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REGULAR RESEARCH ARTICLE

P2X7 Receptors Drive Spine Synapse Plasticity in the Learned Helplessness Model of Depression

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Abstract

Background: Major depressive disorder is characterized by structural and functional abnormalities of cortical and limbic brain areas, including a decrease in spine synapse number in the dentate gyrus of the hippocampus. Recent studies highlighted that both genetic and pharmacological invalidation of the purinergic P2X7 receptor (P2rx7) leads to antidepressant-like phenotype in animal experiments; however, the impact of P2rx7 on depression-related structural changes in the hippocampus is not clarified yet.
Methods: Effects of genetic deletion of P2rx7s on depressive-like behavior and spine synapse density in the dentate gyrus were investigated using the learned helplessness mouse model of depression.
Results: We demonstrate that in wild-type animals, inescapable footshocks lead to learned helplessness behavior reflected in increased latency and number of escape failures to subsequent escapable footshocks. This behavior is accompanied with downregulation of mRNA encoding P2rx7 and decrease of spine synapse density in the dentate gyrus as determined by electron microscopic stereology. In addition, a decrease in synaptopodin but not in PSD95 and NR2B/GluN2B protein level was also observed under these conditions. Whereas the absence of P2rx7 was characterized by escape deficit, no learned helpless behavior is observed in these animals. Likewise, no decrease in spine synapse number and synaptopodin protein levels was detected in response to inescapable footshocks in P2rx7-deficient animals.
Conclusion: Our findings suggest the endogenous activation of P2rx7s in the learned helplessness model of depression and decreased plasticity of spine synapses in P2rx7-deficient mice might explain the resistance of these animals to repeated stressful stimuli.

Keywords: P2rx7, learned helplessness, depression, spine synapse

Introduction

Major depression is a complex psychiatric disorder generated by genetics interacting with environmental factors (Hasin et al., 2005). Among several susceptible genes and chromosomal regions regarding the disorder, early studies identified single nucleotide polymorphisms (SNPs) in the gene encoding the purinergic P2X7 receptor (P2rx7) linked to depression (Lucae et al., 2006; Hejjas et al., 2009; Stokes et al., 2010; Soronen et al., 2011). Although more recent meta-analysis has not confirmed this association (Feng et al., 2014), the effect of SNPs on the phenotype is also affected by environmental factors, and certain genetic variations gain significance only in the coexistence of stressful life events, as it turned out, for example, for the SNPs

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Significance Statement

Previous animal studies show that genetic deletion and pharmacological inhibition of the purinergic P2X7 receptor (P2rx7) leads to an antidepressant phenotype. In this paper, we extended our findings to the learned helplessness mouse model of depression and detected that learned helplessness behavior and consequent reduction in the spine synapse number in the dentate gyrus of hippocampus are absent in P2rx7 knockout mice, suggesting that P2rx7 regulates depressive-like behavior and structural plasticity of spine synapses in response to inescapable shock. The stress-induced change in spine synapse number was accompanied with a decrease in the dendritic spine specific protein synaptopodin level in wild-type mice, while other postsynaptic proteins, such as PSD95 or NR2B/GluN2B, were not subject to regulation by the learned helplessness paradigm.

encoding IL-1β (Kovacs et al., 2016), which might also be true for P2rx7.

P2rx7s are ligand-gated purinergic receptors, activated by high concentrations of ATP, and their activation provides the secondary stimulus for the formation of NLRP3 inflammasome complex responsible for the posttranslational processing of inflammatory cytokines IL-1β and IL-18 (Sperlagh et al., 2006). The P2rx7s are expressed by hematopoietic cells, such as microglia, and under certain conditions also by neurons and astrocytes. They have an important role in the modulation of neurotransmitter release (Anderson and Nedergaard, 2006; Sperlagh et al., 2007), since P2rx7 activation elicits Ca^{2+} influx (Miras-Portugal et al., 2003) followed by increased glutamate and subsequent GABA release (Sperlagh et al., 2002; Papp et al., 2004; Alloisio et al., 2008). P2rx7s are also involved in the activation and proliferation of microglia cells (Monif et al., 2009) and in the regulation of cell death (Skaper et al., 2006), and they are implicated in a variety of central nervous system disorders (Sperlagh et al., 2006; Burnstock, 2008; Sperlagh and Illes, 2014). Recent studies indicate a possible role of P2xr7s in depressive disorders (Basso et al., 2009; Boucher et al., 2011; Csolle et al., 2013a, 2013b; Iwata et al., 2015; Stokes et al., 2015). In these studies, various behavior tests of depression were conducted in P2rx7+/+ and P2rx7-deficient (P2rx7-/-) mice, and the knockout group did not display depressive-like behavior. Behavioral despair was absent and decreased immobility was detected in P2rx7-/- animals in the forced swim and tail suspension tests, respectively (Basso et al., 2009; Boucher et al., 2011; Csolle et al., 2013a; Wilkinson et al., 2014). In addition, lipopolysaccharide-induced decline in sucrose preference was ameliorated in P2rx7-deficient mice compared with P2rx7+/+ values (Csolle et al., 2013b). This antidepressant phenotype could be pharmacologically reproduced using specific P2rx7 antagonists Brilliant blue G and AZ-10606120 in P2rx7+/+ but not in P2rx7-deficient animals. This mood-stabilizing effect of P2rx7 deficiency could not be transferred with bone marrow grafts to P2rx7+/+ animals, indicating the role of P2rx7s expressed by cells of nonhematopoietic origin in this effect (Csolle et al., 2013a). The inhibition of P2rx7 with a selective antagonist alleviated cellular and behavioral effects in more complex, chronic unpredictable stress model of depression as well (Iwata et al., 2015).

Hippocampal atrophy and dysfunction of hippocampus-related functions in depressed patients suggest the involvement of the hippocampus in this mood disorder (Sheline et al., 2003; Kang et al., 2012). As a structural correlate of depression-like behavior, a decrease of spine synapses along with a decreased expression of synapse related genes were also observed in postmortem samples derived from depressed patients (Kang et al., 2012). A similar decrease in spine synapse number was revealed in the hippocampus in the learned helplessness animal model of depression, which is normalized by subsequent antidepressant treatment (Hajszan et al., 2009). This paradigm is widely used in animal behavior studies as an adequate model in the aspect of coping deficit and helplessness in depressive disorders (Chourbaji et al., 2005; Bougarel et al., 2011; Pryce et al., 2012). In the present study, we applied an electron microscopy analysis to see the alteration of these specific neuronal connections in the mouse learned helplessness model of depression in the presence and absence of the purinergic P2rx7. We demonstrated that in P2rx7+/+ animals, inescapable footshocks (IES) led to learned helplessness behavior accompanied by P2rx7 mRNA down-regulation and decreased spine synapse density in the DG of the hippocampus. A reduction in synaptopodin, but not PSD95 and NR2B/GluN2B protein level, was also observed under these conditions. These alterations were absent in P2rx7-/- animals, implicating the role of the receptor in depressive-like behavior and consequent changes in hippocampal synaptic plasticity.

Methods

Animals

All studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory animals and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary, permission no. PEI/001/773-6/2015). Two- to three-month-old male C57Bl/6 P2rx7+/+ and P2rx7-/- mice were kept individually in their cage from the week before the experiment, housed in a light-controlled (12 h on, 12 h off), humidity-controlled (60±10%), and temperature-controlled (23±2°C) room with food and water available ad libitum. All experiments were carried out during the light phase (7:00 AM to 7:00 PM). Homozygous P2rx7+/+ were bred on a background of C57Bl/6J. The original breeding pairs of P2rx7-/- mice (C57BL/6J based) were kindly supplied by Christopher Gabel (Pfizer Inc). Animals contained the DNA construct (P2X7-F1 (5′-CGGCGTGCGTTTTGACATCCT-3′) and P2X7-R2 (5′-AGGGCCCTGCGGTTCTC-3′)) previously shown to produce genetic deletion of P2rx7, and they were genotyped using PCR analysis as described (Solle et al., 2001). All efforts were made to minimize animal suffering and reduce the number of animals used.

Learned Helplessness

P2rx7+/+ and P2rx7-/- animals were randomly assigned to experimental groups of 23 to 27 mice/group. A standard learned helplessness paradigm was used with slight modifications (Chourbaji et al., 2005). In this model, multiple inescapable footshocks (IES) are administered, evoking a helpless condition when animals are unable to avoid a negative situation even though it could be evaded. In our experiment, commercial shuttle boxes (Med Associates) were used. During training, mice received IES in one compartment with the door closed (180 trials, 0.15-mA intensity, 2-second duration, 1 to 15-second intertrial interval) on 2 consecutive days (Figure 1A).

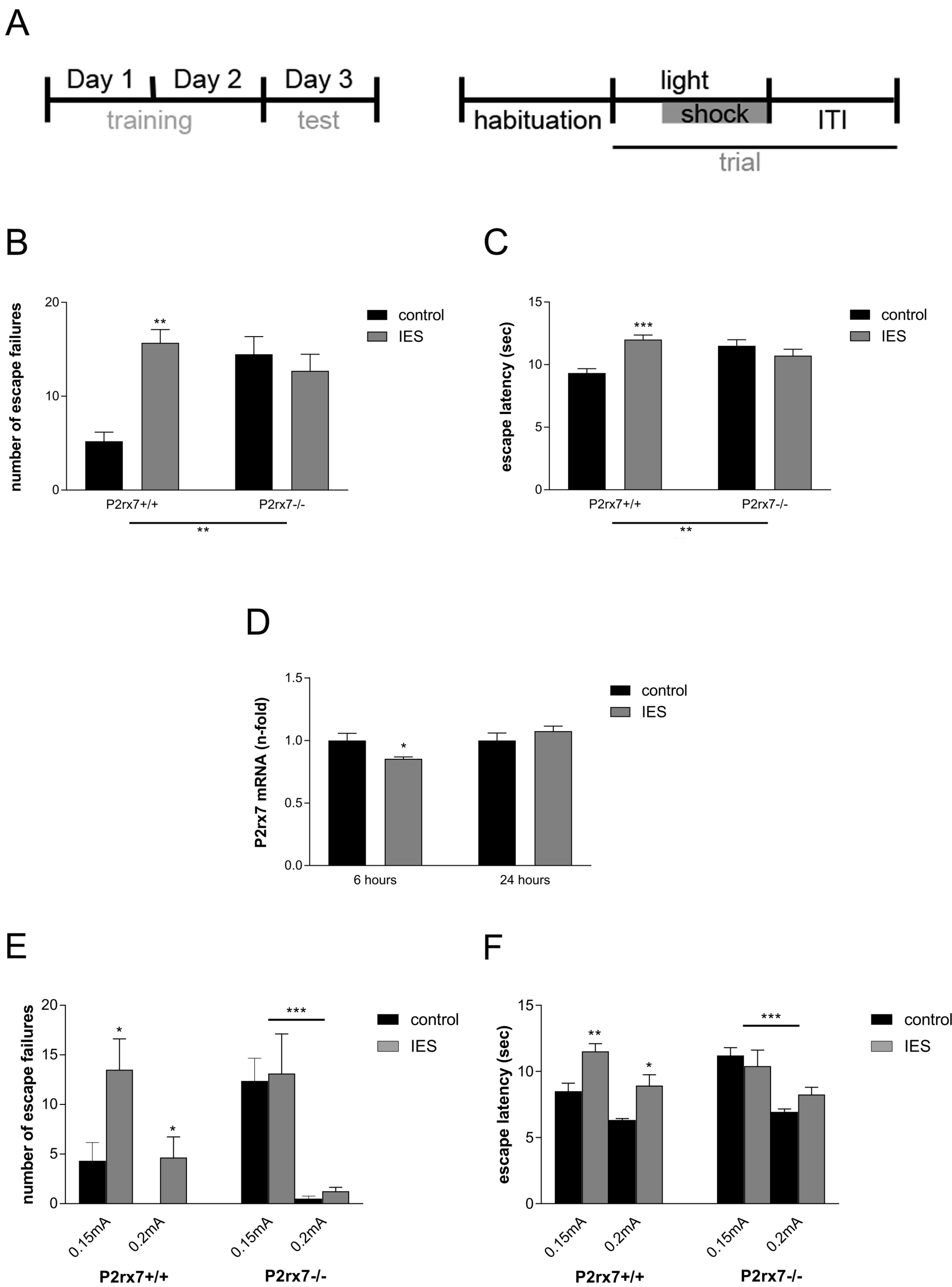

Figure 1. Inescapable footshock (IES) treatment leads to helpless behavior in P2X7 receptor (P2rx7)+/+, but not in P2rx7-/- in learned helplessness model of depression and subsequent time-dependent change in hippocampal expression of P2rx7 mRNA. (A) Schematic illustration of learned helplessness protocol: the first 2 days are the training phase, when mice received IES with the guillotine door closed (2x180 trials). Control animals were exposed to the chambers without IES administration. On the third day active escape was tested, consisting of 30 trials of escapable footshock with the guillotine door being opened at the time of the light onset. In both phases, an initial 5-minute habituation period preceded the first trial. Each trial had randomized intertrial intervals (ITI) as resting phase between shocks. The maximal escape latency was 15 seconds that was equivalent to a failed escape. (B) The IES group of P2rx7+/+ animals developed learned helplessness, indicated by the increased number of failed escapes (**$P < .01$), while in the case of P2rx7-/- mice, no difference could be observed regarding IES treatment, only the elevation of baseline values. (C) Escape latency of IES-treated P2rx7+/+ mice was significantly higher compared with control group (***$P < .001$), whereas in P2rx7-/- groups similar latency values were recorded (n=27). (D) At 6 hours after testing learned helplessness, there was significant dowregulation of P2rx7 mRNA levels, but this effect dissolved after 24 hours (*$P < .05$). (E-F) Higher shock intensity (0.2 mA) during testing triggered active escape behavior not only in P2rx7+/+, but also in P2rx7-/- mice; therefore these genetically deficient animals are not saturated, but are able to respond to shock stress (*$P < .05$, **$P < .01$, ***$P < .001$) (n=6–8).

Control animals were exposed to the box without receiving IES. The test phase consisted of 30 trials of escapable footshock (0.15-mA intensity, 10-second maximum duration, 30-second average intertrial interval), with the door open from the light onset. In both phases, an initial 5-min habituation period preceded the first trial. Escape failures and latencies were recorded automatically for each trial by MED-PC IV software (Med Associates). In another set of experiments, a higher shock intensity (0.2 mA) during testing phase was also investigated with no change in other parameters (n=6–8). Animals were sacrificed either 6 or 24 hours after the testing session for further experiments.

Electron Microscopy

Twenty-four hours after testing learned helplessness, 3 mice/group were subjected to electron microscopy analysis. After CO_2 anesthesia, animals were perfusion fixed transcardially with 4% paraformaldehyde (PFA) containing 0.1% glutaraldehyde, and brains were postfixed overnight in 4% PFA. Throughout the dorsal hippocampus, 100-µm coronal sections were cut by a vibratome and 5 sections were embedded for further work (0.5% osmium tetroxide for 30 minutes, 1% uranyl acetate for 30 minutes, dehydrated in ethanol, and flat-embedded in epoxy resin). Two areas from the molecular layer of DG were sampled in each embedded section (Figure 2A). Then 75-nm consecutive serial sections were made at each sampling site, and digitized electron micrographs were taken for the physical disector in a Hitachi H-7100 transmission electron microscope at 12000x magnification. Appearing asymmetric spine synapses were counted on image pairs depicting identical regions in adjacent ultrasections

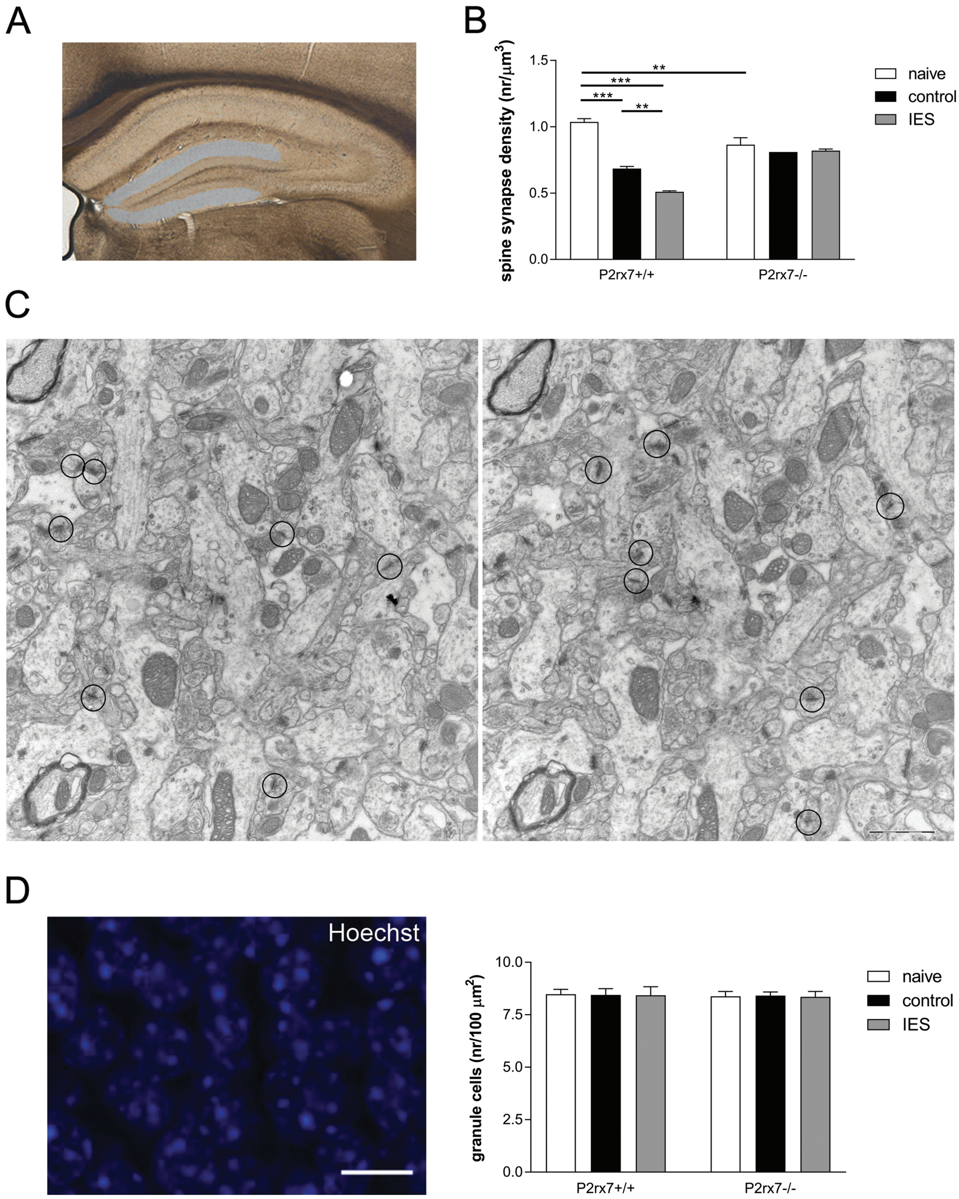

Figure 2. Spine synapse density in the molecular layer of dorsal dentate gyrus (DG) is altered in the learned helplessness paradigm. (A) Sampling sites along the upper and lower blades of DG along the molecular layer were localized in consecutive 100-µm-thick coronal sections, and from the embedded tissue blocks 75-nm serial sections were made for synapse counting. (B) In line with the behavioral findings, inescapable footshock (IES) caused reduced density of spine synapses in P2X7 receptor (P2rx7)+/+ animals (**$P < .01$) compared with controls, but on the other hand, P2rx7-/- mice seemed resistant to synaptic loss after exposure to IES. In addition, comparing synaptic densities in naïve animals, a significant difference was revealed between the 2 genotypes (**$P < .01$). (C) Representation of image pairs of identical regions in adjacent ultrasections and appearing spine synapse identification by postsynaptic densities and lack of cell organelles in P2rx7+/+ naïve hippocampus. The circles in the images show labelled spine synapses that we used for further density calculations. Of 4 consecutive sections, the second and third were used for synapse counting, the first and fourth helped the identification of spine synapses. The average volumetric density (synapse/µm^3) of spine synapses was determined by dividing the total sum of spine synapses counted in all samples taken from an animal by the total disector volume (107.78 x 0.075 x 50 = 404.175 µm^3). (D) Representative image of P2rx7+/+ naïve granule cells. Confocal images were captured to investigate the influence of learned helplessness on granule cells. Hoechst-labelled nuclei of round contours (excluding glial and endothel cells) were counted in several consecutive sections, sampling identical regions of the dorsal hippocampus. Calculations found that granule cell number was not affected by either genotype or learned helplessness regardless of treatment. Bar: 10 µm at 20x magnification.

(Figure 2C). Spine synapses were identified by containing postsynaptic densities and the lack of cell organelles. The average volumetric density (synapse/µm^3) of spine synapses was determined by dividing the total sum of spine synapses counted in all samples taken from an animal. The disector volume was calculated by multiplying the area of image pairs (107.78 µm^2) by thickness of ultrasections (75 nm) and by disector number (50). The number of spine synapses was calculated independently by 2 different investigators (LO and AK).

Immunohistochemistry

Mice (n=3) were anesthetized and perfused transcardially with 4% PFA 24 hours after the evaluation of learned helplessness, then postfixed overnight in 4% PFA at 4°C. Then 40- or 60-µm coronal sections from the dorsal hippocampus were used for immunoreaction. Nucleus staining with 1:10000 Hoechst 33342 (Tocris) in TBS for 2 hours at room temperature (RT) was applied on 40-µm sections. NR2B/GluN2B immunolabeling was performed on 60-µm sections as described previously (Csolle et al., 2013b). Confocal images were acquired at the same depth of the sections with same acquisition parameters with a Nikon C2 confocal system on a Nikon Ni-E microscope equipped with NIS-Elements C software. Brightness and contrast were adjusted using Adobe Photoshop CS3, and average intensity of NR2B/GluN2B immunoreaction was quantified with NIH ImageJ software (U.S. NIH).

Western Blot

Hippocampi dissected 6 or 24 hours after testing learned helplessness were stored at –70°C until further investigation (n=5). Samples were homogenized in 250 µL lysis buffer (containing 1% protease inhibitor) then centrifuged in 4°C 10000 rpm for 10 minutes. Resulting supernatants were used for Western blot after measuring their protein concentrations with BCA protein assay. From each sample, 40 µg protein was loaded and separated by SDS-PAGE (10%) and transferred onto a polyvinylidene difluoride membrane using a MiniProtean-3 apparatus (Bio-Rad). The blot was incubated in blocking solution (1% bovine serum albumin, 5% milk, tris buffered saline solution with Tween® 20 [TBST]) for 2 hours RT, then in primary antibodies (actin, 1:200 goat, synaptopodin 1:200 goat, SantaCruz, PSD95, Abcam) applied overnight at 4°C. After rinsing and washing 3x10 minutes in TBST, horseradish peroxidase-conjugated secondary antibodies were used (RabbitXGoat, 1:5000, GoatXRabbit 1:4000, Millipore) for 2 hours at RT, rinsed and washed in TBST for 3x10 minutes, then in TBS for 5 minutes. The specific immunoreactive bands were detected and visualized by chemiluminescence (Immobilon Western, Millipore) and quantified by densitometric analysis with ImageJ.

RT-PCR

Total RNA samples were isolated and purified from cell lysates of hippocampus (n=4) using the Qiagen RNeasy Lipid Tissue Mini kit according to the manufacturer's instructions. Then 1 µg of total RNA was reverse transcribed using the Tetro cDNA Synthesis Kit (Bioline) with an AB GeneAmp PCR system 2700 instrument in a mixture containing 4 µL of 5x reaction buffer, 1 µL of random hexamer primer, 1 µL of RNase Inhibitor, and 1 µL of 10 mM dNTP mix in a final volume of 18 µL with 0.1% diethylpyrocarbonate-treated distilled water. The reverse transcription reaction was performed at 70°C for 5 minutes, followed by incubation at 25°C for 5 minutes, synthesis at 25°C for 10 minutes, and a final incubation at 42°C for 60 minutes. The expression level of the target gene P2rx7 was determined according to standard protocols using TaqMan Fast Universal PCR Master Mix (2x) and TaqMan Gene Expression Assay Mix (20x) (IDs for target genes: Gapdh Mm99999915_g1 and P2rx7 Mm01199500_m1). The PCR cycling protocol started with denaturation at 95°C for 10 minutes followed by 40 cycles at 94°C for 15 seconds, 64°C for 30 seconds, and 72°C for 10 seconds. P2rx7 expression was normalized to the level of Gapdh as a reference housekeeping gene.

Statistics

All data were presented as the mean ± SEM of n determinations. The statistical analyses were carried out by 2-way ANOVA (factor 1: genotype; factor 2: IES treatment), whereas Fisher's LSD test was used for pairwise comparisons.

Results

Learned Helplessness Is Developed in P2rx7+/+, but Not in P2rx7-Deficient Animals

The learned helplessness paradigm was used to study depressive-like behavior (Figure 1A–C) in drug and test naïve P2rx7+/+ and P2rx7-deficient animals. In P2rx7+/+ mice, IES provoked increased number of escape failures and escape latency values with significant treatment effect on escape failures [$F(1, 100) = 8.057$, $P < .01$] and escape latency [$F(1, 100) = 4.457$, $P < .05$], indicating the development of learned helplessness.

This was accompanied with a time-dependent downregulation of mRNA encoding P2rx7 in P2rx7+/+ mice (Figure 1D). At 6 hours after testing helpless behavior, P2rx7 mRNA decreased significantly ($P < .05$) in the IES group compared with controls. In contrast, 24 hours after testing this effect dissolved, since there was no difference between control and IES animals.

In P2rx7-/- animals, elevated escape failure number and escape latency were found in response to escapable shocks of testing in controls compared with P2rx7+/+ littermates. However, there was no change in failed escapes or escape latencies in P2rx7-deficient animals when exposed to prior IES, that is, these animals did not display learned helplessness. Supporting this assumption, 2-way ANOVA revealed a significant genotype x treatment effect on escape failures [$F(1, 100) = 15.64$, $P < .01$] and also on escape latency values [$F(1, 100) = 15.26$, $P < .01$].

Although higher values of escape failure and latency were measured in P2rx7-/- mice, these animals could not be considered as helpless, since at elevated shock intensity (0.2 mA) during testing they displayed active escape behavior with remarkably low escape failure [$F(1,28) = 26.14$, $P < .001$] and latency values [$F(1,28) = 19.12$, $P < .001$] (Figure 1E–F). This higher shock intensity facilitated escape behavior in P2rx7+/+ mice as well, which responded with much lower escape failure number and escape latency values compared with the original 0.15-mA shock intensity, although the significant difference between the control and IES treated groups was still sustained ($P < .05$) (Figure 1E–F).

Learned Helplessness Decreases Spine Synapse Density and Expression of Synaptopodin in P2rx7+/+ but Not in P2rx7-/- Animals

Depressive-like behavior in animal models is accompanied by synapse loss in the hippocampus (Hajszan et al., 2009). Using electron microscopic stereology, the analysis revealed quantitative alterations in spine synapse number of the examined areas

24 hours following the evaluation of learned helplessness. While in the P2rx7+/+ mice repeated IES evoked a decrease in the spine synapse density in accordance with the observed behavioral alterations, this effect was not observed in the P2rx7-/- groups (Figure 2B). Two-way ANOVA found significant genotype x treatment effect on spine synapse densities [$F(2, 14) = 105.53$, $P < .001$]. We also found significant genotype-related difference in spine synapse density of the DG in naïve animals revealed by Fisher's LSD posthoc test ($P < .001$). No qualitative change in spine synapse morphology was observed either by genotype or treatments.

Because genotype and treatment related alterations in spine synapse number might be due to changes in the number of granule cells, next we determined whether the granule cells also had gone through any quantitative change. Two-way ANOVA found no significant genotype or treatment related effect on granule cell numbers in the hippocampus (Figure 2D). The sampling areas were taken in identical regions of the granule cell layers in both the P2rx7+/+ and in P2rx7-/- mice.

Since reduced hippocampal spine synapse density was revealed in P2rx7+/+ mice exposed to the learned helplessness paradigm, western-blot analysis of synaptic markers was also performed to follow correspondent changes in synaptic proteins. PSD95 and synaptopodin protein levels of the hippocampus were investigated 6 and 24 hours after testing learned helplessness (Figure 3). The structural protein actin was used as a positive control. In case of synaptopodin (Figure 3A–B), a significant decrease in the protein level was detected in the IES-treated group both 6 and 24 hours after testing in the P2rx7+/+, but not in P2rx7-/- animals. Accordingly, significant genotype x treatment effect was revealed by 2-way ANOVA on the level of synaptopodin 6 hours after testing helpless behavior [$F(1, 9) = 8.0276$, $P < .05$], and this change was maintained 24 hours later as well [$F(1, 7) = 7.2509$, $P < .05$].

Statistical analysis found no significant genotype or treatment effect in the amounts of hippocampal PSD95 protein independent of the timing of sample preparation (Figure 3C–D) [$F(1, 13) = 0.3285$, $P = .576$ after 6 hours, $F(1, 4) = 0.0503$, $P = .833$ after 24 hours].

Next, we examined how the NR2B/GluN2B protein level of the DG is altered in the learned helplessness model. We performed NR2B/GluN2B immunostaining 24 hours after testing the behavior (Figure 4A–D). We found difference between the 2 genotypes [$F(1,8) = 29.5615$, $P < .001$]; however, the development of learned helplessness behavior did not influence the intensity of NR2B/GluN2B in either P2rx7+/+ or P2rx7-/- animals [$F = (1,8) = 1.0658$ $P = .33$] (Figure 4E).

Discussion

A growing body of evidence suggests the structural alterations of cortical spine synapse plasticity in major depressive disorder (Gerhard et al., 2016). It has been reported that the number of hippocampal spine synapses are decreased along with the behavioral deficit in the learned helplessness animal model of depression, and both behavioral and structural changes were counteracted by antidepressant treatment (Hajszan et al., 2009). Dendritic spines are rapidly changing protrusions giving place to excitatory synapses and taking an important role in neuronal plasticity (Nimchinsky et al., 2002). Disruption of normal spine synapse distribution can be responsible for cognitive deficit in mood disorders. In depressed patients, smaller hippocampal volume is revealed by in vivo brain imaging (Bremner et al., 2000; Sheline et al., 2003; Huang et al., 2013), and postmortem studies showed dendritic spine reduction as well (Soetanto et al., 2010; Kang et al., 2012). In our present study, we found significant decrease of spine synapse density in the DG of P2rx7+/+ mice subsequent to IES treatment by applying electron microscopy

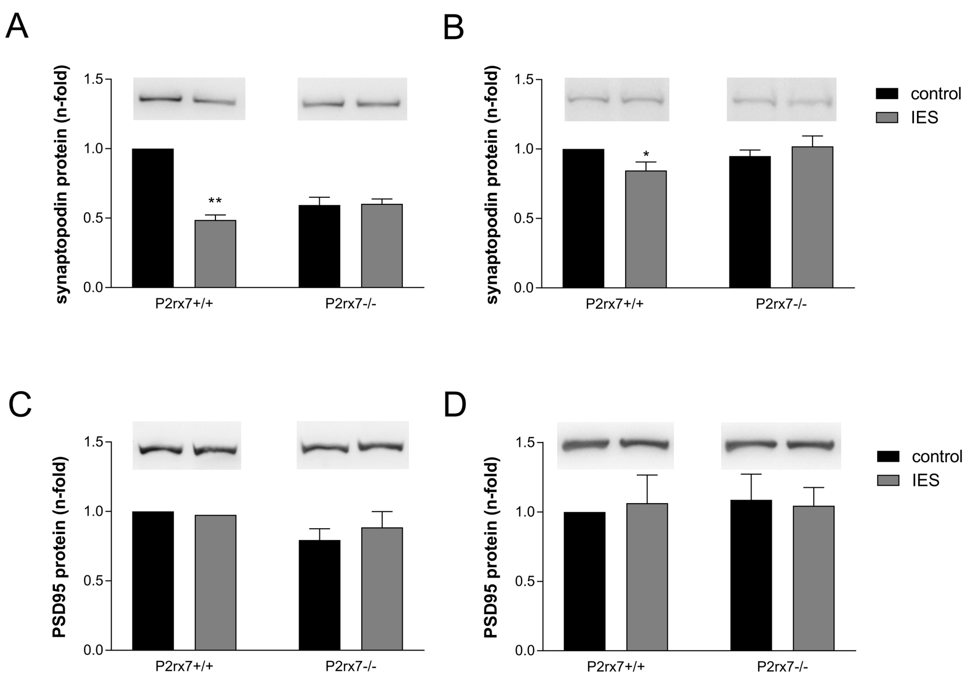

Figure 3. Western-blot analysis revealed time-dependent changes in dendritic spine apparatus synaptopodin protein levels, but not in PSD95 levels in the mouse hippocampus. Sample preparation was performed 6 or 24 hours after testing helpless behavior to examine protein levels in an earlier time point and also when spine synapses were investigated. The graphs show synaptopodin protein levels 6 (A) and 24 hours (B) after testing learned helplessness. After 6 hours, the level of synaptopodin was reduced in the inescapable footshock (IES) group of P2X7 receptor (P2rx7)+/+ animals (**$P < .01$), and this change was maintained in 24 hours as well (*$P < .05$). However, the amount of PSD95 remained unaltered after administration of IES in both genotypes regardless the timing of sample preparation (C–D).

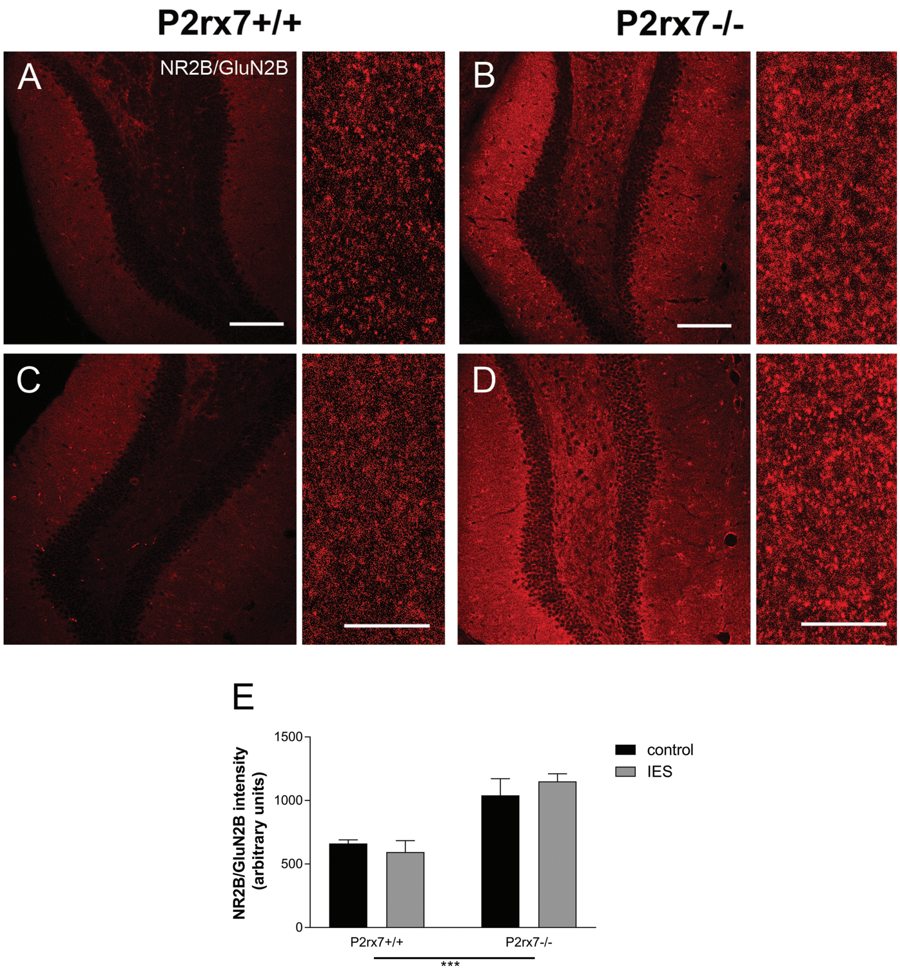

Figure 4. Genotypic difference of NR2B/GluN2B immunostaining in the dentate gyrus (DG) is present in mice involved in the learned helplessness model. Immunofluorescence staining for NR2B/GluN2B on sections from the DG of P2X7 receptor (P2rx7)+/+ (A, C) and P2rx7-/- mice (B, D) after exposed to testing helpless behavior; the staining had more intensity in the sections of P2rx7 deficient mouse, also visible in images acquired at higher magnification (right). Stronger immunofluorescence is present in the hilus region of P2rx7 knockout DG. Bars: 100 µm in original image with 20x magnification, 10 µm in 60x magnification. (E) Average intensity was quantified with NIH ImageJ software and is expressed in arbitrary units (*P < .05).

stereology, which is in line with literature data found in another rodent species (Hajszan et al., 2009). Others have investigated synaptic alterations in CA1 and CA3 regions as well, and CA3 seemed to be more resistant to stress, displaying minor reduction alone (Hajszan et al., 2009). The CA1 region of hippocampus had similar synaptic decline as in the DG; therefore, we decided to examine the latter area alone. Interestingly, P2rx7+/+ control mice, receiving footshocks only during the test period, showed reduced synaptic densities compared with the naïve values. This effect can be attributed to the stress caused by testing learned helplessness, since this phase also involved repeated footshocks, which could cause changes in the delicate mechanism of synaptic plasticity even after 24 hours. In the study of Hajszán et al (Hajszán et al., 2009), naïve animals were not included in the analysis, therefore we could not compare our results with literature data in this respect. As the dendritic spines investigated in our study belong to the proximal dendrites of the granule cells in the DG, we were interested in whether granule cells themselves are affected in the learned helplessness paradigm. We applied nuclei staining at the same time point (24 hours after testing), when spine synapses were examined, but no change in granule cell number was observed in either group of P2rx7+/+ or P2rx7-/- animals. Therefore change in spine synapse density was not a consequence of granule cell loss.

The principal new finding of this study is that learned helplessness behavior and consequent reduction in the spine synapse number in DG of hippocampus are absent in mice genetically deficient in P2rx7. In previous studies, there is agreement in the literature that the lack of P2rx7 results in an antidepressant phenotype using different paradigms to model aspects of depressive behavior (Basso et al., 2009; Boucher et al., 2011; Csolle et al., 2013a, 2013b; Iwata et al., 2015), and P2rx7 antagonist treatment reproduced the effect of genetic deletion. In the present study, we extended these findings to the learned helplessness paradigm. In P2rx7+/+ animals, IES triggered helpless behavior, meaning that mice were unable to avoid the aversive stimulus even if there was an opportunity to escape, because of the previous uncontrollable and unpredictable training. Because in our experiments IES groups were compared with their own control groups within each genotype, the behavior data of all animals are presented without any clustering according to predefined criteria. Hence, learned helplessness was defined as significantly increased escape failure and latency values in the IES groups compared with control groups, which is consistent with other studies (e.g., Hajszan et al., 2009; Dao et al., 2010; Schmidt et al., 2016). Learned helplessness was accompanied with a slight downregulation of P2rx7 mRNA. In chronic restrain stress model, reduced immunolabeling of P2rx7s was found in the hippocampus (Kongsui et al., 2014) and our findings with the learned helplessness paradigm are in line with this study. On the other hand, P2rx7-/- mice did not develop helpless behavior despite being exposed to IES, suggesting that P2rx7 regulates this particular depressive behavior. Although P2rx7 is sensitive to high concentrations of ATP, a recent study showed that extracellular ATP is elevated in a behaviorally relevant concentration in the brain in vivo in the chronic unpredictable stress model of depression (Iwata et al., 2015). Interestingly, escape failure and latency values were higher in the control group of P2rx7-/- mice compared with P2rx7+/+ littermates. This elevated baseline is apparently an inherent change accompanying the genetic inhibition of P2rx7, suggesting an attenuated response to external stress in P2rx7-/- mice in accordance with studies using another type of stress-inducing stimulus such as restraint stress (Csolle et al., 2013a; Li et al., 2016), ovariectomy (Xu et al., 2016), or psychological stress (Iwata et al., 2015). We have applied higher stress intensity in this learned helplessness model to show that P2rx7-/- mice are not saturated and able to respond to shock stress, and both control and IES groups of P2rx7-/- mice displayed active avoidance of shock with moderately reduced values of escape failure and latency (Figure 1E–F).

Behavioral changes were consistent with the electron microscopic stereology measurements. Helpless behavior in P2rx7+/+ mice was accompanied by loss of spine synapses in the DG, suggesting the effect of unpredictable stress on neural plasticity. In the absence of P2rx7, mice were resistant to helplessness, and IES did not elicit changes in spine synapse number.

In addition, to confirm disturbance of spine synapses, the hippocampal synaptic protein levels were also examined with western-blot analysis, and the general synapse marker PSD95 and dendritic spine specific synaptopodin protein quantities were defined 6 or 24 hours after testing learned helplessness. Synaptopodin is an actin-associated structural protein mainly present in spine bearing principal cells, essential in spine apparatus formation that is characteristic only to the most active subpopulation of spines (Noguchi et al., 2005). Therefore, it plays an important role in neural plasticity (Deller et al., 2007). Dendritic spines act as small compartments due to their thin neck, regulating the excessive excitatory inputs that would damage the neuron (Segal, 1995, 2005). The synaptic disturbance triggered by IES manifested in a decreasing amount of synaptopodin in the hippocampus, and this effect was detectable even after 24 hours, when spine synapse density changes were investigated as well. Since the most active spines contain synaptopodin, disruption of spine synapses due to stress could cause the significant decrease in synaptopodin protein levels. Finally, we could not detect change in PSD95 levels in the learned helplessness model; however, because this protein is widely distributed in all hippocampal synapses, the smaller variations due to treatments could be masked.

The pathogenesis of depression has been linked to changes in glutamatergic neurotransmission based on both human (Mitani et al., 2006; Hashimoto et al., 2007; Sanacora, 2008) and preclinical studies (Almeida et al., 2010; Zink et al., 2010). P2rx7 activation leads to glutamate release from hippocampal slices and a consequent decrease in hippocampal BDNF level, which is sensitive to the blockade of NMDA receptors containing the NR2B/GluN2B subunit (Sperlagh et al., 2002; Papp et al., 2004), which implies that P2rx7 activation downregulates BDNF level with the involvement of NR2B/GluN2B. A further consequence of this event could be changes in the plasticity of neurons, which is under the regulation of BDNF, such as adult neurogenesis in the granular layer (Csolle et al., 2013b) and/or a downregulation of spine synapses (present study). NR2B/GluN2B receptors are known to be involved in the regulation of depressive behavior. It has been shown that the rapid antidepressant effect of ketamine (Mathews et al., 2012; Miller et al., 2014; Williams and Schatzberg, 2016) is abolished in the tail suspension test in mice deficient of NR2B/GluN2B in principal cortical neurons (Miller et al., 2014), while the same effect is not changed in the absence of P2rx7 (F. Gölöncsér and B. Sperlágh, unpublished observation) indicating that NR2B/GluN2Bs responsible for this action are downstream from P2rx7 activation.

We also showed an upregulation of NR2B/GluN2B mRNA and elevated intensity of NR2B/GluN2B immunoreactivity in the hippocampus in the absence of P2rx7 (Csolle et al., 2013b), and we have reconfirmed this observation for the DG in this present study. The most likely explanation for the upregulation of NR2B/GluN2B in P2rx7-deficient mice is that it is a compensatory mechanism. Interestingly, however, we did not find alterations of NR2B/GluN2B subunit levels in the DG in response to IES treatment in the learned helplessness paradigm. Because NR2B/GluN2B receptors are uniformly expressed throughout the hippocampus (Shipton and Paulsen, 2014), without restriction to dendritic spines, the changes in their overall expression probably do not follow changes in their expression in the synaptic compartments, confirming the assumption that alterations found in our stereology study are specific to dendritic spine synapses.

In conclusion, our data are the first to show P2xr7-dependent synaptic alterations in the learned helplessness animal model of depression, suggesting a potential structural correlate of the antidepressant effect of genetic deletion of P2rx7. Furthermore, our findings confirm that P2rx7 could be a potential drug target in the treatment of mood disorders.

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Statement of Interest

None.

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