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Sip-1 mutations cause disturbances in the activity of NMDA- and AMPA-, but not kainate receptors of neurons in the cerebral cortex.

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Highlights

▲Sip1 haploinsufficiency causes higher neuronal sensitivity to NMDA- and AMPA-receptors;

▲Sip1 homozygosity causes resistance to activation of NMDA- and AMPA receptors;

▲Sip1 deficiency affects the activity of kainate receptors;

▲The Sip1 mutation causes a changes in glutamatergic neurotransmission of cortical neurons;

Abstract

Smad-interacting protein-1 (Sip1) [Zinc finger homeobox (Zfhx1b), Zeb2] is a transcription factor implicated in the genesis of Mowat–Wilson syndrome (MWS) in humans. MWS is a rare genetic autosomal dominant disease caused by a mutation in the Sip1 gene (aka Zeb2 or Zfhx1b) mapped to 2q22.3 locus. MWS affects 1 in every 50–100 newborns worldwide. It is characterized by mental retardation, small stature, typical facial abnormalities as well as disturbances in the development of the cardio-vascular and renal systems as well as some other organs. Sip1 mutations cause abnormal neurogenesis in the brain during development as well as susceptibility to epileptic seizures. In the current study we investigated the role of the Sip1 gene in the activity of NMDA-, AMPA- and KA- receptors. We showed that a particular Sip1 mutation in the mouse causes changes in the activity of both NMDA- and AMPA- receptors in the neocortical neurons in vitro. We demonstrate that neocortical neurons that have only one copy of Sip1 (heterozygous, Sip1fl/wt), are more sensitive to both NMDA- and AMPA- receptors agonists as compared to wild type neurons (Sip1wt/wt). This is reflected in higher amplitudes of

agonist induced Ca^{2+} signals as well as a lower half maximal effective concentration (EC50). In contrast, neurons from homozygous Sip1 mice (Sip1$^{fl/fl}$), demonstrate higher resistance to these respective receptor agonists. This is reflected in lower amplitudes of Ca^{2+}-responses and so a higher concentration of receptor activators is required for activation.

Keywords: Calcium, glutamate receptors, mutants, neurons, cortex, NMDA-receptors, AMPA-receptors, KA-receptors

1. Introduction.

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Its function is mediated by ionotropic and metabotropic glutamate receptors. There are three major types of ionotropic receptors: NMDA-, AMPA-and kainate receptors (KAR) [20, 22]. They all take part in excitation but kainate receptors, in contrast to NMDA- and AMPA-, preferentially act as modulators. Some authors suggest that kainate receptors are involved in metabotropic signaling pathways [12, 24].

Sip1 is a transcriptional repressor that is involved in the control of multiple signaling cascades in the brain and deletion of Sip1 leads to dramatic disruptions of both neuronal structure and function in all brain regions tested [40]. Sip1 deletion in early brain development causes hippocampal degeneration, disruption of cell differentiation and migration in the cerebral cortex and basal ganglia [26, 27, 6]; down regulation of Sip1 expression results in cognitive disorders such as seizures [33], intellectual disorders [1], memory distortion etc. Sip1 is also required for myelination so its deletion causes abnormal differentiation of oligodendrocytes and abnormal neuronal conduction [43]. Ca^{2+}-signaling plays an important role in regulation of neurotransmitter secretion, excitability, axonal outgrowth, synaptic transmission and neuronal survival [5, 46, 7]. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a powerful activation stimuli for multiple signaling cascades. Pathological increase in $[Ca^{2+}]_i$ can lead to cellular dysfunction and death. One of the most important components of Ca^{2+}- dysregulation is uncontrolled Ca^{2+} influx via glutamate ionotropic AMPAR and KAR. Conversely NMDARs are crucial for induction of epileptogenesis and exitotoxicity [15, 47]. We reasoned that Sip1 might be involved in the control of such an important molecular pathway. We investigated Ca^{2+} signaling in cortical neurons from Sip1 deficient mice and compared them with Ca^{2+} signaling from wild type mice. Our results demonstrate that Sip1 deficient neurons (Sip1$^{fl/fl}$) are resistant to activation by glutamate receptor agonists.

2. Experimental procedures.

This methods have been described in detail previously [17, 18, 38]. In order to address the role of Sip1 in the development of cortical axonal tracts, Sip1 was deleted conditionally in pyramidal neurons using a NexCre mouse line. We tested the genotype of each of the animals involved in the experiment. Genomic DNA was extracted from a tail tip of the animal by the desalting method. Genotyping for Sip1 gene was performed and sizes of the amplification products were 450 b.p. for WT and 600 b.p. for knockout. The cortex and brainstem from neonatal Sip1-mutants mice was dissociated and then incubated with trypsin in the Versene solution. Then, trypsin was inactivated by equal volume of cold B27-supplemented neurobasal medium and cells were doubly centrifuged. The suspension was put on round coverslips covered by Polyethylenimine. 10 DIV cultures were used for the experiments. Cortical and brainstem neurons were isolated from the same mouse. To measure $[Ca^{2+}]_i$, we used a Carl Zeiss Cell Observer with high-speed monochrome CCD-camera AxioCam HSm and high-speed light filters replacing system, Ludl MAC5000. Calcium responses to the ionotropic glutamate receptor agonist were recorded with the double wavelength fluorescent probe Fura-2. To discriminate neuron and astrocytes, short-term application of 35 mM KCl at the end of experiments was used. We determined the amplitudes of Ca^{2+} responses to activators of glutamate receptors as (Δ) – Fmax-Fmin of Fura-2 fluorescence. Amplitudes of Ca^{2+} responses were averaged and used to plot dose-response dependences. Number of animals used for dose-response dependences was 10 for $Sip1^{wt/wt}$, 6 for $Sip1^{fl/wt}$, 4 for $Sip1^{fl/fl}$. ImageJ 2002 and Origin 8.5 software was used to analyse data, create graphs and perform statistical tests. All values are given as mean $\pm$ SEM. Data were statistically compared using Kruskal-Wallis test or paired t-test and were considered significantly different at $p \leq 0.05$.

3. Results.

3.1. Sip1 deficient cortical neurons have higher neuronal sensitivity while Sip1 homozygosity causes resistance to activation of NMDA- receptors.

Previously, we have developed imaging techniques to measure NMDA-, AMPA - and KA -receptors activity [4]. In order to test the hypothesis that Sip1 might be involved in the control of NMDA and AMPAR or KA currents, we analyzed $[Ca^{2+}]_i$ in primary cortical neurons isolated from neonatal Sip1 mutant pups. As a negative control we also used neurons isolated from the brainstem where Sip1 was not deleted. Young cortical neurons were cultured *in vitro* for ten days and then subjected to brief, 30 s applications of ionotropic glutamate receptors agonists – NMDA, 5-Fluorowillardiine and Domoic acid. This led to sharp increases of $[Ca^{2+}]_i$ in the

neurons. After wash out of the agonist, cells quickly restored their basal level of $[Ca^{2+}]_i$. Basal levels of $[Ca^{2+}]_i$ in the cortical neurons and brainstem neurons were 0.25 ± 0.06 a.u. and did not differ between Sip1 mutant and wild type pups. Figure 1 shows the Ca^{2+} responses of cortical (A) and brainstem neurons (B) to sequential increases in the concentration of the NMDAR agonist, N-methyl-D-aspartate, in Mg^{2+}-free medium. Increases in NMDA induces increases in the amplitude of Ca^{2+} responses in both cortical and brainstem neurons. However the amplitude of Ca^{2+} responses was substantially lower in cortical neurons isolated from Sip1$^{fl/fl}$ mutants (fig. 1A blue curve) as compared to ip1$^{fl/wt}$ (fig. 1A, red curve) and Sip1$^{wt/wt}$ (fig. 1A, black curve). As shown in Fig. 1B, the average kinetics of Ca^{2+} responses of neurons from brainstem from Sip1 mutant mice did not differ in amplitude from wild type mice, especially in saturating concentration of agonist.

Based on our experimental data we calculated the dependence of $[Ca^{2+}]_i$ amplitude increases on NMDA concentration (fig. 1C). It was used to calculate NMDA concentration causing half of the maximal increase of $[Ca^{2+}]_i$ in neocortical neurons from Sip1$^{fl/fl}$ mice (EC50=0.425 ± 0.06 µM, Hill coefficient (n) 1 ± 0.05) (fig. 1C, curve 3), Sip1$^{fl/wt}$ мышей (EC50=0.083 ± 0.008 µM, n 0.98 ± 0.06) and Sip1$^{wt/wt}$ mice (EC50=0.097 ± 0.002 µM, n 0.98 ± 0.04). Hill coefficient (n) values are approximately 1 for all groups, indicating a lack of cooperation of the process. Despite similar values of EC50 in cortical neurons of Sip1$^{wt/wt}$ и Sip1$^{fl/wt}$ mice, $[Ca^{2+}]_i$, amplitude increase are significantly higher in Sip1$^{fl/wt}$ (0.57 a.u.), while in wild type mice the maximal value of Ca^{2+} amplitude is 0.48. Ca^{2+} influx can control the expression of key intracellular proteins, regulate neuronal activity and can cause apoptosis of neurons if prolonged. Our data indicate that Sip1$^{fl/wt}$ neurons are more sensitive to NMDAR activation.

3.2. Sip1 haploinsufficiency causes higher neuronal sensitivity while Sip1 homozygosity causes resistance to activation of AMPA receptors.

AMPA receptors can activate agonist dependent desensitization that are differentially act in various neuronal cell types [3]. Therefore it is an experimental challenge to precisely define the amplitude of agonist induced Ca^{2+} response. Therefore we used selective activator of AMPA receptors 5-Fluorowillardiine in the presence of desensitization inhibitor - cyclothiazide, applied before FW. Like in the case of NMDA receptor activation, application of selective agonist of AMPA receptors induced $[Ca^{2+}]_i$ increases in a dose dependent manner in the cortical and brainstem neurons (fig. 2A, B). In Sip1$^{fl/wt}$ maximal Ca^{2+}-responses were registered with 0.03 µM FW (fig. 2A, red curve), while in Sip1$^{wt/wt}$ neurons with 0.5 µM (fig. 2A, black curve), and in Sip1$^{fl/fl}$ neurons with 3 µM only (fig. 2A, blue curve). Low sensitivity of Sip1$^{fl/fl}$ cortical neurons to AMPAR activation can be considered activation resistance. This is also supported by our experimental data that brainstem neurons isolated from the same animals that had intact Sip1

gene. Indeed, these neurons increase $[Ca^{2+}]_i$ with FW concentration as low as 0.01 µM, while cortical neurons isolated from the same animals start reacting with 0.05 µM. Like in the case of NMDARs, the maximal amplitude of Ca^{2+} responses were detected in cortical neurons isolated from $Sip1^{fl/wt}$ mice (0.72), compared to $Sip1^{wt/wt}$ neurons (0.6) and neurons from $Sip1^{fl/fl}$ (0.4). Comparing the dependence of $[Ca^{2+}]_i$ amplitudes to increases of FW concentration (fig. 2C), we demonstrate that $Sip1^{fl/wt}$ cortical neurons have higher sensitivity to AMPAR activation. This is supported by the EC50 parameter 0.0084±0.0015 µM (n=1.87±0.12), that is substantially lower than $Sip1^{wt/wt}$ neurons (0.018±0.007 µM, n=1.97±0.05) and brainstem neurons from the same $Sip1^{fl/wt}$ animals (0.024±0.005 µM, n=1.97±0.03) (Table 1). The FW concentration, that causes half the maximal increase of $[Ca^{2+}]_i$ concentration in the cortical neurons from $Sip1^{fl/fl}$ animals is 0.188±0.011 µM (n=1.96±0.07), while this parameter is 0.028±0.003 µM (n=1.99±0.05) in brainstem neurons from the same mouse. In contrast to NMDAR, the Hill coefficient for AMPAR is approximately 2 which suggest co-operativity of the receptor action, when the channel is open upon binding of two or more molecules of the agonist [10]. It is known that the AMPA-receptors have low conductivity for Ca^{2+} and serve for a cell depolarization through pNa^+ increase. AMPA-receptor open and close fast, while the channel Ca^{2+} permeability is determined by the absence of the GluR2 subunit [30]. Taking that into account, to assess the influence of the Sip1 mutation on activity of potential-dependent Ca^{2+}- channels (Cav) we analyzed signals from the neurons after short-term depolarization using application of 35 mM KCl (Fig. 2D). It can be seen, that Ca^{2+} responses of $Sip1^{wt/wt}$, $Sip1^{wt/fl}$ and $Sip1^{fl/fl}$ brain cortex neurons averaged from several hundreds of cells were not statistically different, as well as neurons from brainstem that were used as controls. Thus, we can conclude that the Sip1 mutation had no effect on functional expression of Cav in brain cortex neurons, and differences in sensitivity to the AMPA-receptor activators are most probably due to expression of GluR2.

3.3. Sip1 deficiency affects the activity of kainate receptors

Kainate receptors (KARs), as well as AMPARs can activate agonist dependent desensitization [37], on the other hand any known agonist of KAR, is also an agonist of AMPARs. Therefore we used the AMPA/KA-receptor agonist – Domoic acid (DA). DA was added after incubation of cells with the AMPAR selective antagonist GYKI-52466 and the desensitization inhibitor KAR – concanavalin A. KAR activation increases $[Ca^{2+}]_i$ in the cortical neurons isolated from both wild type and Sip1 deficient mice. Low amplitude signals were detected with a DA concentration as low as 0.010 µM in neocortical neurons of animals of all genotypes (fig. 3A). The amplitude of Ca^{2+}-signals in $Sip1^{fl/wt}$ neurons (Fig. 3A, red curve) and $Sip1^{wt/wt}$ neurons (Fig. 3A, black curve) caused by sequential increases in DA concentration are 0.62 and almost identical for both

genotypes. However for Sip1$^{fl/fl}$ neurons, like for two previously described ionotropic glutamate receptors, the amplitude is significantly lower and 10 μM of agonist is 0.44 (Fig. 3A, blue curve).

Comparison of dependence of amplitude increases of $[Ca^{2+}]_i$ on DA concentration (Fig. 2B) demonstrates that cortical neurons isolated from Sip1$^{fl/wt}$ mice do not differ from Sip1$^{wt/wt}$ neurons as far as EC50 value is concerned, that equals 0.396±0.06 μM (n=1.73±0.05) and 0.4±0.03 μM (n=1.93±0.07), respectively. Activation of KARs in Sip1$^{fl/fl}$ neurons requires substantially higher DA and EC50 concentrations of 2.5±0.07 μM (n=1.79±0.12) (Fig. 3B). EC 50 values for brainstem neurons from the same mice are 0.51±0.011 μM (Hill coefficient 1.98±0.14) (Fig. 3C).

DISCUSSION

Glutamate is an important and widely abundant neurotransmitter in the CNS that activates three types of cation channels: NMDAR, AMPAR and KAR. Glutamate induced calcium increases cause a wide spectrum of neuronal reactions ranging from synaptic plasticity and regulation of neuronal excitation to neurotoxic effects causing apoptosis. Continuous Ca^{2+} influx into the cytosol through glutamate activated receptors-channels can cause dysregulation and become a key factor of neuronal damage and cell death. [9, 13, 28]. The most significant cause of Ca^{2+} dysregulation is uncontrolled Ca^{2+} influx through Ca^{2+} permeable glutamate-receptors. On the other hand, the amplitude and frequency of Ca^{2+} signals is essential for the control of gene expression that either induce synaptic neuroplasticity or neuronal damage [13, 31, 41, 48]. Sip1 deletion in the CNS causes a wide spectrum of developmental disturbances ranging from hippocampal degeneration [26], abnormal specification of cortical pyramidal neurons and interneurons [25, 36, 39], and abnormal myelination [43]. Interestingly, some of these changes are linked to Ca^{2+} signaling. For example, Sip1 mutation causes dysregulation of Wnt pathway, JNK and CaMKII [44, 26]. Additionally, hippocampal degeneration in Sip1 deficient mice is a result of apoptosis [26] that can also be activated by Ca^{2+}-signals, activated by glutamate receptors [16].

In the current work, we could show that Sip1 mutation causes dramatic changes in glutamate mediated Ca^{2+} signaling in cortical neurons. Table 1 compares the characteristics of calcium signaling of cortical neurons with and without Sip1 protein. We hypothesised that Sip1 haploinsufficiensy causes increased sensitivity of NMDA and AMPA receptors to agonists. Our results support this hypothesis as we find that Sip1$^{fl/wt}$ neurons require lower concentration of agonists to induce half maximal increase of $[Ca^{2+}]_i$. Additionally amplitude of Ca^{2+} response of Sip1$^{fl/wt}$ neurons (ΔF) is 42% higher for NMDA-receptors activation and 51.7% higher for

AMPA receptors activation as compared to wild type cortical neurons. In contrast Sip1^{fl/fl} neurons are resistant to agonists of ionotropic glutamate receptors so that it is required higher concentration of agonists to generate Ca^{2+}-response reaching hundreds of nanomoles and micromoles (Table 1). As far as the amplitude of Ca^{2+}- signals is concerned, it is 68.4%, 48.3% и 43.7% lower for activation of NMDA-, AMPA- and KA-receptors in Sip1^{fl/fl} neurons respectively. Expression of different proteins in neurons is heterogeneous even within one cell population. It is known that the AMPARs are permeable to Ca^{2+} ions when they lack the GluR2 subunit [30]. According to this, differences in EC50 detected for the AMPAR can be explained by the influence of the Sip1 mutation on expression of this subunit. This assumption is confirmed by the absence of significant differences between amplitudes of Ca^{2+} responses for depolarization in neurons with the Sip1 mutations and neurons from brainstem (Fig. 2D). Mowat-Wilson syndrome patients have microcephaly, agenesis of the corpus callosum, cerebral atrophy, and poor hippocampal formation, as well as other non-brain-related congenital defects [49]. In humans Sip1 mutation is dominant, but Sip1 heterozygous mice do not show the same degree of the phenotype penetrance as humans. This suggests that humans are more sensitive to Sip1 defects than mice, or human mutations produce dominant-negative forms of Sip1 protein [26]. This might be a possible explanation of differences in sensitivity of neurons with different doses of Sip1 protein to NMDA- and AMPA-receptor activators. Although EC50 in Sip1[fl/wt] neurons is different from that of the wild type neurons in the range of 0.01 µM, such difference between Sip1^{fl/fl} and wild type neurons is as much a 10 times.

Mutations in transcription factors cause dramatic changes in multiple signaling cascades resulting in metabolic dysfunction. For example mutation of MeCP2 that causes Rett syndrome in humans, induces respiratory disturbances in mice as well as disruption of CO_2 detection [8]. It also causes disruption of Ca^{2+} signaling in astrocytes [39]. Our data suggest that Sip1 haploinsufficiensy does not influence KARs, while homozygousity causes significant resistance. On the other hand ionotropic receptors of this type rather act as modulators in excitatory transmission. The Ca^{2+} conductance of KARs, in contrast to NMDARs- and AMPARs depends on co- and post transcription RNA dependent adenosine deaminase RNA editing. In this case adenosine is converted into inosin in the primary mRNA sequence. Unedited KARs are permeable to Ca^{2+} ions, while the edited form of this channel is permeable for monovalent cations such as potassium and sodium. Mutant mice with disrupted Q/R editing of GluR6, are more sensitive to kainite induced seizures. Moreover, dentate gyrus neurons in these mice can develop NMDA-independent potentiating [42]. It was also reported that in the mice that have either edited or non-edited forms of mRNA that the presence of edited form only does not any behavioral abnormalities. Although increases in kainite induced currents were measured in the

dorsal roots of the spinal cord was detected in such mice, it did not cause changes in sensory stimuli sensitivity [34]. Other authors [21] reported that human patients suffering from epileptic seizures have lower level of RNA editing of GluR5 и GluR6 than healthy control individuals. Our data demonstrate that Sip1 mutation causes significant changes in the activity of ionotropic glutamate receptors in the cerebral cortex. Neurons of Sip1$^{fl/wt}$ mutants were characterized by increased sensitivity to agonists of NMDARs and AMPARs (but not kainate). Whereas glutamate receptor in neurons of Sip1$^{fl/fl}$ mutants have a general signaling insufficiency, expressed by reduced sensitivity (compared to wild-type neurons and brainstem) to agonists and atypically low amplitude Ca^{2+} -responses. Increased sensitivity of neurons derived from Sip1$^{fl/wt}$ to activators of NMDAR and AMPAR on a physiological level may lead to a rapid activation of neuronal networks, excitotoxicity phenomena and induction of cell death processes under moderate stresses (transient hypoxia).

Acknowledgments

References

[1] M.P. Adam, S. Schelley, R. Gallagher, A.N. Brady, K. Barr, B. Blumberg, J.T. Shieh, J. Graham, A. Slavotinek, M. Martin, K. Keppler-Noreuil, A.L. Storm, L. Hudgins. Clinical features and management issues in Mowat-Wilson syndrome. Am. J. Med. Genet. A. (2006).

[2] Y. Aponte, J. Bischofberger, P. Jonas. Efficient Ca2+ buffering in fast-spiking basket cells of rat hippocampus. J Physiol. (2008).

[3] A.F. Ambrósio, A.P. Silva, J.O. Malva, J.F. Mesquita, A.P. Carvalho, C.M. Carvalho. Role of desensitization of AMPA receptors on the neuronal viability and on the [Ca2+]i changes in cultured rat hippocampal neurons. Eur. J. Neurosci. (2000).

[4] A. V. Berezhnov, A.V. Kononov, E.I. Fedotova, V.P. Zinchenko. Image analysis applications for characterization of ionotropic glutamate receptor ligands in cultured neurons. Biochemistry (Moscow) Supp. Series A: Membrane and Cell Biology, (2013).

[5] M.J. Berridge. Neuronal calcium signaling. Neuron (1998).

[6] V. van den Berghe, E. Stappers, B. Vandesande, J. Dimidschstein, R. Kroes, A. Francis, A. Conidi, F. Lesage, R. Dries, S. Cazzola, G. Berx, N. Kessaris, P. Vanderhaeghen, W. van Ijcken,

F.G. Grosveld, S. Goossens, J.J. Haigh, G. Fishell, A. Goffinet, S. Aerts, D. Huylebroeck, E. Seuntjens. Directed migration of cortical interneurons depends on the cell-autonomous action of Sip1. Neuron (2013).

[7] N. Burnashev, A. Rozov. Presynaptic Ca2+ dynamics, Ca2+ buffers and synaptic efficacy. Cell Calcium (2005).

[8] J.M. Bissonnette, L.R. Schaevitz, S.J. Knopp, Z. Zhou. Respiratory phenotypes are distinctly affected in mice with common Rett syndrome mutations MeCP2 T158A and R168X. Neuroscience (2014).

[9] D.W. Choi. Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci Lett. (1985).

[10] J.D. Clements, A. Feltz, Y. Sahara, G.L Westbrook. Activation kinetics of AMPA receptor channels reveal the number of functional agonist binding sites. J. Neurosci. (1998).

[11] S.R. Cobb, E.H. Buhl, K. Halasy, O. Paulsen, P. Somogyi. Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature (1995).

[12] A.C. Flint, R.S. Dammerman, A.R. Kriegstein. Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. PNAS (1999).

[13] L.K. Friedman. Calcium. A role for neuroprotection and sustained adaptation. Mol Interv. (2006).

[14] L. Garavelli, P.C. Mainardi. Mowat-Wilson syndrome. Orphanet J. Rare Dis. (2007).

[15] M. Ghasemi, S.C. Schachter. The NMDA receptor complex as a therapeutic target in epilepsy: a review. Epilepsy Behav. (2011).

[16] M.R. Hara, S.H. Snyder. Cell signaling and neuronal death. Annu. Rev. Pharmacol. Toxicol. (2007).

[17] S. Goebbels, I. Bormuth, U. Bode, O. Hermanson, M.H. Schwab, K.A. Nave. Genetic targeting of principal neurons in neocortex and hippocampus of NEX-Cre mice. Genesis. (2006).

[18] Y. Higashi, M. Maruhashi, L. Nelles, T. Van de Putte, K. Verschueren, T. Miyoshi , A. Yoshimoto, H. Kondoh, D. Huylebroeck. Generation of the floxed allele of the SIP1 (Smad-interacting protein 1) gene for Cre-mediated conditional knockout in the mouse. Genesis. (2002).

[19] L.G. Hilgenberg, M.A. Smith. Preparation of dissociated mouse cortical neuron cultures. J. Vis. Exp. (2007).

[20] M. Hollmann, A. O'Shea–Greenfield, S.W. Rogers, S. Heinemann. Cloning by functional expression of a member of the glutamate receptor family. Nature (1989).

[21] G. Kortenbruck, E. Berger, E.J. Speckmann, U. Musshoff. RNA editing at the Q/R site for the glutamate receptor subunits GLUR2, GLUR5, and GLUR6 in hippocampus and temporal cortex from epileptic patients. Neurobiol Dis. (2001).

[22] D. Lodge. The history of the pharmacology and cloning of ionotropic glutamate receptors. Neuropharmacology (2009).

[23] G. Maccaferri, J.D. Roberts, P. Szucs, C.A. Cottingham, P. Somogyi. Cell surface domain specific postsynaptic currents evoked by identified GABAergic neurones in rat hippocampus in vitro. J. Physiol. (2000).

[24] C.J. McBain, T.J. DiChiara, J.A. Kauer. Activation of metabotropic glutamate receptors differentially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. J. Neurosci. (1994).

[25] G.L. McKinsey, S. Lindtner, B. Trzcinski, A. Visel, L.A. Pennacchio, D. Huylebroeck, Y. Higashi, J.L. Rubenstein. Dlx1&2-dependent expression of Zfhx1b (Sip1, Zeb2) regulates the fate switch between cortical and striatal interneurons. Neuron (2013).

[26] A. Miquelajauregui, T. Van de Putte, A. Polyakov, A. Nityanandam, S. Boppana, E. Seuntjens, A. Karabinos, Y. Higashi, D. Huylebroeck, V. Tarabykin. Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. PNAS (2007).

[27] A. Nityanandam, S. Parthasarathy, V. Tarabykin. Postnatal subventricular zone of the neocortex contributes GFAP+ cells to the rostral migratory stream under the control of Sip1. Dev. Biol. (2012).

[28] K.M. Noh, H. Yokota, T. Mashiko, P.E. Castillo, R.S. Zukin, M.V.L. Bennett. Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. PNAS (2005).

[29] D. Orduz, D.P. Bischop, B. Schwaller, S.N. Schiffmann, D. Gall. Parvalbumin tunes spike-timing and efferent short-term plasticity in striatal fast spiking interneurons. J. Physiol. (2013).

[30] C.L. Palmer, L. Cotton, J.M Henley. The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Pharmacol. Rev. (2005).

[31] M.S. Perkinton, T.S. Sihra, R.J. Williams. Ca^{2+}-permeable AMPA-receptors induce phosphorylation of cAMP response element-binding protein through a phosphatidylinositol 3-kinase-dependent stimulation of the mitogen-activated protein kinase signaling cascade in neurons. J. Neurosci. (1999).

[32] S.R. Platt. The role of glutamate in central nervous system health and disease. Vet. J. (2007).

[33] E.M. Powell. Interneuron development and epilepsy: early genetic defects cause long-term consequences in seizuresand susceptibility. Epilepsy Curr. (2013).

[34] A. Sailer, G.T. Swanson, I. Pérez–Otaño, L. O'Leary, S.A. Malkmus, R.H. Dyck, H. Dickinson–Anson, H.H. Schiffer, C. Maron, T.L. Yaksh, F.H. Gage, S. O'Gorman, S.F. Heinemann. Generation and analysis of GluR5(Q636R) kainate receptor mutant mice. J. Neurosci. (1999).

[35] P.H. Seeburg The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci. (1993).

[36] E. Seuntjens, A. Nityanandam, A. Miquelajauregui, J. Debruyn, A. Stryjewska, S. Goebbels, K.A. Nave, D. Huylebroeck, V. Tarabykin. Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. Nat. Neurosci. (2009).

[37] A.P. Silva, J.O. Malva, A.F. Ambrosio, A.J. Salgado, A.P. Carvalho, C.M. Carvalho. Role of kainate receptor activation and desensitization on the $[Ca2+]i$ changes in cultured rat hippocampal neurons. J. Neurosci. Res. (2001).

[38] E.A. Turovsky, M.V. Turovskaya, A.V. Kononov, V.P. Zinchenko. Short-term episodes of hypoxia induce posthypoxic hyperexcitability and selective death of GABAergic hippocampal neurons. Exp Neurol. (2013).

[39] E. Turovsky, A. Karagiannis, A.P. Abdala, A.V. Gourine. Impaired CO2 sensitivity of astrocytes in a mouse model of Rett syndrome. J Physiol. (2015).

[40] K. Verschueren, J.E. Remacle, C. Collart, H. Kraft, B.S. Baker, P. Tylzanowski, L. Nelles, G. Wuytens, M.T. Su, R. Bodmer, J.C. Smith, D. Huylebroeck. SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. J.Biol. Chem. (1999).

[41] P. Vanhoutte, H. Bading. Opposing roles of synaptic and extrasynaptic NMDA receptors in neuronal calcium signaling and BDNF gene regulation. Curr. Opin. Neurobiol. (2003).

[42] B. Vissel, G.A. Royle, B.R. Christie, H.H. Schiffer, A. Ghetti, T. Tritto, I. Perez–Otano, R.A. Radcliffe, J. Seamans, T. Sejnowski, J.M. Wehner, A.C. Collins, S. O'Gorman, S.F Heinemann. The Role of RNA editing of kainate receptors in synaptic plasticity and seizures. Neuron (2001).

[43] Q. Weng, Y. Chen, H. Wang, X. Xu, B. Yang, Q. He, W. Shou, Y. Chen, Y. Higashi, V. van den Berghe, E. Seuntjens, S.G. Kernie, P. Bukshpun, E.H. Sherr, D. Huylebroeck, Q.R. Lu. Dual-mode modulation of Smad signaling by Smad-interacting protein Sip1 is required for myelination in the central nervous system. Neuron (2012).

[44] R. Winklbauer, A. Medina, R.K. Swain, H. Steinbeisser. Frizzled-7 signalling controls tissue separation during Xenopus gastrulation. Nature (2001).

[45] Y.Yang. Wnt signaling in development and disease. Cell Biosci. (2012).

[46] R. Yuste, A. Majewska, K. Holthoff. From form to function: calcium compartmentalization in dendritic spines. Nat Neurosci. (2000).

[47] X. Zhou, D. Hollern, J. Liao, E. Andrechek, H. Wang. NMDA receptor-mediated excitotoxicity depends on the coactivation of synaptic and extrasynapticreceptors. Cell Death Dis. (2013).

[48] D. Zemke, J.L. Smith, M.J. Reeves, A. Majid. Ischemia and ischemic tolerance in the brain: an overview. Neurotoxicology (2004).

[49] C. Zweier, B. Albrecht, B. Mitulla, R. Behrens, M. Beese, G. Gillessen-Kaesbach, H.D. Rott, A. Rauch."Mowat-Wilson" syndrome with and without Hirschsprung disease is a distinct, recognizable multiple congenital anomalies-mental retardation syndrome caused by mutations in the zinc finger homeo box 1B gene. Am. J. Med. Genet. (2002).

Fig. 1. Action of the NMDA on neurons isolated from brain cortex (A) and brainstem (B) of the Sip1-mutant mice.

A – Application of NMDA in Mg^{2+}-free medium to the neurons of brain cortex isolated from the $Sip1^{wt/wt}$ (black curve, n=118), $Sip1^{fl/wt}$ (red curve, n=153), and $Sip1^{fl/fl}$ (blue curve, n=114). B – Application of NMDA in Mg^{2+}-free medium to the neurons of brainstem isolated from the wild type mice (black curve, n=108), heterozygous Sip1-mice (red curve, n=121), and homozygous Sip1-mice (blue curve, n=124). C – Dependence of the Ca^{2+}-responses amplitude on NMDA concentration in the brain cortex neurons of the $Sip1^{wt/wt}$ (curve 1), $Sip1^{fl/wt}$ (curve 2) and $Sip1^{fl/fl}$ (curve 3). For each concentration, average data are represented, obtained from several hundreds of neurons in 5 independent experiments ±S.E.M. For panels A and B, averaged Ca^{2+}-responses for neurons from individual experiments are represented. Medium replacement with Mg^{2+}-free is shown with arrows. NMDA concentrations are specified with horizontal lines accompanied with correspondent ciphers in the bottom of the graph: 1 – **0,001**, 2 – **0.01**, 3 – **0.1**, 4 – **0.3**, 5 – **0.5**, 6 – **1**, 7 – **3**, 8 – **5**, 9 – **10**, 10 – **30**, 11 – **50**, 12 – **100 μM**.

Fig. 2. Action of the AMPA-receptors activator, 5-Fluorowillardiine (FW), on neurons isolated from brain cortex (A) and brainstem (B) of the Sip1-mutant mice.

A – Application of enhancing FW concentrations in the presence of an inhibitor for desensitization of the AMPA-receptors, cyclothiazide (CTZ, 5 μM), to the neurons of brain cortex isolated from the $Sip1^{wt/wt}$ (black curve, n=107), $Sip1^{fl/wt}$ (red curve, n=137), and $Sip1^{fl/fl}$ (blue curve, n=124). B – Application of enhancing FW concentrations in the presence of an inhibitor for desensitization of the AMPA-receptors, cyclothiazide (CTZ, 5 μM), to the neurons

of brainstem isolated from the Sip1$^{wt/wt}$ (black curve, n=111), Sip1$^{fl/wt}$ (red curve, n=106), and Sip1$^{fl/fl}$ (blue curve, n=113). C – Dependence of the Ca^{2+}-responses amplitude on FW concentration in the brain cortex neurons of the Sip1$^{wt/wt}$ (curve 1), Sip1$^{fl/wt}$ (curve 2) and Sip1$^{fl/fl}$ (curve 3). For each concentration, average data are represented, obtained from several hundreds of neurons in 3 (curve 3) and 5 (curves 1 and 2) independent experiments ±S.E.M. D – amplitudes of Ca^{2+} responses averaged for several hundreds of brain cortex neurons after short-term depolarization using application of 35mM KCl. Significantly different at p<0.05. Differences between Sip1$^{fl/fl}$ and Sip$^{wt/fl}$, as well as between Sip1$^{wt/wt}$ and Sip$^{wt/fl}$, were not reliable. Application of 5 μM cyclothiazide is shown with arrows. FW concentrations are specified with horizontal lines accompanied with correspondent ciphers in the bottom of the graph: 1 – **0,001**, 2 – **0.005**, 3 – **0.01**, 4 – **0.03**, 5 – **0.05**, 6 – **0.1**, 7 – **0.5**, 8 – **1**, 9 – **3**, 10 – **5**, 11 – **10 μM**.

Fig. 3. Effect of the KA-receptors activator, domoic acid (DA), on neurons isolated from brain cortex (A) and brainstem (B) of the Sip1-mutant mice.

A – Application of enhancing DA concentrations in the presence of a selective antagonist of the AMPA-receptors, GYKI-52466 (30μM) and an inhibitor for desensitization of the KA-receptors, concanavalin A (ConA, 200 μg/ml), to the neurons of brain cortex isolated from the Sip1$^{wt/wt}$ (black curve, n=93), Sip$^{wt/fl}$ (red curve, n=117), and Sip1$^{fl/fl}$ (blue curve, n=111). DA concentrations are specified with horizontal lines accompanied with correspondent ciphers in the bottom of the graph: 1 – **0.001**, 2 – **0.01**, 3 – **0.05**, 4 – **0.1**, 5 – **0.3**, 6 – **0.5**, 7 – **1**, 8 – **5**, 9 – **10 μM**. Averaged Ca2+-responses for neurons from individual experiments are represented. B – Dependence of the Ca^{2+}-responses amplitude on DA concentration in the brain cortex neurons of the Sip1$^{wt/wt}$ (curve 1), Sip$^{wt/fl}$ (curve 2) and Sip1$^{fl/fl}$ (curve 3). C - Dependence of the Ca^{2+}-responses amplitude on DA concentration in the brainstem neurons of the Sip1$^{wt/wt}$ (curve 1), Sip$^{wt/fl}$ (curve 2) and Sip1$^{fl/fl}$ (curve 3). It can be observed that sigmoid curves 1 and 2 are almost identical both in neurons from brain cortex and brainstem, being indicative of the absence the Sip1 mutation effect on the KAR of neurons from Sip$^{wt/fl}$
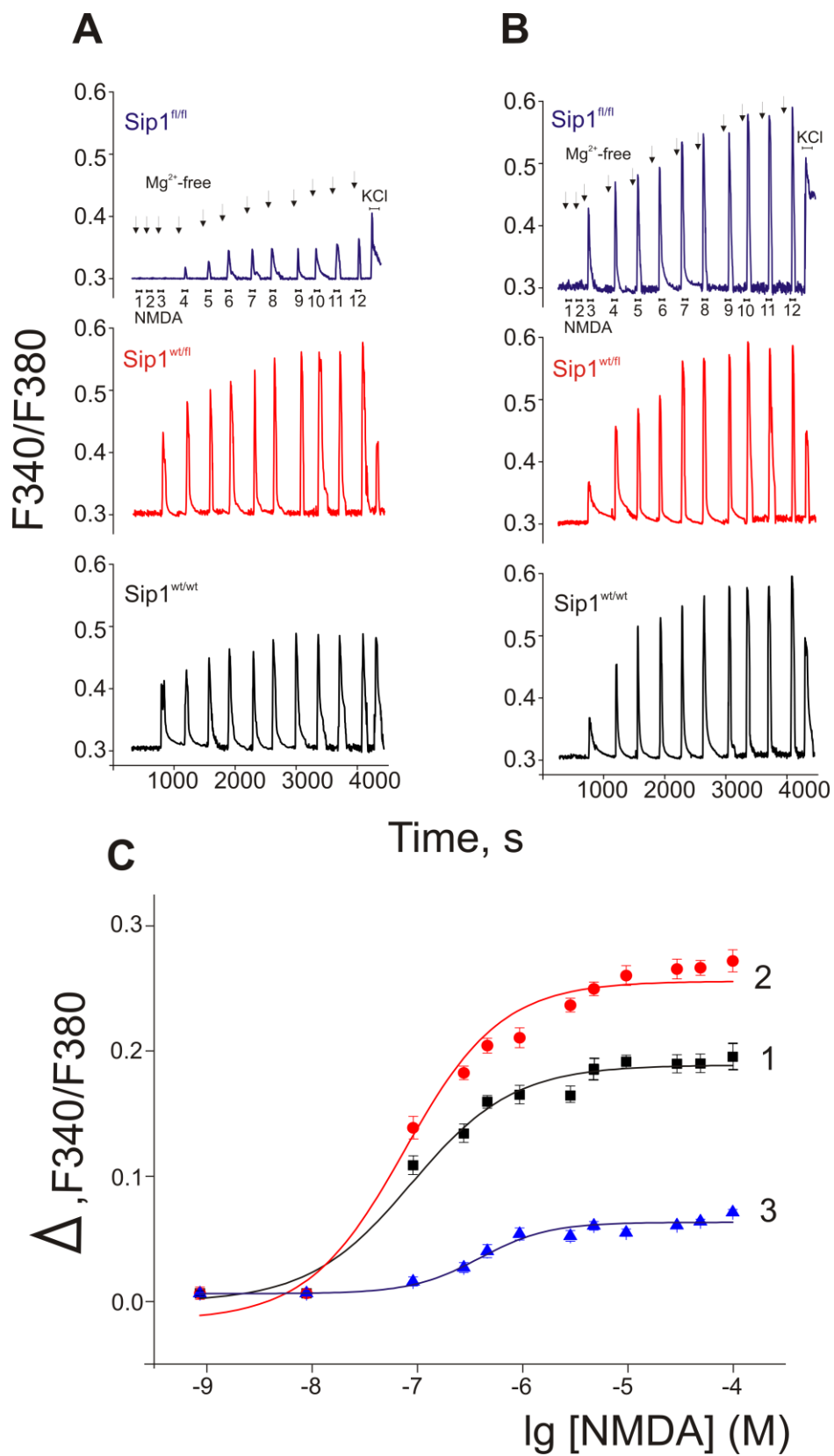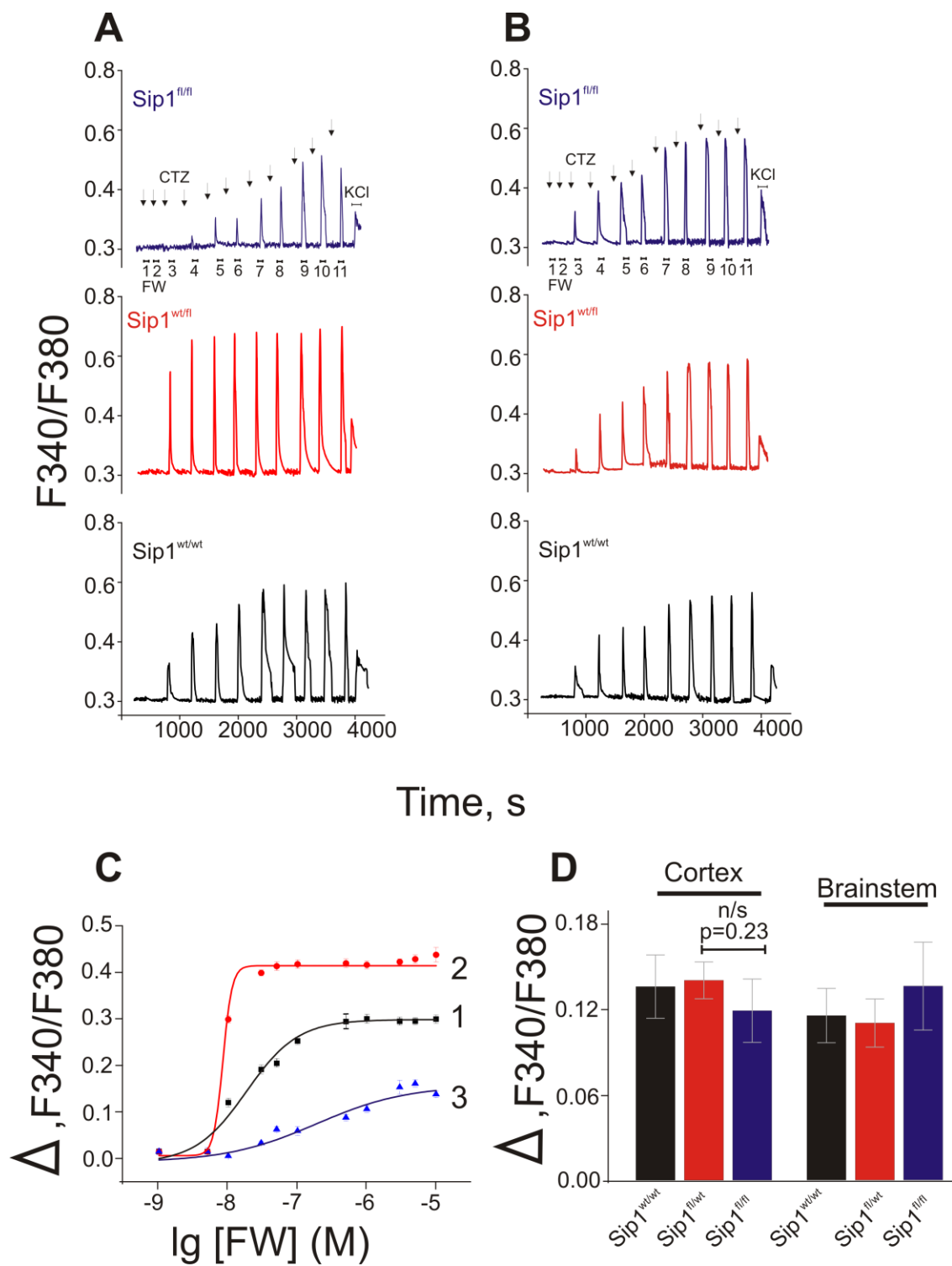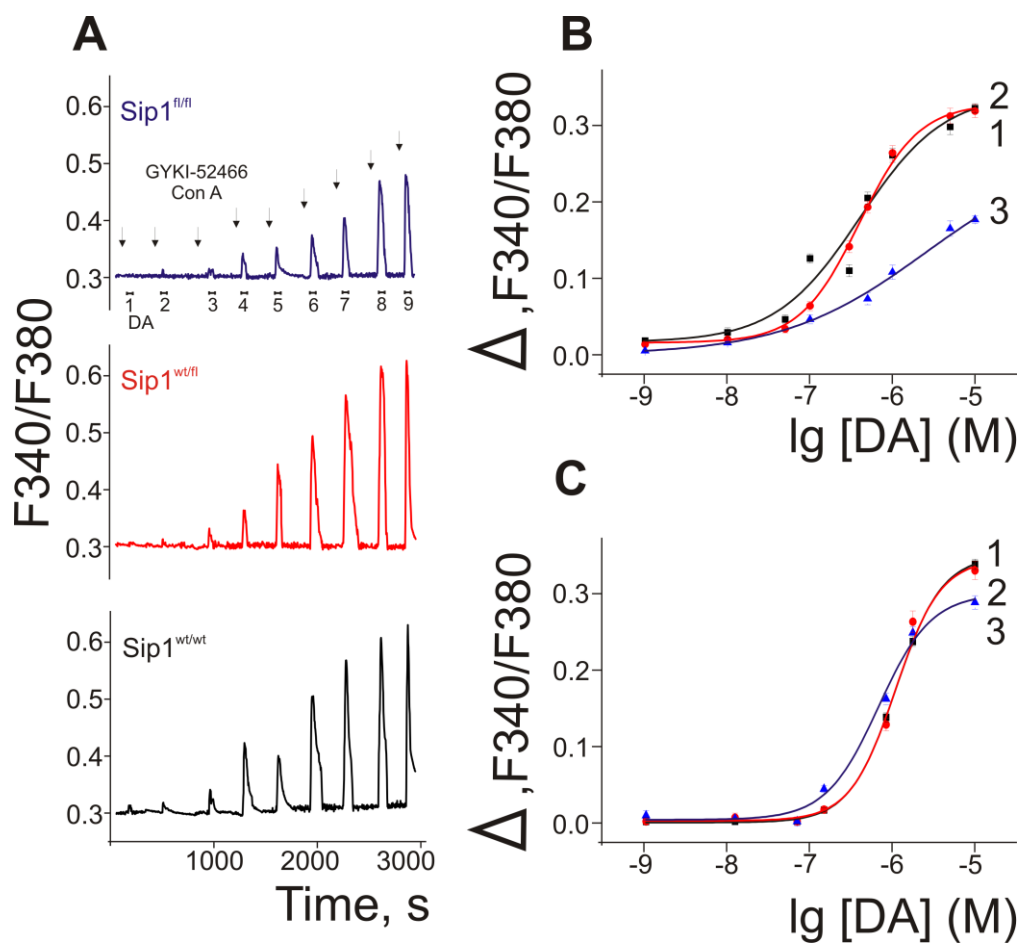
Figure 1.

Figure 2.

Figure 3.

Table 1

	EC50/ΔF (Fmax-Fmin)					
	NMDAR		AMPAR		KAR	
	Cortex	Brainstem	Cortex	Brainstem	Cortex	Brainstem
Sip1wt/wt	0.096μM/0.19	0.222μM/0.3	0.018μM/0.29	0.022μM/0.27	0.404μM/0.32	0.642μM/0.34
Sip1fl/wt	0.083μM/0.27	0.289μM/0.3	0.0084μM/0.44	0.024μM/0.26	0.396μM/0.32	0.636μM/0.34
Sip1fl/fl	0.425μM/0.06	0.197μM/0.27	0.188μM/0.15	0.028μM/0.29	2,55μM/0.18	0.411μM/0.29