SUPPLEMENTAL DATA

Material and methods

HPLC analysis was performed on a LaChrom Elite HPLC system (Hitachi, Armstadt, Germany) equipped with a UV spectrometer. For analysis of radiolabeled compounds, the HPLC eluate, after passage through the UV detector, was led over a shielded 3-inch NaI(Tl) scintillation detector connected to a single channel analyser (Gabi box, Raytest, Straubenhardt, Germany). The output signal was recorded and analyzed using a GINA Star data acquisition system (Raytest). Radioactivity in samples of biodistribution studies, cell uptake experiments and radiometabolite analysis was quantified using an automated gamma counter equipped with a 3-inch NaI(Tl) well crystal coupled to a multichannel analyser (Wallac 2480 Wizard, Wallac, Turku, Finland). Results were corrected for background radiation, physical decay and counter dead time.

Animals were housed in individually ventilated cages in a thermoregulated (~22 °C), humidity controlled facility under a 12h/12h light/dark cycle with access to food and water ad libitum. All animal experiments were conducted according to the Belgian code of practice for the care and use of animals, after approval from the local University Ethics Committee for Animals.

In vitro pharmacology of JNJ-54173717

JNJ-54173717 was assayed for functional antagonism in a calcium flux assay and for binding at rat cortex in membrane binding assay. 1321N1 cells expressing P2X7R orthologues were dissociated 18-24 hours prior to the assay using 0.05% trypsin/EDTA (Invitrogen), and plated at density of 25000 cells/well into PDL coated 96-well black-walled clear bottom plates (Becton-Dickinson, Bedford, MA). On the day of the experiment, cell plates were washed with assay buffer, containing (in mM): 130 NaCl, 2 KCl, 1 $CaCl_2$, 1

$MgCl_2$, 10 HEPES, 5 glucose; pH 7.40. After the wash, dye loading was achieved by adding a 2X Calcium-4 (Molecular Devices) dye solution in the assay buffer. Cells were stained with the Calcium-4 dye in staining buffer for 30 minutes at room temperature in the dark. Test compounds were prepared at 250X the final test concentration in neat dimethylsulfoxide (DMSO). Intermediate 96-well compound plates were prepared by transferring 1.2 μL of the compound into 300 μL of assay buffer. A further 3X dilution occurred when transferring 50 μL/well of the compound plate to 100 μL/well in the cell plate. Cells were incubated with test compounds and dye for 30 minutes. Calcium flux was monitored in FLIPR^{Tetra} as the cells were challenged by adding 50 μL/well of BzATP. The final concentration of BzATP was 250 μM. For radioligand displacement binding, rat cortices were dissected for membrane (P2X7R) preparation. 50 mM Tris-HCl (PH 7.4) was added to the cortex and homogenized for ~30S at high speed. The homogenate was centrifuged at 1500 rpm for 5 mins followed by careful decanting of the supernatant which was centrifuged at 32000g for 30 mins. The resulting membrane pellet was resuspended in ice-cold assay buffer (Tris-HCl + 0.1% BSA). The protein suspension was mixed with 3H-JNJ-54232334, a P2X7R selective ligand *(1)*, with increasing concentration of JNJ-54173717 and incubated for 1 hour at 4 °C. 100 μM of A-804598 was used to define non-specific binding. The assay was terminated by filtration and radioactivity counted.

Radiosynthesis

Carbon-11 was produced *via* a [14N(p,α)11C] nuclear reaction. The target gas, a mixture of N_2 (95%) and H_2 (5%), was irradiated using 18-MeV protons from a Cyclone 18/9 cyclotron (IBA, Louvain-la-Neuve, Belgium) at a beam current of 25 μA. The irradiation was done for about 30 min to yield 11C-methane (11C-CH_4). 11C-CH_4 was then transferred to a home-built recirculation synthesis module and trapped on a Porapak column that was immersed in liquid nitrogen. After flushing with helium, the condensed 11C-CH_4 was converted to the gaseous phase by bringing the Porapak loop to room temperature. 11C-CH_4 was then reacted with vaporous I_2 at 650 °C to convert it to 11C-methyl iodide (11C-

MeI). Subsequently, 11C-MeI was passed over a silver triflate column (6 mm x 50 mm) at 180 °C. The resulting 11C-methyl triflate (11C-MeOTf) was bubbled with a flow of helium through a solution of the precursor (0.2 mg) and Cs_2CO_3 (1-3 mg) in anhydrous dimethylformamide (DMF) (0.2 mL). After stabilization of the radioactivity in the reaction vial the reaction mixture was left at room temperature for 3 min. The crude mixture was diluted with water (0.6 mL) and injected onto an HPLC system (XBridge C_{18} column, 5 µm, 4.6 mm × 150 mm; Waters, Milford, USA) eluted with a mixture of 0.05 M NaOAc (pH 5.5) and EtOH (60:40 v/v) at a flow rate of 1 mL/min. UV detection of the HPLC eluate was performed at 254 nm. The radiolabeled product was collected after 11 min. The collected peak corresponding to the desired radioligand was then diluted with saline (Mini Plasco®, Braun, Melsungen, Germany) to obtain a final ethanol concentration < 10% and the solution was sterile filtered through a 0.22-µm membrane filter (Millex®-GV, Millipore, Billerica, MA, USA).

The tracer identity and the chemical and radiochemical purity of 11C-JNJ-54173717 formulation were analyzed using an analytical HPLC system consisting of an XBridge C_{18} column (3.5 µm, 3.0 mm x 100 mm, Waters) eluted with a mixture of 0.05 M NaOAc (pH 5.5) and CH_3CN (70:30 v/v) at a flow rate of 0.8 mL/min. UV detection was performed at 220 nm.

Biodistribution studies

The biodistribution study of 11C-JNJ-54173717 was performed in healthy non-perfused female Wistar rats (body weight, 185-220 g) at 2, 30 and 60 min after tracer injection (n=3/time point). Rats were anesthetized with isoflurane (2.5% in oxygen at a flow rate of 1 L/min) and injected with 11C-JNJ-54173717 (~19 MBq) via a tail vein. The animals were sacrificed by decapitation at the indicated time points. Blood and major organs were collected in tared tubes and weighed. The radioactivity in the dissected organs and blood was counted using an automated gamma counter. For calculation of total blood radioactivity, blood mass was assumed to be 7% of the body mass.

Rat plasma radiometabolite analysis

Radiometabolites of 11C-JNJ-54173717 in plasma of normal female Wistar rats (n=2) were quantified at 30 min after tracer injection following a previously described procedure *(2)*. The Chromolith C_{18} column was eluted with gradient mixtures of 0.05 M NaOAc (pH 5.5) (A) and CH_3CN (B) (0-4 min: isocratic 0% B and flow rate of 0.5 mL/min; 4-14 min: linear gradient 0% B to 90% B and flow rate of 1 mL/min; and 14-17 min: isocratic 90% B and flow rate of 1mL/min). UV detection was done at 220 nm.

Perfused rat brain radiometabolite analysis

Radiometabolites of 11C-JNJ-54173717 in perfused cerebrum and cerebellum of normal female Wistar rats (n=2) were quantified at 30 min after tracer injection following a previously described procedure *(2)*. Homogenates were analyzed using an analytical XBridge column (C_{18}, 5 µm, 3 mm x 100 mm; Waters) eluted with a mixture of 0.05 M sodium acetate (pH 5.5) and CH_3CN (65:35 v/v) at a flow rate of 0.8 mL/min. UV detection was performed at 220 nm.

Viral vector construction and production

LV transfer plasmids were constructed encoding hP2X7R tagged with eGFP or a triple flag-tag (3flag). The hP2X7R cDNA sequence (GenBank™ accession number Y09561) was amplified by PCR from phP2X7R-eGFP (kindly provided by the research group of Ben Gu of the University of Sydney, Australia *(3)*) using primers DO_1 and DO_2 (Supplemental Table 3). The resulting amplicon was digested with XhoI and MluI, purified and cloned in XhoI-MluI sites of the LV transfer plasmid pCHMWS-eGFP-T2A-fLuc-IRES-PuroR *(4)* yielding pCHMWS-eGFP-hP2X7R-IRES-PuroR. To generate pCHMWS-3flag-hP2X7R–IRES-PuroR, the eGFP cDNA was removed from pCHMWS-eGFP-hP2X7R-IRES-PuroR with XbaI and XhoI and a 3flag epitope tag *(5)* was cloned following adaptor annealing using primers DO_3 and DO_4 (Supplemental Table 3). LV

particles were produced and quality controlled by the Leuven Viral Vector Core as described previously *(6)*, concentrated using Vivaspin™ columns (Vivascience, Hannover, Germany), aliquoted and stored at -80 °C until use, and are referred to as LV_eGFP-hP2X7R and LV_3flag-hP2X7R, for short (Supplemental Fig. 1).

To clone 3flag-hP2X7R in the rAAV transfer plasmid, the 3flag-hP2X7R cDNA was amplified by PCR using pCHMWS-3flag-hP2X7R-IRES-PuroR as a template using primers DO_5 and DO_6 (Supplemental Table 3). The amplified fragment and the rAAV transfer plasmid were digested with XbaI and MluI. The 3flag-hP2X7R cDNA was then ligated downstream of the CaMKII 0.4 promoter *(7)*, yielding pAAV-CaMKII 0.4-3flag-hP2X7R. rAAV vector productions were performed and quality controlled by the Leuven Viral Vector Core as described before *(8)*, and are referred to as rAAV_3flag-hP2X7R (Supplemental Fig. 3).

Cell culture and transduction

SHSY5Y (human dopaminergic neuroblastoma) cells were seeded in a 96-well plate at 20,000 cells per well for LV transduction experiments. Transduced cells were selected using puromycin (10 µg/mL in Dulbecco's Modified Eagle's Medium (DMEM)) and subsequently maintained in DMEM with Glutamax (Gibco BRL, Invitrogen, Merelbeke, Belgium) supplemented with 7% fetal calf serum (FCS, Harlan Sera-Lab, International Medical, Brussels, Belgium) and 10 µg/mL puromycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Western blot analysis

Extracts of transduced SHSY5Y cells were made using 1% sodium dodecyl sulfate (SDS). 10 µg of total protein samples were separated in a self-cast 12.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Watford, United Kingdom). The PVDF membrane was blocked at room temperature using phosphate buffered saline (PBS) containing 5% milk powder and then

at 4 °C overnight with a primary antibody against the eGFP (rabbit, 1:500, in house) and 3flag epitope *(5)* (mouse, 1:500, Sigma, St. Louis, MO, USA) at a dilution of 1:1000. After washing in PBS + 0.1% Triton (3 x 10 min) and blocking with 5% milk powder in PBS, the PVDF membrane was incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody *(5)* at a dilution of 1:10000. The blot was washed in PBS + 0.1% Triton (3 x 10 min) and immunoreactive proteins were visualized using an enhanced chemiluminescence plus (ECL^+) kit (Amersham Biosciences, Uppsala, Sweden).

Immunocytochemistry of transduced SHSY5Y cells

Cells were seeded in LabTec chambers. After fixation with paraformaldehyde 4%, transduced SHSY5Y cells were washed twice with 1x PBS. After permeabilisation of the cell membranes with 0.1% TritonX100 for 5 min, the cells were incubated with a blocking buffer containing 10% horse serum for 20 min, followed by an overnight incubation with primary antibody against eGFP (rabbit, 1:500, in house) and 3flag (mouse, 1:500, Sigma) at 4 °C for LV_eGFP-hP2X7R and LV_3flag-hP2X7R transduced cells, respectively. The next day, cells were washed with PBS and incubated for 1 hour at room temperature with the secondary antibody (goat anti-rabbit Alexa488 and goat anti-mouse Alexa488 (1:500; Molecular Probes, Eugene, USA). Thereafter, the glass plates were mounted on a cover-slip and the expression was analyzed by fluorescence microscopy (Leica DMR, Groot Bijgaarden, Belgium).

Cell uptake experiments

One day before the cell binding study, transduced SHSY5Y cells were plated in a 24-well plate (300,000/well) (n=6). Before starting the study, cells were checked for confluency (should be 90-100%). The incubation medium was removed from the cells and 0.25 mL of fresh medium containing 11C-JNJ-54173717 (1.11 MBq) was added. Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 30 min.

Following incubation and removal of the medium, the cells were washed 3 times with PBS at 4 °C. Next, the cells were lysed with 0.25 mL of lysis buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.2% NP40, 10% glycerol) for 10 min after which the lysate was collected, followed by 0.25 mL rinse using the same solution. The cell fractions (rinse and lysate) as well as the wash fractions (medium and PBS) were collected separately for each well and the radioactivity was measured using a gamma counter. The protein concentration was determined using the Bio-Rad Protein Assay (BioRad, München, Germany) and a spectrophotometer (Bio Synchron-Anthos 2010, Anthos LabTec instruments, Austria) at 595 nm. The tracer uptake was normalized for total protein content in the cell fraction for each individual well and expressed as percentage of total radioactivity per mg of protein.

Stereotactic injection of viral vector

Male Wistar rats (n=7) were anesthetized by intraperitoneal injection of ketamine (75 mg/kg; Ketalar, Pfizer, Brussels, Belgium) and medetomidin (1 mg/kg; Domitor, Pfizer) and positioned in a stereotactic head frame (Stoelting, Wood Dale, IL, USA). Using a 30-gauge needle connected to a 10 µL Hamilton syringe, 4 µL of the hP2X7R vector (rAAV_3flag-hP2X7R) and of the control vector (rAAV_3flag-eGFP) were injected at a rate of 0.4 µL/min into the right and left striatum respectively. The coordinates used for striatal injection of the hP2X7R vector were anteroposterior 0 cm, lateral -0.28 cm and dorsoventral -0.64 cm relative to bregma. The coordinates used for striatal injection of the control vector were anteroposterior 0 cm, lateral +0.28 cm and dorsoventral -0.64 cm relative to bregma. After 10 min of injection (4 µL), the needle was raised slowly. After surgery, anaesthesia was reversed with an intraperitoneal injection of atipamezol (0.5 mg/kg; Antisedan, Orion Pharma, Newburry, Berkshire, UK). Experiments were performed between 5 and 10 weeks after viral vector injection as stable expression of rAAV vectors in the rat brain was demonstrated by Vandeputte et al. for this time period *(9)*.

In vitro autoradiography

Rats (rAAV_3flag-hP2X7R in the right striatum and rAAV_3flag-eGFP in the left striatum; n=4) were sacrificed 5-10 weeks after viral vector injection, brain was removed, rapidly frozen in 2-methylbutane (-40 °C) and stored at -20 °C for at least 24h. Transversal sections (20 µm) of the brain were obtained using a cryotome (Shandon cryotome FSE, Thermo Fisher Scientific, Waltham, USA), mounted on adhesive microscope slides (Superfrost Plus; Thermo Fisher Scientific) and stored at -20 °C. Transversal brain slices (20 µm) were also made from WT Wistar rat (n=3). 20-µm coronal brain sections from WT (n=4) and P2X7R knockout (n=4) mice were provided by Janssen Research & Development (Beerse, Belgium). Brain slices were preincubated in Tris-HCl 50 mM buffer (pH 7.4) for 10 min (2 times) at room temperature and dried. Next, the brain sections were incubated with 11C-JNJ-54173717 (150 kBq in Tris buffer pH 7.4 + 0.1% bovine serum albumin (BSA) for rat brain slices and 100 kBq in Tris buffer pH 7.4 + 0.1% BSA for mouse brain slices) or tracer in the presence of 10 µM of the P2X7R antagonist A-740003 (Santa Cruz Biotechnology, Texas, USA) or with tracer in the presence of authentic reference JNJ54173717 for 20 min. After the incubation, the brain sections were washed twice for 5 min in ice-cold Tris-HCl 50 mM buffer (pH 7.4) with 0.1% BSA. After a quick dip in purified ice-cold water, the slides were dried. Autoradiograms were obtained by exposing the slides overnight to a high-performance phosphor storage screen (super-resolution screen; Perkin Elmer, Waltham, USA). The screens were read using a Cyclone Plus system (Perkin Elmer) and analyzed using Optiquant software (Perkin Elmer). The radioactivity concentration in the autoradiograms is expressed in digital light units (DLU)/mm².

Histology of brain sections

After the microPET scans, in order to verify the expression of hP2X7R in the rat striatum, 3 animals were sacrificed with an intraperitoneal injection of pentobarbital followed by a transcardial perfusion with

4% paraformaldehyde in PBS. The brain was isolated and after overnight post-fixation, 50-μm coronal sections were made using a vibratome (Microm, Walldorf, Germany). Brain sections were treated with 3% hydrogen peroxide and incubated overnight with the primary anti-3flag antibody in 10% donkey serum. Next, the sections were incubated with biotinylated donkey anti-rabbit secondary antibody, followed by incubation with Strept-ABC-HRP complex (Dako, Glostrup, Denmark). Detection was with diaminobenzidine using H_2O_2 as a substrate. Analysis was done by light microscopy.

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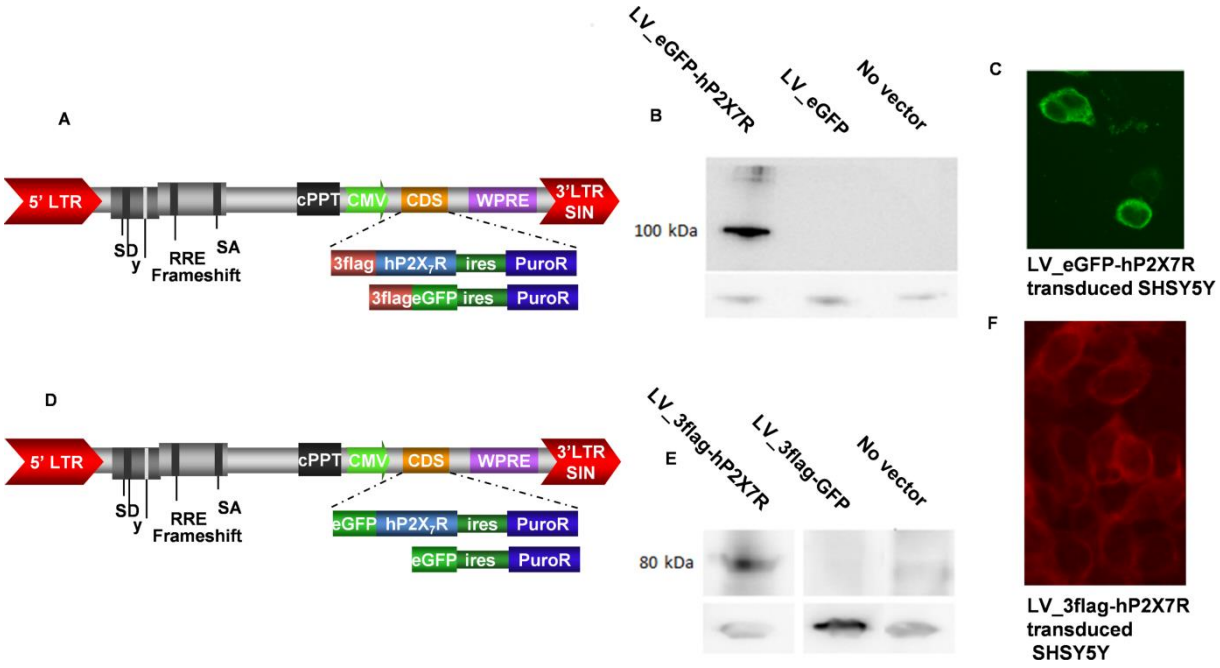
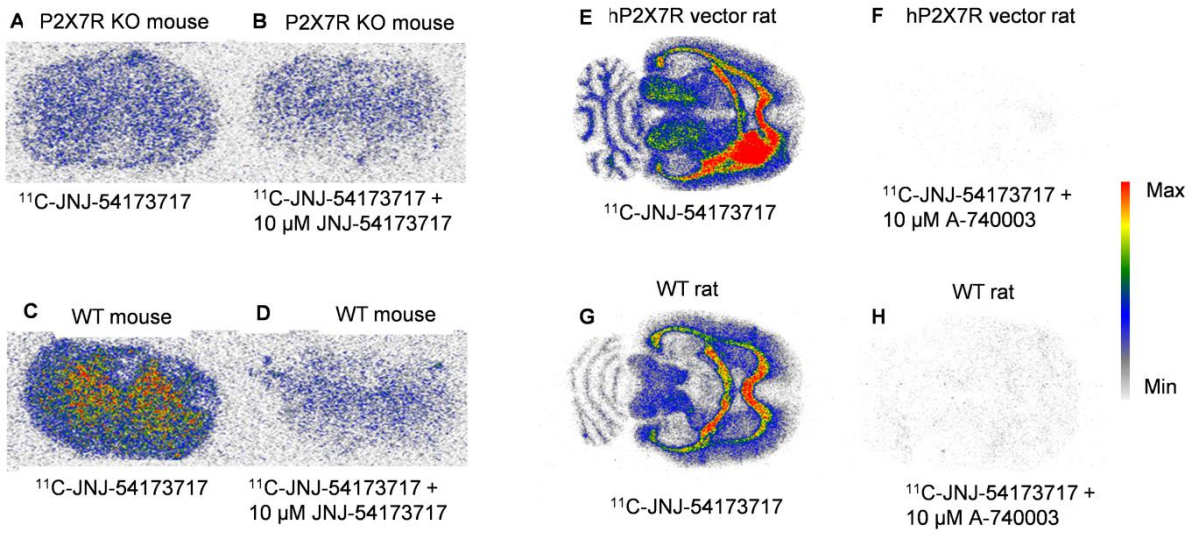
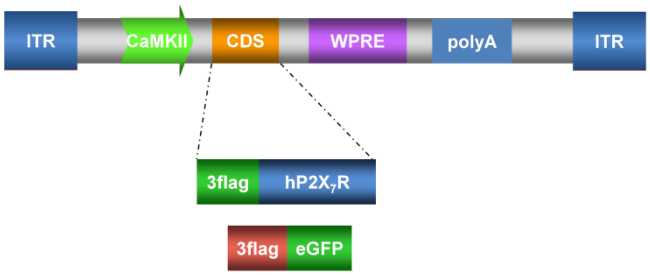


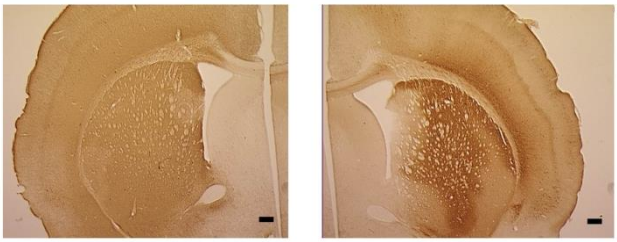
Supplemental figure 1: Viral vector mediated overexpression of hP2X7R in cell culture. The cDNA expression in the lentiviral constructs is driven by the hCMVie promoter and followed by the woodchuck hepatitis posttranscriptional element (WPRE). The promoter is preceded by the central polypurine tract (cPPT). The LV cassette is flanked by the 5' long terminal repeat (LTR) and 3' self-inactivating (SIN) LTR. A: Schematic representation of lentiviral vectors encoding eGFP-hP2X7R and eGFP, linked to puromycin resistance cassette by an IRES sequence. D: Lentiviral vectors encoding 3flag-hP2X7R and 3flag-GFP, linked to puromycin resistance cDNA by an IRES sequence. B and E: Western blot analysis of LV transduced SHSY5Y cells shows expression of LV_eGFP-h P2X7R (~ 100 kDa) and LV_3flag-h P2X7R (80 kDa). C and F: Immunocytology of hP2X7R expression in cell culture after LV transduction. SHSY5Y cells were transduced with LV_eGFP-h P2X7R and LV_3flag-h P2X7R. Cells were stained with an in house anti-eGFP antibody and an anti-3flag antibody for LV_eGFP-h P2X7R and LV_3flag-h P2X7R transduced cells respectively. Expression was detected mainly in the plasma membrane.



Supplemental figure 2: *In vitro* autoradiography binding studies on slices of WT (n=4) and P2X7R knockout (n=4) mouse brain, of rat brain injected with the hP2X7R vector (n=4) and of WT rat brain (n=3) using 11C-JNJ-54173717. A-D: coronal WT and P2X7R knockout mouse brain slices incubated with tracer or with tracer in the presence of 10 µM JNJ-54173717(self-block). E-F 11C-JNJ-54173717 shows specific binding to the rAAV-3flag-hP2X7R injected site which can be blocked with 10 µM A-740003. G-H: transversal brain sections of a WT rat. High displaceable binding is observed on white matter. Images A to D, images E - F, and images G - H are three separate experiments performed with different batches of tracer.



Supplemental figure 3: Schematic representation of recombinant adeno-associated viral vector for overexpression of hP2X7R in the rat brain. Gene expression is driven by the CaMKII promoter for 3flag-hP2X7R and by hCMVie promoter for 3flag-eGFP.The rAAV cassette is flanked by inverted terminal repeats (ITR).



Supplemental figure 4: Histology in hP2X7R rat model. Representative histology image using the 3flag antibody (scale bar = 400 µm; n=3) showing expression of the viral vectors in the right and left striatum.

Supplemental table 1. Biodistribution of 11C-JNJ-54173717 in normal rats at 2, 30 and 60 minutes after tracer injection.

% ID[a]			
	2 min	30 min	60 min
urine	0.06 ± 0.1	0.86 ± 0.0	1.11 ± 0.5
kidneys	4.47 ± 0.8	1.86 ± 0.1	1.78 ± 0.2
liver	34.80 ± 2.1	19.37 ± 0.5	17.08 ± 0.3
spleen + pancreas	1.44 ± 0.4	0.61 ± 0.1	0.61 ± 0.1
lungs	2.06 ± 0.3	0.98 ± 0.1	1.12 ± 0.4
heart	1.34 ± 0.2	0.44 ± 0.0	0.40 ± 0.1
intestines	14.03 ±1.8	23.77 ± 1.1	26.66 ± 9.1
stomach	1.70 ± 0.3	2.14 ± 1.0	5.05 ± 3.4
cerebrum	0.47 ± 0.0	0.28 ± 0.0	0.33 ± 0.1
cerebellum	0.14 ± 0.0	0.06 ± 0.0	0.06 ± 0.0
blood	9.97 ± 2.4	5.15 ±0.6	5.25 ± 0.6
carcass	35.12 ± 4.1	46.83 ± 0.5	43.15 ± 5.3

[a] Percentage of injected dose calculated as (counts per minute (cpm) in organ/total cpm recovered) x 100. Data are expressed as mean ± SD; n=3 per time point.

Supplemental table 2. 11C-JNJ-54173717 concentration in the different rat brain regions and blood at 2, 30 and 60 minutes after tracer injection

SUV[a]			
	2 min	30 min	60 min
striatum	0.78 ± 0.0	0.45 ± 0.0	0.50 ± 0.1
hippocampus	0.74 ± 0.0	0.43 ± 0.0	0.52 ± 0.1
cortex	0.80 ± 0.2	0.58 ± 0.1	0.65 ± 0.1
rest of cerebrum	0.86 ± 0.0	0.46 ± 0.0	0.53 ± 0.1
whole cerebrum	0.83 ± 0.0	0.46 ± 0.0	0.54 ± 0.1
cerebellum	1.02 ± 0.1	0.53 ± 0.0	0.59 ± 0.1
blood	1.42 ± 0.3	0.74 ± 0.1	0.75 ± 0.1

[a] Calculated as (radioactivity in cpm in organ/weight of organ in grams)/(total cpm recovered/body weight rat in grams). Data are expressed as mean ± SD; n=3 per time point.

Supplemental table 3. Primers used for cloning

DO_1	5'-AAAAAAACTCGAGATGCCGGCCTGCTGCAGCTGCAG-3'
DO_2	5'-AAAAAAACGCGTTTAGTAAGGACTCTTGAAGCCACT-3'
DO_3	5'-CTAGGACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGA TTACAAGGATGACGATGACAAGC-3'
DO_4	5'-TCGAGCTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACCGTCA TGGTCTTTGTAGTCCATGGTC-'3
DO_5	5'-AAAAAATCTAGAATGGACTACAAAGACCATGAC-3'
DO_6	5'-AAAAAAACGCGTTTAGTAAGGACTCTTGAAGCCAC-3'