both in vitro (Schmid *et al.* 2008; Shoeb and Ramana 2012; Bozic *et al.* 2015a; Bozic *et al.* 2015b) and in vivo (Sanchez-Ramirez *et al.* 2006; Wu and Ren 2006). This might explain the protective effect of benfotiamine and thiamine on neurogenesis. It should be emphasized that, in contrast to most anti-inflammatory drugs, benfotiamine has no known toxicity or side effects (Stracke *et al.* 2008). We therefore believe that the development of new pharmacological agents (such as thiamine precursors acting at lower concentrations than benfotiamine) represent a promising new strategy to protect the hippocampus from oxidative damage and inflammation, not only in chronic stress and depression but also in neurodegenerative disorders such as Alzheimer's disease. This view is supported by recent clinical study demonstrating anti-depressive effects of thiamine in patients (Ghaleiha *et al.* 2016).

The present results strengthen the view that thiamine could exert physiological effects unrelated to the coenzyme role of ThDP. Those effects would therefore not directly regulate brain energy metabolism but would rather regulate other functions such as neurotransmission, cell signaling and neurogenesis. Although non-coenzyme actions of thiamine appear to be complex and require further investigations, the accumulating evidence points to a potential usefulness in developing new therapeutic approaches, in neuropsychiatric disorders and anti-stress therapy.

Acknowledgments and conflict of interest disclosure

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Legends to figures (374 words)

Fig. 1 Schematic timeline of the experimental protocol. (A) Stress experiment: impact of predator exposure on proliferation of progenitors (Ki67 labeling) and survival of newborn immature neurons (BrdU labeling) in the subgranular zone of the dentate gyrus. (B) Biochemical analysis: impact of predator stress on blood corticosterone concentrations and levels of thiamine derivatives.

Fig. 2 Effect of thiamine and benfotiamine on the proliferation of progenitor cells and short-term survival of new immature neurons in the subgranular zone of the dentate gyrus after predator stress. (A) Number of Ki67-positive cells per mm^3 of cell granular layer in the same groups of animals as in A. (B) Representative photomicrographs of Ki67- labeling in the same groups as in B. (*p<0.05, **p<0.01 as compared to NS-NT; #p<0.05, ###p<0.001 as compared to S-NT). (C) Number of BrdU-positive cells per mm^3 of cell granular layer in non stressed-not treated (NS-NT, n=5), stressed-not treated (S-NT, n=3), stressed-treated with thiamine (S-Thia, n=5) and stressed-treated with benfotiamine (S-BFT, n=5) mice. (D) Representative photomicrographs of BrdU- labeling in NS-NT, S-NT, S-Thia and S-BFT groups.

Fig. 3 Effect of thiamine and benfotiamine predator stress-induced bodyweight loss, anxiety-like behavior and corticosterone levels (A) Body weight was measured in non stressed not treated (NS-NT, n=5), stressed-not treated (S-NT, n=5), stressed-treated with thiamine (S-Thia, n=5) and stressed-treated with benfotiamine (S-BFT, n=5) groups at the beginning (day 1) and end of treatment (day 21). Bodyweight of day 20 are presented as percentage from body weight of day 1. (#p<0.05, ###p<0.001 vs S-NT). (B) A latency of step-down from a platform, a measure of anxiety-like behavior was significantly different between the groups and is increased in S-NT group in comparison to NS-NT group. (p<0.05 vs S-NT). Corticosterone levels in the plasma of non stressed-not treated (NS-NT), stressed-not treated (S-NT), stressed-treated with thiamine (S-Thia) and stressed-treated with benfotiamine (S-BFT) groups sacrificed 6 h **(C)** or 24 h **(D)** after last stress session. (*p<0.05 vs NS-NT).

Fig. 4 Effect of predator stress and treatment with thiamine or benfotiamine on the contents of thiamine derivatives in the blood (A), liver (B) and brain (C, D) of C57BL mice. The animals were sacrificed 24 hours after the last treatment with thiamine, benfotiamine or vehicle. (*p<0.05 vs NS-NT; #p<0.05 vs S-NT, $p<0.05 vs S-Thia).

Figures

Figure 1

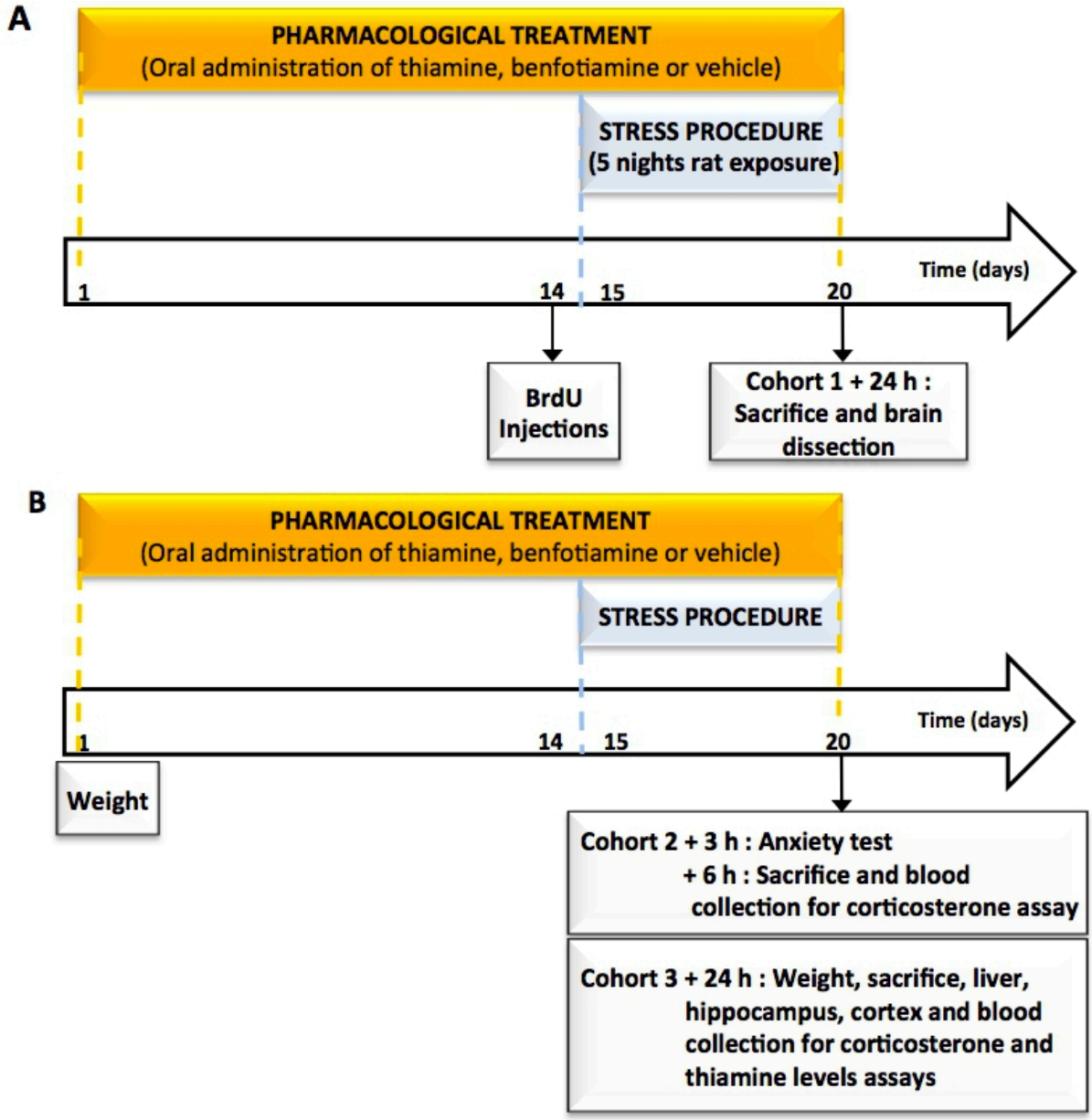

Figure 2

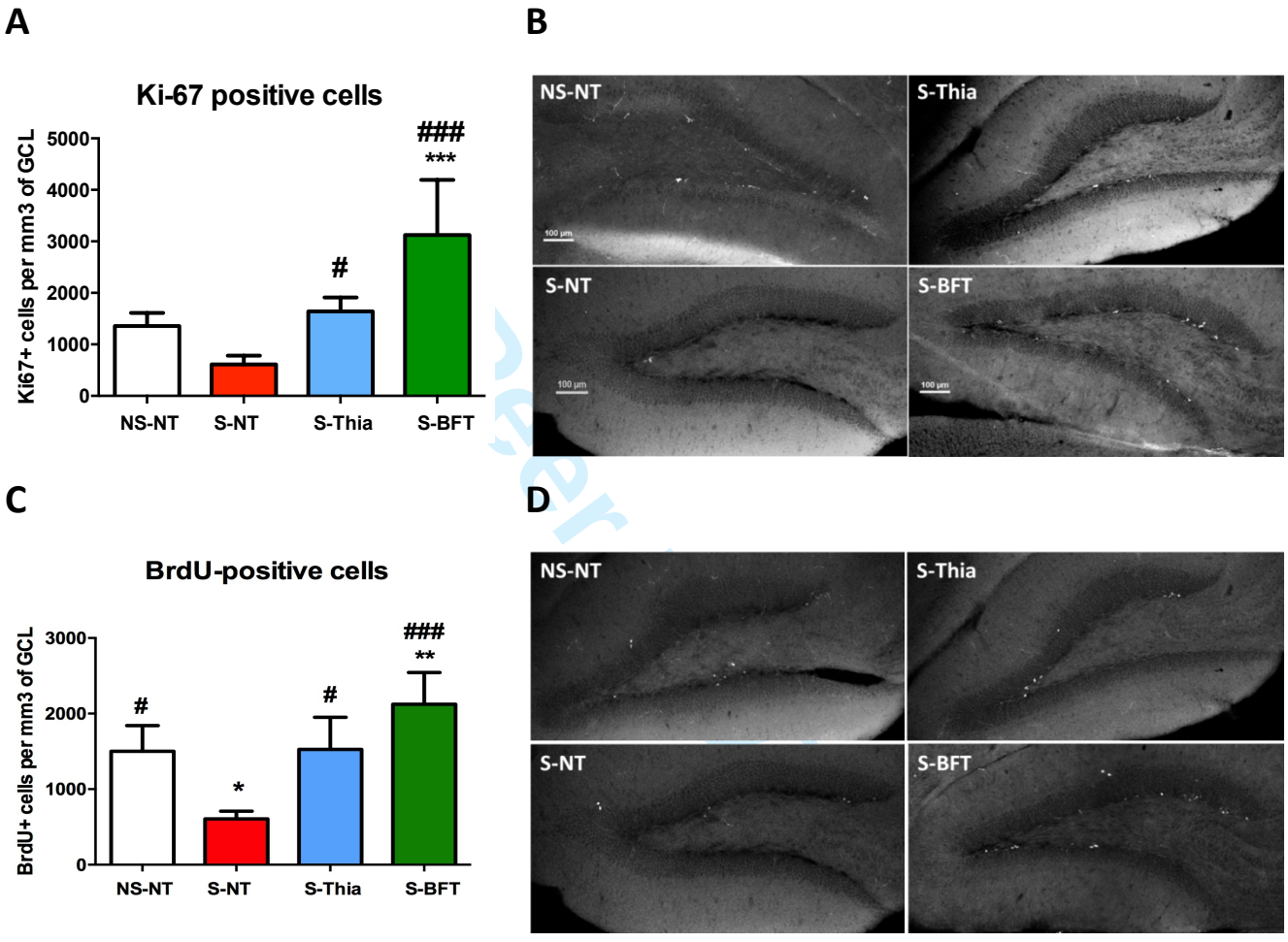
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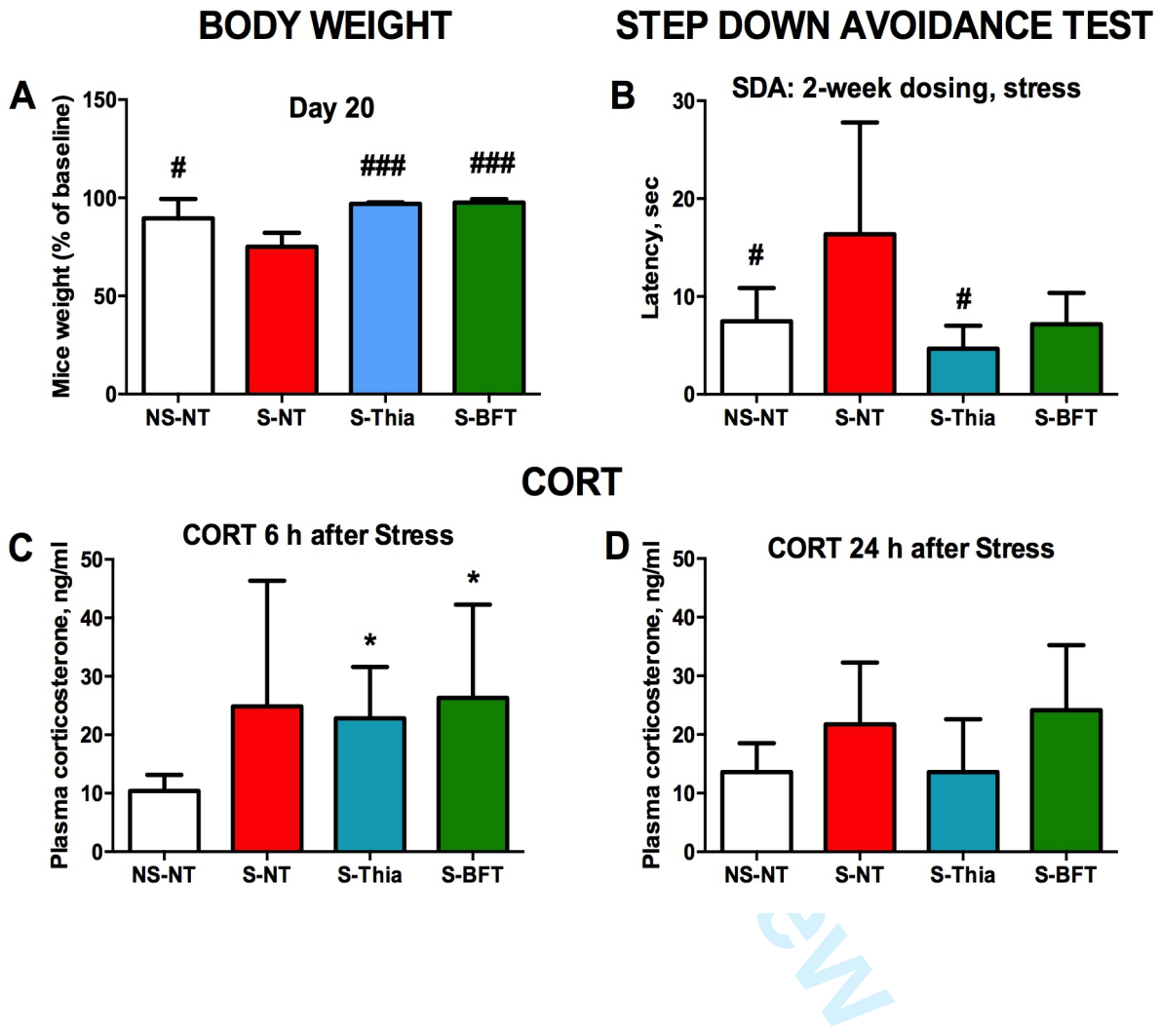
Figure 3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SDA: 2-week dosing, stress
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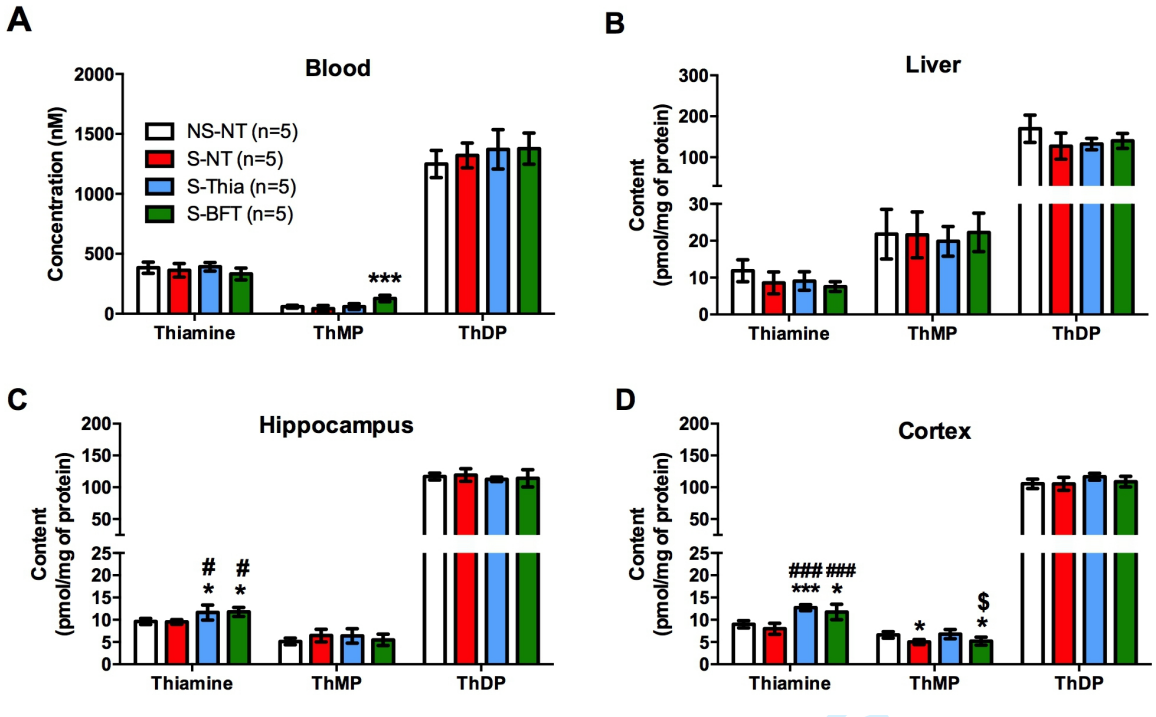
Figure 4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