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Research report

Behavioral profile of $P2X_7$ receptor knockout mice in animal models of depression and anxiety: Relevance for neuropsychiatric disorders

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ABSTRACT

The purinergic $P2X_7$ receptor is a ligand-gated ion channel found on peripheral macrophages and microglia in the nervous system. Activation of $P2X_7$ receptors results in the rapid release of interleukin-1β (IL-1β). Cytokines like IL-1β are suggested to be involved in the pathophysiology of depression. The aim of this study was to behaviorally profile $P2X_7$ receptor knockout (KO) mice in behavioral models of depression- and anxiety-like behaviors. $P2X_7$ receptor KO and wild type (WT) mice were tested in multiple models including; forced swim test, tail suspension test, elevated plus maze, novelty suppressed feeding, spontaneous locomotor activity, and food intake. $P2X_7$ receptor KO mice exhibited an antidepressant-like profile in tail suspension test and forced swim test; an effect that was not associated with changes in spontaneous locomotor activity. In addition, $P2X_7$ receptor KO mice showed higher responsivity to a subefficacious dose of the antidepressant drug imipramine (15 mg/kg) in forced swim test. No significant differences between genotypes were observed in models of anxiety. These data support the relevance of pro-inflammatory cytokines in depressive-like states, and suggest that $P2X_7$ receptor antagonists could be of potential interest for the treatment of affective disorders.

1. Introduction

Depression is recognized as having high prevalence in several medical conditions including infectious, autoimmune and neurodegenerative diseases. Over the last two decades an increasing body of evidence has indicated a strong relationship between depression and immunological dysfunction in depressed patients [5,17,42]. It has been suggested that excessive secretion of macrophage cytokines, interleukin-1β (IL-1β), interferon-α (INF-α) and tumor necrosis factor-α (TNF-α) could be a potential causative factor for the disorder [58]. Several lines of evidence support a role for cytokines in depression: depression is accompanied by signs of immune activation and elevated levels of pro-inflammatory cytokines [32,49,64], pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 may produce "sickness behavior" and mediate many of the physiological and behavioral changes associated with depression [7,16,33,51], cytokines can induce neuroendocrine and neurochemical changes similar to a depressive syndrome [20,26,33,51], and clinical use of cytokines like IFN-α produces depressive-like symptoms that can be attenuated with antidepressant treatments [30].

The purinergic P2X receptors are ligand-gated ion channels activated by adenosine 5′-triphosphate (ATP) and other related nucleotides, and are formed by seven subunits that are encoded by different genes ($P2X_1$–$P2X_7$) [47]. Purinergic receptors are present in mammalian cells such as brain, autonomic and spinal cord neurons, glial cells and immune cells [47]. $P2X_7$ receptors can modulate cell growth, proliferation, cause apoptosis and cell death [1,23]. In addition, the $P2X_7$ receptor can modulate the maturation and release of IL-1β in macrophages and microglia and is involved in general mechanisms implicated in inflammation and progression of neurodegenerative diseases [22,24,25,29,39].

The $P2X_7$ receptor subunit gene in humans is located close to the tip of the long arm of chromosome 12. Previous genetic studies identified a susceptibility locus in the region of chromosome 12q23–24 that suggested a linkage with mood disorders such as bipolar disorder and major depressive disorder [2,14,38]. Recently, an increased vulnerability to bipolar affective disorders associated with single nucleotide polymorphisms in the chromosome 12q24.31 region that encodes for the $P2X_7$ receptor gene has been reported [8,56,57].

In view of the involvement of $P2X_7$ receptors in cytokine release, inflammation, and apoptotic cell death, it is reasonable to hypothesize that $P2X_7$ receptors could play a role in the

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pathology of depression. Considering that cytokines like IL-1β can induce behavioral and physiological changes that resemble depression, that immune activation and altered levels of pro-inflammatory cytokines are associated with major depression, and that $P2X_7$ receptors play an important role regulating the release of pro-inflammatory cytokines, it could be proposed that the inactivation of $P2X_7$ receptors might result in antidepressant-like effects and might represent a potential target for the treatment of depression.

To investigate the role of $P2X_7$ receptors in depression- and anxiety-like behaviors, mutant $P2X_7$ receptor knockout (KO) mice and wild type (WT) mice were tested in several well characterized preclinical paradigms used to evaluate depression- and anxiety-like behaviors.

2. Materials and methods

2.1. Generation of mutant $P2X_7$ receptor knockout mice

The generation of $P2X_7$ receptor gene KO mice has been previously described [31,59]. These mice show altered ATP-stimulated cytokine processing [59] and reduced signs of experimental arthritis [31]. $P2X_7$ receptor mutant mice used in the present studies were purchased from Lexicon Genetics Incorporated (The Woodlands, TX) and were generated based on previously established methodology [59]. The null targeting vector was derived using the Lambda knockout shuttle (KOS) system [65]. The Lambda KOS phage library, arrayed into 96 superpools, was screened by polymerase chain reaction (PCR) using exon 2-specific primers ($P2X_7$ receptor-5 [5′-CTTTGCTTTGGTGAGCGATAAG-3′] and $P2X_7$ receptor-6 [5′-CTGCAAAGGGAAGGTGTAGTC-3′]). The PCR-positive phage superpools were plated and screened by filter hybridization using the 169 bp amplicon derived from primers $P2X_7$ receptor-5 and $P2X_7$ receptor-6 as a probe. Three pKOS genomic clones, pKOS-46, pKOS-51, and pKOS-76 were isolated from the library screen and confirmed by sequence and restriction analysis. Gene-specific arms (5′-TTCCTCTGTCCTCAGCTTTGCTTTGGTGAGCG-3′) and (5′-ATCTCAGCCCTGCGTCCTATCAGAGTGGGTC-3′) were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-76 were co-transformed into yeast, and clones that had undergone homologous recombination to replace a 1496 bp region containing exons 2 and 3 with the yeast selection cassette were isolated. The yeast cassette was subsequently replaced with a LacZ/Neo selection cassette to complete the $P2X_7$ receptor targeting vector. The Not I linearized targeting vector was electrophorated into $129/SvEv^{Brd}$ (Lex-1) embryonic stem (ES) cells. G418/FIAU resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern analysis using a 211 bp 5′ external probe (21/22), generated by PCR using primers $P2X_7$ receptor-21 [5′-TCTTTGCTCAGAACTCTGGG-3′] and $P2X_7$ receptor-22 [5′-AGATATAGACGCAGATGGCC-3′], and a 322 bp 3′ internal probe (23/24), amplified by PCR using primers $P2X_7$ receptor-23 [5′-AGCCTCACAAATTCAGGAGG-3′] and $P2X_7$ receptor-24 [5′-TGTTCGGATGCACATTAGGG-3′]. Southern analysis using probe 21/22 detected an 8.8 kb WT band and 4 kb mutant band in Hind III digested genomic DNA while probe 23/24 detected an 8.8 kb WT band and 8.2 kb mutant band in Hind III digested genomic DNA. Two targeted ES cell clones were microinjected into C57BL/6 (albino) blastocysts. The resulting chimeras were mated to C57BL/6 (albino) females to generate mice that were heterozygous for the $P2X_7$ receptor mutation. These heterozygous mice were then crossed to produce homozygous $P2X_7$ KO offspring, homozygous WT mice and heterozygous $P2X_7$ offspring in the Mendelian ratios of 1:1:2. The homozygous $P2X_7$ KO and WT mice were bred to produce pure breeding colonies of $P2X_7$ KO mice and WT mice.

2.2. Animals

$P2X_7$ KO mice and WT male mice (3–5 months old) were allowed to habituate to our facilities for at least 2 weeks prior the start of the experiments. Mice were group housed (5–10 per cage) and maintained on a 12:12 h light–dark schedule (lights on 6:00, lights off 18:00), in a temperature and humidity controlled environment ($22 \pm 1\,^{\circ}C$, 60–70% humidity). Animals had free access to food and water, unless otherwise specified. Homozygous breeding was used to generate pure colonies of KO and WT mice instead of the heterozygote mating strategy that generates the null mutant, the WT, and heterozygote mice from the same parents and in an identical environment. However, the behavioral data were replicable among independent batches of animals tested in similar assays. Mice from three different cohorts were each tested in more than one behavioral paradigm with at least 5–7 days between tests. Data obtained with the first cohort of mice were replicated with other animal shipments. The results shown are representative of the combined data, except for the forced swim test experiment where the behavioral profile was replicated but due to the experimental design used in the forced swim test + imipramine study, data were not combined, and food consumption experiment that was conducted only once. Mice that received imipramine injection were only from the third shipment and were not tested in additional experiments after the treatment.

Protocols were approved by the Abbott Laboratories Institutional Animal Care and Use Committee (IACUC) in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animals Care (AAALAC). All the studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Research Council (1996).

2.3. Mouse peritoneal macrophages IL-1 release assay

Resident resting macrophages were collected by lavaging the peritoneal cavities of mice with RPMI 1640 Hepes plus 5% of fetal calf serum (FCS, heat-inactivated at 56 °C for 30 min) and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively), immediately before the animals were euthanized. The cells were pelleted and re-suspended to give 2×10^6/ml, and 0.5×10^6 cells were plated in 24 well plates in which the cells were allowed to attach for 2 h. The non-attached cells were washed out of the wells, and the cells were cultured overnight. The next day, 3 μg/ml of lipopolysaccharide (LPS) was added, and the cells were allowed to prime for 2 h. The cells were then challenged with 1–3 mM of the $P2X_7$ receptor agonist 2′,3′-O-(benzoyl-4-benzoyl)-adenosine 5′-triphosphate (BzATP) for 30 min, and the plates were spun and the supernatants collected and analyzed for IL-1β as described in the commercial kit for mouse IL-1β ELISA (Endogen, Pierce Technology, Rockford, IL, USA). Mice used for this assay were not used previously in any behavioral study.

2.4. Mouse tail suspension test

The procedure is similar to the forced swim assay in that it measures "behavioral despair" in mice (i.e. mice stop struggling in the face of an inescapable stressor) [61]. The inescapable stress of suspending a mouse by its tail triggers different behavioral responses in the animal that represent coping strategies to deal with the stress. After an initial period where the mice show vigorous movements attempting to escape, the mice then develop an immobile posture, which has been suggested to represent a state of lowered mood or depressive-like behaviors. Antidepressant treatments significantly increase the time that animals actively engage in escape-oriented behaviors [13,61]. Mice were acclimated to the testing room for at least 1.5 h prior to testing. Mice were tested in individual cages acoustically and visually isolated. Four mice were tested at one time. Mice were suspended from a hook located 38 cm above the floor of the cage, using a piece of tape wrapped around the tail approximately 20 mm from the tip. The hook was attached to a transducer that communicated information about duration and strength of movements to the acquisition interface and the computer. The automated device used for tail suspension test was adapted from other equipment, custom designed in house and extensively validated pharmacologically in our laboratory. The test took 6 min during which immobility time (s) was recorded. Immobility was defined when the mouse hung passively and completely motionless from the hook [13,61].

2.5. Mouse forced swim test

The procedure is based on that described by Porsolt et al. [52]. Mice were habituated to the testing room for at least 1.5 h before the experiment. The forced swim test was conducted as a single trial experiment, by individually placing the mice into cylindrical plastic containers (height, 25 cm; diameter, 18 cm) containing 15 cm of water at 23–25 °C. Containers were non-transparent, preventing the mice from seeing each other.

Test duration was 6 min, and immobility was analyzed during the last 4 min period of the test-swim. Mice were monitored during the testing and, after that, they were removed from the container and left to dry in a heated enclosure before they were returned to their home cages. Test-swims were videotaped and subsequently assessed for immobility time (s). Immobility behavior was described as when the animal remains floating passively in the water without any vertical or horizontal movements, with the exception of minimal movements of the mouse necessary to keep its head above water [50,52]. In the experiment where a subthreshold dose of imipramine (i.e., a dose that does not induce a significant effect in the model) was tested, mice belonging to each genotype were treated either with vehicle (sterile water) or imipramine hydrochloride (15 mg/kg, Sigma, St. Louis, MO, USA) i.p. 30 min before the test.

2.6. Locomotor activity

Mice were habituated to the testing room under dim white light for 1 h before testing. Locomotor activity was recorded by individually placing the mice into automated activity chambers (Versamax from Accuscan, Columbus, OH, USA) measuring 42 cm × 30 cm × 42 cm (length × height × depth). Activity was recorded for 90 min and detected by infrared photo beam sensors. Measures were collected in 5 min bins. Data were expressed as distance moved (cm) as well as distance moved in the periphery versus the center area of the arena. The center of the arena was defined as the area included within 26.25 cm × 26.25 cm. Locomotor activity experiments were carried out between 10:00 and 16:00 h.