Maastricht University

Factors of Learning in Mouse Models of Memory and Evaluation of Effects of Dimebon

Facteurs d'Apprentissage chez les Modèles Murins de Mémoire et Evaluation des Effets du Dimebon

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ABBREVIATIONS LIST

Aβ, Peptide β-amyloid
Ach, Acetycholine
AchE, Acetylcholinesterase
AD, Alzheimer's Disease
ADAS-cog, Alzheimer's Disease Assessment Scale-Cognition
BALB, BALB/c
C57BL, C57BL/6, C57BL/6N
CaMKII, Calcium-Calmoduline Protein Kinase II
CR, Conditioned Response
CS, Conditioned Stimulus
CREB, cAMP reponse element-binding
Dim, dimebon
HD, Hungtington's Disease
I.p., Intraperitoneal
NMDA, N-Methyl-D-Aspartate
NaCl, vehicle
PKA, Protein Kinase A
PKC, Protein Kinase C
PKG, Protein Kinase G
LTP, Long-Term Potentiation
STM, Short-Term Memory
LTM, Long-Term Memory
SDA, Step-Down Avoidance
$T_{1/2}$, Half-life time
UR, Unconditioned Response
US, Unconditioned Stimulus

SUMMARY

1. Factors of learning in mouse models of memory

The impact of several most relevant biological factors in contextual learning in mice was investigated in the first part of our study. It is of general importance to estimate an impact of each of them in animals' acquisition of various learning tasks during practical experimental work. First, factors of strain differences, aging and stress, investigated in our work can confound behavioural testing in memory paradigms. Second, they can be used as a basis of behavioural models of memory deficits and, thus, model pathological conditions in humans.
Step-down avoidance task performed on three strains (namely C57BL/6N, CD1 and BALB/c) has clearly highlighted differences of abilities in learn contextual memory in these strains. The step-down avoidance task was also applied on C57BL/6N old of 3-months or 7-months; older mice displayed lower scores of memory then the young onces, thus, even mild aging impaired contextual learning in this strain. Third, we investigated learning of C57BL/6 mice subjected to chronic stress in a fear conditioning paradigm. Our study showed that the acquisition of this task was disrupted in stressed group, as reflected by decreased scores of freezing behavior. The assessment of investigated here factors of learning provides a possibility to validate animal models of memory and evaluate their sensitivity.

2. Evaluation of effects of dimebon

Dimebon, a heterocyclic compounds previously adopted in clinic as antihistaminic, has recently revealed enhancing cognitive properties in pre-clinical and clinical studies. The aim of the present study was to identify the most optimal dosing and adequate memory test(s), which would be sensitive to the memory enhancing effects of dimebon. Amongst the different studies carried out, dimebon revealed enhancement in memory in the step-down avoidance test, a one trial hippocampus-dependant task, at dose 0.5 mg/kg administered acutely and in the Y-Maze test, a multiple training paradigm at dose 0.1 mg/kg delivered chronically. In conclusion, our study supports therefore that the step-down avoidance and Y-maze paradigms are the most convenient assays allowing a rapid and reliable assessment of effects of drugs with memory enhancing properties such as dimebon and dimebon-like.

RESUME

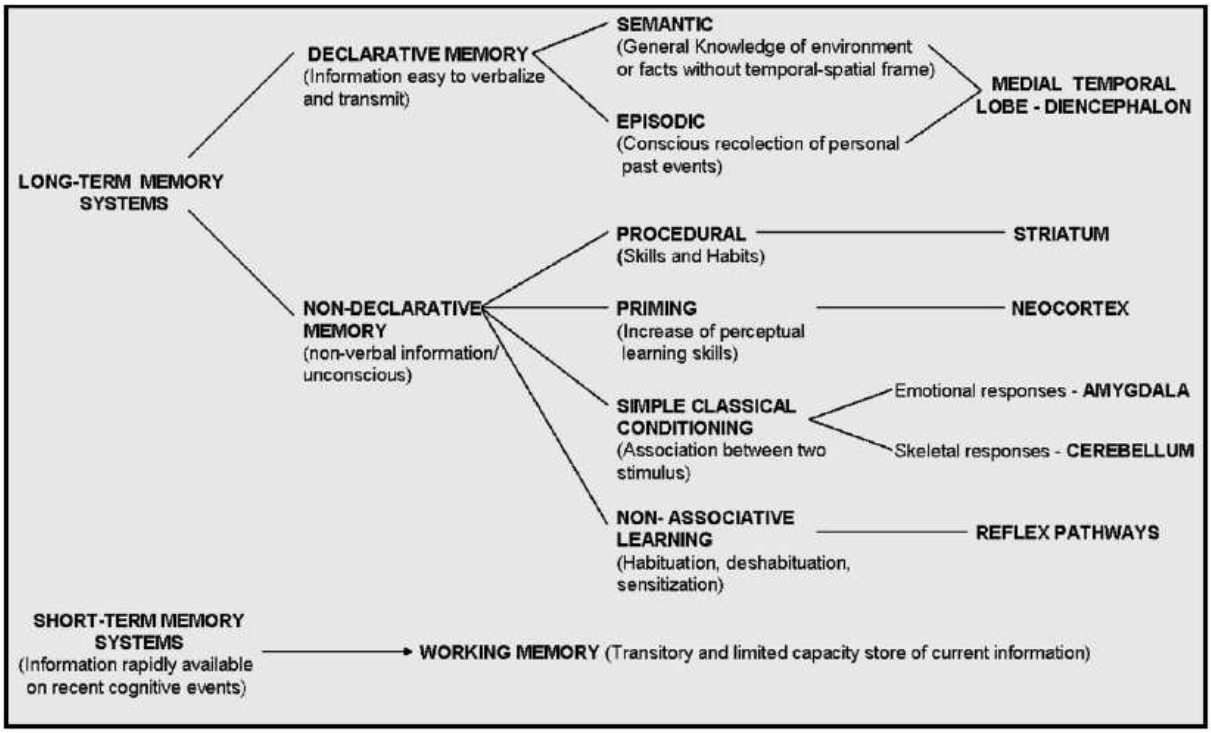
1. Facteurs d'apprentissage chez les modèles murins de mémoire

Les facteurs d'apprentissage sont reconnus pour influencer le comportement des animaux dans les paradigmes de mémoire et d'apprentissage. Identifier et caractériser de tels facteurs permet, d'une part, d'éviter l'interférence de facteurs confondants conduisant à des interprétations erronées de résultats obtenus dans des paradigmes de mémoire chez les animaux. D'autre part, ces facteurs d'apprentissage peuvent servir de base pour développer des modèles reflétant des déficits de mémoire et, par conséquent, des modèles semblables aux pathologies humaines. La première partie de notre étude s'est adressée à l'impact de certains de ces facteurs, c'est-à-dire les différences entre souches de souris, le vieillissement et le stress chronique. Un paradigme d'évitement passif appliqué à différentes souches de souris (respectivement C57BL/6N, CD1 and BALB/c) a clairement souligné des différences dans l'acquisition de la mémoire contextuelle entre ces trois souches. Ce même paradigme a également été appliqué sur des souris C57BL/6N de respectivement 3 et 7 mois; les plus âgées ont présenté des scores d'apprentissage inférieurs à ceux des plus jeunes, suggérant que de minimes différences d'âge peuvent affecter la mémoire contextuelle dans cette souche. Ensuite, des souris C57BL/6 soumises à 2 semaines de stress ont été testées dans un paradigme de conditionnement de peur contextuel. De par leurs faibles scores de freezing, le groupe « stress » a démontré que l'acquisition de la mémoire contextuelle était altérée par le stress. L'évaluation et l'investigation de ces facteurs d'apprentissage fournissent les éléments essentiels à la validation de modèles murins de mémoire et à l'évaluation de leur sensibilité.

2. Evaluation des effets du dimebon

Le dimebon, un anti-histaminique précedemment utilisé pour le traitement des allergies, a récemment révélé des propriétés procognitives dans des études précliniques et cliniques. L'objectif de la seconde partie de notre étude était d'identifier la dose optimale et les tests de mémoire les plus adéquats permettant de détecter les effets procognitifs de ce médicament. Parmi les différentes expériences réalisées, les doses de 0.5mg/kg administrée de façon aiguë, et de 0.1 mg/kg délivrée chroniquement de façon intrapéritonéale à des souris, ont révélé une amélioration de la mémoire respectivement dans une tâche d'évitement passif et dans un labyrinthe en Y. En conclusion, les résultats de nos études suggèrent que ces 2 paradigmes de mémoire sont des tests adéquats pour l'évaluation des médicaments présentant des propriétes procognitives, tels que le dimebon et analogues.

TABLE OF CONTENTS



Scheme 1: Forms of memory and their neurological substrates [From Paul & al., 2009].

1. Introduction

1.1. Definitions of Forms of Learning and Memory

Memory is a complex network of different interrelated functions working together to manage information (Paul & al., 2009). Learning and memory engage several processes, including acquisition, consolidation, retention, and recall of information which are mediated by multiple anatomical and neurochemical mechanism systems. Therefore, the term memory system was introduced as "a brain function whose purpose is to classify, encode, store and recover a wide diversity of information relevant for the subject" (Moscovitch & al., 2005; Paul & al., 2009).

Memory involves two broad forms (scheme 1): "declarative" (or explicit) memory, which refers to the ability to remember personal past experiences (Suzuki and Clayton, 2000), and "non-declarative" (or implicit or procedural) memory, which is the memory about motor or perceptual skills that may not be orally transmitted (e.g. remembering how to use a telephon) (Moscovitch & al., 2005; Paul & al., 2009). Episodic memory ("what, where and when" of an event) and semantic memory, an ability to acquire knowledge and remember facts about the world that may or may not have been acquired trough personal experiences, form the declarative memory (Suzuki & Clayton, 2000).

The structures of the medial temporal lobe, known to be important in declarative memory, include the hippocampus and anatomically related to this brain structure entorhinal, perirhinal and parahippocampal cortices which have a complex functional interrelation (Moscovitch & al., 2005; Suzuki & Clayton, 2000). Declarative, configural (relational) and spatial memories are hippocampus-dependent (Balogh, 2003; Gerlai, 1998; Lorenzini & al., 1996) but implicit memory is not dependent on intact hippocampal function (Fanselow, 2000). Hippocampus is often identified as a key mediating structure in studies examining the pathophysiology of clinical symptoms of deficient memory observed during a number of disorders (Panegyres & al., 2004). This structure appears to be a major target of research in studies of pathophysiological effects of the stress and aging (and the effect of the former on the latter one) (Miller and O'Callaghan, 2005). Furthermore, impairment of the hippocampus-dependent memory is one of the first symptoms of Alzheimer's disease (Dickerson and Sperling, 2008; Miller and O'Callaghan, 2005; Minati & al., 2009). Many studies in rodents have revealed the dependence of declarative memory to hippocampus (Milner & al., 1998).

1.2. Importance of Animal Models in Fundamental and Preclinical Neurosciences Research

The availability of animal models allows to investigate brain-behavior relationship underlying molecular and cellular mechanisms and cognitive processes in normal and pathological conditions. Indeed, the most clinically relevant information derives from human studies, but these studies are limited by degree of invasion which such studies can allow. Not all questions concerning the mechanisms of memory are possible to address in human studies. Animals can be submitted to manipulations which are not applicable in human subjects, such as genetic modifications, lesions of brain structures, pharmacologic treatment and others. Based "*on the evolutionary theory and the assumption that fundamental aspects of the behavior of humans have a genetic basis with a common evolutionary trajectory, i.e., are shared with others animals*" (van der Staay, 2006; Wall & Shani, 2008), animal may serve as model organism for other species including human. Thus, a revelant model in neurobehavioral research is: "*a living organism used to study brain-behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about, these relations in human and/or a species other than the one studied, or in the same species under conditions different from those under which the study was performed*" (van der Staay, 2006). Furthermore, animal models of human cognitive function anticipated to enable the identification of novel chemical entities of therapeutic value, and their molecular, cellular mechanisms of action in drug discrimination paradigm.

It is important though to bear in mind that human and rodents are different species, and even though they share general features, animal models cannot be mechanistically extrapolated to a situation (van der Staay, 2006; Wall & al., 2008). For that reason, animal models of diseases generally can mimic only selected aspects of disease symptoms. Thus, any conclusions suggested by the data obtained in animals models have to be considered with a caution.

1.3. Paradigms of Hippocampus-Dependent Learning in Rodents

A number of animal models of learning and memory has been developed till now. Several behavioral paradigms of hippocampus-dependent memory in mice were shown to be sensitive to a dysfunction in this brain structure (Gerlai, 2001).

1.3.1. Step-Down Avoidance (SDA) Paradigm

In the step-down inhibitory avoidance test, animals learn to suppress exploratory tendency to avoid aversive stimuli (Crawley, 1997; Izquierdo & al., 1999, 2006; Kolata & al., 2008). This paradigm is well-established and it is frequently used to assess learning and hippocampus-dependant memory (Izquierdo & al., 2006) in mice and rats. Usually, one trial is sufficient for an acquisition of the task by an experimental animal: a single association made between a movement (stepping down) in a particular context and an aversive stimulus (a footshock) takes place in seconds and lead to a robust memory (Izquierdo & al., 2006). Retention of this task is called “contextual memory” (Izquierdo & al., 2002). Memory retrieval is facilitated as a function of the degree of similarity between the context of memory retrieval and the context of learning, called “contextual memory retrieval” (Spear, 1973; cited in Martel & al., 2010).

Thus, importantly, the onset of memory consolidation can be determined with high precision in contrast to multiple-trial tasks (Izquierdo & al., 2006). Memory formation of inhibitory avoidance behavior are mediated by a biochemical sequence of events taking place in the rodent hippocampus and basolateral amygdala (Izquierdo & al., 1997, 2006) which are required for the long-term memory (LTM) consolidation. Formation of a short-term memory (STM) requires similar molecular processes that the one mentioned below except that only glutamate AMPA receptors are activated in the hippocampus (for details, see Izquierdo & al., 2002). This cascade involves, first, the activation of different glutamate receptors (NMDA, AMPA and metabotropic) which subsequently enhance activity of protein kinases A (PKA), C (PKC) and G (PKG) and calcium-calmodulin protein kinase II (CaMKII). These protein kinases lead to changes in second messengers and biochemical cascades followed by changes in glutamate receptors subunits (GluR1 and NMDA1) and binding properties and increased expression of constitutive (CREB) and inducible transcriptions (c-fos) factors leading to gene expression and protein synthesis needed for synaptic changes necessary for memory formation. There are two peak of activity in this biochemical sequence: the first one is detected five minutes after the training and the second, which depends on the first one, occurs

2-6h thereafter (Izquierdo & al., 1997, 2002). Hippocampal activity mediated by glutamate NMDA receptors persist at least 3 h after training; memory track is believed to be transmitted to other areas of the brain (entorhinal and posterior parietal cortex) in which a consolidating process takes place. The hippocampus, amygdala, entorhinal cortex, and parietal cortex are involved in retrieval in the first few days after training (Iziquierdo & al., 1997, 2002).

1.3.2. Y-Maze Paradigm

The Y-maze with external cues is a test for measuring spatial / hippocampus-dependant memory. This paradigm is based on the innate tendency of the mice to explore a new environment and also to learn and remember a location of safe or rewarding environment (access to food or water etc…)(Crawley, 2000; Paul & al., 2009). Experimental mice are usually maintained on a food-restricted or water-restricted diet and are trained to choose specific arm of the maze to receive water or food reinforcement (Crawley & al., 2000). Rodents were shown to create a cognitive map in the Y-maze paradigm, which is a strategy of real global representation of the external environment and to locate the target within a maze (Paul & al, 2009).

Generally, spatial memory can be defined as that brain function responsible for recognizing, codifying, storing and recovering spatial information about the arrangement of objects or specific routes (Paul & al., 2009). Spatial cognition is obtained trough exploratory behavior, an instinctive and widely preserved behavior in all animal species, including man (Paul & al., 2009). Experimental evidences point to the hippocampus as a crucial structure involved in the neurobiology of this task (Barkus & al., 2010; Milner & al., 1998; Paul & al., 2009; Sunanda & al., 2000); in particular, dorsal hippocampus was found to be implemented in memory mechanisms of learning in this test (Barkus & al., 2010).

1.3.3. Fear Conditioning Paradigm

Contextual fear conditioning model is a task that is based on measuring of the ability of the mouse to learn and remember an association between an aversive experiment and environment (Crawley, 2000). Fear conditioning takes place when initially innocuous conditioned stimulus (CS, e.g. a new context) is paired with an aversive unconditioned stimulus (US, e.g. electric shock) that reflexively activates unconditioned fear responses (URs, e.g. jumps and screams). Trough CS-US association formation, the CS elicits various conditioned responses (CRs, e.g. freezing) which share similar characteristics to innate fear

response (Kim and Jung, 2006). A single pairing of CS and US is sufficient to induce, in a rat or a mouse, robust association between CS and US (Kim and Jung, 2006; Shimanski & al, 2004).

A large body of evidences from lesion, pharmacological and neurophysiological studies points the amygdala and hippocampus as the keys neural system subserving contextual fear conditioning (Barkus & al., 2010; Fanselow, 2000; Gerlai, 2001; Kim & al., 2006; Philipps and Ledoux, 1992; REF). Lesions of amygdala and hippocampus appeared to interfere with the acquisition of contextual fear conditioning (Barkus & al., 2010; Philipps & Ledoux, 1992). Anatomically, the amygdala receives sensory inputs from diverse areas of the brain (e.g. thalamus, neocortex, olfactory cortex, hippocampal formation) and sends projections to various autonomic and somatomor structures thought to mediate the motor expression of emotional responses (Philipps and Ledoux, 1992). It is generally accepted that sensory information enters the amygdala through its basal and lateral nuclei (Philipps and LeDoux, 1992) in which CS–US association (or elemental learning) formation is believed to take place (Kim and Jung, 2006). These nuclei are interconnected with the central nucleus, which is thought to be the main amygdaloid output structure sending projections to various autonomic and somatomotor centers involved in mediating specific fear responses, e.g. freezing.

According to Philipps and Ledoux (1992), the hippocampus contributes to the fear conditioning as a CS processing channel (as sensory thalamus and sensory cortex) that relay sensory inputs to the amygdala. The hippocampus receives input from cortical areas that integrate information across sensory modalities. On another hand, it has been shown that the hippocampus is a substrate of the formation, storage and consolidation for contextual fear memory (Kim and Jung, 2006; Fanselow, 1999). These two hypotheses are not incompatible. Normal hippocampal function can acquire multiple stimuli and the complex relationships of such stimuli (the context, e.g. a shock chamber), thus hippocampus is involved in the formation of the memory for the integrated representation of the context, i.e. configural, contextual learning (Fanselow, 1999; Gerlai, 2001; Suzuki & al., 2000). Thus, the fear conditioning may be based on two distincts mechanisms, one of them is hippocampus dependent (contextual learning) and the other one is amygdala-dependent (elemental learning) (Gerlai & al., 1998; 2001; Kim and Jung, 2006; Vianna & al., 2004). Some studies suggested that the amygdala is critical for the expression of fear reactions rather than learning and storage of memory and that such function would be achieved by the hippocampus (Vianna & al., 2004; Kim and Jung, 2006).

1.4. Factors of Learning in Mice

Learning and memory can not be measured directly but only via measuring from behavior (Cahill & al., 2001; Cain, 1997). However, *"Behavior is affected by many processes other than learning and memory"* (e.g., age, sex, strain) (Cahill & al., 2001). Current knowledge of the range and the nature of external (experimental parameters)/non-cognitive (intrinsic mice parameters) factors seems to be not complete (Andrews, 1996; D'Mello and Steckler, 1996). These non-cognitive factors, named factors of learning in this study, may exert a major effect on results in cognitive testing. This has important consequences for the interpretation of results and the extrapolation of findings to other areas of research, including to man (Andrew, 1996; Cahill & al., 2001). Identification of factors of learning in experimental animals, and characterization of impact of those factors, such as, in particular, effects of strains differences, aging and stress could enable more correct ethological analyses of behavioral data in future studies and let to define optimal experimental design.

1.4.1. Differences in Memory Abilities between C57BL/6, BALB/c and CD1 Mouse Strains

C57BL/6, CD1 and BALB/c mice are widely used in neurobehavioral research (Belzung & al., 1999; Bolivar & al.; 2000; Clapcote and Roder, 2004; Crawley & al., 1997; Nguyen & al., 2000; Sankoorika & al., 2006; Schneider & al., 2006; Yoshida & al., 2001). However, these strains have differential behavioral phenotype due to their genetic background. The hippocampus-dependant learning was shown to be significantly influenced by a genetic background (Gerlai, 1998; Nguyen & al., 2000; Yang & al., 2004). As C57BL/6 strain is known to have high abilities of learning in various tasks (Crawley, 1997; Gerdai & al., 2001; Nguyen & al., 2000; Schimanski & al, 2004) including the SDA (Crawley & al., 1997). Thus, this mouse strain is often used as a reference strain.

BALB/c and C57BL/6 mice are inbred mice, whereas CD1 mouse is an outbred strain. However, both BALB/c and CD-1 are albinos whereas C57BL/6 mice have pigmented eyes (Michalikova & al., 2010). Mice of C57BL/6, CD1 and BALB/c strains have a similar lifespan (between 24 and 30 months). Various studies investigated the performance of mice in different tasks and have shown that performances in cognitive task are strain-dependant (Crawley & al., 1997; Sik & al., 2003); specific strains of inbred mice may differ importantly in hippocampus-dependant behavioral task (Nguyen & al., 2000). Previous studies have compared obtained results by these three strains in paradigms addressed to different aspects of

behavioral phenotype (Michalikova & al., 2010; Yang & al., 2004; Zarcone & al., 2004). BALB/c mice show the highest level of anxiety-like behavior in several paradigms of anxiety-like behavior (Bolivar & al., 2000; Crawley & al., 1997). The C57BL/6 shows lower levels of anxiety-related measures (Belzung & al., 1997; Crawley & al., 1997; Michalikova & al., 2010) and then CD1 shows the lowest level (Michalikova & al., 2010). BALB/c displays a high exploration and locomotor activity (Kalueff & al., 2005; Schneider & al., 2006; Sık & al., 2003) which is unexpected regarding its high level of anxiety (Crawley & al., 1997). Some studies have shown that locomotor activity in BALB/c is higher (Kalueff & al., 2005; Schneider & al., 2006; Sık & al., 2003), similar (Bolivar & al., 2000; Sankoorikal & al., 2006) or lower than C57BL/6 (Bolivar & al, 2000; Moy 2007). C57BL/6 and BALB/c reveal a relatively high locomotor and exploration activity (Kalueff & al., 2005; Schneider & al., 2006) compared to CD1 strain (Patil & al., 2009). On the other hand, C57BL/6 mice exhibit the highest level of contextual freezing whereas BALB/c show intermediate scores of contextual freezing scores (Balogh, 2003; Schimanski 2004) whereas CD1 mice show impaired ability to perform (Adams & al., 2002) contextual fear conditioning. Similar differences in freezing performance were observed in a rat exposure test (Yang & al., 2004).

1.4.2. Impact of Aging in Learning Abilities in Mice

Age-related differences in learning are well documented in various paradigms in rats (Bizon & al., 2009; Frick & al., 1995; Wyss & al., 2000) and mice (Frick & al., 1999, 2002; Ingram & Jucker, 1999). However, the emergence of genetic engineering in mice has yielded the opportunity to produce genetically mice models of age-associated-diseases such as Alzheimer's disease. As C57BL/6 strain is commonly used as genetic background for transgenic mice lines (Crawley & al., 1997) it is important to characterize age-related behavioral changes which occur in this mouse strain.

Several studies addressed age-related changes in different paradigms in male and female C57BL/6 mice. In male C57BL/6, spatial water maze deficits are evident at 20-26 months of age (Benice & al., 2006; de Fiebre & al., 2006; Fordyce, 1993; Frick & al., 1999, 2002) in the absence of deficits in motor activity or altered anxiety levels (Frick & al., 1999), and have been observed as early as 10 months (Benice & al., 2006). Benice & al. (2006) also demonstrated age-related deficit in passive avoidance memory in C57BL from 10 months to 20 months. Moreover, age-related impairments in spatial learning (Benice & al., 2006, Frick & al., 1999, 2002) and passive avoidance memory (Benice & al., 2006) have been shown to be more pronounced in old females than in males. In contrast, few studies have assessed the

effect of aging in the step-down avoidance task on C57BL/6. C57BL/6 mice showed a steady decline in ability to acquire the avoidance response after 3.5 months (at 6, 12 and 26 months) and showed age-dependant declines in 48h-retention performance by 12 months of age (Forster & al., 1988). C57BL/6 female mice of 20 months-old also displayed impairment in the SDA task compared to 3/4-months-old mice (Pei & al., 2010; Zhaoa & al., 2009). The fact that employed protocol of step-down avoidance enables the differentiation in learning abilities between groups of animals with subtle difference in age in relatively young individuals, reflects high resolution of this paradigm in measuring the hippocampus-dependent memory.

1.4.3. Effects of Chronic Stress on Memory in Mice and Rats

Prolonged exposure to stress can induce neuronal damage in the hippocampus (Conrad & al., 2010; Jayatissa & al., 2008; Magarinos & al., 1995a, b; Watanabe, 1992) in rats. Human studies have also revealed similar kind of interaction between chronic stress and hippocampal alterations (Lupien & al., 1998; Werner & al., 2009).

Chronic stress has been reported to alter a number of cellular and molecular parameters in the hippocampus (Conrad & al., 2010; Jayatissa & al., 2008; Joëls & al., 2007; Kleen & al., 2006; Lupien and Lepage, 2001; Magarinos & al., 1995 b; Mineur & al., 2007; Sandi & al., 2001; Song & al., 2006; Watanabe, 1992) and to impair hippocampus-dependent learning (Conrad & al., 1996; Luine & al., 1994; Magarinos & al., 1995 b; Mineur & al., 2007; Strekalova and Steinbusch, 2010; Sunanda & al., 2000; for review see Conrad, 2010 and Joëls & al., 2007) in rats and mice. However, surprisingly, in previous studies, chronic stress has been shown to increase the level of freezing in fear conditioning in rats submitted to 21-days chronic restraint stress and thus, chronic stress potentiated contextual fear conditioning (Conrad & al., 1999; Sandi & al., 2001). Although several studies have been lead on the effects of chronic stress in learning paradigms in mice (Ducottet & al., 2005; Schwabe & al., 2008; Song & al., 2006; Sterlemann & al., 2009; Strekalova and Steinbusch, 2010) and found impairments in hippocampus-dependant task (Sterlemann & al., 2009; Strekalova and Steinbusch, 2010), the effects of chronic stress in the fear conditioning task were not reported in the literature.

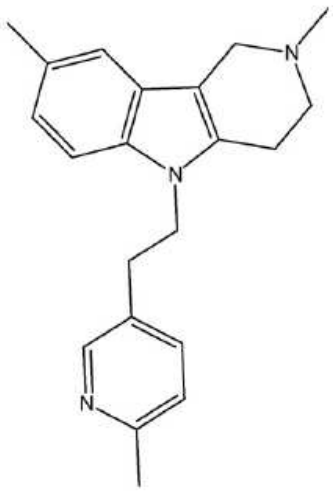


Figure 1. Chemical structure of Dimebon

1.5. Effects of Memory Enhancer Dimebon in Mouse Models of Learning

Dimebon™ (2,8-Dimethyl-5-[2-(6-methylpyridin-3-yl)ethyl]-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole dihydrochloride) (Fig. 1) is a non-selective antihistamine used in Russia since 1983 for allergy treatment (Matveeva, 1983).

1.5.1. Clinical Studies on Dimebon in Treatment of Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by impairment of cognitive function and changes in behavior and personality. Pathological features include: cortical and hippocampal atrophy, accumulation of abnormal fibers in neuronal cell bodies (neurofibrillary tangles composed of a hyperphosphorylated form of the microtubular protein tau), extracellular accumulation of senile plaques made mainly of β-amyloid peptide (Aβ) expressed under two main forms ($A\beta_{40}$ and $A\beta_{42}$), specific loss of cholinergic neurons resulting in a deficiency of the neurotransmitter acetylcholine (Ach) (Celsi & al., 2004; Minati & al., 2009). The currently therapies available for Alzheimer's disease are only symptomatic. To date, four drugs, three cholinesterase inhibitors - donezepil, rivastigmine, galantamine- and a NMDA antagonist - memantine, have been approved for the treatment of symptoms of AD patients but they were not proven to affect the underlying progression of the disease. Because AD is the most frequent neurodegenerative disease in the world, affecting millions of people, it's urgently needed to identify pharmacological treatment able to efficiently cure the disease. Dimebon has been recently demonstrated to be a potentially good candidate for this purpose.

A clinical trial has been performed on 14 patients with Alzheimer's disease and has shown that dimebon treatment improved cognitive functions, psychiatric and emotional symptoms of all treated patients (Bachurin & al., 2001). Recently, 183 patients with mild-to-moderate Alzheimer's disease were enrolled in a randomized, double-blind, placebo-controlled phase II trial in Russia. The patients received oral dimebon, 20mg three times a day and were assessed for different outcomes including cognition (ADAS-cog). Patients treated with dimebon for one year were stabilized on measures of cognition and memory, activities of daily living, behavior and global function, while placebo-treated patients declined significantly (Doody & al., 2008). Dimebon had so demonstrated efficacy in a phase 2 trial of patients with Huntington's disease (HD) conducted by Medivation and Huntington Study Group (DIMOND; Kieburtz, 2009). The phase III clinical trial is investigating currently (Hung, 2008).

1.5.2. Prelinical Studies on Memory Enhancer Dimebon

Several animal studies also showed potential beneficial effects of dimebon. In a long-term experiment, C57BL/6 21-months-old female mice were treated with dimebon (1.5 mg/kg per day orally *ad* libitum) for 14 months. The dimebon-treated mice were observed to have a better "quality" of life than control mice and lived more than 35 months as compared to 33 months for the control animals (Bachurin & al., 2007). Rats acutely treated orally with dimebon at doses 0.05, 0.5 or 5 mg/kg demonstrated an increase of cognition in short-term memory in a novel object recognition task (Giorgetti & al., 2010). In a rat social recognition task, Schaffhauser and co-authors (2009) showed that a 10 mg/kg i.p. acute dosed improved short-term memory. A chronic administration of dimebon (1 mg/kg daily i.p. injected during 10 days) demonstrated cognition and memory-enhancing properties in the active avoidance test in a rat AF64A cholinergic-lesion model (Lermontova & al., 2000). In this model, AFA64A, a neurotoxic analogue of acetylcholine, is injected in lateral cerebral ventricles and causes neurodegenerative disorders similar to those characteristic of AD (Hanin, 1996). To date the mechanism of action of dimebon remains poorly understood.

1.5.3. Hypothesized Mechanisms of Action of Dimebon

In vitro, dimebon exhibits a broad pharmacological profile, it has been demonstrated able to bind to no less than 61 neurotransmitter and neuropeptide receptors, 7 ions channels, 5 neurotransmitter transporters and other targets with different affinity (Giorgetti & al., 2010; Wu & al., 2008). Dimebon has been reported to be a weak (IC_{50} = 10 µM) antagonist of N-methyl-D-aspartate (NMDA) receptor (Bachurin & al., 2001; Giorgetti & al., 2010; Wu & al., 2008) and a weak (IC_{50}= 8-42µM) competitive and reversible inhibitor of acetylcholinesterase (AchE) *in vitro* and *in vivo* (Bachurin & al., 2001; Giorgetti & al., 2010; Grigoriev & al., 2003). Moreover, dimebon has been shown to act as a positive modulator of AMPA receptors at low concentrations (1-20 µM) and as an inhibitor at higher concentrations (40-50 µM) (Grigoriev & al., 2003). Studies (Giorgetti & al., 2010; Protter & al., 2010) suggested that memory enhancing effects of dimebon is unlikely to be due to AchE or NMDA receptor, which are validated target of current AD therapeutics.

Other targets for which dimebon have been found to have higher affinity may contribute to the cognitive effects of dimebon. Recently, Wu and co-authors (2008) and Giorgetti andco-authors (2010) showed that dimebon binds to numerous targets when tested at 10 µM, with moderate to high affinity. Indeed, dimebon inhibits with relatively high affinity ($K_i \leq 900$ nM)

to inhibit α-adrenergic receptors (α1A, α1B, α1D, α2A, α2B and α2C), histamine receptors (H1 and H2), serotonin receptors (5-HT2A, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6 and 5-HT7), dopamine receptors (D1, D2S, D3) and imidazoline receptor (I2) (Giorgetti & al., 2010; Wu & al., 2008). Interactions with these receptors may underlie the memory enhancing and / or other potential effects of dimebon.

Recent study suggested that dimebon may act via a reduction of production or accumulation of abnormal protein aggregates (Yamashita & al., 2009). However, and surprisingly enough, acute dimebon treatment has also been found to elevate Aβ extracellular levels during *in vitro* and *in vivo* studies (Steele & al., 2009). Furthermore, another investigation showed that dimebon protected neurons in the cerebellum cell culture from the neurotoxic action of β-amyloid fragment (Aβ25-35) in a dose-dependent way (EC50 = 25 μM) (Lermontova & al., 2001). One hypothesis for Alzheimer's disease is that neurotoxic β-amyloid peptide is thought to affect glutamate reuptake; glutamate which in turn, induces a neurotoxic effect by activation of NMDA receptors and increasing intracellular of Ca^{2+} (Bachurin & al., 2001; Harkany & al., 1999) into neurons. This deleterious influx of calcium ion into neurons triggers intracellular events, a pathologic activation of the permeability transition and an irreversible opening of mitochondria pores, that eventually causes cell death (Moreira & al., 2010; Narahashi & al., 2004; Nirogi & al., 2009). Several lines of evidence suggest that mitochondrial dysfunction have a central role in neurodegenerative disease including AD and HD (Moreira & al., 2010). Previous studies showed that dimebon blocks opening of the mitochondrial permeability transition pores induced by Aβ25-35 and MPP+ (Bachurin & al., 2003) and stabilize mitochondrial membrane function (Bernales & al., 2009; Protter & al., 2009). Furthermore, dimebon prevented the mitochondrial lipid peroxidation induced by butylhydroxyperoxide and ferrous ions (Bachurin & al., 2003, 2007). Dimebon has been shown to induce neurite outgrowth from cortical, hippocampal and spinal cord neurons for which a normal mitochondrial function is necessary (Bernales, 2009; Protter, 2009). In an *in vitro* Huntington's disease assay, dimebon showed significant protective effects from glutamate-induced apoptosis (Wu & al., 2008). Moreover, dimebon has been reported to stabilize glutamate-induced Ca^{2+} signals (Bachurin & al., 2001; Lermontova & al., 2001; Wu & al., 2008) and then neuronal Ca^{2+} homeostasis (Moreira & al., 2010). Given that mitochondrial function and Ca^{2+} homeostasis are interdependent (Celsi & al., 2009), dimebon is currently thought to operate by Ca^{2+} modulation level and by mitochondrial mechanism of action that may prevent neuronal death and thus, the cognitive decline (Bachurin & al., 2001, 2003; Moreira & al., 2010; Narahashi & al., 2004; Wu & al., 2008).

In addition to cognitive enhancive properties, dimebon also displays neuroprotective effects and these two properties have both to be considered in beneficial effects in clinical trials. Dimebon could be the first pharmacological agent that modifies the development of molecular and cellular pathogenesis of proteinopathies. To investigate mechanisms and targets of dimebon by biomedical imaging technique positron emission tomography (PET), a Carbon-11-labeled dimebon has been synthetized as a new potential PET agent. In the future, this radioligand should allow to monitor activity of enzyme and receptor in a non-invasive way in AD and HD response to dimebon treatment using PET (Minghzhang, 2010). Given the success and the multitargeting ability of dimebon, several analogues have been synthetized (Ivachtchenko chtenko & al., 2009, 2010), which were predicted to have even higher pharmacological activity than original molecule.

2. Objectives and Plan of the Studies

The goal of the first part of the study was to assess an impact of most common factors of learning in mouse models of learning: genetic background, aging and experiencing stress. To do so, following experiments were carried out:

1. We examined the performance of three strains of 3-months old male mice BALB/c, C57BL/6 and CD-1 in the step-down avoidance task. C57BL/6, CD1 and BALB/C mice demonstrated different behavioral phenotype in previous studies, particularly in anxiety, freezing response and locomotor activity, suggesting that we should expect different results in the SDA test between these three strains.

2. We performed the step-down avoidance task on 3-months and 7-months mice in order to investigate the effects of subtle aging on hippocampus-dependent memory in C57BL/6 mice and to validate the high resolution of the step-down avoidance.

3. To date no study in mice has investigated the effects of chronic stress in contextual fear conditioning. Given hippocampal altered function and deficits in hippocampus-dependent learning induced by chronic stress in rats and mice, we hypothesized that chronic stress could impair learning in an applied subsequent contextual fear conditioning task. C57BL/6 mice were first submitted to social defeat and exposure to rats in small container in order to induce a chronic stress. We investigated whether chronic stress impaired fear conditioning task and whether mice will also show a high level of freezing (like rats) in the fear conditioning task after submission to a chronic stress procedure.

The aim of the second part of the present study was to identify the most optimal dosing and the most optimal behavioral test which would be sensitive to the memory enhancing effects of dimebon. We focused on a step-down avoidance task, a one-trial paradigm, applied to mice injected acetuly intraperitoneally with different doses of dimebon (0.1 mg/kg and 0.5mg/kg). Moreover, because memory enhancer properties of Dimebon can be strain-dependent, we performed the SDA task on CD1 mice treated with dose 0.1mg/kg of dimebon. In addition, we used Y-maze test, a paradigm with a multiple training protocol on C57BL/6 mice daily injected with 0.1 mg/kg dimebon in order to contrast this data with results obtained in a single training paradigm.

3. Material and Methods

3.1. Animal and Housing Conditions

In this study, we used mice of different strains and ages: 3-months-old male C57BL/6N and 7-months-old male C57BL/6N (C57BL/6); 3-months-old male CD1 mice; 3-months-old male BALB/c mice.

All mice were housed individually on a reverse 12/12h light/dark cycle (light off at 10 am, red light during the dark phase of the cycle) in temperature (22 ± 1ºC) and humidity (55%±15%) controlled. These conditions were applied fourteen days prior to the behavioral experiments. The cages were opaque plastic boxes with a metal cover fitted with openings for delivering food and liquid. Food and water were available *ad libitum* except during the Y-Maze test (*see 2.5.3. Y-Maze test*). Cages were changed once per week. Wistar male rats, used for the rat exposure stress, were kept in the same conditions as those of the mice, in a different room. All animals were obtained from Charles River (Südfeld, Germany). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals upon approval by the local governmental body of animal care and welfare.

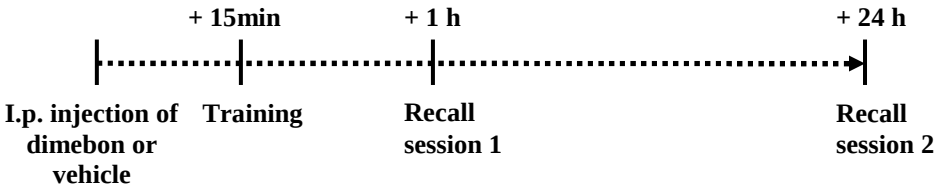
3.2. Design of Studies

3.2.1. Study 1: Evaluation of Factors of Learning in Mice

In Study 1, we investigated the effects of some most relevant factors of learning, such as strain differences, aging, and chronic stress exposure by using behavioral paradigms of contextual memory in mice.

Evaluation of strain differences: The SDA test has been applied to compare contextual learning in the three most commonly used laboratory mouse strains: BALB/cmice (n=21), C57BL/6(n=16) and CD1 (n=17) mice. All mice were three months old. A subpopulation of these animals was also used in Study 2 (see below step-down avoidance on C57BL/6and on CD1). Learning of this task was assessed in a recall session 24 hours after a single training session (see 2.5.2 step-down avoidance).

Evaluation of effects of aging: In this experiment, in order to assess the effects of aging on contextual learning, the step-down avoidance (SDA) task was performed on 7-months-old (n=8) and 3-months-old C57BL/6(n=16) mice. The data of the latter group was also used in Study 2 (see below step-down avoidance on C57BL/6). The hippocampus-dependent learning



Scheme 2. Evaluation of effects of dimebon in step-down avoidance paradigm:Timeline of experiment

Table 1. Design of Study 2: Evaluation of effects of Dimebon in mouse models of learning

Behavioral Test	Strain	Dose	Treatment
Step-down avoidance test	CD1	0.1 mg/kg	acute
	C57BL/6N	0.1 mg/kg	acute
	C57BL/6N	0.5 mg/kg	acute
Y-Maze Test	C57BL/6N	0.1mg/kg	chronic

was assessed in a recall session 24 hours after a single training session *(see 2.5.2 step-down avoidance).*

Evaluation of effects of chronic stress: Chronically stressed mice were submitted to the fear conditioning test to determine the effect of stress on contextual learning. In order to induce chronic stress, 3-months-old C57BL/6(n=33) mice were submitted to the ethological chronic stress procedure for 2 weeks.

3.2.2. Study 2: Evaluation of Effects of Dimebon in Mouse Models of Learning

Step-down avoidance on C57BL/6: To investigate possible effects of Dimebon on learning in the SDA task, we performed two experiments: fifteen minutes prior the training session, 3-months-old C57BL/6 mice were injected intraperitoneally either with Dimebon in with a dose of 0.1mg/kg (n=9) or 0.5mg/kg (n=16) or with vehicle (n=8 and n=17, respectively to two experiments). Memory performance was assessed in two recall sessions: one hour after training in order to assess short-term memory, and twenty-four hours after training in order to study long-term memory (Scheme 1) (Izquierdo & al., 2002; Strekalova & al., 2001, 2002).

Step-down avoidance on CD1: Because effects of memory enhancive properties of dimebon can be strain-dependent and may appear when weak learning scores are observed, we additionally used CD1 strain, which is characterized by lower abilities for hippocampus-dependent memory (Adams & al., 2002). The step-down avoidance paradigm has been applied on CD1 mice after have been injected either with 0.1mg/kg Dimebon (n=16) or vehicle (n=17) with the same design as that used on C57BL/6mice (*see above*).

Y-Maze test on C57BL/6: In order to investigate the effects of a daily administration of Dimebon on hippocampus-dependent spatial learning, the Y-Maze test was carried out on 3-months-old C57BL/6mice (n=16). During the five days of testing in Y-Maze, mice received single daily injection of 0.1mg/kg Dimebon (n=8) or vehicle (n=8) fifteen minutes prior the first trial.

3.3. General Conditions of Testing

The testing room was protected from noise and all groups of mice were tested during the dark period of the light cycle. The person performing the experiments was blind to the treatment until the end of the behavioral tests. Mice were randomly assigned to vehicle and dimebon-treated group. Vehicle/control and treated/stress groups were evaluated alternately in a same test in order to minimize possible influence of the environment. Majority of the experiments were recorded on videotape.

3.4. Testing and Procedures

3.4.1. Protocol of Learning and Memory Paradigms

3.4.1.1. Step-down avoidance paradigm

The protocol of this test was applied as described elsewhere in the literature (Strekalova & al., 2001; Strekalova and Steinbusch, 2010). The step-down is a passive avoidance task used to evaluate hippocampus-dependent memory in rodents. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The step-down apparatus (Evolocus LLC Tarrytown, NY, USA) consisted of a plastic box (25 cm x 25 cm x 50 cm; Technosmart, Rome, Italy) with translucent walls placed on a stainless-steel grid floor (33 rods 2 mm in diameter) wired to a customized shock generator. This shocker was used to deliver an alternative electric current (AC, 50 Hz). The apparatus was illuminated with a white light (25 lux).

The test included a single training session and one or two recall session(s), depending on the design. In the training session, the mouse was introduced into a transparent cylinder on a square wooden platform (7 cm x 7 cm x 1.5 cm), which was placed on the metal grid floor. After 30 seconds, the cylinder was removed allowing to animal to move freely. The baseline latency is the time taken by the mouse to step down with four paws on the grid floor and was measured by an experimenter with a stopwatch. Immediately after step down, a single electric footshock (0.5 mA; 2 s) was given to the mouse which was promptly returned to its home cage.

During the recall session, the mouse was subjected to the same procedure, with the exception of the footshock. Latency of step-down increased between training and recall session showing that the mice have acquired the context/the task. The latency of stepping down measured during recall sessions is taken as a parameter of contextual memory. Latency

of step-down was measured until 180 seconds had elapsed. Mice that showed latencies of more than thirty seconds in the recall session were considered good learners. Handling of mice was excluded between the training and the recall session to avoid the interference of a new context with contextual memory acquired from the test. As short latencies of escape behavior were shown to reflect panic responses which confound learning scores in this test (Strekalova & al., 2001), mice displaying latencies of step down greater than or equal to one second were excluded from the analysis.

3.4.1.2. Y-Maze paradigm

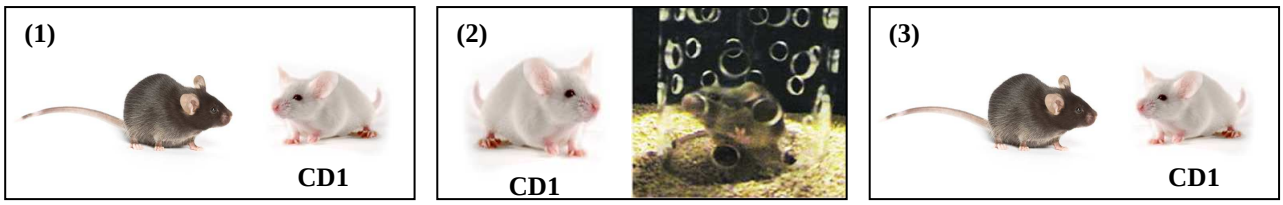
The apparatus was a maze Y-shaped in black Plexiglas and consisted of three arms with an angle of 120° between each of the two arms. Each arm was 6 cm ×40 cm ×10 cm (width × length × height) (Open Science, Russia). Visual cues were placed around the maze on the walls of the room (at a distance of 100 cm from the maze) to allow a spatial orientation. A bottle of water was placed on the left or on the right arm of the Y-Maze.

This protocol was described elsewhere in research literature (Gorenkova & al., 2005; Sunanda & al., 2000). Mice were water-deprived 18 hours before the training session. For the test, mice were positioned at the end of one arm (randomly assigned) and were trained to reach a drinking bottle on one of the other two arms. Half of each group was trained to reach a bottle on a right side and another half to reach it on a left side.

Two 15-minutes sessions were performed per day spaced apart by one hour. Bottle's position remained the same between each trial. Mice were left a cut-off of ten minutes to reach the bottle. If the mice were not drinking at the end of the trial, they obtained free access for water in their home cages for one hour. The latency to reach the bottle, defined as the time passed between the placement of the mouse in the Y-Maze and the point from mouse started sniffing the bottle, was scored by an experimenter with a stopwatch. From trial 2 of day 1, mice were scored in choosing the correct arm, the one containing the bottle, in each trial. Then the percentage of correct choices along with the days was calculated. Drinking time was scored from the point mice started to drink till the moment they stopped. They were removed from the Y-Maze immediately after ceased drinking. The behavior of mice was scored by visual observation. The latency to reach the bottle and the percentage of the correct choices and were taken as indicators of learning abilities in this test. In addition the duration of drinking was measured to assess anxiety and motivation levels.

A

B



Scheme 3. Chronic stress procedure. (A) Exposure to rats in small containers: C57BL/6 3-months-old mouse was introduced every day into cylindrical containers and placed into the rat cage, during 15h from 18h00 to 9h00. **(B)** Social defeat procedure: C57BL/6 mouse was placed inside of a home cage containing an aggressive CD1 mouse for 5 minutes (1). C57BL/6 mouse was then introduced into the small container, which was placed inside the CD1 cage for 3 hours (2). C57BL/6 mouse was over again placed in the CD1 mouse home cage for 5 min (3).

4.3.1.3. Fear conditioning test

The fear conditioning test allowed to measure the ability of mice to learn and to remember an association between an aversive stimulus with a contextual environment. The apparatus (Evolocus LLC Tarrytown, NY, USA) consisted of a plastic box (25 cm x 25cm x 50cm; Technosmart, Rome, Italy) with translucent walls placed on a stainless-steel grid floor (33 rods 2 mm in diameter) wired to a customized shock generator. This shocker was used to deliver an alternative electric current (AC, 50 Hz). In the training session, the mouse was placed directly on the gridfloor and received an electric footshock (0.8 mA, 2s). The mouse was returned immediately in its cage.

In the recall session, performed 24 hours later, the mouse was placed in the same context, no electric shock was applied. Mice were kept undisturbed (no handling) between the training and the recall sessions, in order to avoid any interference with formation of contextual memory. Freezing is a typical response displayed by the rodents when they are re-exposed to the context in which they had previously experienced brief inescapable shock (Sandi & al., 2001). The freezing behavior, defined by a complete immobilization except the respiration, was scored every 10 seconds during 180 seconds by visual observation. Data were presented as the percentage of time spent freezing during the entire session [100x(time spent freezing/duration of session] that was taken as a measure of learning . The behavior was also recorded on video.

3.4.2. Chronic Stress Experiment

3.4.2.1. Application of chronic stress procedure

To assess the effect of chronic stress on contextual learning in the fear conditioning task, mice were submitted to a social defeat (11h00 – 15h00) and rat exposure in small containers (18h00 – 9h00) during 2 weeks (*see below).* During the stress procedure, mice of control group were kept in their home cages. During the entire study, control and stressed groups were housed in the same room.

3.4.2.2. Characteristics of stressors (scheme 3)

Exposure to rats in small containers: C57BL/6 3-months-old mice *(n=9)* were introduced every day into cylindrical containers and placed into the rat cage, during 15h (over-night, from 18h00 to 9h00). During the weekends, mice were placed in their home cage on the top of rat cages. Containers are made from customized transparent plastic, size 15cm x

Ø 8 cm, with small holes in plastic covers (Ø < 0.5cm), which can ensure protection of the mice from the rats on one hand, and visual and odor contact, on another.

Social defeat: A C57BL/6 mouse was placed inside of a home cage containing an aggressive CD1 mouse, for five minutes. This stress condition was peformed twice: once before the placement of the small container containing the C57BL/6 mouse (*see above exposure to rat in small containers*) inside the CD1 cage for three hours, and the second time after this period. Animals were carefully observed during the test; in case of excessive aggressive attacks, the procedure was interrupted immediately.

3.4.3. Drug and Drug Administration

Dimebon,(2,3,4,5-tetrahydro-2,8-dimethyl-5-[2-(6-methyl-3-pyridinyl)ethyl]-1H-pyrido[4,3-b]indole (pK_a = 6.8), was generously offered by drug inventors from the Institute of Physiologically Active Compounds, RAS, Chernogolovka, Moscow region, Russia.

Dimebon was dissolved into sterile 0.9% saline (vehicle) solution at concentration of 0.01 mg/mL or 0.05mg/mL (respectively doses of 0.1mg/kg and 0.5mg/kg) to be delivered in an amount of 0.01ml/g of body weight by intraperitoneal (i.p.) injection. For the acute treatment with Dimebon, the solution was prepared extemporaneously and given in a single injection (dose 0.1mg/kg or 0.5mg/kg) fifteen minutes before training. For the chronic treatment, the solution was prepared on the first day of the testing week and was daily administered (dose 0.1mg/kg) fifteen minutes before the test.

3.5. Statistical Analysis

Data were analyzed with a statistical software package (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA). Data obtained step-down avoidance and fear conditioning tests were treated with non-parametric analysis, even though the populations were Gaussian, since several values were measured arbitrarily in this paradigm due to the cut-off in the behavioral scoring (as observation period is elapsed). Repeated measurements in the SDA task were evaluated by the non-parametric Wilcoxon test; independent data sets were evaluated by the non-parametric Mann-Withney *U* test except for the data from the three strains, which were analyzed by the Kruskal-Wallis ANOVA test. By our definition, animals which showed latencies of step down greater than or equal to thirty seconds in the recall session(s), were considered as good learners. The relative number of good learners was compared between the groups by the Fischer's exact test and by the Chi-

squared test when more than two groups were analyzed. Data obtained in the fear conditioning paradigm were also analyzed by the non-parametric Mann-Withney *U* test.

The statistical analysis for the data obtained in the Y-Maze test was carried out by one-way analysis of variance (ANOVA) for independent data and by repeated measures ANOVA techniques for paired data when data values had normal distribution, in order to detect significant differences in and between the groups. Then, repeated data were analyzed by using the Tukey's multiple comparison test and independent data measurements were assessed by an unpaired t-test. When the data values had not normal distribution, the Kruskal–Wallis-ANOVA and Mann–Whitney *U*-test were applied. The level of confidence was set to 95% ($P<0.05$). All tests were one-tailed.

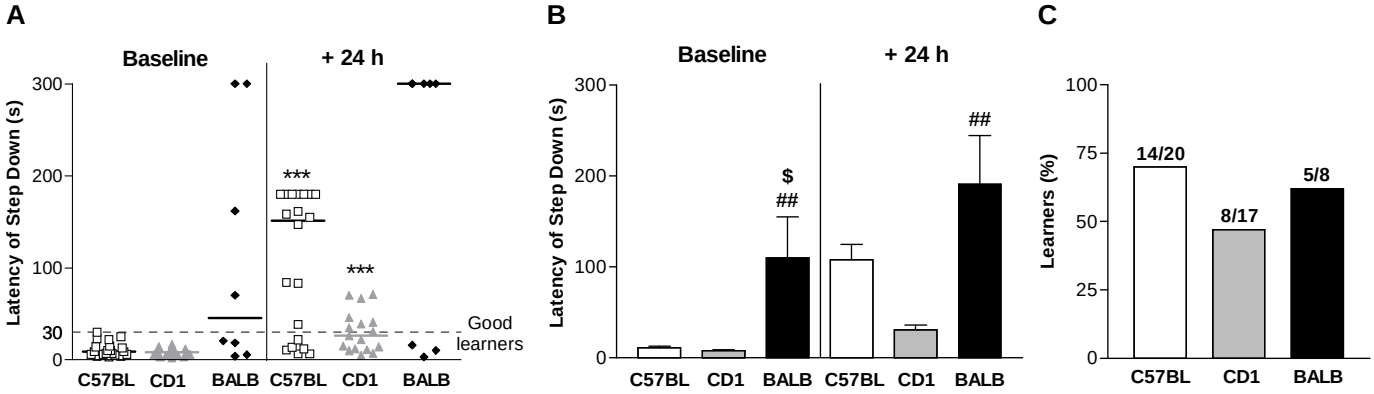


Figure 1. Effects of strain differences (C57BL/6, CD1 and Balb/c) on learning performance in the step-down avoidance test. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The latency to step down (time to step down completely on the grid floor) was scored in C57BL/6 (*n*=20, C57BL, open symbols), CD1 (*n*=17, CD1, grey symbols) and BALB/c (*n*=8, BALB, close symbols) mice. **(A)** Individual data of step-down latencies obtained in each strain of mice during training (Baseline) and recall (+24h) session. Bars indicate medians of the groups and dotted line indicates the threshold (30 s) of good learners. **(B)** Each column represents the mean (± SEM) of training (baseline) or recall (+ 24 h) session latencies. **(C)** Percentage of good learners in the recall session was not significantly different between the three groups ($P > 0.05$, χ^2 test). Numbers of good learners and all of animals tested per group are indicated above the bars. According to exclusion criteria (step-down latency ≤ 1 second, *see the text*), some of the C57BL/6 mice (*n*=4) were excluded from the analysis. ***$P < 0.001$ vs training (Wilcoxon); ##$P < 0.01$ vs CD1 mice (Kruskal-Wallis test, $P < 0.05$); \$ $P < 0.05$ vs C57BL/6 mice (Kruskal-Wallis test, $P < 0.05$).

4. Results

3.6. Study 1: Evaluation of Factors of Learning in Mice

3.6.1. Three Mouse Strains Displayed Different Abilities to Learn Contextual Memory

As follows from Fig. 1, the baseline latencies were similar in C57BL/6 and CD1 mice ($P > 0.05$, Kruskal-Wallis test), while this parameter was significantly higher in the BALB/c mice in comparison to two other strains ($P < 0.05$ vs C57BL/6 mice and $P < 0.01$ vs CD1, Kruskal-Wallis test; Fig. 1B). During the training session, half of the BALB/c mice (n=4) displayed a latency higher than thirty seconds, a taken criterion of task acquisition, while C57BL/6 (*n=1*) and CD1 (*n=0*) mice did not reveal such behavior. At the recall session, latencies of step down of all three strains increased significantly as compared to the training session ($P = 0.0001$ for C57BL/6, $P = 0.0004$ for CD1, $P = 0.015$ for BALB/c vs training session, Wilcoxon; Fig. 1A) indicating that all mice acquired the task. Even though high percentage of BALB/c can be classified as good learners in the recall session (62%), they are not indicative for good learning abilities because they already showed these values of step down in the training session (Fig. 1C), i.e. they are likely to be due to high anxiety, a feature characteristic for BALB/c strain (Bolivar & al., 2000; Crawley & al., 1997; Michalikova & al., 2010). Thus, this strain is rather inconvenient for testing contextual memory in the step-down avoidance task. Similarly, whereas 70% of C57BL/6 mice could be characterized as good learners, less than 50% of CD1 mice were assigned to this group (Fig. 1C).

Together, C57BL/6 mice had highest scores of learning of step-down avoidance, BALB/c strain showed poor learning parameters and CD1 demonstrated intermediate memory scores. These data are not in line with other reports (Crawley & al., 1997) where BALB/c showed better learning of avoidance task compared to C57BL/6 (data concerning CD1 mice compared to the others strains in this task were not found). These results suggested remarkable strain differences in acquisition of hippocampus-dependent contextual learning between three tested mouse lines. They also suggested that the step-down avoidance in C57BL/6 mice is an efficient paradigm of testing contextual learning in mice.

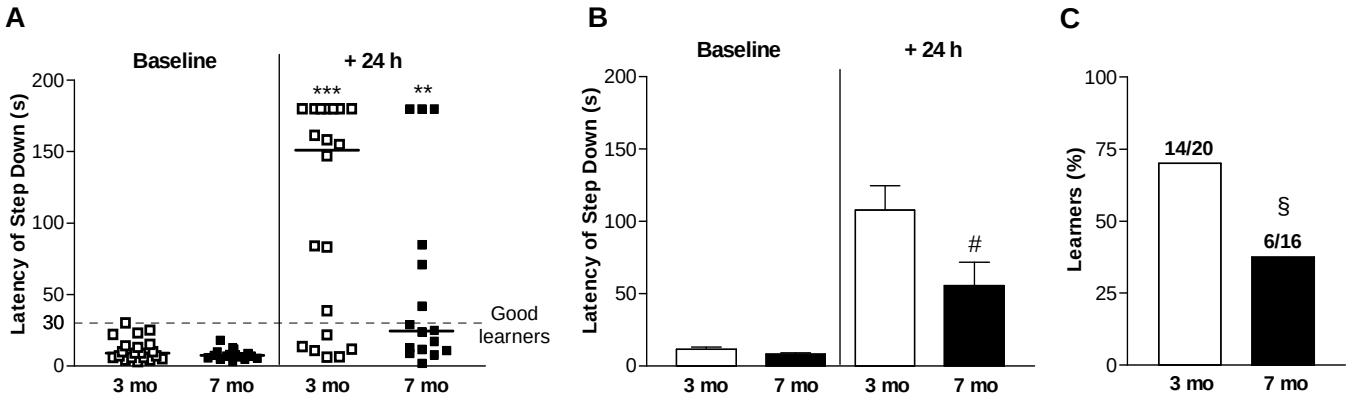


Figure 2. Effects of aging on learning in the step-down avoidance task. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The latency to step down (time to step down completely on the grid floor) was scored in 3-months old (n=20, 3 mo, open symbols) and 7-months old (n=16, 7 mo, close symbols) C57BL/6 mice. **(A)** Individual data showing latencies of step down measured during the training session (Baseline) and during the recall (+ 24 h) session. Bars indicate medians of the groups and dotted line indicates the threshold (30 s) of good learners. **(B)** Each column represents the mean (± SEM) of training (Baseline) or recall (+ 24 h) session latencies. **(C)** Percentage of good learners in 3 months-old mice had tendency to be significantly higher than among 7 months-old mice ($0.05 < \S P \leq 0.1$ vs 3-months-old mice (Fischer's exact test)). Numbers of good learners and all of animals tested per group are indicated above the bars. According to exclusion criteria (step-down latency ≤ 1 second, *see the text*), 3-months-old mice (n=4) and 7-monthd-old mice (n=2) were excluded from the analysis. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs training (Wilcoxon); $0.05 < \#P \leq 0.1$ vs 3-months-old mice (Mann-Withney U test).

3.6.2.

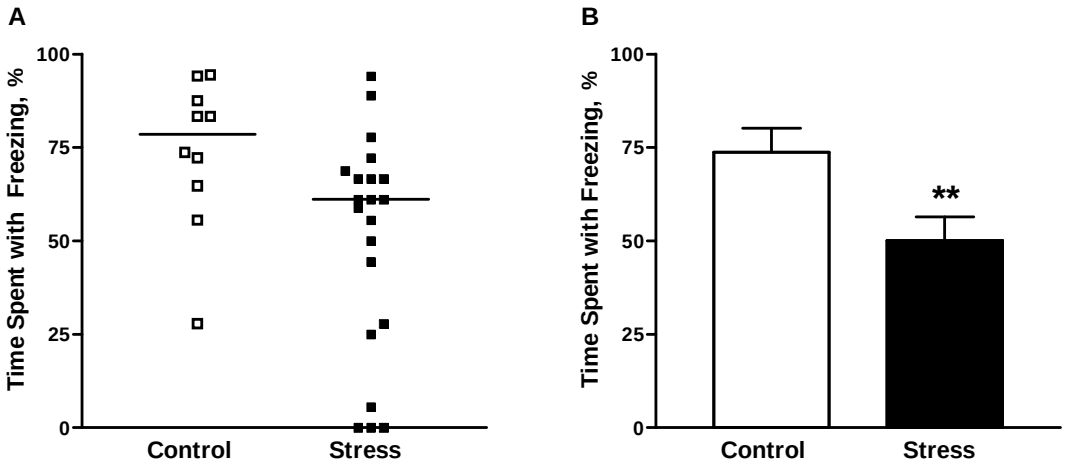


Figure 3. Effects of chronic stress on learning in fear conditioning task. Control (n=10, open symbols) and chronic stressed (n=21, close symbols) mice were trained with a single foot shock in a contextual environment. Fear conditioning was tested 24 h later in the training context by scoring of freezing bouts every 10 seconds during 3 minutes. The percentage of time spent freezing during the entire session [100x(time spent freezing/duration of session] was taken as a measure of learning. **(A)** Individual data of percentage of time spent freezing obtained in control and chronic stressed group. Bars indicate medians of the groups. **(B)** Each column represents the mean (± SEM) of percent time freezing during the 3-min test interval in control and stress group. $^{**}P < 0.01$ (Mann-Withney U test).

Aging Impaired Learning in the Step-Down Avoidance Task

Latencies of step down measured in training and recall session in 3-months old and 7-months old C57BL/6 are shown in Fig. 2. The baselines latencies were similar in 3 months-old mice and 7-months-old mice ($P > 0.05$, Mann-Whitney *U* test; Fig. 2A-2B) suggesting that the age difference did not affect the locomotion and the anxiety under applied experimental conditions. However, latencies of step down in both groups were significantly increased during the recall session in comparison to the values recorded during training ($P_{3m.o.}= 0.0001$ and $P_{7m.o.}= 0.001$ vs training session, Wilcoxon; Fig. 2A) showing that all animals acquired the task. During the recall session, the latency of step down had tendency to be significantly higher in 3-months than in 7-months old animals (P = 0.055 vs 3 months-old, Mann-Whitney *U* test, Fig. 2B). In addition, 14 of 20 animals (70%) from group of 3-months-old were considered as good learners according to a taken criterion (animals displaying latencies greater than or equal to thirty seconds), whereas only 6 of 16 animals among 7 months-old animals (37.5%) were assigned to this group (Fig. 2C). Notably, the percentage of good learners in older mice was lower (<50%) from that of 3-months old animals. Thus, subtle aging does impair contextual learning of male C57BL/6 mice in the step-down avoidance paradigm.

3.6.3. Chronic Stress Disrupted the Contextual Fear Conditioning

Mice were first submitted to a fifteen days chronic stress procedure, and the contextual memory in mice was evaluated in the fear conditioning test after the termination of chronic stress. In the training session, the mouse was placed directly on the gridfloor and receives an electric footshock. In the recall session, the mouse was placed in the same context without any electric shock and the freezing behavior was scored and taken as a measure of learning. Fig.3. shows that the percentage of time spent freezing was significantly higher in control group (74%) than in stress group (50%) ($P = 0.009$, U=49.00, Mann-Whitney *U* test). These data suggested that the contextual learning was impaired in the stress group and therefore that the stress impaired the contextual learning in the fear conditioning task.

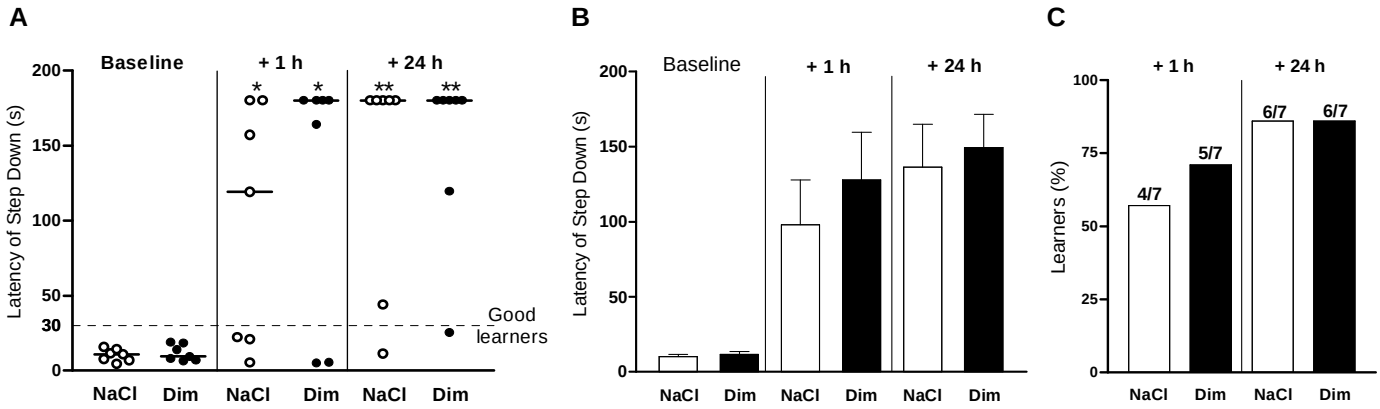


Figure 4. Evaluation of learning in the Step-down avoidance on C57BL/6 mice treated by Dimebon at dose 0.1mg/kg. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The latency to step down (time to step down completely on the grid floor) was scored 15 minutes (Baseline) after the intraperitoneal injection of vehicle (*n*=7, NaCl, open symbols) or either Dimebon (*n*=7, Dim, close symbols). Learning performance was assessed in two recall sessions, 1 h later for the assessment of short-term memory and 24 h later for the assessment of long-term memory. **(A)** Individual data showing latencies of step down measured during the training session (baseline) and during the two recall (+ 1, + 24 h) sessions in vehicle and Dimebon-treated group. Bars indicate medians of the groups and dotted line indicates the threshold (30 s) for good learners. **(B)** Each column represents the mean (± SEM) of training (baseline) or recall (+ 1 h, + 24 h) session latencies. **(C)** Percentage of good learners in the two recall session was not significantly different between the vehicle and the control group. Numbers of good learners and all of animals tested per group are indicated above the bars. According to exclusion criteria (step-down latency ≤ 1 second, *see the text*), one control and two treated mice (*n*=2) were excluded from the analysis. **P* < 0.05 and ***P* < 0.01 vs training (Wilcoxon).

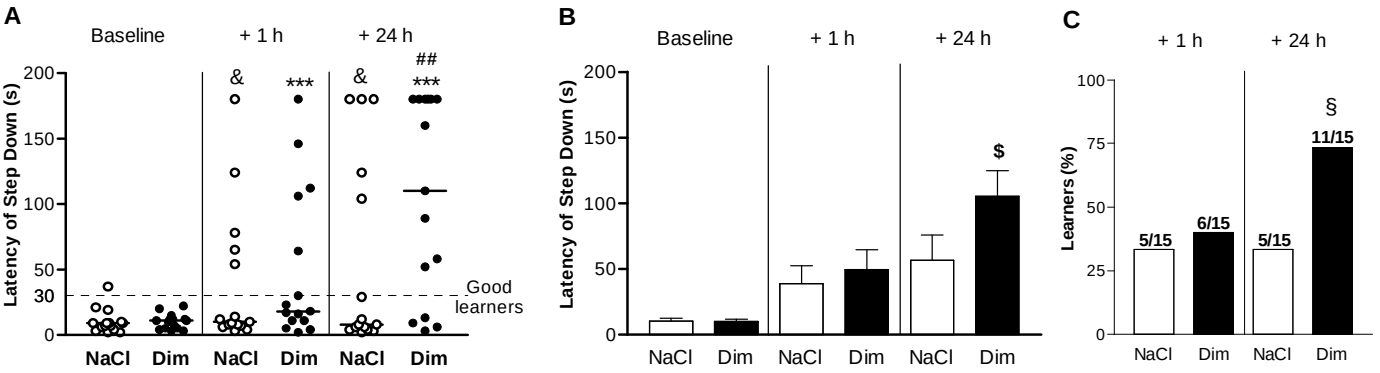


Figure 5. Evaluation of learning in the Step-down avoidance on C57BL/6mice treated by Dimebon at dose 0.5mg/kg. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The latency to step down (time to step down completely on the grid floor) was scored 15 min (Baseline) after the intraperitoneal injection of vehicle (*n*=15, NaCl, open symbols) or either Dimebon (*n*=15, Dim, close symbols). Learning performance was assessed in two recall sessions, 1 h later for the assessment of short-term memory and 24 h later for the assessment of long-term memory. **(A)** Individual data showing latencies of step down measured during the training session (Baseline) and during the two recall sessions (+ 1, + 24 h) in vehicle- and Dimebon-treated group. Bars indicate medians of the groups and dotted line indicates the threshold (30 s) of good learners. **(B)** Each column represents the mean (± SEM) of training (Baseline) or recall (+ 1 h, + 24 h) sessions latencies. **(C)** Percentage of good learners in the two recall sessions. Numbers of good learners and all of animals tested per group are indicated above the bars. According to exclusion criteria (step-down latency ≤ 1 second, *see the text*), one control and two treated mice were excluded from the analysis. 0.5 < &*P* <0.1 and ****P* < 0.001 vs training (Wilcoxon); ##*P* < 0.01 vs first recall session (Wilcoxon); $*P* < 0.05 vs vehicle-treated group in the second recall session (Mann-Withney *U* test); §*P* < 0.05 vs vehicle-treated group in the second recall session (Fischer's exact test).

3.7. Study 2: Evaluation of Effects of Dimebon in Mouse Models of Learning

3.7.1. Dimebon Enhanced Learning in a One-Trial Step-Down Avoidance Paradigm

Step-down avoidance on C57BL/6 Mice treated with 0.1mg/kg Dimebon: Mice were injected with Dimebon at dose 0.1mg/kg or with vehicle and then submitted to the training of the SDA after fifteen minutes. Learning performance was assessed in two recall sessions, one hour and twenty-four hours later. As follows from Fig. 4A-4B, vehicle and Dimebon-treated group showed similar baseline latencies values ($P > 0.05$, Mann-Whitney *U* test) indicating that Dimebon did not affect the locomotion and the anxiety. Moreover, latencies of step down were increased for vehicle and Dimebon-treated group at the first recall session ($P = 0.02$ and $P = 0.04$ respectively vs training, Wilcoxon), but also at the second session ($P = 0.008$ and $P = 0.008$ respectively, Wilcoxon) in comparison to the training session (Fig. 4A) suggesting that mice all acquired the task. However, no significant difference was found between the groups whether for one hour or twenty-four hours after the training session ($P > 0.05$, Mann-Whitney *U* test, Fig 4B). Furthermore, percentages of good learners were not significantly different between vehicle- and Dimebon-treated group in the first recall session ($P > 0.05$, Fischer's exact test) and were even identical in the second recall session (Fig. 4C). Thus, Dimebon injected acutely at dose 0.1 mg/kg intraperitoneally did not affect contextual learning.

Step-down avoidance on C57BL /6N Mice treated with 0.5mg/kg Dimebon: The same design as in the previous study (*see above*) was used in dose 0.5mg/kg of Dimebon. Baseline latencies of step down of vehicle- and dimebon-treated mice were similar ($P > 0.05$, Mann-Whitney *U* test; Fig. 5A-5B). Surprisingly, latencies of step down of vehicle-treated group had only tendency to be significantly higher in the first recall (P=0.052 vs training, Wilcoxon) and in the second recall session (P=0.063 vs training, Wilcoxon) in comparison to the training session (Fig. 5A). Moreover, less than the half (33%) from vehicle were classified as good learners in both recall sessions (Fig. 5C). Latencies of step-down of Dimebon-treated group significantly increased in the first and the second recall session ($P = 0.0008$ and $P = 0.0002$ respectively, Wilcoxon; Fig. 5A) compared to the training session suggesting the acquisition of the task by the Dimebon-treated mice. Moreover, Dimebon-treated mice remained significantly longer on the platform in the second recall session compared to the first recall

session ($P < 0.01$ vs first recall session, Wilcoxon; Fig. 5A). In addition, on one side, Dimebon-treated group displayed latencies of step down significantly higher compared to

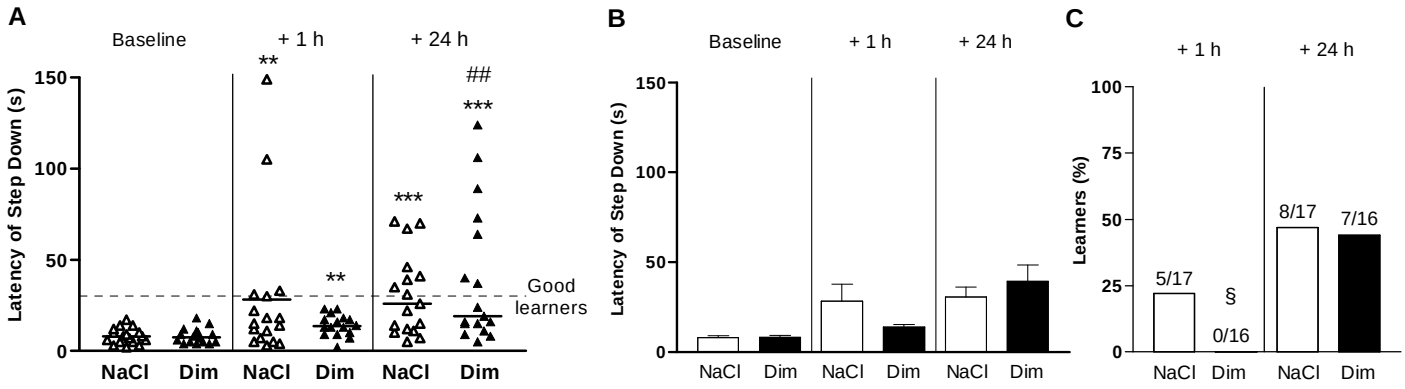


Figure 6. Evaluation of learning in the Step-down avoidance on CD1 mice treated at dose 0.1mg/kg of Dimebon. Mice were trained not to step down from a wooden platform onto a grid floor to avoid an electric footshock. The latency to step down (time to step down completely on the grid floor) was scored 15 min (Baseline) after the intraperitoneal injection of vehicle (n=17, NaCl, open symbols) or either Dimebon (n=16, Dim, close symbols). Learning performance was assessed in two recall sessions, 1 h later for the assessment of short-term memory and 24 h later for the assessment of long-term memory. **(A)** Individual data showing latencies of step down measured during the training session (baseline) and during the two recall sessions (+ 1, + 24 h) in vehicle and Dimebon-treated group. Bars indicate medians of the groups and mean when the values had not a normal distribution (vehicle-treated group in the first recall session). Dotted line indicates the threshold (30 s) of good learners. **(B)** Each column represents the mean (± SEM) of training (baseline) or recall (+ 1 h, + 24 h) sessions latencies. **(C)** Percentage of good learners in the two recall sessions. Numbers of good learners and all of animals tested per group are indicated above the bars. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs training (Wilcoxon); $^{\#\#}P < 0.01$ vs first recall session (Wilcoxon); §$P < 0.05$ vs vehicle-treated group in the second recall session (Fischer's exact test).

those of the vehicle-treated group ($P < 0.05$, Mann-Whitney U test; fig 5B) and, on the other side, the percentage of good learners was also significantly higher ($P < 0.05$, Fischer's exact test; Fig. 5C). These results suggested that Dimebon injected acutely at dose 0.5 mg/kg intraperitoneally enhanced long-term contextual learning. However, considering the results reported for the vehicle-treated group, this interpretation would be taken with precaution.

3.7.2. Dimebon Did Not Affect Contextual Learning in CD1 Mice

CD1 mice were treated with Dimebon at dose 0.1mg/kg according to the design used on the C57BL/6 mice injected with the same dose. Mice treated with Dimebon or vehicle revealed significant increase of latencies of step down in the recall session one hour after training ($P = 0.003$ and $P = 0.004$ respectively, vs training, Wilcoxon; Fig. 6A), but also twenty-four hours after training ($P = 0.0004$ and $P = 0.0003$ respectively, vs training, Wilcoxon, Fig. 6A). However, less than the half from each group were classified as good learners (Fig. 6C), which was defined, for recall, as mice displaying latencies greater than or equal to thirthy seconds. Latencies of step down were similar in vehicle and Dimebon treated-group as well in the training session ($P > 0.05$, Mann-Whitney *U* test; Fig. 6A-6B) as in the two recall sessions ($P > 0.05$, Mann-Whitney U test, Fig. 6A-6B). The percentage of learners in Dimebon-treated mice was zero in the first recall session whereas one third of vehicle-treated mice were considered as good learners (Fig. 6C). Moreover, percentage of good learners in the dimebon-treated group was lower than in the vehicle-treated group not only one hour after training period but also twenty-four hours later (Fig. 6C) suggesting that Dimebon may significantly affect the CD1 mice behavior under applied experimental conditions. On one hand, these data suggested that CD1 strain was not a good model of learning in the step-down avoidance, as previously noticed (*see Study 1)*. On the other hand, these results indicated that Dimebon did not affect the learning in the step-down avoidance on CD1 mice treated at dose 0.1mg/kg

3.7.3. Chronic Administration of Dimebon during Multiple Training Protocol Accelerates Learning in the Y-Maze

We used the Y-Maze to assess the effects of daily administration of Dimebon on spatial learning. The latency to reach the bottle and the percentage of correct choices (choosing the correct arm) were taken as indicators of learning abilities in this test. In addition, duration of drinking was measured to assess anxiety and motivation levels.

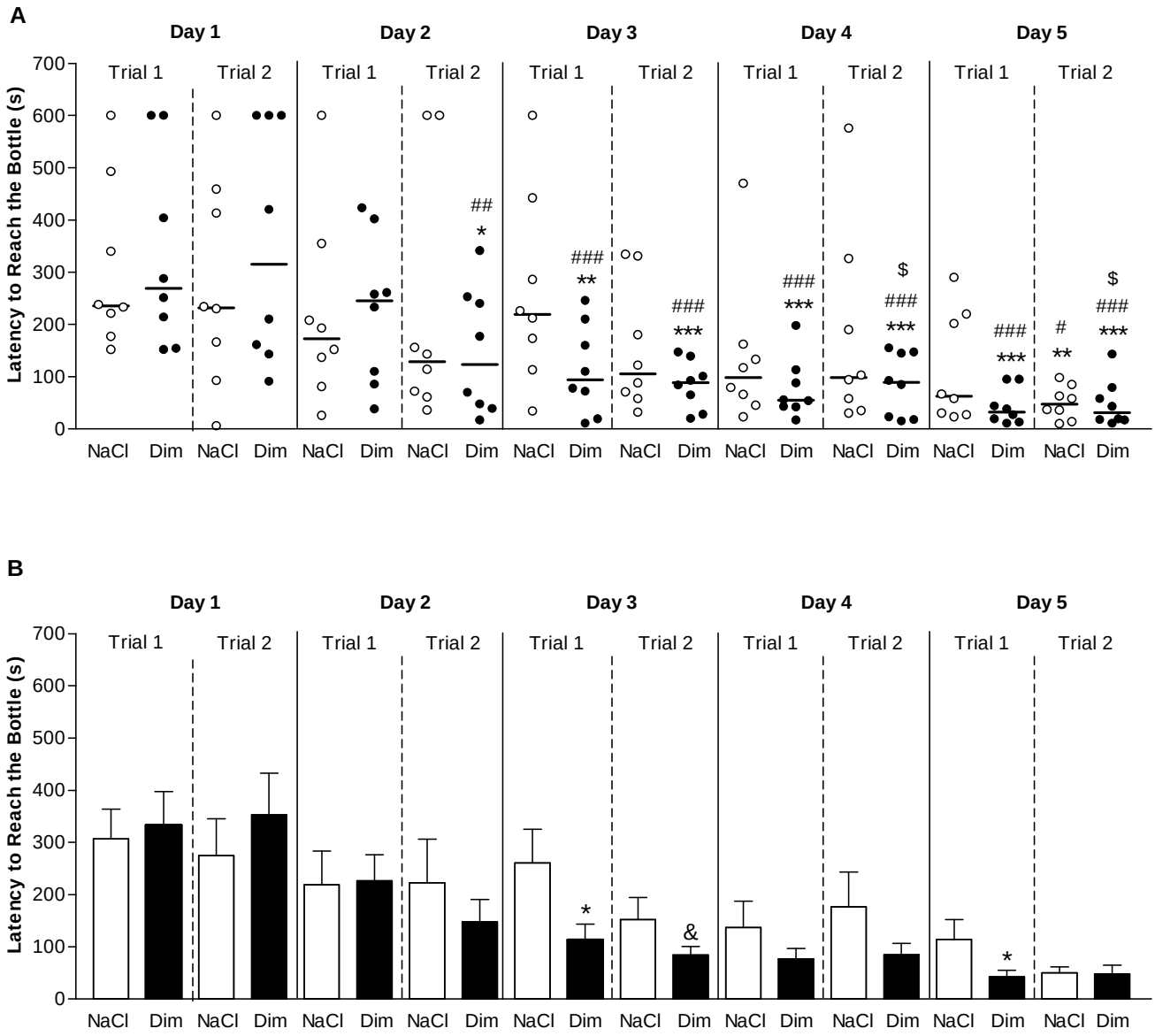


Figure 7. Effects of daily administration of Dimebon on spatial learning in Y-Maze test on C57BL/6N: latencies to reach the bottles. Mice were trained to reach a drinking bottle on the left or right arm of the Y-maze after being water-deprived. Visual cues were placed around the maze to allow a spatial orientation. Two trials per day spaced apart by 1 h were performed on each mouse. C57BL/6N mice received a single daily injection with vehicle (*n*=8, NaCl, open symbols) or either Dimebon at dose 0.1mg/kg (*n*=8, Dim, close symbols) 15 minutes prior the first trial during 5 days.

(A) Individual data showing latencies of vehicle and Dimebon-treated group to reach the bottle in trial 1 and 2 along with 5 days of testing were used as a measure of learning. Bars represent the medians. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs Trial 1 Day 1 respectively in each group; #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.01 vs Trial 2 Day 1 respectively in each group; $*P* < 0.05 vs Trial 1 Day 2 respectively in each group. All statistical comparisons were obtained by a Tukey's posthoc test after repeated ANOVA test (*P* < 0.001) revealed significant differences inside of each group.

(B) Each column represents the mean (± SEM) of latencies values to reach the bottle in vehicle (NaCl) and Dimebon-treated group (Dim) in each trial during the 5 days of testing. **P* < 0.05 vs vehicle in the same trial (Unpaired T-test); 0.5 < &*P* <0.1 vs vehicle in the same trial (Unpaired T-test).

The repeated ANOVA test revealed significant differences in latencies to reach the bottle along with learning trials and days in vehicle- ($P = 0.006$, $F = 2.92$, $R^2 = 0.29$) and dimebon-treated group ($P < 0.0001$, $F = 2.92$, $R^2 = 0.29$) (Fig.7A) suggesting that all mice acquired the task. Treatment with Dimebon caused a strong reduction in the latencies to reach the bottle every day starting from trial 2 day 2 compared to day 1 (Fig. 7A, * and # symbols). The latencies to reach the bottle of vehicle-treated group were significantly shorter from trial 2 day 5 in comparison to day 1 (Fig. 7A, * and # symbols). Thus, Dimebon-treated mice showed a decrease in latencies starting from day 2 whereas vehicle-treated group demonstrated decreased latencies in day 5, in comparison to day 1 (Fig. 7A). Moreover, in contrast to vehicle- treated group, Dimebon-treated mice displayed a significant decrease in the latencies to reach the bottle in trials 2 of day 4 and 5 compared to day 2 (Fig. 7A, $ symbols). On the other side, comparison between each group in the same trial each day by an unpaired t-test provided more information. Latencies of Dimebon-treated group were significantly shorter in trial 1 of day 3 and day 5 as compared to vehicle-treated group ($P = 0.03$ and $P = 0.04$ respectively, vs vehicle-treated group, unpaired t-test; Fig. 7B, * symbols). Latencies to reach the bottle in Dimebon-treated group also had tendency to be significantly shorter in trial 2 of day 3 as compared to vehicle-treated group ($P = 0.08$ vs vehicle-treated group, unpaired t-test; Fig. 6A Fig. 7B, & symbol). Given that latencies to reach the bottle of both groups were similar at the end of testing (Fig. 7B), together these findings indicated that Dimebon daily administered accelerated learning in this task. Moreover, the percentage of correct choice (fig. 9) was significantly higher in treated mice (77.78%) than in non-treated mice (52.78%) ($P = 0.001$, Fischer's exact test) strengthening the learning enhancive properties of Dimebon.

Friedman test revealed significant differences in drinking time between trials along with days respectively in Dimebon-treated group and vehicle-treated group ($P < 0.0001$; Fig. 8A). Vehicle- and dimebon-treated group spent significantly longer time while drinking during day 5 in comparison to day 1 and 2 (Fig. 8A, * and # symbols). Duration of drinking in dimebon-treated group also increased in trial 1 of day 4 as compared to day 1 (Fig. 8A, * symbols), that is earlier than vehicle-treated group (Fig. 8A). However, drinking time in both groups increased from day 1 to day 5 (Fig. 8A), suggesting that mice acquired the task and perhaps, became less anxious in the testing environment. On the other hand, duration of drinking was significantly higher in Dimebon-treated group than in vehicle-treated group (Fig. 8B): in trial 1 day 1 ($P = 0.01$, $U = 11.0$, Mann-Whitney U test), in trial 1 and 2 day 2 ($P = 0.003$, $U = 7.5$

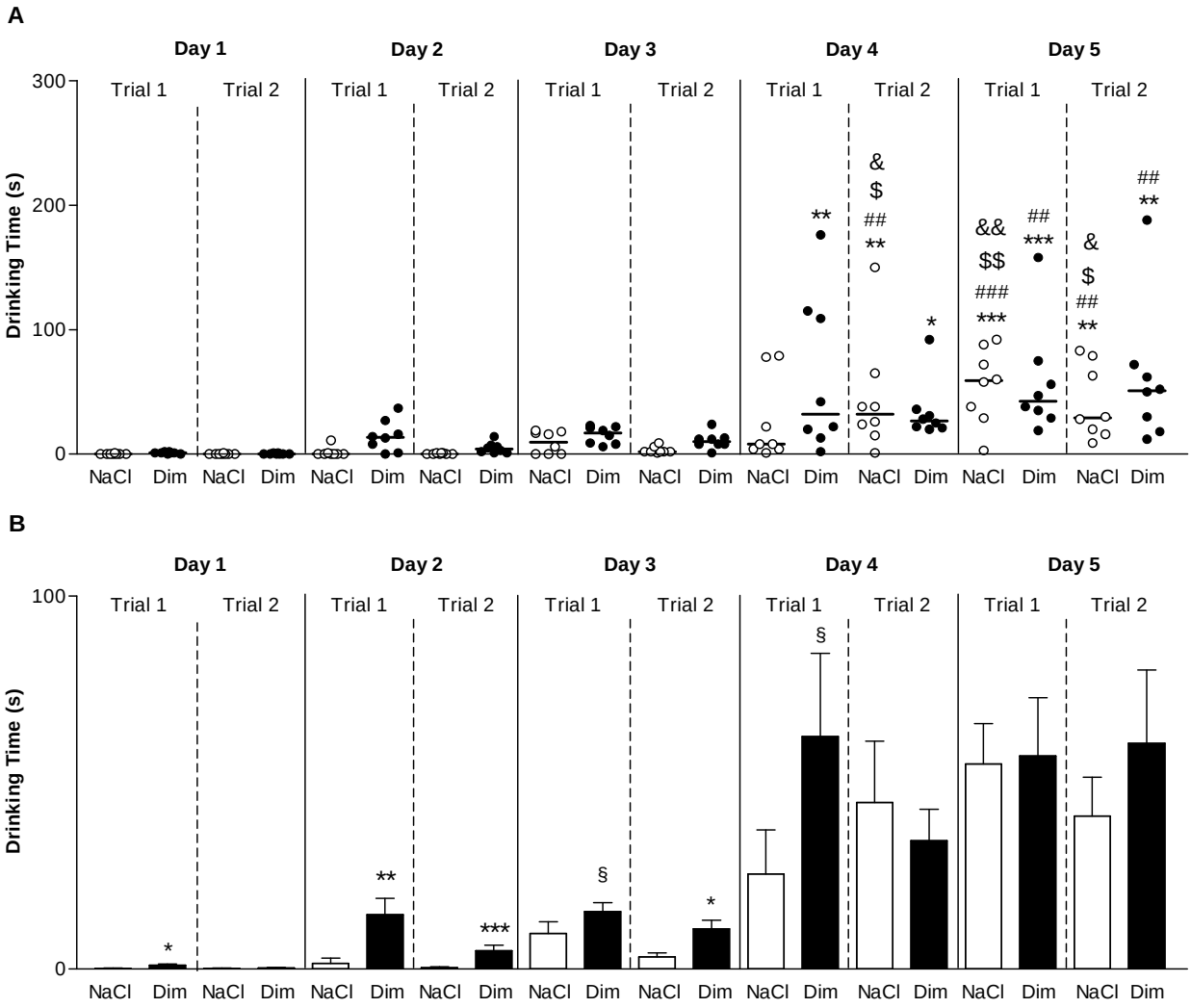


Figure 8. Effects of daily administration of Dimebon on spatial learning in Y-Maze test on C57BL/6N: drinking time. Time spent drinking in the two trials of each day in control and treated group was measured to assess anxiety and motivation level.

(A) Individual data showing time spent drinking by vehicle and Dimebon-treated group to reach in trial 1 and 2 along with 5 days of testing were used as a measure of anxiety. Bars represent the medians except in Trial 1 and 2 Day 1 where bars represent the means, values being not distributed normally. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs Trial 1 Day 1 respectively in each group; ##*P* < 0.01 and ###*P* < 0.01 vs Trial 2 Day 1 respectively in each group; $*P* < 0.05, $$*P* < 0.01 vs Trial 1 Day 2 respectively in each group; &*P* < 0.05, &&*P* < 0.01 vs Trial 2 Day 2 respectively in each group). All statistical comparisons were obtained by a Dunn's posthoc test after Friedman test revealed significant differences inside of each group (*P* < 0.0001).

(B) Each column represents the mean (± SEM) of time spent drinking in vehicle (NaCl) and Dimebon-treated group (Dim) in each trial during the 5 days of testing. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs vehicle in the same trial (Mann-Withney *U* test); 0.5 < §*P* <0.1 vs vehicle in the same trial (Mann-Withney *U* test). Mann-Withney *U* test has been applied after Kruskal-Wallis test revealed significant differences (*P* < 0.0001).

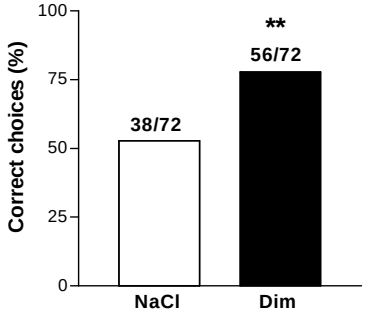


Figure 9. Effects of daily administration of Dimebon on spatial learning in Y-Maze test on C57BL/6N: Percentage of correct choices, consisted in choosing the arm in which was placed the bottle, in vehicle (NaCl) and Dimebon-treated group (Dim). Numbers of good choices executed by all mice along with the 5 days of testing and maximum number of good choices per group are indicated above the bars. ***P* < 0.01, Fischer's exact test.

and $P = 0.0005$, $U = 3.0$; Mann-Whitney U test), in trial 2 day 3 ($P = 0.01$, $U = 10.5$, Mann-Whitney U test).

On the other hand, duration of drinking was significantly higher in Dimebon-treated group than in vehicle-treated group (Fig. 8B): in trial 1 day 1 ($P = 0.01$, $U = 11.0$, Mann-Whitney U test), in trial 1 and 2 day 2 ($P = 0.003$, $U = 7.5$ and $P = 0.0005$, $U = 3.0$; Mann-Whitney U test), in trial 2 day 3 ($P = 0.01$, $U = 10.5$, Mann-Whitney U test). Time spent drinking by Dimebon-treated mice had tendency to be higher in trial 1 day 3 ($P = 0.06$, $U = 17.0$, Mann-Whitney U test) and in trial 1 day 4 ($P = 0.06$, $U = 17.5$, Mann-Whitney U test) (Fig. 8B). However, from trial 2 day 4, duration of drinking time was similar in each group ($P > 0.05$, Mann-Whitney U test). Thus, during the first days, exception of trial 2 day 1, duration of drinking was higher in Dimebon-treated mice compared to vehicle-treated mice which that could be interpreted by a possible anxiolytic effect of Dimebon (Fig. 8B). These results suggested that a chronic treatment with Dimebon at dose 0.1 mg/kg enhance learning and memory in mice but also could have an anxiolytic effect.

4. Discussion and Outlooks

4.1. Factors of Contextual Learning in Mice

4.1.1. Comparison of Performance of Three Mouse Strains in a Step-Down Avoidance Task

C57BL/6, CD1 and BALB/c displayed different abilities to acquire the contextual memory, thus, suggesting that the hippocampus-dependent learning is influenced by the genetic background, which is in line with the literature (Gerlai, 1998; Nguyen & al., 2000; Yang & al., 2004). Previous studies, carried out on the same mouse strains, were focused on different behavioural phenotype: open field locomotion (Michalikova & al., 2010), behaviour in a rat exposure test (Yang & al., 2004) and disk pressing in a food-reinforcement paradigm (Zarcone & al., 2004). However, to our knowledge, no reports are available, which compare a performance of C57BL6N, CD1 and BALB/c mice in a step-down avoidance task. C57BL/6 mice showed the highest scores of learning in this paradigm, thus, suggesting that employed study design can be appropriate for studies on learning and memory in various experimental conditions; other studies suggest good learning abilities of this mouse strain as well (Crawley, 1997; Gerlai & al., 2001; Nguyen & al., 2000; Schimanski & al, 2004). At the same time, CD1 mice showed intermediate level of learning of step-down avoidance test in our experiments. Mice of BALB/c strain showed the lowest ability to acquire the step down avoidance task that may be due, at least partly, to its high level of anxiety or / and high locomotor activity.

Anxiety and exploration activity and their effects are important issues that may affect animals' performance in the step-down avoidance task in a non-specific way. Further experiments are needed to analyze an impact of anxiety, locomotor and exploratory activity in memory abilities and performance of tested here mouse strains in a step-down avoidance model. Another possibility would be to assess their performance in a different test for the hippocampus-dependent memory (e.g. contextual fear conditioning). Comparison of strains of mice provides a useful and simple tool to study the genetic basis of behaviour and may help to identify molecular mechanisms of normal and abnormal behaviour (Crawley, 1997; Shimanski & al., 2004).

4.1.2. Learning Differences in 3-Months and 7-Months Old C57BL/6 Mice Could Be Due To Changes in Hippocampus

The step-down avoidance paradigm enabled to reveal age-related differences in learning between relatively young 3-months old and 7 months-old C57BL6/N mice. On one hand, these results are in agreement with those reported by Foster and co-authors (1988) who demonstrated a steady decline in the ability to acquire the SDA task in 6-months old C57BL6N mice, in comparison to 3.5-months old animals of this strain. We suggest that changes occurring in the hippocampal structure and/ or the plasticity of the /hippocampal formation might underlie such differences in the learning (see Ingram & Jucker, 1999). Present study provided more information in the age-related learning in the C57BL/6 strain, which correlation with aging-related changes in structural and functional plasticity of the hippocampus could be addressed in further studies. On the other hand, present finding confirms a high sensitivity of the step-down avoidance in detection of minor changes in the hippocampus-dependent learning, as memory deficits during subtle differences in age are often difficult to detect.

4.1.3. Experienced Stress in Mice Induce a Reduction of Freezing in Fear Conditioning Contrasting to Rats

The contextual fear conditioning learning was disrupted in chronically stressed C57BL/6 mice, as compared to control non-stressed group. This finding is consistent with previously reported deficits in the hippocampus-dependent learning and evidences altered hippocampal functional and structural plasticity detected in chronic stressed models (Conrad & al., 1996, 2010; Jayatissa & al., 2008; Joël & al., 2007; Kleen & al., 2006; Luine & al., 1994; Lupien & al., 2001; Magarinos & al., 1995 b; Mineur & al., 2007; Sandi & al., 2001; Song & al., 2006; Strekalova & Steinbush, 2010; Sunanda & al., 2000; Watanabe, 1992; for review, see Conrad & al., 2010 and Joël & al., 2007). However, the fact that chronically stressed mice demonstrated a reduced level of freezing compared to the control group contradicts the data reported by other researchers, who described enhanced freezing behaviour in the fear conditioning task in stressed rats (Conrad & al., 1999; Sandi & al., 2001). Sandi and co-authors (2001) suggested that potentiated freezing response observed in stressed animals, reflects an altered reaction to the conditioning procedure, instead of memory facilitation. On another hand, stressors we used on mice were not the same as those applied on rats in this study (restraint stress procedure) that could result in differences in a stress load between stress

paradigms and thus,lead to different effect on memory. Rats and mice have been shown to have different strategies and scores of learning of in the same paradigms of hippocampus-dependent learning (Whishaw and Tomie, 1996), suggesting that the opposite freezing behaviour between mice and rats could be related to a specie-specific component that may additionally contribute to a discrepancy in observed stress-induced changes. Although Strekalova and Steinbusch (2010) found similar data, further studies are necessary to assess the hippocampus-dependent learning during stress in a more systematic way.

4.2. Step-down Avoidance and Y-Maze Task as Models of Learning for Assessment of the Effects of Dimebon

4.2.1. Memory-Enhancing Effects of Dimebon and Potential Mechanisms of Action

Baseline latencies in the SDA test were similar in vehicle- and dimebon-treated groups for all doses tested, suggesting that dimebon administration does not affect anxiety-like behaviour nor locomotor activity of treated mice. However, CD1 mice demonstrated impaired learning in the first recall session in comparison to the training that could be interpreted as a strain effect and needs further confirmation. .

Mice of CD1 and C57BL/6 strains treated by dimebon in a dose 0.1 mg/kg did not reveal enhanced memory scores in a step-down avoidance task. Taking into account dosing used in this study, our result is rather unexpected since dimebon was reported to enhance memory in a lower dose, namely of 0.05 mg/kg (orally administered); however, this effect was observed in a protocol of short-term learning of a novel object recognition task (Giorgetti & al., 2010), which has distinct mechanisms (Bertaina-Anglade, 2007). Such discrepancies might be due to different way of drug administration. However, to our knowledge, few studies have assessed the pharmacokinetics of dimebon in rodents (Tishcenkova & al., 1991) . The pharmacokinetic of dimebon in human has been studied by Pfizer but data were not published in the literature. Tishcenkova and co-authors (1991) studied the pharmacokinetic in rats and rabbits with i.p. and oral administration of different doses, but it is not correlated to any evaluation in a learning paradigm. However, Giorgetti & al. (2010) have revealed, in rats, dimebon plasma concentration from 0.15 nM to 13.9 nM and brain concentration from 1.7 to 172 nM 50 minutes following acute oral administration of cognition-enhancing doses (0.05 to 5 mg/kg) used in a novel object recognition task. Nirogi and co-authors (2009) have developed a liquid chromatography-tandem mass spectrometry method for the quantification

of dimebon in rat plasma and brain tissue which could be used in future studies to determine pharmacologically active concentration in plasma and brain in rats correspondiing to cognition-enhancing doses. Moreover, no study addressed whether dimebon is an active compound or whether it need to be metabolized to be active. Furthermore, although several studies have revealed presence of dimebon in brain, the way whereby dimebon could go/pass trough the blood brain barrier has not yet been addressed. Further studies are necessary to address these questions. In our study, dimebon was injected 15 min before the training session and according to the pharmacokinetic data was not supposed be in the brain at the first and second recall sessions. Thus, possible effecst of dimebon can be account for its interference with the acquisition / early consolidation phase of memory formation, but not a memory retrieval.

Dimebon enhanced long-term memories in a one-trial step-down avoidance paradigm in C57BL/6 mice treated acutely with a dose 0.5 mg/kg. This finding is consistent with the previously reported data on memory enhancing properties of dimebon in animal models learning (Giorgetti & al., 2010; Lermontova & al., 2000; Schaffhauser & al., 2009). Taking into account data obtained in a vehicle-treated group, it would be of advantage to repeat the study.

Chronic administration of dimebon during multiple training protocole was found to accelerate learning of C57BL6N mice in the Y-Maze. In contrast to acute treatment situation, chronically delivered dose of dimebon of 0.1 mg/kg was efficient in enhancing memory abilities of tested mice that was reflected by a more rapid reduction of a latency to reach the bottle with water that in control group. Also, dimebon-treated mice showed higher percentage of correct choices, as compared to a vehicle-treated group. Interestingly, the analysis of drinking behaviour in experimental groups revealed increased time spent while drinking in dimebon-treated animals that may be interpreted as a sign of anti-anxiety effects of this drug. Of note, antagonists of NMDA receptors have been shown to induce anxiolytic effects (Barkus & al., 2009) that may explain anxyolitic effects of dimebon, which possess such activity. This effect may contribute / mediate the memory-enhancing action of dimebon revealed in the Y-maze paradigm. However, this hypothesis needs to be tested experimentally.

Memory enhancing effects observed in our study could be mediated via AMPA and NMDA receptors, which are known to play a crucial role in consolidation of the hippocampus-dependent long-term memory (Izquierdo & al., 1997, 2002) on one hand, and be an important mechanism of action of dimebon, on another one. However, Gorgetti & al. (2010) suggested cognition-enhancing properties of dimebon are unlikely mediated by an

antagonism on NMDA receptors because the concentration of dimebon in the brain (14 nM for an oral dose of 0.5mg/kg) and this affinity (as compared to other targets) for the NMDA receptor were too low for mediating this mechanism. However, Bachurin & al. (2001) demonstrated that at dose of 42mg/kg was sufficient to inhibit anti-NMDA activity of dimebon was sufficient to prevent NMDA-induced seizures in mice. Thus, the activity of dimebon on NMDA receptors is likely dose-dependant and dependent of way of administration. Besides, Grigoriev & al. (2003) demonstrated that dimebon act as an inhibitor of AMPA receptors at concentration equal or greater than 40-50 µM in in vitro study. Moreover, as already noticed, others molecular mechanisms of dimebon could mediate memory enhancing effect of this molecule found in our study. For example, consolidation of memory in the step-down avoidance task is modulated by, among others, dopamine and 5HT1A receptors (Izquierdo & al., 1997) that are demonstrated target of dimebon (Giorgetti & al., 2010 and Wu & al., 2010). Furthermore, antagonism of 5-HT6 receptor has been proposed to mediate these cognitive-enhancing effects (Schaffhauser & al., 2009). Interestingly, 5-HT6 receptor is reported as having a little role in other behaviour as anxiety (Mitchell & al., 2005). Further studies assessing pharmacokinetic related to pharmacologically active dose demonstrated in learning paradigm and potential targets are necessary to determine mechanisms of action of Dimebon.

4.2.2. Models of learning to assess the memory enhancing effects of dimebon and potential analogues

In conclusion, our study suggest the step-down avoidance and Y-maze paradigms to be the convenient tasks for rapid and reliable assessment of effects of drugs with memory enhancing properties, such as dimebon. Additional tests with described here study design could help in demonstrating the reproducibility of obtained here data. In order to validate the the dose 0.5mg/kg as the most optimal dose, it would be important to carry out additional experiments with similar dose and higher doses of this compound, inasmuch as dose of 0.1mg/kg failed to show effect of dimebon. Our study suggests that employed here experimental design could be useful in the future screening of functional activity of analogues of dimebon. Also, continuation or application of this study design could enable a pharmacological analysis of the mechanisms of action of dimebon. This hopefully can eventually lead to better understanding of the mechanism of the curative effects of the treatment used in patients with Alzheimer's disease.

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