

Online supplementary document

Animals

Male and female GAERS, Wistar Hannover (WH) and NEC rats aged 14 days (P14; respectively $24 \pm 3.5\text{g}$, $30 \pm 1\text{g}$ and $28 \pm 2\text{g}$), 21 days (P21; respectively $35 \pm 3.5\text{g}$, $46 \pm 2.5\text{g}$ and $40 \pm 4\text{g}$) and 90 days (P90; respectively $260 \pm 17\text{g}$, $306 \pm 3\text{g}$ and $322 \pm 39\text{g}$) were used in this study. GAERS and NEC were obtained from the University Grenoble Alpes animal facilities and WH were purchased from Charles River (L'Arbresle, France). Animals were maintained in cages with food and drink *ad libitum*. Temperature ($22 \pm 1^\circ\text{C}$) and lighting (12 h/12 h cycle; light period from 7 a.m. to 7 p.m.) were controlled. All animal procedures fulfilled both ARRIVE (Animal Research: Reporting of In Vivo Experiments) and Basel declarations (<http://www.basel-declaration.org>) guidelines, including the 3R concept. Experiments were performed in accordance with the rules of the European Community Council directive of September 22, 2010 (2010–63) and the French guidelines on the use of living animals in scientific investigations, with the approval of the “Grenoble-Institut des Neurosciences ethical committee”, French Ministry of Higher Education and Research agreement number 004 (01/04/2009).

Autoradiographic procedures

Rats were anaesthetized by injection of sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused with saline (0.9%). Brains were rapidly removed from the skull, frozen in -20 to -40°C isopentane2-methylbutane, and stored at -80°C . Frozen brains were cut into coronal sections ($14 \mu\text{m}$ thick) using a cryostat and thaw-mounted onto Superfrost slides (Fisher Scientific, Hampton, NH) and kept at -80°C . In the following experiments, three slides (up to nine sections) were used per animal

Online supplementary document

from the different structures, for each autoradiographic assay. For each age (P14, P21 and P90), seven animals of each strain (GAERS, NEC and WH) were used.

To label D3R, [¹²⁵I]7OH-PIPAT autoradiography was performed with a protocol adapted from Burriss *et al.*, 1994 and Stanwood *et al.*, 2000.^{1,2} To dissociate dopamine from the receptors, sections were preincubated (4 min, 3 times at room temperature (RT)) in a solution consisting of 50 mM HEPES buffer (pH=7.4), 1 mM EDTA, 0.28 mM ascorbic acid, bovine serum albumin (BSA) 1g.L⁻¹, 100 μM guanosine 5'-triphosphate (GTP, to inhibit fixation to D2 and 5HT1A receptors) and 25 μM 1,3-di(2-tolyl)guanidine (DTG, to protect sigma receptors). To estimate total binding, incubation (60 min, RT) with 0.3 nM [¹²⁵I]7OH-PIPAT (PerkinElmer, Courtaboeuf, France) was performed in preincubation solution. Nonspecific binding was determined in the presence of 10 μM DA. The reaction was stopped by washing (4 times 15 min, 4 °C) the slides in 50 mM HEPES buffer (pH=7.4). After a dip in ice-cold deionized water (4°C, 5 sec.), sections were dried and put in contact with Biomax MR Films for up to 6 days in light-proof X-ray cassettes. The films were developed by immersion at RT using Kodak GBX Developer (60 sec.), rinsed in tap water (60 sec.), fixed using Kodak GBX Fixer (60 sec.) and rinsed in deionized water (Kodak processing chemicals for autoradiography films, Sigma-Aldrich). Total and non-specific [¹²⁵I]7OH-PIPAT binding was estimated based on an average of at least six measures from each brain region. After digitization of films (Capture NX2 Nikon Software), optical density values were derived from the autoradiograms of [¹²⁵I]-7-OH-PIPAT binding with Autoradio V4.03 software (SAMBA Technologies, Meylan, France).

To label DAT sites, slices were preincubated in 50 mM Tris-HCl buffer (pH 7.5) containing 450 mM NaCl, 0.02% BSA and 1 μM cis-flupentixol for 30 min at

Online supplementary document

RT. Slices were then incubated for 20 h at 4 °C in Tris–HCl buffer containing 2 nM of [3H]-GBR12935 (1-[2-(diphenyl-methoxy)ethyl]-4-(3-phenylpropyl)-piperazine-3H, SA 43.5 Ci/mmol, PerkinElmer, Courtaboeuf, France).^{3,4} Nonspecific binding was determined in the presence of 10 µM GBR 12909 dihydrochloride. The reaction was stopped by dipping the slides first in ice-cold desionized water (10 sec., 4°C) and slides were then washed in 50 mM Tris-HCl buffer (3 times, 5 min, 4 °C). After a dip in ice-cold desionized water (10 sec, 4°C), sections were dried and transferred into cassettes apposed to Fuji BAS phospho-imaging plate (*BAS-TR2025, Fuji Imaging Plates*) with calibrated [³H] standards for up to 7 days. Sections were scanned and images were analysed using a Bio-image Analyzer Phosphoimager BAS5000 (Fuji Photo Film, Japan).

Total and non-specific binding was estimated based on an average of at least six measures from each brain region (striatum, nucleus accumbens core and shell, motor cortex, somatosensory cortex, islands of Calleja, olfactory tubercles). Values of optical densities measured from each structure analyzed were expressed as a mean ± standard error of the mean (SEM) of relative optical density (ROD) after removing of the corresponding value for non-specific binding. Data were analyzed for each brain structure by Kruskal-Wallis tests with Prism 6.0 software (Graphpad, La Jolla, USA). Mean to mean comparisons were carried out with the Dunn's method.

Dopamine transporter SPECT imaging

SPECT/CT imaging

6 GAERS, 5 NEC and 6 WH were used in this study. One hour following DatScan intravenous injection to restrained animals (47 ± 6 MBq), Single-Photon Emission Computed Tomography (SPECT) and Computed Tomography (CT) acquisitions were

Online supplementary document

performed under gas anesthesia (Isoflurane 2%, Baxter) using dedicated preclinical camera (nanoSPECT/CT, Bioscan, Washington, USA).⁵ CT acquisition time was 4 min using the following acquisition parameters: 55kvp, 240 projections and 1000ms per projection. The helicoidal SPECT acquisition was performed with 4 heads equipped with multi-pinholes collimators (9 x 1.2 mm diameter pinhole per head) using 24 projections and 30 min of acquisition. CT and SPECT acquisitions were reconstructed and fused using dedicated software (InVivoScope, Invicro, Boston, USA).

MRI imaging

Magnetic Resonance Imaging (MRI) was performed at 4.7T (Avance III console; Bruker – Grenoble MRI facility IRMaGE) in three additional animals - i.e., one animal of each group (GAERS, WH and NEC) - in order to obtain a representative anatomical brain imaging from each group, as described before.⁶ These images were compared to a standardized MRI template of the WH strain⁷ showing no major differences in volume and radioactive tracer uptake in the selected volumes of interest (VOIs) (data not shown).

In vivo quantification

SPECT/CT and MRI images were fused using the appropriate MRI template and volumes of interest (VOIs) were drawn on various brain regions, including the striatum, using dedicated software (PMOD, PMOD technologies, Zürich, Switzerland). The results were then corrected for background and decay and expressed as a target to cerebellum ratio.⁸

Ex vivo quantification

Online supplementary document

Following SPECT/CT imaging, animals were euthanized under deep anesthesia (sodium pentobarbital; 100 mg/kg i.p.) by cardiac puncture. The brain was then quickly excised and immediately frozen in liquid nitrogen-cooled isopentane. Twenty micrometer (20 μ m) thick coronal slices were cut at distinct levels of the brain (HM 505E, Microm), and autoradiographic imaging was performed using a phosphor-Imager (BASS-5000, Fujifilm). Regions of interest (ROIs) were drawn on various brain regions and internal references using dedicated software (Image Gauge, Fujifilm). Results were corrected from background and decay and expressed as a target to cerebellum ratio. Histograms presented in figures thus show the mean \pm SEM of ROD. Data were analyzed for each brain structure by Kruskal-Wallis tests with Prism 6.0 software (Graphpad, La Jolla, USA). Mean to mean comparisons were carried out with the Dunn's method.

EEG recordings

Surgery

All animals were implanted under general anaesthesia, using xylazine (Rompun®, Centravet, Lapalisse, France) 5 mg/kg i.p. and ketamine (Clorketam1000®, Vetoquinol, Centravet, Lapalisse, France) 40 mg/kg i.p., with four single-contact cortical stainless steel electrodes placed bilaterally over the frontal and parietal cortex, and an additional one over occipital cortex for reference, connected to a female microconnector and fixed to the skull with acrylic cement. Pain was relieved by a subcutaneous administration of carprofen (Rimadyl, Centravet, Lapalisse, France; 5 mg/kg). Animals were allowed a week for recovery during which time they were handled daily for habituation.

Online supplementary document*Acute drug effects on seizure*

LFPs were monitored using Coherence® (Deltamed, Paris, France) in response to acute injections of quinpirole (DA receptor agonist with a greater affinity for D3 than for D2 receptors;⁹ 12.5, 25 and 50 µg/kg, s.c.; Sigma–Aldrich, Germany), (+)-PD128907 hydrochloride (D3R agonist; 0.1 mg/kg, s.c.; Sigma–Aldrich, Germany), SR21502 (D3R antagonist; 7.5 and 15mg/kg, s.c.; generous gift from S. Anantha), SB277011-A (D3R antagonist; 3 and 9 mg/kg, s.c.; Sigma–Aldrich, Germany), nafadotride (D3R antagonist; 0.5 and 1 mg/kg, generous gift from P. Sokoloff), amphetamine (dopamine releasing agent; 0.3, 0.6 and 0.9 mg/kg; Sigma–Aldrich, Germany), aripiprazole (atypical antipsychotic; 3, 5 and 10 mg/kg, i.p., Abilify, Otsuka) or NaCl (0.9%). All compounds were dissolved in NaCl 0.9% except aripiprazole (Tween-80 2.5%), and injected in a volume of 1 ml/kg. Number, cumulated duration and mean duration of SWD were quantified for twenty minutes of reference period and then for up to 80 min after injection.

Chronic effects of aripiprazole on seizure

We administered aripiprazole (3 mg/kg, i.p.), or vehicle (Tween-80 2.5%), daily to rat pups from birth (P0) to P90.¹⁰ Aripiprazole was used here for its propensity to increase tonic DA release and to decrease phasic DA release upon chronic administration.¹¹ The dose of aripiprazole (3.0 mg/kg) was determined because of (i) its lack of effect on SWD in adult GAERS when injected acutely (present study) and (ii) the decrease of dopamine release reported in the nucleus accumbens at this dose by others¹² At P17, rat pups were implanted as previously described and we monitored LFP using Coherence® (Deltamed) for one-hour sessions at P26, P27, P28, P29, P33, P40, P47, P53 and P90. Recordings were always done between 08:00 and

Online supplementary document

10:00 a.m., before the daily injection of APZ. Because APZ has also been described as a partial agonist of D3R^{13,14} rats were killed at P90 and [¹²⁵I]7OH-PIPAT autoradiography was performed (see above), to measure a possible change in the expression of these receptor.

Data were expressed as mean \pm SEM of numbers and cumulative durations of SWD(s) for 20 min periods and analyzed with Prism 6.0 software (Graphpad, La Jolla, USA). Within each period the means between the test conditions were compared using a non-parametric analysis of variance (Friedman test). Paired comparisons versus control conditions (vehicle only) were made using a non-parametric test for related samples (Wilcoxon test).

Quinpirole-induced yawns

The doses of quinpirole were determined in pilot studies, to act preferentially on D3 receptors as assessed by modification in yawns, and to not induce rearing and other motor behaviors that would prevent animal's state of quiet wakefulness and seizure occurrence at each age.¹⁵⁻¹⁷ Yawns were defined as a prolonged (1 s), wide opening of the mouth followed by a rapid closure. On the day of testing, rats were transferred from their home cage to a test chamber (30x15x15-cm clear box rodent cage) and allowed to habituate for a period of 20 min. Observations began thereafter and the total number of basal spontaneous yawns was recorded for one hour (baseline). Quinpirole (12.5, 25 or 50 μ g/kg, s.c.) or NaCl (0.9%, s.c.) were injected thereafter and the total number of yawns was recorded for a further period of one hour. Data were expressed as mean number \pm SEM of cumulated number of yawns/hour and analyzed for each strain of rats by Kruskal-Wallis tests with Prism 6.0 software

Online supplementary document

(Graphpad, La Jolla, USA). Mean to mean comparisons were carried out with the Dunn's method.

³H-DA synaptosomal uptake

Synaptosomal preparation.

Fresh crude synaptosomes were prepared as previously described¹⁹ with minor modifications. Male rats (P90 or P14 ± 1d pups) were killed by decapitation and striata were dissected at 4°C then homogenized in 15 volumes (gm tissue/ml) of ice-cold 0.32 M sucrose in a tapered glass tissue grinder with a Teflon pestle (clearance of the cylindrical section 0.1-0.15 mm). The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was centrifuged at 12000 x g for 15 min. The second pellet was resuspended in 500 µl sucrose (0.32 M) and protein concentration was determined by Bradford assay (Biorad).

Dopamine uptake.

Uptakes were performed as previously described²⁰ with some modifications. A MultiScreen HTS vacuum manifold with 96-well plates (Merck Millipore) was used with 15 µg of striatal synaptosomes in uptake buffer (125 ul final volume). Thirty nanomolar of ³H-dopamine (Perkin Elmer) was used with increasing concentration of unlabeled substrate (70 to 2970 nM) for the characterization of DAT kinetic parameters. Entrapped radioactivity was counted by liquid scintillation spectrometry (MicroScint 20, Perkin Elmer). K_M and V_{Max} values were determined by non-linear regression analysis (curve fit, Binding-Saturation, One Site-Specific binding, GraphPad Prism software 5.0, San Diego, CA, USA). Data were analyzed with two-tailed Student's t-tests analysis, with significance set at p value < 0.05.

Online supplementary document***Amphetamine pretreatment***

For pretreatment assay, synaptosomes, obtained as previously described, were pretreated with NaCl (9%) or amphetamine (2 μ M) for 10 min at 25°C, washed twice, and then loaded with 10 nM [³H]dopamine for 5 min at 25°C. Radioactivity count was performed as previously described, and dopamine release after amphetamine injection was determined by subtracting results after amphetamine to results after NaCl pretreatment. Data were expressed as the mean \pm SEM of relative counts/minute. and analyzed for each strain by Kruskal-Wallis tests with Prism 6.0 software (Graphpad, La Jolla, USA). Mean to mean comparisons were carried out with the Dunn's method.

Microdialysis

To further investigate whether dopamine extracellular content was different between GAERS and non-epileptic animals, we performed *in vivo* microdialysis in adult rats. In this experiment and the followings we decided to focus on the comparisons between GAERS and NEC to avoid the possibility that occurrence of SWD in WH interfere with our measurements. Indeed, occasional spontaneous SWD have been reported in WH.²¹⁻²⁴ Since our previous and present data emphasized the role of DA within the AcbC in the modulation of seizure occurrence in GAERS,^{25,26} we positioned the microdialysis probe in this structure.²⁷⁻²⁹

Probe implantation

In 16 animals (8 GAERS and 8 NEC, 3 to 5 months old), concentric microdialysis probes (Hospal membrane: 2 mm active length, 0.2 mm o.d., molecular mass cutoff of

Online supplementary document

40 kDa) were implanted in the right AcbC (A/P: 1.5 mm from bregma; M/L: -2 mm; D/V: -8 mm) under ketamine (Clorketam1000®, Vetoquinol, Centravet, Lapalisse, France) 40 mg/kg i.p. and xylazine (Rompun®, Centravet, Lapalisse, France) 5 mg/kg i.p. anesthesia.³⁰ The active membrane of the probes (2 mm) was thus located within the AcbC (centrally to its mediolateral axis and extending from -6 to -8 mm in the dorsoventral axis, Figure 4D).

Dialysate collection

Microdialysis was performed 12-14 h after probe implantation by perfusing the probe with artificial cerebrospinal fluid (aCSF, mM): NaCl 149, KCl 2.8, MgCl₂ 1.2, CaCl₂ 1.2, Glucose 5.4, Na₂HPO₄ 1.2 and NaH₂PO₄ 0.27 (pH 7.4). aCSF was made fresh before each experiment and the solution was then filter-sterilized through a 0.22-mm cellulose acetate membrane. The probes were perfused (1 µl/min) and sampling was performed every 15 min.³⁰ One-hour equilibration period was allowed before collection of 26 successive samples using a 4°C refrigerated sample microcollector (Microsampler 820, Univentor, Phymep, Paris). The collection schedule was adapted from Carboni *et al.*, 2003²⁹ : 1-h resting period and 6 consecutive basal reference values, followed by a 10-sample collection immediately after amphetamine injections (0.6 mg/kg, s.c.), followed by a 10-sample collection after transient 15 min substitution of the aCSF by aCSF-K⁺, i.e. with a high K⁺ concentration (NaCl 91.8 mM, KCl 60 mM) before artificial cerebrospinal fluid was restored. Samples were stored at -80°C until biochemical analysis. Upon completion of the experiments, rats were sacrificed with an overdose of pentobarbital (100 mg/kg., i.p.). Their brain was frozen, sliced in 20-µm sections and stained with cresyl violet. Each injection site and microdialysis probe placement was localized with reference to the atlas of Paxinos

Online supplementary document

and Watson 2007³¹ (Fig 4D).

Biochemical analysis

Dialysates collected for amino acids analysis were frozen at -80°C for subsequent analysis. The high performance liquid chromatography (HPLC) system includes a LC-10AD pump (Shimadzu, France), a refrigerated auto-injector (FAMOSTM Micro Autosampler, Bionex, France) equipped with a 25 μl Hamilton syringe and a Valco switching valve, a reverse-phase C18 microcolumn (Aquasil 150x1mm ; 3 μm , ThermoHypersil, France), a Decade detector equipped with a VT03 electrochemical flow cell (Antec, Netherlands). Data were acquired and processed using a CLAS VP software (Shimadzu, France). Extracellular dopamine, DOPAC_{ext} and HVA_{ext} were measured in the collected fractions by electrochemical detection with the potential of the working electrode maintained at 0.75 V after HPLC separation (C18 reverse-phase microcolumn (Aquasil 150x1mm ; 3 μm , ThermoHypersil, France) maintained at 32°C ; mobile phase containing 50 mM KH_2PO_4 , 3 mM KCl, 0.57 mM octane sulphonic-acid, 0.205 mM EDTA, pH 4.0 and 5% acetonitrile; flow rate at 60 $\mu\text{l}/\text{min}$). Catecholamine peaks were identified based on retention time. Extracellular catecholamine concentrations were estimated by rationing peak areas of each substance and their respective external standard. The running time for each determination was 25 min with a detection threshold of 1 to 3 nM for the three substances. The relative recovery of the probes was determined using a 0.1 ng/ μl of standard solution. For 3,4-dihydroxy-phenylacetic acid (DOPAC), dopamine (DA) and homovanillic acid (HVA), as averaged over 6 consecutive samples from 2 different probes, the relative recoveries were $7.16 \pm 0.67\%$, $8.40 \pm 0.39\%$ and $6.86 \pm 0.66\%$, respectively.³² The extracellular concentrations were calculated from the

Online supplementary document

concentrations found in the dialysated sample corrected from the microdialysis yield. The basal dopamine levels (nM) were calculated from the first six samples (before amphetamine injection) and compared between the two strains using Mann-Whitney test. For a given time period, extracellular dopamine levels (nM) were compared between GAERS and NEC using a 2-way repeated measures Anova followed by Sidak's multiple comparison test. Results from treatments showing significant overall changes were subjected to post hoc Sidak's multiple comparison test with significance set at p value < 0.05 .

Brain tissue monoamines and BDNF determination

18 aged-matched adult rats were used in this study (6/strains). Animals were killed by decapitation between 09:00 and 12:00 am in order to minimize the circadian influences on catecholamines³³ and BDNF metabolism.³⁴ The brains were rapidly removed after decapitation, and coronal sections (1 mm thick) were prepared at -18°C using a brain matrix. Slices were dissected at -18° to collect samples from both hemispheres for dopamine and BDNF determination.^{35,36} Up to four 2 mm diameter punches (Harris micro-punch® allowing collection of up to 12 mg of tissue per hemisphere) were collected from the following structures: primary somatosensory cortex, motor cortex, ventrobasal thalamus, striatum, nucleus accumbens, amygdala and dorsal hippocampus. The substantia nigra pars compacta and ventral tegmental area region were microdissected as a whole using a scalpel. Samples were collected in safe-lock Eppendorf tubes and frozen by immersion in liquid nitrogen and stored at -80°C until quantitation.

Monoamine assay:

Punches for monoamine assay were dissolved in 1% lysis buffer (0.1 mM HClO_4

Online supplementary document

70%, 0.1 mM EDTA and 0.01 mM cysteine). After sonication (30 s) and centrifugation (20000 g, 40 min, 4°C), supernatant was filtered (22 µm) and stored at -80°C until HPLC analysis (see above) and pellet stored until protein assay. In addition to DA, DOPAC and HVA, tissue serotonin (5HT) and metabolite 5-hydroxy indole acetic acid (5-HIAA) were also determined. Pellets were dissolved overnight by adding 2% NaOH 0.4 mM at 4°C. After centrifugation (18500 g, 20 min, at 4°C), supernatant were used for proteins quantitation using the micro Bicinchoninic Acid (BCA) Protein Assay (Promega, Biorad, France). Briefly, samples were added to duplicate wells, mixed with the BCA Working Reagent and incubated for 30 min at 37°C. Absorbance was measured at 562 nm and the protein concentration in each sample was determined using a bovine serum albumin standard curve. Duplicate measurements for each sample were averaged. For each sample, monoamine content was calculated as ng of monoamine per mg of total sample proteins. Data were expressed as mean ± SEM and analyzed for each brain structure by Kruskal-Wallis tests with Prism 6.0 software. Mean to mean comparisons were carried out with the Dunn's multiple comparison method.

BDNF measurement by enzyme-linked immunosorbent assay (ELISA):

BDNF levels was determined by ELISA as adapted from Angelucci *et al.*, 2000³⁷ and Elfving *et al.*, 2010.³⁴ Briefly, for extraction of BDNF, the brain tissue samples were homogenized mechanically and with ultrasonication in 5% w/v of ice-cold extraction buffer containing 137 mM NaCl, 20 mM Tris-HCl (pH 7.2), 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1.536 µM aprotinin, 2.1 µM leupeptin, 0.5 mM sodium vanadate, pH 8.0. The homogenates were centrifuged (18,500 g, 20 min, 4°C), and the supernatant was collected and stored at -80°C until quantitation of BDNF. BDNF protein levels were determined using a heterologous two-site sandwich ELISA

Online supplementary document

kit (Promega, France) following manufacturer's recommendations. Briefly, ninety-six-well plates were coated with anti-BDNF monoclonal antibody (overnight, 4°C) incubated in the presence of carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate; pH = 9.7). Then, after incubation for 1 h with block and sample buffer, samples were added to triplicate wells and serial dilution of recombinant BDNF standard were added to duplicate wells in each plate to generate a standard curve. After incubation for 2 h, anti-BDNF polyclonal antibody was added and incubated for 2 h. Subsequently, plates were incubated in a solution containing HRP conjugated anti-IgY antibody for 1 h. Finally, a hydrogen peroxide solution was added together with a peroxidase substrate (TMB One Solution) and plates were incubated for 10 min. Reactions were stopped by adding 1 N hydrochloric acid and absorbance was then measured at 450 nm using a Wallace 1420 Victor microplate reader (PerkinElmer). The level of BDNF protein in each sample was determined using the standard curve and triplicate determinations for each sample were averaged. The content of total sample proteins was determined in each supernatant using the Bicinchoninic Acid (BCA) protein assay kit (Promega, France) following the manufacturer's instructions (see above). For each sample, BDNF content was normalized and calculated as pg of BDNF per mg of total sample proteins. Data were expressed as mean \pm SEM and analyzed for each brain structure by Kruskal-Wallis tests with Prism 6.0 software. Mean to mean comparisons were carried out with the Dunn's method.

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Online supplementary document

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Online supplementary document

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