

# 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, adrenocorticotrophic 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 P2rx7 (Surprenant *et al.* 1996) has distinct structural, functional and pharmacological features within the P2X receptor family (Jarvis & Khakh, 2009; Sperlág *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

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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)-1 $\beta$  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 Ca<sup>2+</sup> influx (Miras-Portugal *et al.* 2003), followed by increased glutamate and subsequent  $\gamma$ -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; Grigoriou-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<sup>-/-</sup>*) 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<sup>-/-</sup>* 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<sup>+/+</sup>*) and knockout mice (*P2rx7<sup>-/-</sup>*), which were housed in a light-controlled (12-h light/dark cycle; lights on 07:00 hours), humidity-controlled (60  $\pm$  10%) and temperature-controlled (23  $\pm$  2  $^{\circ}$ C) room with food and water available *ad libitum*. Homozygous *P2rx7<sup>+/+</sup>* mice were bred on a background of C57BL/6J. The original breeding pairs of *P2rx7<sup>-/-</sup>* mice (C57BL/6J-based) were kindly supplied by Christopher Gabel (Pfizer Inc., USA). The animals contained the DNA construct [P2X7-F1 (5'-CGGCGTGCGTTTTGACATCCT-3') and P2X7-R2 (5'-AGGGCCCTGCGGTTCTC-3')], previously shown to produce the genetic deletion of P2rx7 (Solle *et al.* 2001). Offspring of this mouse line were cross-bred with *P2rx7<sup>+/+</sup>* 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  $\times$  45  $\times$  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<sup>+/+</sup>* and *P2rx7<sup>-/-</sup>* 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 ( $22 \pm 0.5$  °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-1 $\beta$ experiments*

All animals were given an i.p. injection of sterile saline (0.9% NaCl) or bacterial lipopolysaccharide (LPS, 250  $\mu$ g/kg i.p.) with an injection volume of 0.1 ml/mouse). Animals were killed by decapitation 6 h after LPS injection. The amygdalae were collected, frozen on dry ice and stored at  $-70$  °C until further investigation. The IL-1 $\beta$  assays were performed as described previously (Csölle & Sperlágh, 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 amygdalae and striata were frozen in liquid nitrogen. The weighted frozen tissue was

homogenized in ice-cold 0.1 M perchloric acid containing theophylline (10  $\mu$ M; 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.

#### [<sup>3</sup>H]Dopamine release experiments

[<sup>3</sup>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 at 3000 g for 20 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% O<sub>2</sub>/5% CO<sub>2</sub> 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<sup>–8</sup> M; Sigma) medium in the second fraction.

#### Hormone assays

Adrenocorticotrophic hormone (ACTH) and corticosterone in the trunk blood and in acutely isolated pituitaries were measured by radioimmunoassay in 50- $\mu$ l unextracted samples as described previously (Zelena *et al.* 2008).

#### Bone marrow chimeras

Bone marrow chimeras were generated by transplantation of *P2rx7<sup>+/+</sup>* or *P2rx7<sup>–/–</sup>* 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- $\mu$ m 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  $\mu$ g/kg) and killed by decapitation 6 h after injection. Microarray profiling on amygdala samples was performed in the Agilent Microarray Core Facility (Semelweis 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=rzwxlikqskwayfk&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  $\pm$  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-1 $\beta$ , 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*<sup>+/+</sup> 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*<sup>-/-</sup> mice were less prone to develop depression-like behaviour than *P2rx7*<sup>+/+</sup> mice (Fig. 1a). During the first trial, the duration of immobility was similar in the two genotypes (92.43  $\pm$  21.12 s, for wild-type *vs.* 118.38  $\pm$  16.46 s for *P2rx7*<sup>-/-</sup>; genotype:  $F_{1,18} = 0.05$ ,  $p < 0.8$ ). In *P2rx7*<sup>+/+</sup> mice, the time spent in immobility increased over time during the first trial ( $F_{3,36} = 3.61$ ,  $p < 0.02$ ). In *P2rx7*<sup>-/-</sup> mice, parallel changes were seen, but these were not significant ( $F_{3,36} = 0.30$ ,  $p > 0.8$ ). When the two genotypes were considered together, the time  $\times$  genotype interaction was significant ( $F_{3,54} = 2.92$ ,  $p < 0.04$ ) and significant

time-related changes were seen in *P2rx7*<sup>+/+</sup> mice only.

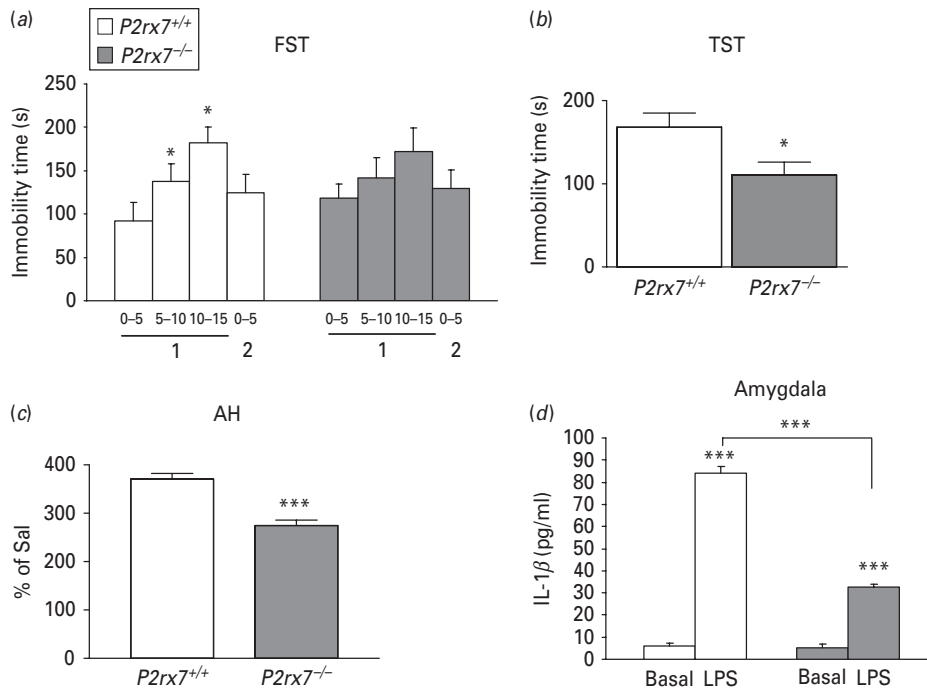
The basal time of immobility in the TST was 167.90  $\pm$  17.13 s ( $n = 9$ ) in *P2rx7*<sup>+/+</sup> mice (Fig. 1b). In line with previous data (Basso *et al.* 2009), *P2rx7*<sup>-/-</sup> mice spent significantly less time with immobility (i.e. they exhibited antidepressant-like phenotype in this test as well; 110.24  $\pm$  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*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice (120.17  $\pm$  24.71 m,  $n = 9$  and 156.53  $\pm$  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-1 $\beta$  6 h after saline administration was 6.06  $\pm$  1.18 pg/ml in the amygdalae of *P2rx7*<sup>+/+</sup> mice (Fig. 1d,  $n = 4$ ). Systemic LPS administration (250  $\mu$ g/kg i.p.) caused a significant elevation in IL-1 $\beta$  levels (83.94  $\pm$  3.01 pg/ml,  $n = 8$ ,  $F_{1,28} = 45.2$ ,  $p < 0.001$ , Fig. 1d). The IL-1 $\beta$  level in the amygdalae of saline-treated *P2rx7*<sup>-/-</sup> mice was 5.29  $\pm$  1.48 pg/ml ( $n = 4$ ), not significantly different from *P2rx7*<sup>+/+</sup> mice ( $p > 0.05$ , Fig. 1d). The LPS-induced elevation of IL-1 $\beta$  level was significantly attenuated in the amygdalae of *P2rx7*<sup>-/-</sup> mice (32.53  $\pm$  1.55 pg/ml,  $n = 8$ ; genotype:  $F_{1,28} = 183.3$ ,  $p < 0.001$ ; interaction:  $F_{1,28} = 42.55$ ,  $p < 0.001$ , 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*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice. The basal level of norepinephrine was elevated in the absence of *P2rx7* in the amygdala (genotype:  $F_{1,27} = 3.30$ ,  $p = 0.08$ ; interaction:  $F_{1,27} = 6.52$ ,  $p = 0.016$ ; *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  $\pm$  13.79,  $n = 7$  and 18.21  $\pm$  3.83 pmol/mg protein,  $n = 8$  in *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice, respectively; interaction:  $F_{1,27} = 0.05$ ,  $p > 0.8$ ). Likewise, basal dopamine and 5-HT levels were not affected by the genetic deletion either in the amygdala (Fig. 2a-c,

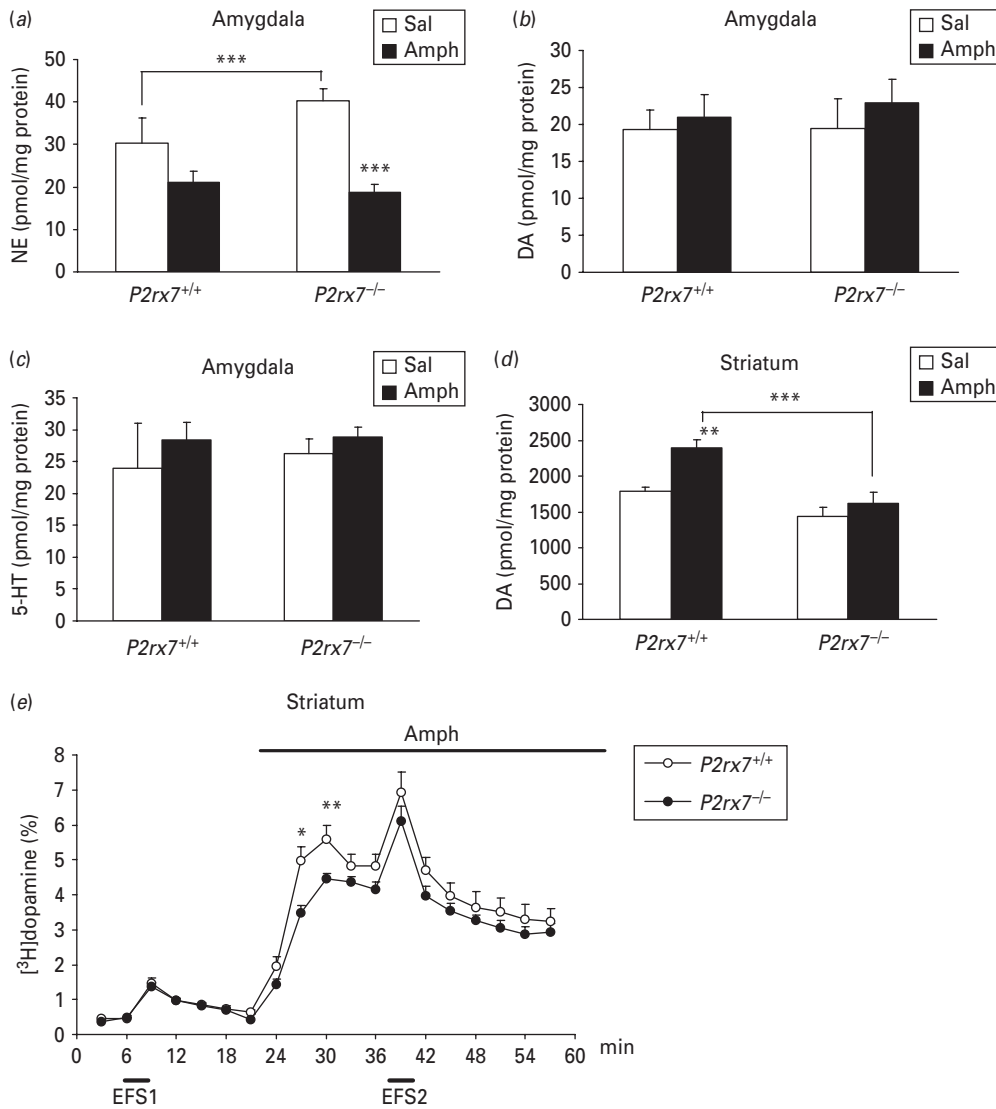


**Fig. 1.** Genetic deletion of *P2rx7*s in mice leads to an antidepressant-like phenotype in the forced swim test (FST) and tail suspension test (TST) and mood stabilizing-like phenotype in amphetamine-induced hyperlocomotion test (AH), but does not affect basal interleukin (IL)-1 $\beta$  levels in the amygdala. (a) *P2rx7*<sup>-/-</sup> 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*<sup>+/+</sup>, 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*<sup>-/-</sup> mice. Mice were placed into the open field arena for a 30-min habituation period and then injected with i.p. saline (Sal) or D-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*<sup>+/+</sup>, Student's  $t$  test. Drug- and test-naive male homozygous mice (*P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup>, aged 2–3 months) weighing approximately 30 g were used in the experiments. (d) IL-1 $\beta$  protein level in the amygdala of *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice after Sal and lipopolysaccharide (LPS) treatment. The IL-1 $\beta$  protein level was similar in the amygdalae of Sal-treated *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice. Injection of LPS (*E. coli*; 250  $\mu$ g/kg i.p.) significantly increased the level of IL-1 $\beta$  in the amygdalae of *P2rx7*<sup>+/+</sup> mice 6 h after treatment. IL-1 $\beta$  protein level was less elevated in the amygdalae of *P2rx7*<sup>-/-</sup> mice in response to systemic endotoxin. The levels of IL-1 $\beta$  were quantified in the supernatants by ELISA. Data are given as the mean level of cytokines  $\pm$  S.E.M., expressed in pg/ml. \* Indicates significant differences between Sal- and LPS-treated and between *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice ( $n=4$  per group, \*\*\*  $p<0.001$ , two-way analysis of variance).

dopamine:  $F_{1,27}=0.05$ ,  $p>0.8$ ; 5-HT:  $F_{1,27}=1.07$ ,  $p>0.3$ ) or in the striatum of saline-treated mice (Fig. 2d and data not shown). Consistent with the observed increase in locomotor activity, amphetamine treatment caused an elevation in the dopamine content of the striatum of *P2rx7*<sup>+/+</sup> mice (Fig. 2d,  $F_{1,27}=10.08$ ;  $p<0.004$ ), a reduction of norepinephrine level in the amygdala ( $F_{1,27}=17.17$ ,  $p<0.001$ ), without changing the 5-HT and dopamine levels in the amygdala (Fig. 2a–c, 5-HT:  $F_{1,27}=0.23$ ;  $p>0.6$ , dopamine:  $F_{1,27}=0.43$ ,  $p>0.5$ ) and with a tendency for lower norepinephrine levels in the striatum ( $26.4\pm 3.23$ ,  $n=7$  and  $12.2\pm 3.07$  pmol/mg protein,  $n=8$  in *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice, respectively; genotype:  $F_{1,27}=$

$3.36$ ,  $p=0.07$ ). In line with these findings, when acute striatal slices treated *in vitro* with amphetamine (30  $\mu$ M), a profound increase in [<sup>3</sup>H]dopamine efflux was observed (Fig. 2e). Both the amphetamine-induced increase in dopamine content (Fig. 2d; interaction:  $F_{1,27}=2.91$ ,  $p=0.09$ , *post-hoc* comparison by Fischer's LSD test:  $p<0.001$ ) and [<sup>3</sup>H]dopamine efflux (Fig. 2e) were significantly attenuated in *P2rx7*<sup>-/-</sup> mice, without the alteration of basal and electrical field stimulation-evoked [<sup>3</sup>H]dopamine release (Fig. 2e).

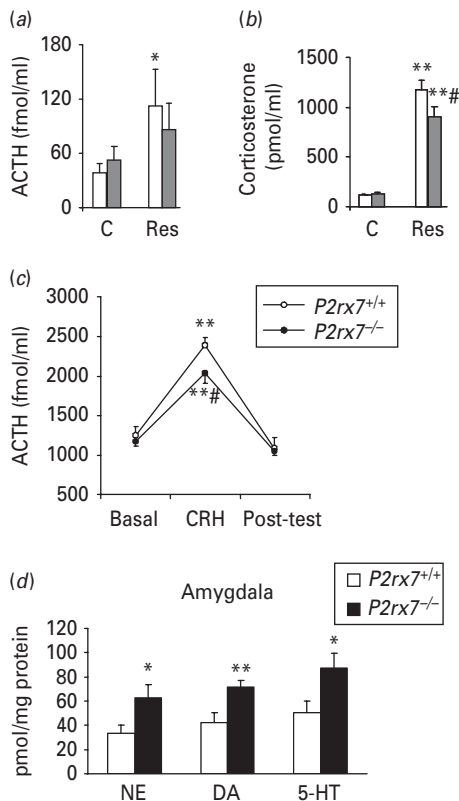
Body, thymus and adrenal gland weights remained unchanged by *P2rx7* gene disruption (Supplementary Table S1). Similarly, resting ACTH and corticosterone levels were not affected by genotype (Fig. 3a,b;



**Fig. 2.** Changes in the content of norepinephrine (NE) (a), dopamine (DA) (b), 5-HT (c) in the amygdala; dopamine content in the striatum (d) and release of [<sup>3</sup>H]dopamine (e) in acute striatal slices in the absence of P2rx7. (a–d) *P2rx7<sup>+/+</sup>* and *P2rx7<sup>-/-</sup>* mice were treated with saline (Sal) or amphetamine (Amph, 2.5 mg/kg i.p.) and 30 min after the treatment were decapitated. NE, DA and 5-HT levels were analysed by high performance liquid chromatography in the amygdala and striatum and are expressed in pmol/mg protein. \* Indicates significant differences between *P2rx7<sup>+/+</sup>* and *P2rx7<sup>-/-</sup>* mice and between Sal- and Amph-treated groups, as indicated ( $n=7-8$ /group,  $**p<0.01$ ,  $***p<0.001$ , two-way analysis of variance followed by Fischer's LSD test). (e) Basal, electrical field stimulation (EFS)- and Amph-induced [<sup>3</sup>H]dopamine efflux from striatal slices of *P2rx7<sup>+/+</sup>* and *P2rx7<sup>-/-</sup>* mice. Striatal slices were incubated with [<sup>3</sup>H]dopamine and superfused with Krebs' solution. EFS (20 V, 2 Hz, 240 shocks) and Amph (30  $\mu$ M) were applied as indicated by the horizontal bars. The efflux of [<sup>3</sup>H]dopamine is expressed as fractional release, which represents the tritium content in a sample as a percentage of the actual total tritium content. Amph-induced [<sup>3</sup>H]dopamine release was significantly decreased in striatal slices of *P2rx7<sup>-/-</sup>* mice, whereas basal and electrical stimulation-induced efflux remained unchanged.  $n=8$ /group,  $*p<0.05$ ,  $**p<0.01$ , Student's *t* test.

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<sup>+/+</sup>*

animals only ( $p=0.03$ ; Fig. 3a). In addition, the corticosterone increase was significantly lower in *P2rx7<sup>-/-</sup>* mice, than in *P2rx7<sup>+/+</sup>* mice ( $p=0.048$ ; Fig. 3b). In isolated *in vitro* pituitaries, CRH treatment significantly increased ACTH secretion (Fig. 3c). However,



**Fig. 3.** *P2rx7*<sup>-/-</sup> mice [grey bars in (a) and (b)] respond with a decreased elevation of adrenocorticotrophic hormone (ACTH) and corticosterone in the plasma (a, b) and pituitary (c) and increased norepinephrine (NE) level (d) in the amygdala to stress. Hormonal secretion was evaluated after 30 min of restraint. Plasma levels of ACTH (a) and corticosterone (b) were measured by radioimmunoassay of trunk blood ( $n=9-10$ ). The resting ACTH and corticosterone levels were not affected by genotype. Restraint significantly increased the level of both hormones, but the elevation of ACTH levels reached the level of significance in *P2rx7*<sup>+/+</sup> animals (clear bars) only ( $p=0.03$ ). (c) The ACTH response to  $5 \times 10^{-8}$  M corticotropin-releasing hormone (CRH) was studied *in vitro* ( $n=10$  per group). Fifteen-min fractions were collected. CRH treatment significantly increased ACTH secretion. This effect was dependent on the genotype and there was a significantly smaller response in *P2rx7*<sup>-/-</sup> mice. C, control; Res, exposed to 30-min restraint. *Post-hoc* comparisons: \*  $p < 0.05$ ; \*\*  $p < 0.01$  vs. control; #  $p < 0.05$  vs. *P2rx7*<sup>+/+</sup>. (d) The level of NE, dopamine (DA) and 5-HT was analysed by high performance liquid chromatography in the amygdalae of *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice after 30 min restraint. The results are expressed as pmol/mg protein. \* Represents a significant difference between *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> animals,  $n=10-12$ /group, \*  $p < 0.05$ , \*\*  $p < 0.01$ , Student's *t* test.

this effect depended on genotype ( $F_{1,17}=4.65$ ,  $p=0.045$ ), with a significantly smaller response in *P2rx7*<sup>-/-</sup> mice (Fig. 3c). After restraint stress, norepinephrine,

dopamine and 5-HT levels were all elevated in the amygdala of *P2rx7*<sup>-/-</sup> mice when compared to *P2rx7*<sup>+/+</sup> littermates (Fig. 3d).

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

Next, we asked whether the behavioural phenotype detected in *P2rx7*<sup>-/-</sup> 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*<sup>+/+</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice is not related to *P2rx7*s expressed on cells of haematopoietic origin

To test whether the alteration of behavioural phenotype detected in *P2rx7*<sup>-/-</sup> mice was due to the lack of *P2rx7*s 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*<sup>+/+</sup> or *P2rx7*<sup>-/-</sup> mice (expressing the CD45.2 leukocyte 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*<sup>+/+</sup> or *P2rx7*<sup>-/-</sup> 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 *P2rx7*s 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*<sup>+/+</sup> mice and of bone marrow chimeras engrafted with *P2rx7*<sup>+/+</sup> bone marrow cells (CD45.1/*P2rx7*<sup>+/+</sup>) 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*<sup>-/-</sup> mice (Fig. 5d). Microglia cells of bone marrow chimeras engrafted with *P2rx7*<sup>-/-</sup> bone marrow cells (CD45.1/*P2rx7*<sup>-/-</sup>) 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 mice 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*<sup>-/-</sup> mice was not transferred to *P2rx7*<sup>+/+</sup> recipients with the engraftment of the *P2rx7*<sup>-/-</sup> bone marrow cells.

#### Alteration of the gene expression profile in the amygdalae of *P2rx7*<sup>-/-</sup> mice

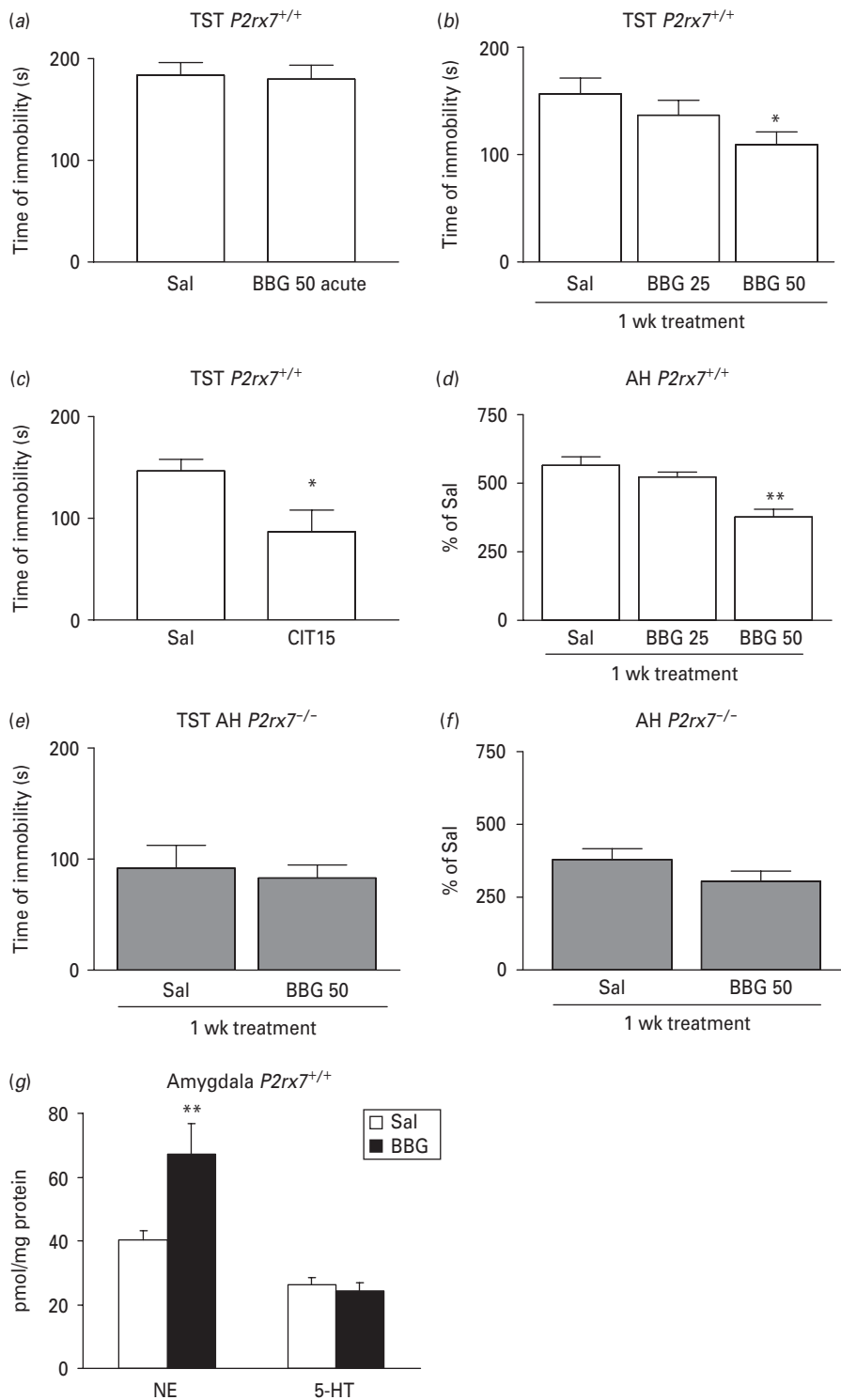
Because previous experiments and literature data indicated that monoamine transmitter levels and c-Fos expression (Boucher *et al.* 2011) are regulated by P2rx7 in the amygdala, we next examined how global gene expression is changed in the absence of P2rx7 in this region and LPS treatment was used as a reference stimulus. Four amygdala samples/group collected from *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> 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  $\geq 2.0$  (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*<sup>-/-</sup> 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  $\alpha_2$  adrenergic receptor (*Adra2a*), the CB<sub>1</sub> cannabinoid receptor (*Cnr1*), the D<sub>2</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 subunits of the glycine receptor (*Glr2*, *Glr3*; Table 1a). In contrast, four validated genes of the 29, including  $\beta$  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<sup>-/-</sup>* 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<sup>-/-</sup>* 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.

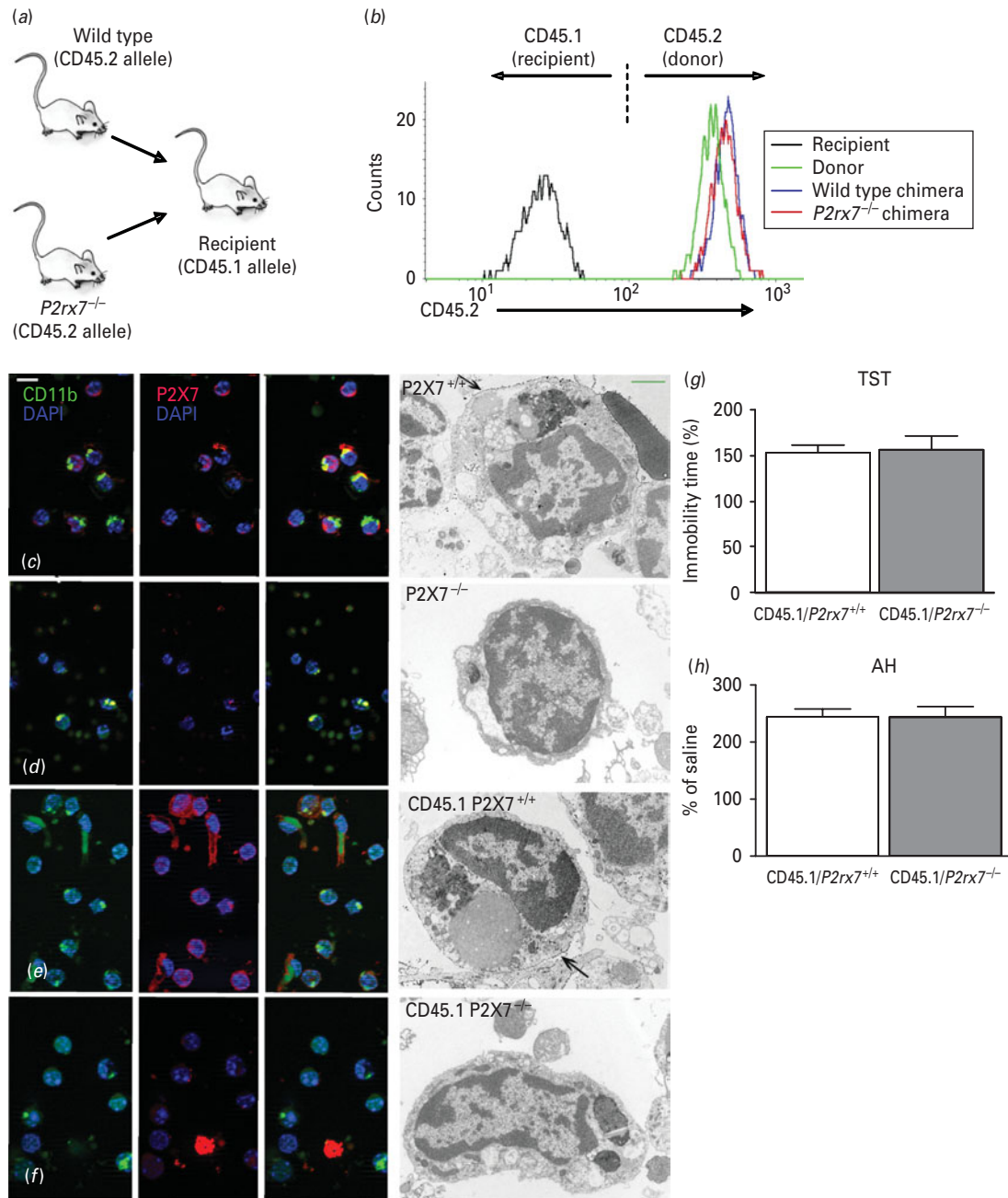
Changes in behaviour were accompanied by corresponding alterations in brain monoamine levels in the amygdala and the striatum of *P2rx7<sup>-/-</sup>* mice. In line with the decreased behavioural despair in the FST and decreased immobility in the TST, an increase in basal norepinephrine level was found 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<sup>-/-</sup>* mice.

*P2rx7<sup>-/-</sup>* mice responded with a decreased elevation of plasma ACTH and corticosterone in response to restraint stress, which suggests that *P2rx7<sup>-/-</sup>* 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<sup>-/-</sup>* mice after restraint, which indicates an interaction between stress and the brain monoaminergic system, which is regulated by P2rx7.

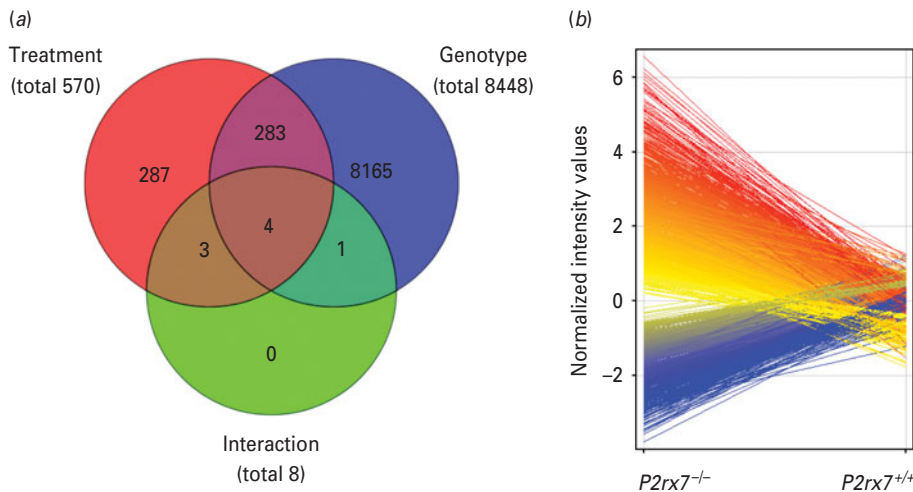
Importantly, we could reproduce the effect of genetic deletion by the systemic application of a specific antagonist of P2rx7s. BBG is a non-toxic and fairly selective antagonist of P2rx7s, which is able to penetrate the blood–brain barrier (Peng *et al.* 2009). Although it was active only using a sub-acute application, BBG dose-dependently decreased immobility

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antidepressant, decreased basal immobility in the TST in *P2rx7<sup>+/+</sup>* mice ( $n=11$ /group,  $*p<0.05$  vs. Sal, Student's *t* test). (d) Effect of 1-wk daily treatment with BBG (25–50 mg/kg i.p.) or Sal on amphetamine induced hyperactivity in *P2rx7<sup>+/+</sup>* mice. ( $n=10$ –12/group,  $**p<0.001$  vs. Sal, one-way ANOVA followed by Dunnett's test). Citalopram and BBG were administered i.p. 30 min before testing at the doses (mg/kg) indicated on the abscissa. Sal-treated mice were injected with an equal volume of Sal. (e) Effect of sub-acute, 1-wk treatment with BBG (50 mg/kg i.p.) on basal immobility in *P2rx7<sup>-/-</sup>* mice. Mice were treated with BBG for 7 d or with Sal and then submitted to the TST ( $n=8$ –10/group). (f) Effect of 1-wk daily treatment with BBG (50 mg/kg i.p.) or Sal on amphetamine-induced hyperactivity in *P2rx7<sup>-/-</sup>* mice ( $n=10$ /group). Note that the basal immobility (e) and amphetamine-induced hyperactivity (f) is lower in Sal-treated *P2rx7<sup>-/-</sup>* mice than in Sal-treated *P2rx7<sup>+/+</sup>* mice. (g) Sub-acute, 1-wk treatment with BBG (50 mg/kg i.p.) elevates NE, but not 5-HT levels in the amygdala in *P2rx7<sup>+/+</sup>* mice, when compared to Sal-treated mice. Tissue content of NE and 5-HT were analysed by high performance liquid chromatography and are expressed in pmol/mg protein,  $n=11$ –12,  $**p<0.01$ , Student's *t* test.



**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 [(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



**Fig. 6.** Summary of results of the whole genome microarray analysis performed on amygdala samples of *P2rx7*<sup>+/+</sup> and *P2rx7*<sup>-/-</sup> mice subjected to saline or lipopolysaccharide (LPS) treatments ( $n=4$ /group). (a) Venn diagram represents the main effects of the two-way analysis of variance with Benjamini–Hochberg’s multiple correction comparison test for unequal replications and using the selection filters of >2.0-fold change: LPS treatment (red circle), genotype (blue circle) and their interaction (green circle). Numbers inside the compartments represent the number of transcripts that are significant for that effect. The intersections of the sets represent significantly changed genes for each of the effects involved in the intersection. Significance threshold was 0.05 and the  $p$  value computation was asymptotic. In the primary microarray analysis, the expression of a total of 8739 transcripts was found to be significantly altered with the applied statistical filters. These 8739 genes sum up from the genes significantly affected by treatment independently from genotype ( $287+283=570$ , illustrated by the numbers in those areas of the red circle, which is covered only by the red area, and covered by the red and blue areas), by genotype independently from treatment ( $8165+283=8448$ , illustrated by the numbers in those areas of the blue circle, which is covered only by the blue area, and covered by the red and blue areas) and by their interaction (4, as illustrated by the numbers within the intersection of the red/blue and green circles), i.e.  $287+283+8165+4=8739$ . The intersection of the red and green circles represents three transcripts, which were not only significantly affected by LPS treatment but may also be altered by genotype, whereas the intersection of the blue and green circles represents one transcript, which is not only significantly affected by knockout genotype but may also be altered by LPS treatment. (b) Deficiency of the P2rx7 had the most profound effect on the expression profile, changing the expression of 8448 transcripts. Among them, 3133 were increased (red) and 5315 were decreased (blue), as illustrated by the deviation of the lines according to the normalized intensity values.

in the TST and attenuated hyperactivity elicited by amphetamine in *P2rx7*<sup>+/+</sup> mice. Consistent with the involvement of P2rx7 in these effects, an identical BBG treatment was ineffective in both tests in *P2rx7*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice. The majority of brain microglial cells derived from *P2rx7*<sup>-/-</sup>/CD45.1 mice were negative for the P2rx7,

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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*<sup>-/-</sup> 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*<sup>+/+</sup> and CD45.1/*P2rx7*<sup>-/-</sup> 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
(a)					
<i>Adra2a</i>	11 551	Adrenergic receptor $\alpha$ 2a (NM_007417)	2.72	1.34 $\pm$ 0.38 $p=0.0171$	Mm00845383_s1
<i>Adrb1</i>	11 554	Adrenergic receptor $\beta$ 1 (NM_007419)	4.77		Mm00431701_s1
<i>Ak5</i>	229 949	Adenylate kinase 5 (NM_001081277)	5.71		Mm00461978_m1
<i>Aplp2</i>	11 804	Amyloid $\beta$ (A4) precursor-like protein 2 (NM_009691)	5.55		Mm00507819_m1
<i>Bdnf</i>	12 064	Brain derived neurotrophic factor isoform 1 (NM_007540)	3.89		Mm01334047_m1
<i>Casp8</i>	12 370	Caspase 8 (NM_009812)	9.57		Mm00802247_m1
<i>Chkb</i>	12 651	Choline kinase $\beta$ (NM_007692)	4.160		Mm00432498_m1
<i>Cnr1</i>	12 801	Cannabinoid receptor 1 (NM_007726)	2.081	1.31 $\pm$ 0.28 $p=0.0087$	Mm01212171_s1
<i>Ddc</i>	13 195	Dopa decarboxylase (NM_016672)	2.051	1.58 $\pm$ 0.84 $p=0.0249$	Mm00516688_m1
<i>Drd2</i>	13 489	Dopamine receptor 2 (NM_010077)	2.37	2.44 $\pm$ 0.47 $p=0.0120$	Mm00438545_m1
<i>Gabarapl1</i>	57 436	GABA <sub>A</sub> receptor-associated protein-like 1 (NM_020590)	4.47		Mm00457880_m1
<i>Gabra1</i>	14 394	GABA <sub>A</sub> receptor subunit $\alpha$ 1 (NM_010250)	4.130		Mm00439046_m1
<i>Gabra5</i>	110 886	GABA <sub>A</sub> receptor subunit $\alpha$ 5 (NM_176942)	7.79		Mm00621092_m1
<i>Gabrb2</i>	14 401	GABA <sub>A</sub> receptor subunit $\beta$ 2 (NM_008070)	2.141	2.51 $\pm$ 1.12 $p=0.0036$	Mm00549788_s1
<i>Gabrb3</i>	14 402	GABA <sub>A</sub> receptor subunit $\beta$ 3 (NM_008071)	2.041	1.34 $\pm$ 0.55 $p<0.0005$	Mm00433473_m1
<i>Gabrg1</i>	14 405	GABA <sub>A</sub> receptor subunit $\gamma$ 1 (NM_010252)	2.55	3.38 $\pm$ 1.19 $p=0.0005$	Mm00439047_m1
<i>Gabrg2</i>	14 406	GABA <sub>A</sub> receptor subunit $\gamma$ 2 (NM_008073)	2.43	2.04 $\pm$ 0.89 $p=0.0012$	Mm00433489_m1
<i>Gad1</i>	14 415	Glutamic acid decarboxylase 1 (NM_008077)	2.171	1.10 $\pm$ 0.06 $p=0.0009$	Mm00725661_s1
<i>Gfra1</i>	14 585	Glial cell line derived neurotrophic factor family receptor $\alpha$ 1 (NM_010279)	2.231	1.82 $\pm$ 0.54 $p=0.0308$	Mm00833897_m1
<i>Gja1</i>	14 609	Gap junction protein $\alpha$ 1 (NM_010288)	4.097		Mm00621092_m1
<i>Gjb6</i>	14 623	Gap junction protein $\beta$ 6 (NM_001010937)	4.48	1.40 $\pm$ 0.28 $p<0.0016$	Mm00433661_s1
<i>Gjc1</i>	14 615	Gap junction protein $\gamma$ 1 (NM_008122)	2.161	1.247 $\pm$ 0.11 $p=0.0050$	Mm01253027_m1
<i>Gjd2</i>	14 617	Gap junction protein $\delta$ 2 (NM_010290)	4.61		Mm00439121_m1
<i>Gje1</i>	76 743	Gap junction membrane channel protein $\epsilon$ 1 (NM_080450)	2.00	1.49 $\pm$ 0.18 $p=0.0022$	Mm00519120_s1
<i>Glr2</i>	237 213	Glycine receptor $\alpha$ 2 subunit (NM_183427)	2.34	1.30 $\pm$ 0.25 $p=0.0157$	Mm00439140_m1
<i>Glrb</i>	14 658	Glycine receptor $\beta$ subunit (NM_010298)	5.089	1.16 $\pm$ 0.25 $p=0.0018$	Mm00439140_m1
<i>Gria2</i>	14 800	Glutamate receptor, ionotropic, AMPA2 ( $\alpha$ 2)	2.59	1.13 $\pm$ 0.05 $p<0.0002$	Mm00442822_m1
<i>Gria4</i>	14 802	Glutamate receptor, ionotropic, AMPA4 ( $\alpha$ 4), transcript variant 1 (NM_019691)	2.74	1.41 $\pm$ 0.66 $p=0.0014$	Mm00444754_m1
<i>Grm7</i>	108 073	Glutamate receptor, metabotropic 7 (NM_177328)	35.81	1.07 $\pm$ 0.16 $p=0.0317$	Mm01189424_m1
<i>Gstm4</i>	14 865	Glutathione S-transferase $\mu$ 4 (NM_026764)	3.34		Mm00728197_s1

Table 1 (cont.)

Symbol	Gene ID	Gene name (accession number)	Agilent (FC)	Real-time PCR (RQ)	TaqMan ID
<i>Klhl12</i>	240 756	Kelch-like 12 ( <i>Drosophila</i> ) (NM_153128)	6.98	1.19 ± 0.31 <i>p</i> = 0.0074	Mm00462323_m1
<i>Narg1l</i>	66 897	NMDA receptor regulated 1-like (NM_025832)	6.98	1.77 ± 0.21 <i>p</i> = 0.0032	Mm00462323_m1
<i>Ncam1</i>	17 967	Neural cell adhesion molecule 1 (NM_010875)	3.310		Mm00456815_m1
<i>Npy5r</i>	18 168	Neuropeptide Y receptor Y5 (NM_016708)	6.68		Mm02620267_s1
<i>P2ry1</i>	18 441	Purinergic receptor P2Y, G-protein coupled 1 (NM_008772)	3.89	1.47 ± 0.73 <i>p</i> = 0.0467	Mm00435471_m1
<i>Pcdh10</i>	18 526	Protocadherin 10 (NM_001098171)	14.93		Mm00477987_s1
<i>Pcmt1</i>	18 537	Protein-L-isoaspartate (D-aspartate) O-methyltransferase 1 (NM_008786)	2.251	1.23 ± 0.28 <i>p</i> = 0.0073	Mm00476600_m1
<i>Pde1a</i>	18 573	Phosphodiesterase 1A calmodulin-dependent (NM_016744)	2.191	1.24 ± 0.74 <i>p</i> = 0.0123	Mm00450244_m1
<i>Plp1</i>	18 823	Proteolipid protein (myelin) 1 (NM_011123)	9.031	1.51 ± 0.18 <i>p</i> = 0.0033	Mm00456892_m1
<i>Ryr3</i>	20 192	Ryanodine receptor 3 (BC116740)	2.80	1.79 ± 0.22 <i>p</i> < 0.0002	Mm01335482_m1
(b)					
<i>Adcy8</i>	11 514	Adenylate cyclase (NM_009623)	17.56		Mm00507722_m1
<i>Adrbk1</i>	110 355	Adrenergic receptor kinase β1 (NM_130863)	5.55		Mm00804778_m1
<i>Adrbk2</i>	320 129	Adrenergic receptor kinase β2 (NM_177080)	4.38		Mm00622037_m1
<i>Aloxe3</i>	23 801	Arachidonate lipoxygenase 3 (NM_011786)	5.121		Mm004778628_m1
<i>Anxa7</i>	11 750	Annexin A7 (NM_009674)	3.94		Mm00477549_m1
<i>Avp</i>	11 998	Arginin vasopressine (NM_009732)	57.00		Mm00437761_g1
<i>Bai1</i>	107 831	Brain-specific angiogenesis inhibitor 1 (NM_174991)	2.52		Mm00558144_m1
<i>Bdrkb1</i>	12 061	Bradykinin receptor β1 (NM_007539)	27.79	1.60 ± 0.43 <i>p</i> = 0.0498	Mm00432059_s1
<i>Crhr2</i>	12 922	Corticotropin releasing hormone receptor 2 (NM_007539)	5.110	5.89 ± 0.43 <i>p</i> = 0.0040	Mm00438303_m1
<i>Drd4</i>	13 491	Dopamine receptor 4 (NM_007878)	4.580		Mm004382893_m1
<i>Gabrd</i>	14 403	GABA <sub>A</sub> receptor subunit delta (NM_008072)	2.72		Mm00433476_m1
<i>Gabbr1</i>	14 408	GABA <sub>C</sub> receptor, subunit rho (NM_008075)	4.123		Mm00433499_m1
<i>Gcat</i>	26 912	Glycine C-acetyltransferase (2-amino-3 ketobutyrate-coenzyme A ligase) (NM_013847)	25.45		Mm00496962_m1
<i>Glul</i>	14 645	glutamate-ammonia ligase (glutamine synthetase) (NM_008131)	4.56		Mm00725701_s1
<i>Grik1</i>	14 805	Glutamate receptor, ionotropic, kainate 2 (NM_010348)	12.130		Mm00446882_m1
<i>Grik5</i>	14 809	Glutamate receptor, ionotropic, kainate 5 (γ2) (NM_008168)	9.95		Mm00435701_m1
<i>Grin2b</i>	14 812	Glutamate receptor, ionotropic, NMDA2B (ε2) (NM_008171)	2.77	1.10 ± 0.13 <i>p</i> = 0.0062	Mm00433820_m1
<i>Grin2d</i>	14 814	Glutamate receptor, ionotropic, NMDA2D (ε4) (NM_008171)	7.168		Mm00433822_m1
<i>Grm1</i>	14 816	Glutamate receptor metabotropic 1 (NM_016976)	33.64		Mm00810231_s1
<i>Grm5</i>	108 071	Glutamate receptor metabotropic 5 (NM_001081414)	6.110		Mm00690332_m1

**Table 1** (*cont.*)

Symbol	Gene ID	Gene name (accession number)	Agilent (FC)	Real-time PCR (RQ)	TaqMan ID
<i>Nrxn2</i>	18 190	Neurexin II (NM_020253)	7.131	1.28 ± 0.18 <i>p</i> < 0.0328	Mm01236851_m1
<i>P2rx2</i>	231 602	Purinergic receptor P2X, ligand-gated ion channel, 2 (NM_153400)	11.180		Mm00462952_m1

GABA,  $\gamma$ -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 of mood-related behaviour were also independent from microglial P2rx7s that might regulate IL-1 $\beta$  production (Sperlágh & Illes, 2007*b*). Moreover, the basal IL-1 $\beta$  level was not subject to regulation by P2rx7, either in the amygdala (present study) or in the serum and hippocampus (Csölle & Sperlágh, 2010), which is against the role of IL-1 $\beta$  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<sup>-/-</sup> 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, D<sub>2</sub> receptors were also down-regulated in the amygdala of P2rx7<sup>-/-</sup> 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  $\alpha$ -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<sup>-/-</sup> 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<sup>-/-</sup> 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



**Table 2.** Genes that were up-regulated by lipopolysaccharide (LPS) treatment in the mouse amygdala

Symbol	Gene ID	Gene name (accession number)	Agilent (FC)	Real-time PCR (RQ)	TaqMan ID
<i>Ifit1</i>	15 957	Interferon-induced protein with tetratricopeptide repeats 1 (NM_008331)	> 1000	25.77 <i>p</i> < 0.0001	Mm00515153_m1
<i>Casp4</i>	12 363	Caspase 4, apoptosis-related cysteine peptidase (NM_007609)	> 1000	19.24 <i>p</i> = 0.0007	Mm00432307_m1
<i>Ccl5</i>	20 340	Chemokine (C—C motif) ligand 5 (NM_013653)	> 1000	745.12 <i>p</i> = 0.0002	Mm01302428_m1
<i>Ccl9</i>	20 308	Chemokine (C—C motif) ligand 9 (NM_011338)	> 1000	20.22 <i>p</i> = 0.0001	Mm00441260_m1
<i>Cxcl10</i>	15 945	Chemokine (C—X—C motif) ligand 10 (NM_021274)	> 1000	518.39 <i>p</i> = 0.0002	Mm99999072_m1
<i>Cxcl16</i>	66 102	Chemokine (C—X—C motif) ligand 16 (NM_023158)	55.71	7.94 <i>p</i> = 0.0007	Mm00469712_m1
<i>Gbp2</i>	14 469	Guanylate nucleotide binding protein 2 (NM_010260)	> 1000	68.13 <i>p</i> = 0.0001	Mm00494575_m1
<i>Gpr65</i>	14 744	G-protein coupled receptor 65 (NM_008152)	> 1000	10.44 <i>p</i> = 0.0021	Mm02619732_s1
<i>Igtp</i>	16 145	Interferon $\gamma$ induced GTPase (NM_018738)	> 1000	10.43 <i>p</i> = 0.0002	Mm00497611_m1
<i>Iigp1</i>	60 440	Interferon inducible GTPase 1 (NM_021792)	> 1000	46.86 <i>p</i> = 0.0001	Mm00649928_s1
<i>Il1rn</i>	16 181	Interleukin 1 receptor antagonist (NM_001039701)	> 1000	> 1000 <i>p</i> = 0.0001	Mm01337566_m1
<i>Il4ra</i>	16 190	Interleukin 4 receptor, $\alpha$ (NM_001008700)	23.58	6.81 <i>p</i> < 0.0001	Mm00439634_m1
<i>Irgm</i>	15 944	Immunity-related GTPase family (NM_008326)	> 1000	21.93 <i>p</i> = 0.0024	Mm00492596_m1
<i>Isg20</i>	57 444	Interferon-stimulated protein (NM_020583)	> 1000	12.29 <i>p</i> = 0.0001	Mm00469585_m1
<i>Mpa2l</i>	100 702	Macrophage activation 2 like (NM_194336)	> 1000	61.41 <i>p</i> = 0.0001	Mm00843395_m1
<i>Saa1</i>	20 208	Serum amyloid A 1 (NM_009117)	> 1000	34.91 <i>p</i> = 0.0026	Mm00656927_g1
<i>Tlr2</i>	24 088	Toll like receptor 2 (NM_0119057)	> 1000	36.57 <i>p</i> = 0.0004	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  $\mu$ g/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 *p* < 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, *p* < 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.

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

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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