The absence of P2X7 receptors (P2rx7) on non-haematopoietic cells leads to selective alteration in mood-related behaviour with dysregulated gene expression and stress reactivity in mice

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Abstract

The purpose of this study was to explore how genetic deletion and pharmacological antagonism of the P2X7 receptor (P2rx7) alter mood-related behaviour, gene expression and stress reactivity in the brain. The forced swim test (FST), tail suspension test (TST) and amphetamine-induced hyperlocomotion (AH) tests were used in wild-type (P2rx7+/+) and P2rx7-deficient (P2rx7−/−) mice. Biogenic amine levels were analysed in the amygdala and striatum, adrenocorticotropic hormone (ACTH) and corticosterone levels were measured in the plasma and pituitary after restraint stress. Chimeric mice were generated by bone marrow transplantation. A whole genome microarray analysis with real-time polymerase chain reaction validation was performed on the amygdala. In the absence of P2rx7s decreased behavioural despair in the FST, reduced immobility in the TST and attenuated amphetamine-induced hyperactivity were detected. Basal norepinephrine levels were elevated in the amygdala, whereas stress-induced ACTH and corticosterone responses were alleviated in P2rx7−/− mice. Sub-acute treatment with the selective P2rx7 antagonist, Brilliant Blue G, reproduced the effect of genetic deletion in the TST and AH test in P2rx7+/+ but not P2rx7−/− mice. No change in behavioural phenotype was observed in chimeras lacking the P2rx7 in their haematopoietic compartment. Whole genome microarray analysis indicated a widespread up- and down-regulation of genes crucial for synaptic function and neuroplasticity by genetic deletion. Here, we present evidence that the absence of P2rx7s on non-haematopoietic cells leads to a mood-stabilizing phenotype in several behavioural models and suggest a therapeutic potential of P2rx7 antagonists for the treatment of mood disorders.

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Introduction

P2X7 receptors (P2rx7) belong to the ionotropic P2X receptors that are sensitive to ATP and other purine and pyrimidine nucleotides. The homo-oligomeric P2X7 (Surprenant et al. 1996) has distinct structural, functional and pharmacological features within the P2X receptor family (Jarvis & Khakh, 2009; Sperlah et al. 2006): (1) its intracellular carboxyl-terminal domain is longer than those of other P2X receptor subunits; (2) it has several splice variants that display different functionality in P2rx7+/+ and knockout mice lines (Nicke et al. 2009); (3) its persistent activation elicits the opening of a membrane pore permeable to
high molecular weight substances; (4) it needs high micromolar concentrations of ATP to be activated.

P2rx7s are widely distributed in different cells, including cells of haematopoietic origin, neurons, microglia and astrocytes. Accordingly, P2rx7s are involved in the regulation of different aspects of the host-defence reaction (Chen & Brosnan, 2006; di Virgilio et al. 2009). A major immunomodulatory function of P2rx7 activation is that it acts as a necessary co-stimulus for the post-translational processing and subsequent release of the pro-inflammatory cytokine interleukin (IL)-1β in peripheral immune cells in response to bacterial endotoxin (Ferrari et al. 2006; Solle et al. 2001).

The primary role of P2rx7s in the brain is the modulation of neurotransmitter release (Anderson & Nedergaard, 2006; Sperlagh et al. 2006, 2007a). The activation of P2rx7s elicits Ca2+ influx (Mirao-Portugal et al. 2003), followed by increased glutamate and subsequent γ-aminobutyric acid (GABA) release (Alloisio et al. 2008; Papp et al. 2004; Patti et al. 2006; Sperlagh et al. 2002). By regulating the activation and proliferation of microglia and the subsequent pathological process leading to neuronal death (Monif et al. 2009; Skaper et al. 2006), P2rx7s might also act as a ‘danger signal’ and contribute to neurodegenerative and neuroplasticity events underlying a variety of central nervous system disorders from Alzheimer’s disease to mood disorders (Burnstock, 2008; Skaper et al. 2010; Sperlagh et al. 2006).

Gene polymorphism studies have revealed that non-synonymous single nucleotide polymorphisms in the human P2X7 gene (P2RX7) affect receptor function (Roger et al. 2010; Stokes et al. 2010) and are associated with bipolar disorder (Barden et al. 2006; Hejjas et al. 2009; McQuillin et al. 2009; Nagy et al. 2008; Soronen et al. 2011) and major depressive disorder (Lucae et al. 2006; Soronen et al. 2011; but see Green et al. 2009; Grigoroiu-Serbanescu et al. 2010; Lavebratt et al. 2010; Viikki et al. 2011). These mutations might underlie the susceptibility to genetically related mood disorders (Harvey et al. 2007; Sluyter et al. 2010). Moreover, recent studies found an antidepressant phenotype in mice genetically deficient in P2rx7s (Basso et al. 2009; Boucher et al. 2011). Nevertheless, it is not known, how P2rx7 affects mania-like behaviour and whether behavioural alterations detected in the absence of P2rx7s can be reproduced by pharmacological blockade. In addition, the mechanism, whereby the activity of P2rx7 leads to alterations in mood also remains to be explored.

We report here that, in addition to the known antidepressant-like effect, the genetic deletion of P2rx7 (P2rx7−/−) results in a mood stabilizing-like phenotype and P2rx7 antagonists reproduce these behavioural changes. The lack of P2rx7s also leads to decreased stress response to restraint stress and region-specific alterations in brain monoamine levels, in particular in the amygdala, an intrinsic part of the limbic system. Experiments performed on bone marrow chimeric mice demonstrated that behavioural alterations detected in P2rx7−/− mice are not related to P2rx7s on haematopoietic cells. Therefore, a whole genome microarray analysis was performed in the amygdala, which revealed widespread alterations in the expression of genes responsible for synaptic signalling and neuroplasticity, in the absence of P2rx7s.

Method

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 IEM HAS. This study used 2- to 3-month old (approx. 30 g), drug- and test-naive male wild-type (P2rx7+/+) and knockout mice (P2rx7−/−), which were housed in a light-controlled (12-h light/dark cycle; lights on 07:00 hours), humidity-controlled (60 ± 10%) and temperature-controlled (23 ± 2 °C) room with food and water available ad libitum. Homozygous P2rx7+/+ mice 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., USA). The animals contained the DNA construct [P2RX7-F1 (5′-CGGCCGTGGCTTTTGACATCGT-3′) and P2RX7-R2 (5′-AGGGCCCCGTGGTCTTCG-3′), previously shown to produce the genetic deletion of P2rx7 (Solle et al. 2001)]. Offspring of this mouse line were cross-bred with P2rx7+/+ mice and the resulting heterozygotes were used as breeding stock for the F1 generation offspring employed in the behaviour studies. The genotypes of animals were confirmed by polymerase chain reaction (PCR) analysis, as described previously (Solle et al. 2001). Mice carrying the CD45.1 allele on the C57BL/6J genetic background were obtained from the Jackson Laboratory (USA).

Behaviour experiments

Mice establish strong dominance hierarchies in both their natural habitats and the laboratory (Capanna et al. 1984; Ferrari et al. 1998; Poshivalov 1980). Therefore, to avoid confounds from social status, subjects were kept in individual cages (15 × 45 × 20 cm) for 1 wk...
before experimentation. In contrast to rats, isolation does not increase but slightly decreases depression-like behaviour in mice, at least in the Swiss strain of this species (Hilakivi et al. 1989).

All experiments and treatments were carried out during the light phase (07:00–19:00 hours). P2rx7+/+ and P2rx7−/− mice were submitted to testing in alternation. Except for the elevated plus maze (EPM) test, which immediately followed the open field (OF) test, the animals used in this study were subjected to behaviour tests only once. Treatments with the P2rx7 antagonist, Brilliant Blue G (BBG; Sigma-Aldrich, Hungary) or the selective serotonin reuptake inhibitor, citalopram (Sigma-Aldrich), were applied i.p. 30 min before testing. The doses were chosen based on literature data (Diaz-Hernandez et al. 2009) and preliminary tests.

In the forced swim test (FST), the OF test and the EPM test, behaviour was video-recorded and analysed later with a computer-based event recorder by an experimenter blind to the genotype.

**Forced swim test**

Behavioural despair was analysed as described previously (Porsolt et al. 1977). Each mouse was placed in a transparent glass cylinder filled with water (22 ± 0.5 °C) and submitted to 15 min of forced swim, divided into three equal trial periods (5 min each) and followed by a single 5-min test period the next day. Fresh water was used for each mouse and four animals were tested simultaneously. After the test period, the mice were removed from the cylinder, dried with paper towels and a clean towel was left in the home cage for 1 h to avoid cooling. The time of immobility (floating) was expressed in seconds.

**Automated tail suspension test (TST)**

This test was performed using an automated tail suspension device (BIO-TST2, Bioseb, France), following the method described in Cryan et al. (2005), which was connected to a computer that recorded the activity of the animals during test sessions. Three mice were individually suspended by the tail onto the hooks of the device using adhesive tape (distance from tip of tail was 1–2 cm). The automatic measurements were started within 5–10 s after placing the last animal into the chamber and each measurement lasted 6 min. During the test, the animals showed several escaping behaviours with temporary periods of immobility. The threshold was set at level 6. The time of immobility was expressed in seconds. In accordance with the observations of Mayorga & Lucki (2001), a proportion of the animals (8.9% on average, 0–16%, depending on the experiment) displayed tail-climbing behaviour. The data of these animals were post-hoc excluded from the calculations.

**OF test and EPM test**

For technical details, see Supplement 1 (available online).

**Amphetamine-induced hyperlocomotion (AH) in the OF test**

Experiments were performed in the light phase under dimmed lights (~3 lx). At least 3 d before the tests, animals were transferred to the experimental room.

Each animal was placed in the centre of a non-transparent Plexiglas arena (dimensions: 40 × 40 × 40 cm) for a habituation period of 30 min and then removed for 2 min into their home cages for i.p. saline or d-amphetamine-sulfate (equivalent to 2.5 mg/kg free base, A5880; Sigma-Aldrich, Hungary) treatment. Immediately after amphetamine (or saline) injection, each mouse was placed back into the box and the locomotor activity of the animals was recorded for 90 min using a video camera positioned above the arena. To measure locomotor activity, video files were analysed offline by converting them into single frames (25 frames/s) and a custom-written motion tracking algorithm was applied within the image processing software ImageJ. The total distance (m) was provided for the 90 min of the experiment. Hyperactivity induced by amphetamine was expressed in percentage of locomotor activity measured in saline-treated mice under an identical period.

**IL-1β experiments**

All animals were given an i.p. injection of sterile saline (0.9% NaCl) or bacterial lipopolysaccharide (LPS, 250 µg/kg i.p.) with an injection volume of 0.1 ml/mouse). Animals were killed by decapitation 6 h after LPS injection. The amygdala were collected, frozen on dry ice and stored at −70 °C until further investigation. The IL-1β assays were performed as described previously (Csöllé & Sperlagh, 2010).

**High performance liquid chromatography (HPLC) analysis of endogenous biogenic amine levels**

After various treatments (saline/2.5 mg/kg amphetamine i.p.; 30 min restraint; saline/50 mg/kg, d BBG i.p. for 7 d), animals were killed by decapitation and native amygdala and striata were frozen in liquid nitrogen. The weighted frozen tissue was
homogenized in ice-cold 0.1 M perchloric acid containing theophylline (10 μM; internal standard) and 0.5 mM sodium metabisulphite. The suspension was centrifuged at 300 g for 10 min at 4 °C. The perchloric anion was precipitated by addition of 1 M KOH, removed by centrifugation and the protein content of the pellet was determined according to the method of Lowry et al. (1951). The supernatant was kept at −20 °C until analysis. Biogenic amines were measured by a liquid–liquid, two-dimensional reversed phase and ion pair-reversed phase chromatographic separation, as described earlier (Baranyi et al. 2006) using a Gilson liquid chromatographic system (Gilson Medical Electronics Inc., USA) equipped with an Applied Biosystems 785/A UV and BAS CC-4 amperometric detector in a cascade line. Data were expressed as pmol per mg protein.

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[H]Dopamine release experiments
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\[H\]Dopamine release experiments on acute striatal slices were performed according to the method described in Milusheva et al. (2010). For the detailed protocol of release experiments, see Supplement 2.

Stress studies

Restraint stress consisted of placing mice for 30 min in ventilated polyethylene tubes (inner diameter: 2.5 cm; length: 10 cm), closed with plastic tape (Lolait et al. 2007), and was immediately (within 15 s) followed by decapitation. Controls were decapitated at the same time under basal conditions. Trunk blood was collected in ice-cold sodium-EDTA (20%) during the light phase between 09:00 and 13:00 hours and centrifuged for 10 min at 4 °C. The plasma was stored at −20 °C until the hormone assays were performed. The pituitaries were dissected into four pieces and incubated at 37 °C in 1 ml Dulbecco’s minimal essential medium (DMEM, pH 7.4; Sigma) containing 2.5% bovine serum albumin in a 95% O₂/5% CO₂ atmosphere (one gland/tube) for 60 min. After a further pre-incubation with fresh DMEM for 60 min, 15 min samples were collected three times with corticotropin-releasing hormone (CRH)-containing (5 × 10⁻⁸ M; Sigma) medium in the second fraction.

Hormone assays

Adrenocorticotropic hormone (ACTH) and corticosterone in the trunk blood and in acutely isolated pituitaries were measured by radioimmunoassay in 50-μl unextracted samples as described previously (Zelena et al. 2008).

Bone marrow chimeras

Bone marrow chimeras were generated by transplantation of P2rx7+/+ or P2rx7−/− bone marrow cells (carrying the CD45.2 allele) into lethally irradiated recipients carrying the CD45.1 allele as described previously (Jakus et al. 2009). The repopulation of the peripheral leukocyte compartment by donor-derived cells was tested 8 wk after transplantation by flow cytometric analysis of blood samples stained for the granulocyte-specific Gr1 and the donor-specific CD45.2 markers (Jakus et al. 2009). Percent repopulation was defined as the percentage of CD45.2-positive cells among Gr1-positive cells with typical forward and side-scatter characteristics.

Preparation of microglia

Microglia was prepared following the method described by Racz et al. (2008), with slight modifications. Briefly, single-cell suspensions from mouse brain were generated using a commercially available enzymatic kit (Miltenyi Neural Tissue Dissociation kit ‘P’; Miltenyi Biotec, Germany) and filtered through an 83-μm sieve. Percoll (GE Healthcare, UK) gradients of 75/25% were performed for fractionation of cells at 800 g for 25 min and 800 g for 10 min and brain mononuclear cells were collected from the interface.

Immunohistochemistry

Light and electronmicroscopic immunostaining for P2rx7s and the microglia marker CD11b was performed using standard protocols described earlier (Csölle et al. 2008). For the detailed protocol of immunohistochemistry, see Supplement 3.

Microarray experiments and real-time PCR validation

Mice were randomly assigned to experimental groups and injected i.p. with sterile saline (0.9% NaCl) or LPS (250 μg/kg) and killed by decapitation 6 h after injection. Microarray profiling on amygdala samples was performed in the Agilent Microarray Core Facility (Semmelweis University, Budapest; http://www.dgci.sote.hu/microarray). The complete microarray expression data are deposited at NCBI’s Gene Expression Omnibus (Edgar et al. 2002) through GEO series accession number GSE21218 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token= rwxlikqskwayfk&acc=GSE21218). The microarray data were further analysed using the bioinformatics analysis tool GeneCodis (Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009).
Real-time PCR validation

TaqMan low-density arrays (TLDA microfluidic card; Applied Biosystems, USA) were performed on 92 selected genes of interest and four housekeeping genes (18S rRNA, Gapdh, Hprt, B2m) (Vandesompele et al. 2002).

See Supplement 4 for the technical details of the microarray and real-time PCR experiments.

Statistics

All data shown are the means ± s.e.m. of n determinations. OF and EPM data were evaluated by analysis of variance (ANOVA). TST and AH data were analysed by one-way ANOVA followed by Dunnett’s test. Hormone levels, monoamine contents, IL-1β, microarray and PCR data were analysed by two-way ANOVA. The FST and in vitro ACTH secretion was analysed by repeated measures ANOVA. The Student’s t test and Fischer’s LSD test were used for pairwise comparisons. Unless otherwise stated, statistical analyses were performed using the Statistica program (Statsoft, USA). The level of significance was set at p < 0.05.

Results

Genetic deletion of P2rx7 leads to mood-stabilizing behavioural phenotype

To model depressive-like behaviour, occurring during major depressive disorder and the depressive pole of bipolar disorder, FST and TST were used. In the FST, we have used the widely used behavioural despair paradigm, originally described by Porsolt et al. (1977), which involves subjects being forced to swim twice on two consecutive days. P2rx7+/+ mice displayed a time-dependent increase in the time of immobility during consecutive 5-min periods of the first trial, indicating that they developed behavioural despair, a well-known depressive-like behaviour (Fig. 1a). P2rx7−/− mice were less prone to develop depression-like behaviour than P2rx7+/+ mice (Fig. 1a). During the first trial, the duration of immobility was similar in the two genotypes (92.43 ± 21.12 s, for wild-type vs. 118.38 ± 16.46 s for P2rx7−/−; genotype: F1,18 = 0.05, p < 0.8). In P2rx7+/+ mice, the time spent in immobility increased over time during the first trial (F3,54 = 3.61, p < 0.02). In P2rx7−/− mice, parallel changes were seen, but these were not significant (F3,54 = 0.30, p > 0.8). When the two genotypes were considered together, the time × genotype interaction was significant (F3,54 = 2.92, p < 0.04) and significant time-related changes were seen in P2rx7+/+ mice only.

The basal time of immobility in the TST was 167.90 ± 17.13 s (n = 9) in P2rx7+/+ mice (Fig. 1b). In line with previous data (Basso et al. 2009), P2rx7−/− mice spent significantly less time with immobility (i.e. they exhibited antidepressant-like phenotype in this test as well; 110.24 ± 15.84 s, n = 11, p < 0.05, Fig. 1b). Neither basal locomotor activity nor anxiety-like behaviour, examined in the OF and EPM tests, was affected by the genetic disruption of the P2rx7 gene (Supplementary Fig. 1a, b).

As a behavioural model of manic pole of bipolar disorder (Einat, 2007), the AH test was used in our study. The locomotor activity of saline-treated mice, expressed as the total covered distance during the test period, was similar in P2rx7+/+ and P2rx7−/− mice (120.17 ± 24.71 m, n = 9 and 156.53 ± 18.48 m, n = 10, respectively, p > 0.05). By contrast, amphetamine (2.5 mg/kg i.p.) induced hyperactivity was significantly attenuated in mice genetically deficient in P2rx7 (Fig. 1c).

Neurochemical changes and stress-induced hormone response in the absence of P2rx7

The level of IL-1β 6 h after saline administration was 6.06 ± 1.18 pg/ml in the amygdalae of P2rx7+/+ mice (Fig. 1d, n = 4). Systemic LPS administration (250 µg/kg i.p.) caused a significant elevation in IL-1β levels (83.94 ± 3.01 pg/ml, n = 8, F1,18 = 45.2, p < 0.001, Fig. 1d). The IL-1β level in the amygdalae of saline-treated P2rx7−/− mice was 5.29 ± 1.48 pg/ml (n = 4), not significantly different from P2rx7+/+ mice (p > 0.05, Fig. 1d). The LPS-induced elevation of IL-1β level was significantly attenuated in the amygdalae of P2rx7−/− mice (32.53 ± 1.55 pg/ml, n = 8; genotype: F1,18 = 183.3, p < 0.001; interaction: F1,18 = 42.55, p < 0.001, Fig. 1d).

To explore neurochemical correlates of changes in behaviour, the level of norepinephrine, dopamine and serotonin (5-HT) was also analysed in the amygdala and striatum of saline and amphetamine (2.5 mg/kg i.p.) treated P2rx7+/+ and P2rx7−/− mice. The basal level of norepinephrine was elevated in the absence of P2rx7 in the amygdala (genotype: F1,17 = 3.30, p = 0.08; interaction: F1,17 = 6.52, p = 0.016; post-hoc comparison by Fischer’s LSD test, p < 0.005), whereas no change in basal norepinephrine level was found in the striatum (29.37 ± 13.79, n = 7 and 18.21 ± 3.83 pmol/mg protein, n = 8 in P2rx7+/+ and P2rx7−/− mice, respectively; interaction: F1,17 = 0.05, p > 0.8). Likewise, basal dopamine and 5-HT levels were not affected by the genetic deletion either in the amygdala (Fig. 2a–c,
Fig. 1. Genetic deletion of P2rx7s in mice leads to an antidepressant-like phenotype in the forced swim test (FST) and tail suspension test (TST) and mood stabilizing-like phenotype in amphetamine-induced hyperlocomotion test (AH), but does not affect basal interleukin (IL)-1β levels in the amygdala. (a) P2rx7−/+ mice failed to develop the depression-like behaviour typical to the FST. The time of immobility is expressed in seconds. * Indicates significant changes from immobility values observed during min 0–5 of the first day, n = 20/group; p < 0.05. (b) Genetic disruption decreased basal immobility in the TST (n = 9–11, * p < 0.05 vs. P2rx7+/+, Student’s t test). The immobility time is expressed in seconds. The total test period was 360 s. (c) Amphetamine-induced hyperactivity is significantly attenuated in P2rx7−/− mice. Mice were placed into the open field arena for a 30-min habituation period and then injected with i.p. saline (Sal) or ν-amphetamine-sulfate (2.5 mg/kg). Locomotor activity was assessed for 90 min immediately after the injection and expressed as the percentage of Sal-treated mice, n = 9–10, *** p < 0.001 vs. P2rx7+/+. ** Student’s t test. Drug- and test-naive male homozygous mice (P2rx7+/+ and P2rx7−/−, aged 2–3 months) weighing approximately 30 g were used in the experiments. (d) IL-1β protein level in the amygdala of P2rx7+/+ and P2rx7−/− mice after Sal and lipopolysaccharide (LPS) treatment. The IL-1β protein level was similar in the amygdalae of Sal-treated P2rx7+/+ and P2rx7−/− mice. Injection of LPS (E. coli; 250 μg/kg i.p.) significantly increased the level of IL-1β in the amygdalae of P2rx7−/− mice 6 h after treatment. IL-1β protein level was less elevated in the amygdalae of P2rx7−/− mice in response to systemic endotoxin. The levels of IL-1β were quantified in the supernatants by ELISA. Data are given as the mean level of cytokines ± S.E.M., expressed in pg/ml. * Indicates significant differences between Sal- and LPS-treated and between P2rx7+/+ and P2rx7−/− mice (n = 4 per group, *** p < 0.001, two-way analysis of variance).

|                      | P2rx7+/+ | P2rx7−/− |
|----------------------|----------|----------|
| Immobility time (s)  | 0–5      | 0–5      |
|                      | 1        | 1        |
|                      | 2        | 2        |
|                      | 0–5–10   | 0–5–10   |
|                      | 15–20    | 15–20    |
|                      | 30–45    | 30–45    |

The levels of dopamine and 5-HT were not affected by genotype (Fig. 3a, b;
ACTH: $F_{1,34} = 0.1$, $p > 0.9$; corticosterone: $F_{1,33} = 1.06$, $p > 0.3$. Restraint stress increased the level of both hormones (ACTH: $F_{1,34} = 6.9$, $p = 0.013$; corticosterone: $F_{1,33} = 272.7$, $p < 0.001$), but the elevation of ACTH levels reached the level of significance in $P2rx7^{+/+}$ animals only ($p = 0.03$; Fig. 3a). In addition, the corticosterone increase was significantly lower in $P2rx7^{-/-}$ mice, than in $P2rx7^{+/+}$ mice ($p = 0.048$; Fig. 3b). In isolated in vitro pituitaries, CRH treatment significantly increased ACTH secretion (Fig. 3c). However,
P2rx7 reached the level of significance in level of both hormones, but the elevation of ACTH levels not affected by genotype. Restraint significantly increased the restraint. Plasma levels of ACTH (stress. Hormonal secretion was evaluated after 30 min of increased norepinephrine (NE) level (d). The ACTH response to 5 min restraint significantly increased ACTH secretion. This effect and corticosterone in the plasma (decreased elevation of adrenocorticotropic hormone (ACTH) treatment significantly increased ACTH secretion. This effect difference between P2rx7 expressed as pmol/mg protein. * Represents a significant P2rx7 liquid chromatography in the amygdalae of P2rx7 mice (Fig. 3). After restraint stress, norepinephrine, dopamine and 5-HT levels were all elevated in the amygdala of P2rx7+/− mice when compared to P2rx7+/+ littermates (Fig. 3d).

**P2rx7 antagonist treatment reproduces the effect of genetic deletion**

Next, we asked whether the behavioural phenotype detected in P2rx7−/− mice could be reproduced by the systemic administration of a P2rx7 antagonist. BBG, a selective P2rx7 antagonist, was ineffective upon acute application (50 mg/kg i.p.), but it dose-dependently (25–50 mg/kg i.p.) inhibited depressive-like behaviour after a 1-wk sub-acute treatment in the TST (Fig. 4a, b). Citalopram (acute treatment, 15 mg/kg i.p.), which was used as a reference compound, elicited a 40.6% decrease in the immobility time in the TST in P2rx7+/+ mice (Fig. 4c). Sub-acute (Fig. 4d), but not acute (data not shown) BBG treatment (25–50 mg/kg i.p.) also reproduced the effect of genetic deletion in the AH test and elevated norepinephrine levels in the amygdala (Fig. 4g). When sub-acute BBG treatment was applied in P2rx7−/− mice, no significant change either in immobility in the TST (Fig. 4e) or in AH (Fig. 4f) was detected.

**Alteration of behavioural phenotype of P2rx7−/− mice is not related to P2rx7s expressed on cells of haematopoietic origin**

To test whether the alteration of behavioural phenotype detected in P2rx7−/− mice was due to the lack of P2rx7s on cells of haematopoietic origin, we generated bone marrow chimeras that lacked the P2rx7 only in their haematopoietic compartment (Fig. 5a). Bone marrow cells harvested from P2rx7+/+ or P2rx7−/− mice (expressing the CD45.2 leucocyte marker) were injected i.v. into lethally irradiated congenic recipients carrying the CD45.1 marker. The efficiency of the reconstitution was tested 8 wk after transplantation by flow cytometric analysis of the expression of the donor-specific CD45.2 marker in peripheral blood leukocytes. As shown in Fig. 5b, CD45.2 was present on granulocytes of the donor strain but not on those of the recipient strain. Importantly, granulocytes isolated from bone marrow chimeras used for the behavioural studies, engrafted with either P2rx7+/+ or P2rx7−/− bone marrow cells, were practically exclusively (>99%) of donor (CD45.2-positive) origin. The recruitment of donor-derived cells was also assessed by immunocytochemical staining of P2rx7s in microglia cells isolated from the brains of intact mice and bone marrow chimeras. As shown by fluorescent and electron microscopic images, the cell membranes of
microglia (i.e. cells positive for the microglia marker CD11b) of intact P2rx7+/+ mice and of bone marrow chimeras engrafted with P2rx7+/+ bone marrow cells (CD45.1/P2rx7+/+) showed intense P2rx7 immunoreactivity (Fig. 5c, e). Because not all microglia are positive for CD11b and not only microglia express the P2rx7, CD11b-negative/P2rx7-positive and CD11b-positive/P2rx7-negative cells were also found in the preparation. In contrast, no P2rx7 immunoreactivity was observed on microglial cells isolated from intact P2rx7−/− mice (Fig. 5d). Microglia cells of bone marrow chimeras engrafted with P2rx7−/− bone marrow cells (CD45.1/P2rx7−/−) also displayed scarce P2rx7 immunoreactivity, whereas other non-haematopoietic cells of the preparation were stained with the antibody against the P2rx7 (Fig. 5f). Bone marrow chimeric subjects were subjected to the TST and AH as described above. No difference was found in the time of immobility in either the TST (Fig. 5g) or in the hyperlocomotion in the AH (Fig. 5h), showing that the mood stabilizing-like phenotype found in P2rx7−/− mice was not transferred to P2rx7+/+ recipients with the engraftment of the P2rx7−/− bone marrow cells.

**Alteration of the gene expression profile in the amygdala of P2rx7−/− mice**

Because previous experiments and literature data indicated that monoamine transmitter levels and c-Fos expression (Boucher et al. 2011) are regulated by P2rx7 in the amygdala, we next examined how global gene expression is changed in the absence of P2rx7 in this region and LPS treatment was used as a reference stimulus. Four amygdala samples/group collected from P2rx7+/+ and P2rx7−/− mice 6 h after LPS (250 μg/kg i.p.) or saline treatment were subjected to whole genome microarray analysis. Out of the 41041 transcripts printed on the microarrays, the expression of a total of 8739 transcripts was significantly altered with the applied statistical filters (>2.0-fold change; p < 0.05; Benjamini-Hochberg’s multiple correction; Fig. 6a). These transcripts were sorted according to their main effect, namely, genotype and treatment, as illustrated in the Venn diagram in Fig. 6a. Deficiency of P2rx7 had the most profound effect on the expression profile, changing the expression of 8448 transcripts (3133 increased, 5315 decreased, Fig. 6b) independently from the treatment, followed by the effect of LPS, which affected the expression of 570 transcripts, (415 increased, 155 decreased) independently from the genotype. Four transcripts, as illustrated by the red/blue/green intersection in the Venn diagram, were differentially altered by the treatment, depending on the genotype.

To further understand the functional impact of the biological processes affected by P2rx7 deficiency, microarray data were further subjected to gene ontology analysis. Because the total number of modified genes affected only by P2rx7 deletion (8165) was relatively large, 588 genes showing the top rank of up- and down-regulation were selected for gene ontology analysis by GeneCodis (Supplementary Table S2a). This allowed us to investigate the association of the 588 genes using the defined algorithm to determine biological annotations or combinations of annotations that were over-represented with regard to a reference list of *Mus musculus* deposited in the NCBI GenBank database. The GeneCodis analysis generated a relationship of 373 annotation groups characterized with the Gene Ontology (GO) Biological Process, including transport, ion transport, signal transduction, synaptic transmission, G-protein coupled receptor protein signalling pathway, ATP synthesis coupled proton transport, transcription, regulation of transcription and GABA signalling pathway (Supplementary Table S2b, c). The genes involved in each annotation group are listed in Supplementary Table S2 with reference to their involved GO categories. The annotation groups were further sorted using a permutation test for p value correction.

Using the data generated by the GO analyses and searching biological plausibility for depression (e.g. synaptic transmission and neuroplasticity), we chose 60 genes for further validation out of the 8165 transcripts that were changed significantly by P2rx7 deficiency, regardless of the effect of LPS treatment, and showed a fold change (FC) of ≥2.0 (Table 1). Among the 287 transcripts that had a significant LPS treatment effect, regardless of the effect of genotype, 17 transcripts were selected that had biological plausibility in inflammatory and immune responses (Table 2).

Microarray experiments were validated by assessing the expression of genes of interest using quantitative TLDA real-time PCR, applying both sample and treatment validation. Significant down- or up-regulatory effects of P2rx7 deficiency were confirmed for 29 genes (Table 1a,b), which corresponded to 48% of the total genes tested by real-time PCR. Among these genes, 25 genes were down-regulated in P2rx7−/− mice, including genes encoding various subunits of the GABA<sub>A</sub> receptor (*Gabrb2, Gabrb3, Gabrg2, Gabrg3*), ionotropic glutamate receptors (*AMPA2, AMPA4*), the metabotropic glutamate receptor 7 (*Grm7*), the α₂ adrenergic receptor (*Adra2a*), the CB<sub>1</sub> cannabinoid receptor (*Cnr1*), the D<sub>4</sub> dopamine
Fig. 4. The selective P2rx7 antagonist Brilliant Blue G (BBG) exhibits antidepressant-like activity in the tail suspension test (TST), decreases amphetamine-induced hyperactivity and elevates norepinephrine (NE) level in the amygdala in P2rx7+/+, but not P2rx7−/− mice. (a) The effect of acute BBG treatment on basal immobility in P2rx7+/+ mice (n = 13–15). (b) Effect of sub-acute, 1-wk treatment with BBG on basal immobility in P2rx7+/+ mice. Mice were treated with BBG for 7 d with the daily doses indicated on the abscissa or with saline (Sal) and then submitted to the TST (n = 13–15, * p < 0.05 vs. Sal, one-way analysis of variance (ANOVA), followed by Dunnett’s test). (c) Citalopram (Cit; 15 mg/kg), the potent selective serotonin reuptake inhibitor...
receptor (Drd2), dopa decarboxylase (Ddc) and sub-units of the glycine receptor (Gln2, Ghrb; Table 1a). In contrast, four validated genes of the 29, including β bradykinin receptor (Bdkrb1) and the NMDA2B ionotropic glutamate receptor (Grin2b), were up-regulated (Table 1b). All genes selected for the validation of endotoxin treatment were significantly up-regulated in the real-time PCR assay (Table 2).

Discussion

According to the current view, major depressive disorder and bipolar disorder are caused by plastic alterations of distributed networks involving multiple neurotransmitters and pathways (Stone et al. 2008). There is still much controversy concerning the changes in the brain that underlie the therapeutic actions of antidepressants and mood stabilizers. Moreover, despite the emerging knowledge of the pathophysiology of mood disorders, a relatively high proportion of patients do not respond to existing medications (Martinowich et al. 2009; Sanacora et al. 2008), which urges the discovery and validation of new potential therapeutic targets. One such target could be the P2rx7s. Therefore, we tested this hypothesis by the evaluation of the genetic deletion and pharmacological antagonism of P2rx7s in several behavioural paradigms used for testing antidepressants and mood-stabilizing drugs. P2rx7−/− mice did not develop behavioural despair in the FST and reduced immobility was detected in the TST. In addition, we report here for the first time an attenuated response of P2rx7−/− mice in the AH test, a widely used model of the manic pole of bipolar disorder. Because there was no difference between the two genotypes in the OF and EPM tests, the observed changes in behaviour could not be accounted for by changes in spontaneous locomotor activity or anxiety. These observations confirm and extend previous data, which showed decreased immobility in the TST (Basso et al. 2009) and decreased floating time in the FST on the second day (Boucher et al. 2011) after genetic deletion. However, partly differing from the study of Basso et al., in our experiments there was no significant difference in the basal immobility in the FST between the two genotypes, which might be explained by different housing conditions and purely homozygous mouse strain of a different origin used by the above study.

Changes in behaviour were accompanied by corresponding alterations in brain monoamine levels in the amygdala and the striatum of P2rx7−/− mice. In line with the decreased behavioural despair in the FST and decreased immobility in the TST, an increase in basal norepinephrine level was found in the amygdala, which could be reproduced by P2rx7 antagonist treatment. As a neurochemical correlate of decreased hyperlocomotion, amphetamine-induced elevation of dopamine content and release in the striatum were also alleviated in P2rx7−/− mice.

P2rx7−/− mice responded with a decreased elevation of plasma ACTH and corticosterone in response to restraint stress, which suggests that P2rx7−/− mice react with an attenuated response to external stress, the opposite of what is found in depressive patients. Functional P2rx7s are expressed on pituitary cells (Chung et al. 2000), which, together with our in vitro results, suggests a direct ATP effect on ACTH secretion. Nevertheless, an indirect action through other neuronal pathways converging on the hypothalamic–pituitary–adrenal axis cannot be excluded either. Interestingly, norepinephrine, dopamine and 5-HT levels were higher in the amygdala of P2rx7−/− mice after restraint, which indicates an interaction between stress and the brain monoaminergic system, which is regulated by P2rx7.

Importantly, we could reproduce the effect of genetic deletion by the systemic application of a specific antagonist of P2rx7s. BBG is a non-toxic and fairly selective antagonist of P2rx7s, which is able to penetrate the blood–brain barrier (Peng et al. 2009). Although it was active only using a sub-acute application, BBG dose-dependently decreased immobility.
Fig. 5. Mood-stabilizing phenotype is not detectable in chimeric mice transplanted with the bone marrow of P2rx7−/− mice. (a) General scheme of bone marrow transplantation. (b) Flow cytometric analysis of the expression of the donor-specific CD45.2 allele on peripheral blood granulocytes of an intact C57BL/6j mouse (donor), a CD45.1-expressing congenic mouse (recipient) and bone marrow chimeras engrafted with P2rx7+/+ and P2rx7−/− bone marrow cells (P2rx7+/+ and P2rx7−/− chimeras, respectively). (c–f) Immunocytochemical demonstration of the P2rx7 in mouse microglial cells. Co-localization of P2rx7s (labelled red) with microglial labelling CD11b (green) was found in microglia cells retrieved from P2rx7+/+ mouse brain tissue (c) and in chimeras transplanted with P2rx7+/+ bone marrow [(c) CD45.1/P2rx7+/+, (e) CD45.1/P2rx7−/−]. Microglial cells of P2rx7−/− animals (d) did not show P2rx7 immunoreactivity. In chimeras transplanted with P2rx7−/− bone marrow [(f), CD45.1/P2rx7−/−], the P2rx7 (labelled red) was not found on microglial cells (labelled green) but was in some unidentified cells and cell debris in the cell suspension/fraction. Electron microscopy supported this finding. The cell membrane of morphologically characterized microglial cells – dark nuclei, either oval or bean shaped, electron dense cytoplasm, long cisternae of granular endoplasmic reticulum and large inclusions of phagocytosed material in the cell bodies that
in the TST and attenuated hyperactivity elicited by amphetamine in \( P2rx7^{+/+} \) mice. Consistent with the involvement of \( P2rx7 \) in these effects, an identical BBG treatment was ineffective in both tests in \( P2rx7^{-/-} \) mice.

Recent studies have shown that certain behavioural patterns, such as pathological grooming, can be transferred from one animal to another by immune cell transplantation (Chen et al. 2010). Circulating cytokines, including IL-1\( \beta \), are important mediators of depressive-like behaviour (Dantzer et al. 2008) and, therefore, peripheral IL-1\( \beta \) could be a mediator of \( P2rx7 \) activation on mood-related changes. In bone marrow chimera mice transplanted with the \( P2rx7^{-/-} \) bone marrow, the changes in behaviour in the TST and AH could not be reproduced despite the >99% reconstitution of donor cells among recipient leukocytes. These data suggest that the lack of \( P2rx7 \) expressed on peripheral immune cells is not responsible for the behavioural phenotype found in \( P2rx7^{-/-} \) mice.

The majority of brain microglial cells derived from \( P2rx7^{-/-} \) mice were negative for the \( P2rx7 \), are commonly found in old animals – were covered by diaminobenzidine precipitates, demonstrating \( P2rx7 \) immunoreactivity (c and e rows). Cell processes of unidentified cells also showed \( P2rx7 \) immunoreactivity (e). Microglial cells of \( P2rx7 \) gene knockout animals (d) or microglial cells from CD45.1/\( P2rx7^{-/-} \) animals (f) were free of precipitate.

(g, h) Basal immobility time in the tail suspension test (g) and amphetamine-induced hyperactivity (h) were not different in CD45.1/\( P2rx7^{+/+} \) and CD45.1/\( P2rx7^{-/-} \) mice. Mice were submitted to behaviour tests 8 wk after engraftment (\( n = 13–14 \) /group).
Table 1. Genes that were up- and down-regulated by the deficiency of P2rx7 in the mouse amygdala

| Symbol | Gene ID | Gene name (accession number) | Agilent (FC) | Real-time PCR (RQ) | TaqMan ID |
|--------|---------|-------------------------------|--------------|--------------------|-----------|
| Adra2a | 11 551  | Adrenergic receptor α2a (NM_007417) | 2.72         | 1.34 ± 0.38        | Mm00845383_s1 |
| Adrb1  | 11 554  | Adrenergic receptor β1 (NM_007419) | 4.77         |                    | Mm00431701_s1 |
| Ak5    | 229 949 | Adenylate kinase 5 (NM_001081277) | 5.71         |                    | Mm00461978_m1 |
| Aplp2  | 11 804  | Amyloid β (A4) precursor-like protein 2 (NM_009691) | 5.55 | | Mm00507819_m1 |
| Bdhf   | 12 064  | Brain derived neurotrophic factor isoform 1 (NM_007540) | 3.89 | | Mm01334047_m1 |
| Casp8  | 12 370  | Caspase 8 (NM_009812) | 9.57 | 1.58 ± 0.84 | Mm00516688_m1 |
| Chkb   | 12 651  | Choline kinase | 4.160 | 1.31 ± 0.28 | Mm01212171_s1 |
| Cnr1   | 12 801  | Cannabinoid receptor 1 (NM_007726) | 2.081 | 0.84 | Mm00549788_s1 |
| Ddc    | 13 195  | Dopa decarboxylase (NM_001081277) | 2.051 | 1.58 ± 0.84 | Mm00516688_m1 |
| Drd2   | 13 489  | Dopamine receptor 2 (NM_010077) | 2.37 | 2.44 ± 0.47 | Mm00438545_m1 |
| Gabarapl1 | 57 436  | GABA A receptor-associated protein-like 1 (NM_020590) | 4.47 | | Mm00457880_m1 |
| Gabra1 | 14 394  | GABA A receptor subunit 1 (NM_010250) | 4.130 | | Mm00439046_m1 |
| Gabra5 | 110 886 | GABA A receptor subunit 5 (NM_176942) | 7.79 | | Mm00621092_m1 |
| Gabrb2 | 14 401  | GABA A receptor subunit β2 (NM_008070) | 2.141 | 1.12 | Mm00549788_s1 |
| Gabrb3 | 14 402  | GABA A receptor subunit β3 (NM_008070) | 2.041 | 0.89 | Mm00549788_s1 |
| Gabrg1 | 14 405  | GABA A receptor subunit γ1 (NM_010252) | 2.55 | 3.38 ± 1.19 | Mm00439047_m1 |
| Gabrg2 | 14 406  | GABA A receptor subunit γ2 (NM_008073) | 2.43 | 2.04 ± 0.89 | Mm00433489_m1 |
| Gad1   | 14 415  | Glutamic acid decarboxylase 1 (NM_008077) | 2.171 | 1.30 ± 0.06 | Mm00725661_s1 |
| Gfra1  | 14 585  | Gli cell line derived neurotrophic factor family receptor α1 (NM_010279) | 2.231 | 1.82 ± 0.54 | Mm00833897_m1 |
| Gja1   | 14 609  | Gap junction protein 1 (NM_010288) | 4.097 | | Mm00621092_m1 |
| Gjlb   | 14 623  | Gap junction protein β6 (NM_001010937) | 4.48 | 1.40 ± 0.28 | Mm00433661_s1 |
| Gjc1   | 14 615  | Gap junction protein γ1 (NM_008122) | 2.161 | 1.247 ± 0.11 | Mm01253027_m1 |
| Gja2   | 14 617  | Gap junction protein δ2 (NM_010290) | 4.61 | | Mm00439121_m1 |
| Gjc1   | 76 743  | Gap junction membrane channel protein ε1 (NM_0080450) | 2.00 | 1.49 ± 0.18 | Mm00519120_s1 |
| Glra2  | 237 213 | Glycine receptor α2 subunit (NM_183427) | 2.34 | 1.30 ± 0.25 | Mm00439140_m1 |
| Glrb   | 14 658  | Glycine receptor β subunit (NM_010298) | 5.089 | 1.16 ± 0.25 | Mm00439140_m1 |
| Gria2  | 14 800  | Glutamate receptor, ionotrophic, AMPA2 (α2) | 2.59 | 1.13 ± 0.05 | Mm00442822_m1 |
| Gria4  | 14 802  | Glutamate receptor, ionotrophic, AMPA4 (α4), transcript variant 1 (NM_019691) | 2.74 | 1.41 ± 0.66 | Mm00444754_m1 |
| Grin7  | 108 073 | Glutamate receptor, metabotropic 7 (NM_177328) | 35.81 | 1.07 ± 0.16 | Mm01189424_m1 |
| Gstm4  | 14 865  | Glutathione S-transferase μ4 (NM_026764) | 3.34 | | Mm00728197_s1 |
| Symbol | Gene ID | Gene name (accession number) | Agilent (FC) | Real-time PCR (RQ) | TaqMan ID |
|--------|---------|------------------------------|-------------|-------------------|-----------|
| Klhl12 | 240 756 | Kelch-like 12 (Drosophila) (NM_153128) | 6.98        | 1.19 ± 0.31       | Mm00462323_m1 |
| Narg1l | 66 897  | NMDA receptor regulated 1-like (NM_025832) | 6.98        | 1.77 ± 0.21       | Mm00462323_m1 |
| Ncam1  | 17 967  | Neural cell adhesion molecule 1 (NM_010875) | 3.310       | p = 0.0032 | Mm00456815_m1 |
| Npy5r  | 18 441  | Purinergic receptor P2Y, G-protein coupled 1 (NM_008772) | 3.89        | 1.47 ± 0.73       | Mm00435471_m1 |
| P2ry1  | 18 526  | Protocadherin 10 (NM_01098171) | 14.93       | p = 0.0074 | Mm00477987_s1 |
| Pcdk10 | 18 537  | Protein-L-isoaspartate (D-aspartate)-O-methyltransferase 1 (NM_008786) | 2.251       | 1.23 ± 0.28       | Mm00476600_m1 |
| Adcy8  | 11 514  | Adenylate cyclase (NM_009623) | 17.56       | p = 0.0073 | Mm00507722_m1 |
| Adrbk1 | 11 355  | Adrenergic receptor kinase β1 (NM_130863) | 5.55        | p = 0.0123 | Mm00804778_m1 |
| Adrbk2 | 320 129 | Adrenergic receptor kinase β2 (NM_177080) | 4.38        | p < 0.0002 | Mm0062037_m1 |
| Aloxe3 | 23 801  | Arachidonate lipooxygenase 3 (NM_011786) | 5.121       | 5.89 ± 0.43       | Mm00478628_m1 |
| Anxa7  | 11 750  | Annexin A7 (NM_009674) | 3.94 | p = 0.0049 | Mm00475749_m1 |
| Avp    | 11 998  | Arginin vasopressine (NM_009732) | 57.00        | p = 0.0002 | Mm00437761_g1 |
| Bai1   | 107 831 | Brain-specific angiogenesis inhibitor 1 (NM_174991) | 2.52        | 5.89 ± 0.43       | Mm00558144_m1 |
| Bdrb1  | 12 061  | Bradykinin receptor β1 (NM_007539) | 27.79       | 1.60 ± 0.43       | Mm00432059_s1 |
| Cnr2   | 12 922  | Corticotropin releasing hormone receptor 2 (NM_007539) | 5.110       | 5.89 ± 0.43       | Mm00438303_m1 |
| Dn14   | 13 491  | Dopamine receptor 4 (NM_007878) | 4.580 | p = 0.0049 | Mm004382893_m1 |
| Gabrd  | 14 403  | GABA A receptor subunit delta (NM_008072) | 2.72        | p = 0.0049 | Mm00433476_m1 |
| Gabrr1 | 14 408  | GABA C receptor, subunit rho (NM_008075) | 4.123 | p = 0.0049 | Mm00433499_m1 |
| Gcat   | 26 912  | Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) (NM_013847) | 25.45 | 5.89 ± 0.43 | Mm00496962_m1 |
| Glul   | 14 645  | Glutamate-ammonia ligase (glutamine synthetase) (NM_008131) | 4.56 | p = 0.0049 | Mm00725701_s1 |
| Grik1  | 14 805  | Glutamate receptor, ionotropic, kainate 2 (NM_010348) | 12.130 | p = 0.0049 | Mm00446882_m1 |
| Grik5  | 14 809  | Glutamate receptor, ionotropic, kainate 5 (NM_008168) | 9.95 | p = 0.0049 | Mm00435701_m1 |
| Grin2b | 14 812  | Glutamate receptor, ionotropic, NMDA2B (NM_008171) | 2.77 | 1.10 ± 0.13 | Mm00433820_m1 |
| Grin2d | 14 814  | Glutamate receptor, ionotropic, NMDA2D (NM_008171) | 7.168 | p = 0.0049 | Mm00433822_m1 |
| Gm1    | 14 816  | Glutamate receptor metabotropic 1 (NM_016976) | 33.64 | p = 0.0049 | Mm00810231_s1 |
| Gm5    | 108 071 | Glutamate receptor metabotropic 5 (NM_001081414) | 6.110 | p = 0.0049 | Mm00690332_m1 |
Table 1 (cont.)

| Symbol | Gene ID | Gene name (accession number) | Agilent (FC) | Real-time PCR (RQ) | TaqMan ID |
|--------|---------|-------------------------------|--------------|-------------------|-----------|
| Nrxn2  | 18190   | Neurexin II (NM_020253)       | 7.131        | 1.28±0.18         | Mm01236851_m1 |
| P2rx2  | 231602  | Purinergic receptor P2X, ligand-gated ion channel, 2 (NM_153400) | 11.180 | p<0.0328 | Mm00462952_m1 |

GABA, γ-aminobutyric acid; NMDA, N-Methyl-D-aspartate.
The table shows the comparison of gene expression data from microarray and real-time polymerase chain reaction (PCR) studies. The detected changes in the expression level are expressed in fold change (FC) in the microarray study and relative quantity (RQ) in the PCR study. Among the representative list of 60 genes of interest, 39 were down-regulated (a) and 21 were up-regulated (b) exclusively by P2rx7 deletion in the mouse amygdala using the selection filters of >2.0-FC in the microarray study. Significant differences were calculated by a two-way analysis of variance (ANOVA) followed by Benjamini–Hochberg’s multiple correction, with p<0.05. Significant down- or up-regulatory effects of P2rx7 deficiency were confirmed for 29 of the genes by TaqMan-based quantitative real-time PCR (TLDA). Samples of RNA from the microarray experiment (n=4) and from an independent experiment (n=4) were measured for each group on TLDA and the results were evaluated by RealTime StatMiner software. (a) Twenty-five genes among the total of 39 were changed significantly (two-way ANOVA, p<0.05) and showed similarity in size and direction by both methods. (b) Four out of 21 genes were changed significantly (two-way ANOVA, p<0.05) and showed similarity in size and direction by both methods. The TaqMan ID gives the product number of Applied Biosystems Gene Expression Assay used for the validation of the corresponding gene.

which implies that the observed changes in mood-related behaviour were also independent from microglial P2rx7s that might regulate IL-1β production (Sperlagh & Illes, 2007b). Moreover, the basal IL-1β level was not subject to regulation by P2rx7, either in the amygdala (present study) or in the serum and hippocampus (Csöllé & Sperlagh, 2010), which is against the role of IL-1β as a mediator of behavioural changes shown here.

Consequently, P2rx7s expressed on other cell types, most likely on neurons or astrocytes, are responsible for P2rx7-related changes in mood. Our findings also indicated P2rx7-dependent changes in monoamine levels in the amygdala, which is an intrinsic part of the neuronal circuitry regulating emotions and the site of action of antidepressants (Castro et al. 2010). Moreover, a recent study (Boucher et al. 2011) showed that c-Fos expression induced by a repeated FST in the amygdala was attenuated by the genetic deletion of P2rx7. Therefore, to gain insight into the impact of P2rx7s on gene expression in this region, a whole genome microarray analysis was performed. Here we should note that the amygdala is a heterogeneous brain nucleus, comprising anatomically and functionally distinct subnuclei. Among them, the basolateral amygdala has primarily been associated with emotional processing, whereas our analyses were performed on the whole amygdala. Our data show that the expression of a surprisingly high number of genes (>8000) was changed significantly by genetic deletion of P2rx7, which is in line with the results of Boucher et al. (2011), who found a relatively selective decrease of c-Fos activation in the basolateral amygdala of P2rx7−/− mice after repeated FST. Twenty-nine out of the 60 genes selected for validation were significantly down- or up-regulated in the real-time PCR assay. The majority of these genes have biological plausibility in synaptic signalling and neuroplasticity and could be linked to the pathogenesis of depression. Interestingly, a down-regulation of the dopa decarboxylase enzyme was found in the absence of P2rx7, which indicates that elevated norepinephrine levels in the amygdala are not due to increased synthesis but decreased metabolism or utilization. In addition, D2 receptors were also down-regulated in the amygdala of P2rx7−/− mice. Consistent with the presumed dysfunction of glutamatergic transmission and consequent neuroplasticity changes in depression (Sanacora, 2010; Sanacora et al. 2008), a down-regulation of different α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and metabotropic glutamate receptor subunits (Gria 2, Gria 4, Grm 7) and the glial cell-derived neurotrophic factor was detected in P2rx7−/− mice. In contrast, an up-regulation of the NR2B subunit of N-methyl-D-aspartate receptors (Grin2b) and CRHR2 receptors (crhr2) were found in P2rx7−/− mice. These changes in gene expression profile might also underlie the behavioural alterations and decreased stress reactivity found in the absence of P2rx7. Genes related to GABAergic transmission were
also affected by genetic deletion, causing a subtype-specific up- and down-regulation of various GABA<sub>A</sub> receptor subunits and the down-regulation of the GABA synthesizing enzyme, glutamic acid decarboxylase. These changes are consistent with previous results showing that the activation of P2rx7s in the brain leads to an increased glutamate and GABA release from nerve terminals (Papp et al. 2004; Patti et al. 2006; Sperlagh et al. 2002) and astrocytes (Duan et al. 2003). Therefore, it is tempting to speculate that the

| Symbol | Gene ID | Gene name (accession number) | Agilent (FC) | Real-time PCR (RQ) | TaqMan ID |
|--------|---------|-------------------------------|------------|-------------------|-----------|
| Ift1   | 15957   | Interferon-induced protein with tetratricopeptide repeats 1 (NM_008331) | >1000       | 25.77             | Mm00515153_m1 |
| Casp4  | 12363   | Caspase 4, apoptosis-related cysteine peptidase (NM_007609) | >1000       | 19.24             | Mm00432307_m1 |
| Ccl5   | 20340   | Chemokine (C—C motif) ligand 5 (NM_013653) | >1000       | 745.12            | Mm0102428_m1 |
| Ccl9   | 20308   | Chemokine (C—C motif) ligand 9 (NM_011338) | >1000       | 20.22             | Mm00441260_m1 |
| Cxcl10 | 15945   | Chemokine (C—X—C motif) ligand 10 (NM_021274) | >1000       | 518.39            | Mm99999072_m1 |
| Cxcl16 | 66102   | Chemokine (C—X—C motif) ligand 16 (NM_023158) | 55.71       | 7.94              | Mm00469712_m1 |
| Gbp2   | 14469   | Guanylate nucleotide binding protein 2 (NM_010260) | >1000       | 68.13             | Mm00494575_m1 |
| Gpr65  | 14744   | G-protein coupled receptor 65 (NM_008152) | >1000       | 10.44             | Mm02619732_s1 |
| Igt1   | 16145   | Interferon y induced GTPase (NM_018738) | >1000       | 10.43             | Mm00497611_m1 |
| Igrp1  | 60440   | Interferon inducible GTPase 1 (NM_021792) | >1000       | 46.86             | Mm00649928_s1 |
| I1nr   | 16181   | Interleukin 1 receptor antagonist (NM_001039701) | >1000       | >1000             | Mm01337566_m1 |
| I4ra   | 16190   | Interleukin 4 receptor, a (NM_001008700) | 23.58       | 6.81              | Mm00439634_m1 |
| Irgm   | 15944   | Immunity-related GTPase family (NM_008326) | >1000       | 21.93             | Mm00492596_m1 |
| Isg20  | 57444   | Interferon-stimulated protein (NM_020583) | >1000       | 12.29             | Mm00469585_m1 |
| Mpa2l  | 100702  | Macrophage activation 2 like (NM_194336) | >1000       | 61.41             | Mm00843395_m1 |
| Saa1   | 20208   | Serum amyloid A 1 (NM_009117) | >1000       | 34.91             | Mm00656927_g1 |
| Thr2   | 24088   | Toll like receptor 2 (NM_0119057) | >1000       | 36.57             | Mm00442346_m1 |

The table shows the comparison of gene expression data from microarray and real-time polymerase chain reaction (PCR) studies. Seventeen genes were selected among the exclusively up-regulated genes by systemic LPS (250 μg/kg i.p.) in the mouse amygdala (P2rx7<sup>+/−</sup> and P2rx7<sup>−/−</sup>) using the selection filters of >2.0-fold change (FC) in the microarray study. Significant differences were calculated by two-way analysis of variance (ANOVA) followed by Benjamini–Hochberg’s multiple correction, with <i>p</i> < 0.05. These changes were validated by TaqMan-based quantitative real-time PCR (TLDA). The detected changes in the expression level are expressed in FC in the microarray study and relative quantity (RQ) in the PCR study. The change of all 17 genes was statistically significant (two-way ANOVA, <i>p</i> < 0.05) and showed similarity in direction by both methods. Samples of RNA from the microarray experiment (n = 4) and from an independent experiment (n = 4) were measured for each group on TLDA and the results were evaluated by RealTime StatMiner software. The TaqMan ID gives the product number of Applied Biosystems Gene Expression Assay used for the validation of the corresponding gene.
increased activation due to gain-of-function mutations of the P2RX7 gene (Stokes et al. 2010) may lead to glutamatergic over-activation. This over-activation would lead to depressive symptoms, whereas the lack of these effects in the absence of the P2rx7 results in compensatory changes in the expression of glutamate and GABA receptors. This assumption is strengthened by observations from animal and human studies, indicating that excessive glutamate transmission might be involved in the pathophysiology of depressive disorders. These include abnormalities in the level of glutamate (Hashimoto et al. 2007), genetic polymorphisms (Mundo et al. 2003), the differential expression of glutamate receptor subunits in bipolar and major depressive disorders (Meador-Woodruff et al. 2001; Toro & Deakin, 2005), the antidepressant properties of glutamate receptor antagonists and modulators (Sanacora et al. 2008) and the regulation of glutamate receptor subunits by clinically used antidepressants (Nowak et al. 1998; Skolnick et al. 1996).

As a control stimulus, gene expression changes were also examined after a relatively modest LPS stimulus. This treatment elicited the significant change of 570 transcripts with >2FC, which is comparable to the gene expression changes found by others in the brain after systemic endotoxin treatment (Marques et al. 2009; Marsh et al. 2009; Mastronardi et al. 2007; Perreau et al. 2007). Therefore, genes encoding chemokines and other signalling proteins of immune response were primarily up-regulated by the endotoxin.

In conclusion, our data show that the genetic deletion and pharmacological antagonism of P2rx7s leads to selective alteration in mood-related behaviour and related changes in brain monoamine levels, stress reactivity and gene expression. Although the precise translation of P2rx7 function to behavioural changes needs further investigation, our data may open up new avenues in the treatment of mood disorders.

**Note**

Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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**Statement of Interest**

None.

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