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Abstract: Neocortical and striatal TRPV1 (vanilloid or capsaicin) receptors (TRPV1Rs) are excitatory ligand-gated ion channels, and are implicated in psychiatric disorders. However, the purported presynaptic neuromodulator role of TRPV1Rs in glutamatergic, serotonergic or dopaminergic terminals of the rodent forebrain remains little understood. With the help of patch-clamp electrophysiology and neurochemical approaches, we mapped the age-dependence of presynaptic TRPV1R function, and furthermore, we aimed at exploring whether the presence of CB1 cannabinoid receptors (CB1Rs) influences the function of the TRPV1Rs, as both receptor types share endogenous ligands.
We found that the major factor which affects presynaptic TRPV1R function is age: by post-natal day 13, the amplitude of capsaicin-induced release of dopamine and glutamate is halved in the rat striatum, and two weeks later, capsaicin already loses its effect. However, TRPV1R receptor function is not enhanced by chemical or genetic ablation of the CB1Rs in dopaminergic, glutamatergic and serotonergic terminals of the mouse brain.
Altogether, our data indicate a possible neurodevelopmental role for presynaptic TRPV1Rs in the rodent brain, but we found no cross-talk between TRPV1Rs and CB1Rs in the same nerve terminal.

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Presynaptic $TRPV_1$ vanilloid receptor function is age- but not CB_1 cannabinoid receptor-dependent in the rodent forebrain

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Keywords

CB_1 cannabinoid receptor; dopamine; glutamate; serotonin; striatum; $TRPV_1$ vanilloid receptor.

Abstract

Neocortical and striatal $TRPV_1$ (vanilloid or capsaicin) receptors ($TRPV_1Rs$) are excitatory ligand-gated ion channels, and are implicated in psychiatric disorders. However, the purported presynaptic neuromodulator role of $TRPV_1Rs$ in glutamatergic, serotonergic or dopaminergic terminals of the rodent forebrain remains little understood. With the help of patch-clamp electrophysiology and neurochemical approaches, we mapped the age-dependence of presynaptic $TRPV_1R$ function, and furthermore, we aimed at exploring whether the presence of CB_1 cannabinoid receptors (CB_1Rs) influences the function of the $TRPV_1Rs$, as both receptor types share endogenous ligands.

We found that the major factor which affects presynaptic $TRPV_1R$ function is age: by post-natal day 13, the amplitude of capsaicin-induced release of dopamine and glutamate is halved in the rat striatum, and two weeks later, capsaicin already loses its effect. However, $TRPV_1R$ receptor function is not enhanced by chemical or genetic ablation of the CB_1Rs in dopaminergic, glutamatergic and serotonergic terminals of the mouse brain.

Altogether, our data indicate a possible neurodevelopmental role for presynaptic $TRPV_1Rs$ in the rodent brain, but we found no cross-talk between $TRPV_1Rs$ and CB_1Rs in the same nerve terminal.

Abbreviations

3Rs, replacement, reduction, refinement; 4-AP, 4-aminopyridine; 7D, 14D, 29D, 60D, 7-, 14-, 29- and 60-day-old; ACEA, arachidonyl-2'-chloroethylamide; aCSF, artificial cerebrospinal fluid; ARC, American Radiolabeled Chemicals; ARRIVE, Animal Research: Reporting *In Vivo* Experiments; AUC, area-under-the-curve; BCA, bicinchoninic acid; BSA, bovine serum albumin; CB_1R, cannabinoid

receptor type 1; DMSO, dimethyl sulfoxide; DPM, disintegration per minute; DTT, dithiothreitol; ECF, Enhanced Chemi-Fluorescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; GABA, γ-aminobutyric acid; Felasa, Federation for Laboratory Animal Science Associations; FR%, fractional release %; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); KHR, Krebs-HEPES-Ringer; KO, knockout; LiGTP, lithium guanozine triphosphate; MgATP, magnesium adenosine triphosphate; MAO B, monoamine oxidase B; NADA, N-arachidonyl dopamine; NO, nitric oxide; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; RTX, resiniferatoxin; SDS, sodium dodecyl sufate; SEM, standard error of the mean; sEPSCs, spontaneous excitatory postsynaptic currents; TBS-T, Tris-buffered saline with Tween 20; Tris, tris(hydroxymethyl)aminomethane; $TRPV_1R$ and $TRPV_4R$, transient release potential receptor vanilloid type 4, WT, wild-type.

1. Introduction

A major peripheral transducer of noxious stimuli into neuronal activity is the transient receptor potential Na^+/Ca^{2+} channel subfamily vanilloid member 1 ($TRPV_1$), also termed as the capsaicin receptor (Nagy et al., 2008; Di Marzo and De Petrocellis, 2010). $TRPV_1R$ channels are also expressed in the brain beyond the central endings of the sensory pathways, and are believed to participate in the fine-tuning of several types of synapses (Mezey et al., 2000; Roberts et al., 2004; Grueter et al., 2010; Matta and Ahern, 2011; Mori et al., 2012). Apart from the (anti)nociceptive pathways, functional experiments pointed out presynaptic location for the $TRPV_1R$ in the forebrain (see Table 1 as well as Matta and Ahern, 2011), while other studies also found it post-synaptically (Tóth et al., 2005; Cristino

et al., 2006; Marsch et al., 2007; Li et al., 2008; Maccarrone et al., 2008; Mulder et al., 2011; Grueter et al., 2010; Zschenderlein et al., 2011).

The $TRPV_1R$ receptor has been shown to directly modulate glutamate release both outside (Matta and Ahern, 2011) and inside the forebrain (see Table 1). Interestingly, about one third of the studies failed to detect presynaptic $TRPV_1R$-mediated modulation of excitatory amino acid release in the rat forebrain (see Table 1). If we compare these studies with the ones reporting $TRPV_1R$-modulation, a disparity in the age of the rats used becomes overt: Ignoring variations in strains and brain areas, we estimated the mean ± SEM of the age of the rats as 23.3 ± 2.6 days (n=10 studies) for the existence and 48 ± 1 days (n=5) for the absence of $TRPV_1R$-mediated modulation of excitatory amino acid release (see third column in Table 1). In the mice, similar age discrepancies are calculated for glutamate release modulation, namely 25 ± 10 (n=3) vs. 59 ± 9 days (n=3). This puts forward the interesting hypothesis that the activity of presynaptic $TRPV_1Rs$ may decline in the first weeks of post-natal life, suggesting a neurodevelopmental role for the $TRPV_1R$.

GABAergic, dopaminergic and serotonergic terminals are additional purported candidates to be modulated by $TRPV_1Rs$, as central $TRPV_1Rs$ are implicated in mood disorders, anxiety, panic responses, depression and psychosis (Moreira and Wotjak; 2010; Chahl, 2011; Hayase, 2011; Casarotto et al., 2012; Moreira et al., 2012, Micale et al., 2013). Yet no direct presynaptic $TRPV_1R$ modulation of dopamine and GABA release has been observed so far in the adult rodent brain (see Table 1), while to our knowledge, presynaptic $TRPV_1R$ modulation of serotonergic terminals in the brain has never been looked for.

The $TRPV_1R$ receptor can be activated by several endogenous lipids including arachidonoylethanolamine (anandamide) and N-arachidonoyl dopamine (NADA) (Di Marzo and De Petrocellis, 2010). Interestingly, anandamide and NADA can also activate the metabotropic

cannabinoid receptor CB_1 (Walker et al., 2002). The CB_1 receptor (CB_1R) is a major modulator of synaptic plasticity (Katona and Freund, 2012), and a growing body of evidence supports that the inhibitory metabotropic CB_1R and the excitatory ionotropic $TRPV_1R$ influence behavior in a diametrically opposite manner (Moreira and Wotjak , 2010; Casarotto et al., 2012; Moreira et al., 2012; Riebe et al., 2012).

Theoretically, anandamide and NADA can activate both receptors at the same nerve terminal, resulting in inhibition and excitation in the same time. Accordingly, CB_1Rs can negatively control $TRPV_1Rs$ in a Ca^{2+}-dependent manner, preventing the contrasting co-activation of the ionotropic partner (Marinelli et al., 2005; Oshita et al., 2005; Wu et al., 2005; Mahmud et al., 2009; Yang et al., 2013).

With the help of previously optimized techniques, here we sought answer to the possible age- and CB_1R-dependence of presynaptic $TRPV_1R$ function in the neocortex and the striatum.

2. Materials and Methods

2.1. Electrophysiology

2.1.1. Subjects and slice preparation

Coronal slices of young Wistar rats of either sex (8-14 days old; 24–30 g weight) at the level of the striatum were prepared according to the guidelines, and with the approval, of the Ethical Board of Semmelweis University, Budapest, Hungary, based on the Declaration of the European Communities Council Directives (86/609/ECC). In brief, rat pups were decapitated. The brain was quickly removed and placed in ice-cold, oxygenated (95% O_2 plus 5% CO_2) artificial cerebrospinal fluid (aCSF, pH 7.4) of the following composition (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 25 and glucose 11. Two-hundred-µm-thick coronal slices were cut from a block of tissue containing the striatum in ice-cold aCSF using an MA752 tissue cutter (Campden Instruments, England). Slices were transferred to a holding chamber, where they were equilibrated in oxygenated aCSF for at least 1 h at 36 ºC, before recordings began. Then, a single slice was placed in a recording chamber and superfused with oxygenated aCSF at a rate of 2.5-3 ml/min at room temperature (20-22 ºC). The slices were allowed to recover for at least 15 min before the start of individual experiments.

2.1.2. Patch-clamp recording

Membrane currents of striatal medium spiny neurons were recorded by procedures similar to those described by Edwards et al. (1989) and by us (Wirkner et al., 2004). The cells were visualized with an upright interference contrast microscope and a ×40 water immersion objective (Axioskop 2 FS; Carl Zeiss, Germany). Patch pipettes (tip resistance, 4-7 MΩ) were prepared by a puller (Narishige PP-83, Narishige, Japan) from borosilicate capillaries and were filled with intracellular solution of the following composition (mM): K-gluconate 140, NaCl 10, $MgCl_2$ 1, HEPES [N-(2-

hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)] 10, EGTA [ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid] 11, MgATP 1.5 and LiGTP 0.3; pH 7.3 adjusted with KOH. The medium spiny neurons included in the data were selected on the basis of having a diameter smaller than 15 μm and a stable resting membrane potential of at least -75 mV (Calabresi et al., 1987).

Membrane currents and membrane potentials were recorded by means of a patch-clamp amplifier (Axopatch 200B, Molecular Devices, USA). After establishing whole cell access the system was left for 5-10 min to allow for the settling of diffusion equilibrium between the patch pipette and the cell interior, before spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at a holding potential of -80 mV in the voltage-clamp mode. Bicuculline (10 μM) was present in the external medium to pharmacologically isolate the excitatory postsynaptic currents from the GABA-mediated inhibitory currents. A 5-min control period was followed by the application of capsaicin (1 μM; 5 min) or its vehicle control, DMSO, and subsequently by a 10-min washout period. DMSO failed to affect any of the three parameters (data not shown).

Currents were filtered at 5-10 kHz with the inbuilt lowpass-filter of the patch-clamp amplifier. Data were then sampled at 10 kHz and stored on-line with a PC using the pClamp 10.0 software package (Molecular Devices) that was also used for data analysis. Average values of amplitude, half-width and frequency of sEPSCs were calculated for the 5-min control (predrug) period and were taken as 100%. The same three parameters during the 5-min period of capsaicin perfusion were normalized to the predrug period and analyzed as detailed under *2.2.3.*

2.2. Neurochemistry

2.2.1. Subjects

All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the guidelines of EU (86/609/EEC), FELASA, and the National Centre for the 3Rs (the ARRIVE; Kilkenny et al., 2010), and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of Coimbra. We also applied the principles of the ARRIVE guideline for the design and the execution of the *in vitro* pharmacological experiments (see below) as well as for data management and interpretation, according to McGrath et al., 2010.

CB_1R null-mutant (knockout) male mice (Ledent et al., 1999) and their wild-type littermates on CD-1 background were genotyped by tail snips, housed until 16 weeks of age as detailed above, and sacrificed and used in pairs (one WT and one KO). Male Wistar rats (10-14 weeks old) were obtained from Charles River (Barcelona, Spain). All rodents were housed under controlled temperature (23 ± 2 ºC), subject to a fixed 12 h light/dark cycle, with free access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort. The animals used to perform the *in vitro* studies were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine.

2.2.2. Synaptosomal experiments

Purified nerve terminals, termed synaptosomes (Whittaker et al., 1964), represent excellent tool to study presynaptic processes free of polysynaptic and glial influences (Raiteri and Raiteri, 2000).

2.2.2.1. Partially purified synaptosomes (P2 fraction) for release experiments

This preparation was obtained as previously described (Ferreira et al., 2009, 2012). Briefly, the caudate-putamen region without the nucleus accumbens (hereafter simply: striatum) and the frontal cortices were quickly dissected out into 2 mL of ice-cold sucrose solution (0.32 M, containing 5 mM

HEPES, pH 7.4). After homogenization with a Teflon homogenizer, and centrifugation at 5,000 *g* for 5 min, the supernatant was collected and centrifuged at 13,000 *g* for 10 min to obtain the P2 synaptosomal fraction.

2.2.2.2. [^{3}H]serotonin single label release experiments from cortical synaptosomes, and [^{3}H]dopamine/ [^{14}C]glutamate release assay from striatal nerve terminals

Synaptosomes were then diluted to 0.5 mL with Krebs-HEPES solution (in mM: NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, HEPES 15, ascorbic acid 100, pH 7.4, 37°C), containing the MAO B inhibitor, pargyline (10 µM), and besides, for the [^{3}H]dopamine/ [^{14}C]glutamate experiments, the glutamate decarboxylase inhibitor, aminooxyacetic acid (100 µM) to prevent [^{14}C]glutamate metabolism. Under these conditions, cortical synaptosomes were incubated with 5-[1,2-^{3}H]hydroxytryptamine creatinine sulfate (final concentration, 300 nM) (American Radiolabeled Chemicals, [ARC] Inc; Saint Louis, MO, USA) in the presence of reboxetine (30 nM) and GBR12783 (100 nM) to prevent the uptake of [^{3}H]serotonin into noradrenergic and dopaminergic terminals. Striatal synaptosomes were co-incubated with L-[^{14}C(U)]glutamic acid (final concentration, 30 µM) (ARC Inc) and [7,8-^{3}H(N)]dopamine (200 nM) (ARC Inc) for 10 min. A 16-microvolume chamber perfusion setup was filled with the preloaded synaptosomes (rat, ~0.24 mg protein/65 µL/chamber; mouse, ~0.17 mg protein/130 µL/chamber) which were trapped by layers of Whatman GF/B filters and superfused continuously at a rate of 0.8 ml/min at 37°C until the end of the experiment. After a 10-min washout period, 2-min samples were collected for liquid scintillation assay.

2.2.2.2.1. Protocol for rat striatal synaptosomes

After collecting three 2-min samples as baseline, the striatal synaptosomes of rats of different age (post-natal days 7, 14, 29 and 60; 8 animal/group) were perfused with capsaicin or vehicle (0.1% DMSO) for 3 min, and were washed thereafter for more 5 min (see Fig. 2A-C). Major release data including resting release levels, S_1, S_2/S_2 values and filter content can be found in Table 2.

2.2.2.2.2. Protocol for mouse cortical and striatal synaptosomes

After collecting four 2-min samples as baseline, the evoked release of the transmitters was stimulated twice with 4-aminopyridine (4-AP; 300 µM) for 2 min, with a 10-min interval (S_1, S_2; see Figs. 3A,B; 4A). Vehicle (0.1% DMSO) or $TRPV_1R$ agonists, capsaicin (1 µM) (Tocris), resiniferatoxin (100 nM) or arachidonyl-2'-chloroethylamide (ACEA, 1 µM) (Abcam) were added 4 min before the 2^{nd} stimulation (S_2). In parallel channels, no 4-AP-stimulation was applied to test the $TRPV_1R$ ligands' own effect on the baseline (Fig. 3C,D). Treatments were applied in duplicate, each averaged as n = 1. The CB_1R antagonists, AM251 (500 nM) (Tocris) and O-2050 (500 nM) (Tocris) were given 10 min before sample collection; therefore such treatment has no direct consequence for the S_2/S_1 ratios or for the basal release. Major release data including resting release levels, S_1, S_2/S_2 values and filter content can be found in Table 2. In a separate study, we investigated the Ca^{2+} dependence of our transmitter release models, measuring both the resting and the KCl (20 mM, 1 min) -evoked release of the neurotransmitters in a modified Krebs'-HEPES medium containing 100 nM $CaCl_2$ and 10 mM $MgCl_2$. Low calcium diminished the resting [^{3}H]dopamine release by 27.1±3.1% (n=12, $p<0.001$) and the KCl-evoked release by 89.5±0.8% (n=12, $p<0.001$), and also lessened the resting and the KCl-evoked release of [^{14}C]glutamate by 22.1±1. % (n=12, $p<0.001$) and 73.3±1.5 % (n=12, $p<0.001$), respectively, compared to normocalcemia. As for [^{3}H]serotonin, according to our previous publication

(Ferreira et al., 2012), the evoked release of [^{3}H]serotonin was 47.2±2.4% (n=6, $p<0.001$) and the resting release was 18.2±2.1% (n=6, $p<0.01$) Ca^{2+}-dependent.

2.2.2.2.3. β-counting

After the experiment, the radioactivity content of each samples and the filters with the trapped synaptosomes were counted by a single or a dual-label protocol using a Tricarb β-counter (PerkinElmer), and DPM values were expressed as fractional release (FR%), *i.e.* the percent of actual content in the effluent as a function of the total synaptosomal content.

2.2.2.2.4. Release data interpretation

For the dual-label [^{3}H]dopamine/ [^{14}C]glutamate experiments in rat striatal synaptosomes since no 4-AP stimulation was applied, the vehicle control results (FR%) were subtracted from the capsaicin-stimulated FR% data. The curves obtained are displayed in Fig. 2A-C. Fig. 2D displays the area-under-the-curve (the sum of deviation from zero FR% changes) for capsaicin's effect.

For the rest of the release experiments in mice, since there were two types of controls, namely drug-naïve 4-AP-stimulated and drug-naïve non-stimulated controls, we present instead the original mean±SEM FR% plots for 4-AP-stimulated (Figs. 3A,B and 4A) as well as for the unstimulated (Fig. 3C,D) conditions.

Areas under the curve were calculated for the effect of both 4-AP and the $TRPV_1R$ agonists. $TRPV_1R$ agonist effects were statistically compared to a hypothetical value of zero. S_2/S_1 ratios under treatment were normalized to the vehicle control S_2/S_1 ratio, and then were statistically compared to a hypothetical value of 100%.

2.2.2.3. Synaptosomes purified by a 45% Percoll gradient for Western blotting

This preparation was obtained as previously described (Rebola et al., 2005). Briefly, the two striata from one animal were homogenized in an ice-cold sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% BSA and 10 mM HEPES (pH 7.4). The homogenate was spun at 3,000 *g* for 10 min at 4 ºC and the supernatant spun again at 14,000 *g* for 12 min. The pellet (P2 fraction) was resuspended in 1 mL of Percoll 45% (v/v) made up in Krebs-HEPES-Ringer (KHR) medium (in mM: NaCl 140, EDTA 1, KCl 5, glucose 5 and HEPES 10, pH 7.4) and spun again at 14,000 *g* for 2 min. The synaptosomes (top layer) were then removed and washed once with KHR medium at 14,000 *g* for 2 min. The synaptosomal pellet obtained was solubilized in 5% SDS supplemented with 100 µM PMSF, 2 mM DTT and a protease inhibitor cocktail. The protein concentration was then determined using the bicinchoninic acid (BCA) protein assay reagent and the samples added to a 1/6 volume of 6× SDS-PAGE sample buffer [30% (v/v) glycerol, 0.6 M dithiothreitol (DTT), 10% (w/v) SDS and 375 mM Tris–HCl, and 0.012% bromophenol blue, pH 6.8] and the volume adjusted with milliQ water to normalize for a maximum of 2 µg/µL.

2.2.2.4. Western blotting

The samples were denaturated by boiling at 95 ºC for 5 min and 80 µg protein loads were separated by SDS-PAGE electrophoresis, using 10% polyacrylamide resolving gels and 4% polyacrylamide concentrating (stacking) gels, under reducing conditions at 80-120 mV. Prestained precision protein standards (Biorad) were run simultaneously with the samples to help identify the proteins of interest. The proteins in the gel were then electrophoretically transferred (1A current, for 1.5 h at 4ºC with constant agitation) to previously activated polyvinylidene difluoride (PVDF) membranes (0.45 µm).

After blocking for 1 h at room temperature with 5% essential fatty acid free bovine serum albumin (BSA) in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6) containing 0.1% Tween 20 (TBS-T), to prevent nonspecific binding, the membranes were incubated overnight at 4°C with the primary antibody (rabbit anti-$TRPV_1R$ at 1:500, Millipore) diluted in TBS-T with 1% BSA. After three washing periods of 15 min with TBS-T, the membranes were incubated with the appropriate alkaline phosphatase-tagged secondary antibody diluted in TBS-T containing 1% BSA, for 2 h at room temperature. After three 15-min washes with TBS-T, the membranes were incubated with Enhanced Chemi-Fluorescence (ECF) substrate and visualized in a VersaDoc 3000 imaging system with the assistance of Quantity One software. The membranes were then re-probed and tested for β-actin immunoreactivity to confirm that similar amounts of protein were applied to the gels.

2.2.3. Data treatment

All data represent mean ± SEM of $n \geq 5$ observations (5 animals). Pooled data were tested for normality by the Kolmogorov-Smirnov normality tests. If data exhibited Gaussian distribution, statistical significance was calculated by one-sample *t*-test against a hypothetical value of 100 (representing either pretreatment period or vehicle control) or of 0 (no effect on baseline). A $p < 0.05$ was accepted as significant difference.

2.3. Materials and Chemicals

Phenylmethanesulfonyl fluoride (PMSF), DL-dithiothreitol (DTT), protease inhibitor cocktail (leupeptin, pepstatin A, chymostatin and antipain), 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane), pargyline, bovine serum albumin (BSA), aminooxy acetic acid, pargyline, Tween-20, DMSO and Whatman GF/C filters were obtained from Sigma (Sintra, Portugal). Sodium dodecyl sulphate (SDS)

and the Quantity one software were from Bio-Rad (Amadora, Portugal). Polyvinylidene difluoride (PVDF) membranes, pre-stained precision protein standards and enhanced chemifluorescence substrate (ECF) were purchased from Amersham Biosciences (Amadora, Portugal). Bicinchoninic acid (BCA) protein assay, ascorbic acid and inorganic reagents were bought from Merck-Millipore, Darmstadt, Germany. 4-aminopyridine, ACEA, AM251 were purchased from Abcam Biochemicals (Cambridge, U.K.). *E*-capsaicin, capsazepine and O-2050 were from Tocris Bioscience (Bristol, U.K.) Non-water soluble materials were dissolved in DMSO, aliquoted and kept at -20ºC until use.

3. Results and Discussion

3.1. Capsaicin increases the frequency of post-synaptic excitatory currents in the striatum

Lack of $TRPV_1R$-triggered release of radiolabeled neurotransmitters from synaptosomal preparations (D'Amico et al., 2004; Köfalvi et al., 2005, 2007; Cannizzaro et al., 2006; Ferreira et al., 2009) can be a result of the age of the animals used and/or of the lack of temporal resolution of the technique itself, i.e. that a fast-desensitizing capsaicin effect (Baamonde et al., 2005) is diluted in the minute-scale sampling process. To avoid any of these, we first used a positive control, i.e. tight-seal voltage clamp recording of glutamatergic input in medium spiny neurons of the striatum of rat pups. As Fig. 1 demonstrates, the perfusion of capsaicin (1 μM) triggered a sustained 29.2±8.1% increase in spontaneous firing rate of glutamatergic terminals ($n = 5$, $p < 0.01$) which slowly returned to baseline upon washout (not shown), while having no effect either on the amplitude or the half-width of the currents, indicating presynaptic rather than post-synaptic modulation of glutamatergic connections. This indicates to us that presynaptic functional $TRPV_1Rs$ with apparently low desensitization rate do

exist in the glutamatergic terminals in the developing striatum. Capsaicin at the concentration of 10 μM already induced a considerable desensitization during its perfusion, hence reducing the amplitude of its effect to 21±18.6% facilitation of basal activity (n =5, $p > 0.05$).

3.2. Age-dependence of presynaptic $TRPV_1R$ functionality in the striatum

This prompted us to test the effect of capsaicin on the release of [^{3}H]dopamine and [^{14}C]glutamate in striatal synaptosomes of rats of four post-natal ages. As Fig. 2 illustrates, 3-min perfusion of capsaicin (1 μM) triggered a rapid and sustained increase in the release of both [^{3}H]dopamine and [^{14}C]glutamate over the vehicle control, which was the greatest in the 7-day-old rats, and declined thereafter. Age-effect size curves reveal that the response to capsaicin drops to 50% of the theoretical maximum on post-natal day 13 for both [^{3}H]dopamine and [^{14}C]glutamate. Furthermore, Western-blotting analysis of the striatal nerve terminals reveals an overt decrease in synaptic $TRPV_1R$ density from post-natal day 14 to post-natal day 29 (Fig. 2E).

Of note, synaptosomal preparations usually contain significant percentages of presynaptic nerve terminals bearing still their post-synaptic partners, and functional $TRPV_1Rs$ have been found also post-synaptically in the striatum (Maccarrone et al., 2008; Grueter et al., 2010). Therefore, there is a certain chance that post-synaptic $TRPV_1R$ triggered the production of a retrograde messenger (e.g. NO) that could facilitate [^{3}H]dopamine and [^{14}C]glutamate release presynaptically. However, post-synaptic $TRPV_1R$ expression is not age-dependent as the mean age of the animals in the above two studies is 44 days. Our data therefore rather argue for the presynaptic $TRPV_1R$ functionality which declines in the first weeks of post-natal life, at least in the striatum. This is not the first such report: Maione and colleagues (2009) also found that $TRPV_1R$ immunoreactivity and the $TRPV_1R$-mediated synaptic plasticity in retinocollicular glutamatergic terminals are at their maximum in the second week of post-

natal life, and decline in the forthcoming weeks, until disappearing in the adult brain. The authors speculated that $TRPV_1Rs$ participate in axonal guidance and target selection. Interestingly, anandamide, the endogenous activator of $TRPV_1Rs$, has been shown to participate in the synaptogenesis and target selection of GABAergic interneurons in the embryonic mouse brain, via the activation of presynaptic CB_1Rs (Berghuis et al., 2007). Another recent study reports probable developmental role of $TRPV_1R$ in the ependymal cells of the embryonic rat brain (Jo et al., 2013). To test this developmental hypothesis, Zavitsanou and colleagues (2010) injected rats with capsaicin at neonatal day 2, and at 15-16 weeks of post-natal age, differences in the density of dopamine, muscarinic, cannabinoid and serotonin receptors were detected, indicating that neonatal $TRPV_1Rs$ have long-term impact on the neuromodulator system of the adult brain.

The present electrophysiological and neurochemical evidence do not necessarily argue for glutamate release modulation at cortico- or thalamostriatal terminals: it is known that glutamate serves as a co-transmitter to dopamine in mesolimbic efferents, helping axonal guidance and target selection (Descarries et al., 2008). It is therefore plausible that the effect of capsaicin seen by us is restricted to such developing terminals in the striatum, thus capsaicin can evoke sustained release of glutamate and dopamine from nerve terminals of the same mesolimbic origin.

3.3. CB_1R-dependence of presynaptic $TRPV_1R$ functionality in the adult brain

The presence of CB_1Rs in the same nerve terminal may also mask presynaptic $TRPV_1R$ function (Marinelli et al., 2005; Oshita et al., 2005; Wu et al., 2005; Mahmud et al., 2009; Yang et al., 2013). Alternatively, since the $TRPV_1R$ is also a voltage-gated ion channel, $TRPV_1R$ function may prevail under depolarization instead of at resting membrane potential, in the adult brain. To test these, we measured the effect of $TRPV_1R$ agonists both on the resting and the 4-AP-stimulated release of

[^{3}H]serotonin from frontocortical nerve terminals as well of [^{3}H]dopamine and [^{14}C]glutamate from striatal nerve terminals, under genetic and pharmacological ablation of the CB_1R.

3.3.1. [^{3}H]dopamine and [^{14}C]glutamate release in mouse striatal nerve terminals

We found previously that the evoked release of [^{14}C]glutamate was greater in the neocortex of CB_1R KO mice than that in the WT mice (Ferreira et al., 2012), and we observed now a 36.1 ± 10.2% ($p < 0.05$) greater evoked release of [^{14}C]glutamate in the striatal synaptosomes of the CB_1R KO mice when comparing the S_1 values (Fig. 3B). This is the first report on that the evoked release of glutamate is greater in the striatum of the CB_1R KO mice. In contrast, the evoked release of [^{3}H]dopamine was similar between the WT and the CB_1R KO mice (KO: 105.8 ± 9.4% of control, $n = 6$; $p > 0.05$) which is in accordance with the lack of functional CB_1Rs in striatal dopaminergic terminals (Köfalvi et al., 2005).

Nicotine (1 nM - 10 µM) in our hands is capable of stimulating the efflux of dopamine under resting conditions (Ferreira et al., 2009; Garção et al., 2013), which serves as a positive control for the present study. However, capsaicin (1 µM) failed to affect either the resting or the 4-AP-stimulated release of [^{3}H]dopamine and [^{14}C]glutamate in the striatum of both mouse strains (Fig. 3A-G). For curiosity, we tested capsaicin in the presence of the $TRPV_1R$ antagonist, capsazepine (1 µM) to allow unmasking any possible off-target capsaicin action. For instance, an inhibition of glutamate release by capsaicin similar to the one seen by Benninger et al. (2008) in the hippocampi of both the WT and the $TRPV_1R$ KO mice would mask $TRPV_1R$-mediated facilitation. Yet, the lack of capsaicin effect persisted in the presence of capsazepine (Fig. 3E).

Resiniferatoxin (RTX, 100 nM), another highly selective and potent $TRPV_1R$ agonist (Szolcsányi et al., 1990) also failed to affect either the resting release (Fig. 3F,G) or the 4-AP-evoked release of

[^{3}H]dopamine and [^{14}C]glutamate (data not shown; $p > 0.05$). Chemical instead of genetic ablation of the CB_1R also failed to unmask presynaptic $TRPV_1Rs$: capsaicin (1 μM) in the presence of the CB_1R antagonist, AM251 (500 nM) did not produce change ($p > 0.05$) either on the resting release (Fig. 3F,G) or on the 4-AP-evoked release of [^{3}H]dopamine and [^{14}C]glutamate (data not shown) in the WT mice.

3.3.2. [^{3}H]serotonin release in mouse frontocortical nerve terminals

Similarly to our previous report (Ferreira et al., 2012), the evoked release of [^{3}H]serotonin (comparing the S_1 values) was 11.8 ± 3.9% greater ($n = 6$, $p < 0.01$) in the CB_1R knockout (KO) mice (Figs. 4A). This with the above data indicate that CB_1Rs are tonically negatively coupled to the release of serotonin and glutamate as both nerve terminal types are positive for specific CB_1R immunoreactivity (Köfalvi et al., 2005; Häring et al., 2007; Ferreira et al., 2012).

Besides capsaicin, this time we tested a non-metabolizable analogue of anandamide, ACEA, which is also a hybrid CB_1R/ $TRPV_1R$ agonist to stimulate [^{3}H]serotonin release. Yet, neither ACEA (1 μM) nor capsaicin (1 μM) triggered statistically significant change ($p > 0.05$) in the resting release of [^{3}H]serotonin from cortical synaptosomes of WT mice in the absence or the presence of another specific CB_1R antagonist, O-2050 (500 nM) (Fig. 4C). ACEA also had no effect on the resting [^{3}H]serotonin release in the CB_1R KO mice, respectively (Fig. 4C). Interestingly, ACEA statistically significantly increased the evoked release of [^{3}H]serotonin in the CB_1R KO but not in the WT mice (by 12.8 ± 3.8%, $n = 7$, $p < 0.05$; Fig. 4A,B). In contrast, capsaicin failed to facilitate the evoked release of [^{3}H]serotonin in both mouse strains (Fig. 4B). Additionally, under CB_1R blockade with O-2050 in the WT mice, neither ACEA nor capsaicin produced effect on the evoked release (Fig. 4B). Capsaicin is a well-established and potent agonist for the $TRPV_1R$ (Caterina et al., 1997). ACEA, however, possesses

additional putative binding sites including the $TRPV_4R$ channel (Vay et al., 2012) and intracellular targets (Köfalvi, 2008). Hence, these conflicting data here do not support the hypothesis that CB_1Rs keep putative $TRPV_1Rs$ silent in serotonergic terminals. The facilitatory effect of ACEA in the CB_1R KO mice is novel information, and may represent developmental compensation for the lack of CB_1Rs. Notwithstanding, the determination of the underlying cannabinoid receptor subtype responsible for such ACEA effect is beyond the scope of this study.

Altogether, the second part of the study refuted the presynaptic neuromodulator role for $TRPV_1Rs$ in the investigated terminals. The lack of such neuromodulation is likely not due to a putative negative control by CB_1Rs on $TRPV_1Rs$.

4. Conclusions

Presynaptic metabotropic CB_1 cannabinoid receptors have long been known to modulate the release of amino acid and monoamine transmitters (Katona et al., 1999; Nakazi et al., 2000; Balázsa et al., 2008; Ferreira et al., 2012). The endocannabinoids, anandamide and NADA can activate the ionotropic $TRPV_1R$ besides the CB_1R. Our aim was to investigate the little understood nature of presynaptic functional $TRPV_1Rs$. Here we report for the first time that capsaicin triggers transmitter release in forebrain synaptosomes. This role of $TRPV_1Rs$ is age dependent, and disappears in the first few weeks of post-natal life. Thus, our work is one of the few pioneer studies (Maione et al., 2009; Zavitsanou et al., 2010) which propose a neurodevelopmental role for the presynaptic $TRPV_1Rs$.

Although our data is not in favor of, but is also not inconsistent with the idea of functional presynaptic $TRPV_1Rs$ in the adult brain. What we can safely conclude based on the previous literature (see e.g. Table 1) and on our present results is that $TRPV_1Rs$ do not posses major presynaptic neuromodulator role in the adult rodent forebrain. This means that presynaptic $TRPV_1Rs$ at

considerably decreased density in the adult brain still can have other roles, for instance, in axon guidance in immature synapses. To stretch this idea, recent data suggest the involvement of anandamide and $TRPV_1R$ signaling in depression (Micale et al., 2013). Hence, it is feasible that misregulation of axon guidance and target selection by impaired $TRPV_1R$ signaling contributes to the genesis of depression in the adult.

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animal	transmitter	age (D)	brain area	$TRPV_1$ effect?	reference

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Figure Legends

Fig. 1. Capsaicin facilitates spontaneous glutamate release onto medium spiny neurons in the striatum of 8-14 day-old rats. A) Representative recording at whole-cell voltage clamp mode shows that the frequency of glutamatergic firing increases upon perfusion of the $TRPV_1R$ agonist capsaicin (1 μM). B) Quantification of major parameters of synaptic transmission reveals statistically significant presynaptic modulation as the frequency rather than the amplitude or the desensitization rate of the glutamatergic current changes upon capsaicin perfusion. Data represent the mean±SEM of n=5 observations (animals); $^{**}p < 0.01$.

Fig. 2. Capsaicin facilitates the release of glutamate and dopamine from striatal synaptosomes of rat pups only. Synaptosomes were loaded with the respective radiolabeled neurotransmitters, and trapped in GF/B filters in microvolume release chambers, and superfused thereafter. Two-min samples of the effluents were measured for radioactivity. Diagrams representing the averaged net release curves of [^{3}H]dopamine and [^{14}C]glutamate from striatal synaptosomes of A) 7, B) 14 and C) 60 day-old rats. Stimulation with capsaicin (1 μM) occurred since the 6th min of sample collection for 3 min, as indicated by the horizontal bar. Net curves were obtained after subtracting the vehicle control data from the capsaicin-treated data. D) Age-effect-size curves reveal that the effect of capsaicin falls to 50% of its theoretical maximum by the 13th post-natal day. Data represent the mean±SEM of n=8 observations (animals); $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; $^{***,\#\#\#}p < 0.001$

E) Representative Western-blotting image illustrates that by the 29th post-natal day, the disappearance of $TRPV_1R$ immunoreactivity from purified striatal nerve terminals is almost complete.

Fig. 3. $TRPV_1Rs$ do not affect the release of glutamate and dopamine in striatal synaptosomes of adult WT and CB_1R KO mice. Release diagrams representing the averaged release curves of A) [^{3}H]dopamine and B) [^{14}C]glutamate from striate synaptosomes of wild-type (WT) and CB_1R knockout (KO) mice. Synaptosomes were loaded with the respective radiolabeled neurotransmitters, and trapped in GF/B filters in microvolume release chambers, and superfused thereafter. Two-min samples of the effluents were measured for radioactivity. Depolarization with 4-aminopyridine (300 μM; 2 × 2 min) is marked as S_1 and S_2. In those synaptosomes which were not challenged with 4-AP, capsaicin (1 μM) bath-applied from min 12 of the sample collection period, failed to affect the resting release of C,D,F) [^{3}H]dopamine and G) [^{14}C]glutamate in the WT and CB_1R KO mice. Treatment with the selective $TRPV_1R$ agonist, resiniferatoxin (RTX, 100 nM) or capsaicin in the presence of the CB_1R antagonist, AM251 (500 nM) also failed to alter the resting release values. E) Capsaicin also had no effect on the 4-AP evoked release of both transmitters. Capsazepine (1 μM), a $TRPV_1R$ antagonist did not unmask off-target effect for capsaicin on the 4-AP-evoked release of glutamate. Data represent mean ± S.E.M. from 6 animals in duplicate. n.s.: not significant.

Fig. 4. $TRPV_1Rs$ do not affect the release of serotonin in frontocortical synaptosomes of adult WT and CB_1R KO mice. Release diagrams representing the averaged release curves of A) [^{3}H]serotonin from frontocortical synaptosomes of wild-type (WT) and CB_1R knockout (KO) mice. Synaptosomes were loaded with [^{3}H]serotonin under the blockade of noradrenalin and dopamine transporters, then trapped in GF/B filters in microvolume release chambers, and superfused thereafter. Two-min samples of the effluents were measured for radioactivity. Depolarization with 4-aminopyridine (300 μM; 2 × 2 min) is marked as S_1 and S_2. B) Neither capsaicin (1 μM) nor the hybrid CB_1R/ $TRPV_1R$ agonist, ACEA (1 μM), both bath-applied from min 12 of the sample collection, altered the 4-AP-evoked release of [^{3}H]serotonin in the WT mice under CB_1R blockade. However,

ACEA increased the evoked release of [^{3}H]serotonin in the CB_1R KO mice by ~13%, suggesting the recruitment of a novel ACEA-sensitive receptor in the serotonergic terminals of the genetically altered animals. C) In those synaptosomes which were not challenged with 4-AP, ACEA and capsaicin did not stimulated [^{3}H]serotonin release neither on their own, nor in the presence of another selective CB_1R agonist, O-2050 (500 nM). All bars represent the mean ± S.E.M. of data obtained from n = 6 animals in duplicate. *$p < 0.05$.

Highlights

1. Capsaicin triggers glutamate and dopamine release in the striatum of young rats only

2. $TRPV_1R$ function is not enhanced under chemical and genetic ablation of CB_1Rs.

3. Our data reveal a possible neurodevelopmental role for the $TRPV_1R$ in the rodent brain

Figure 1
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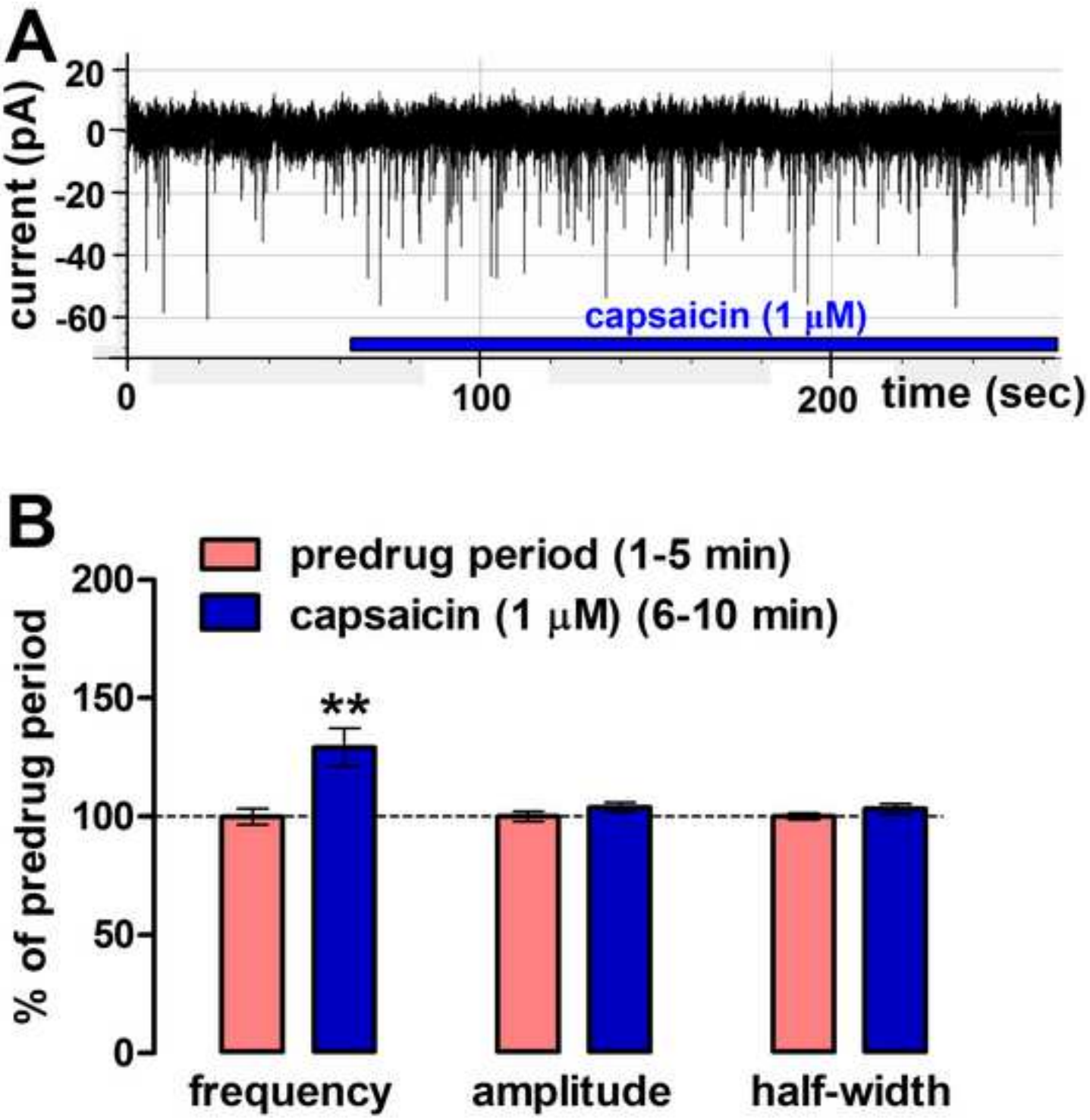

Figure 2

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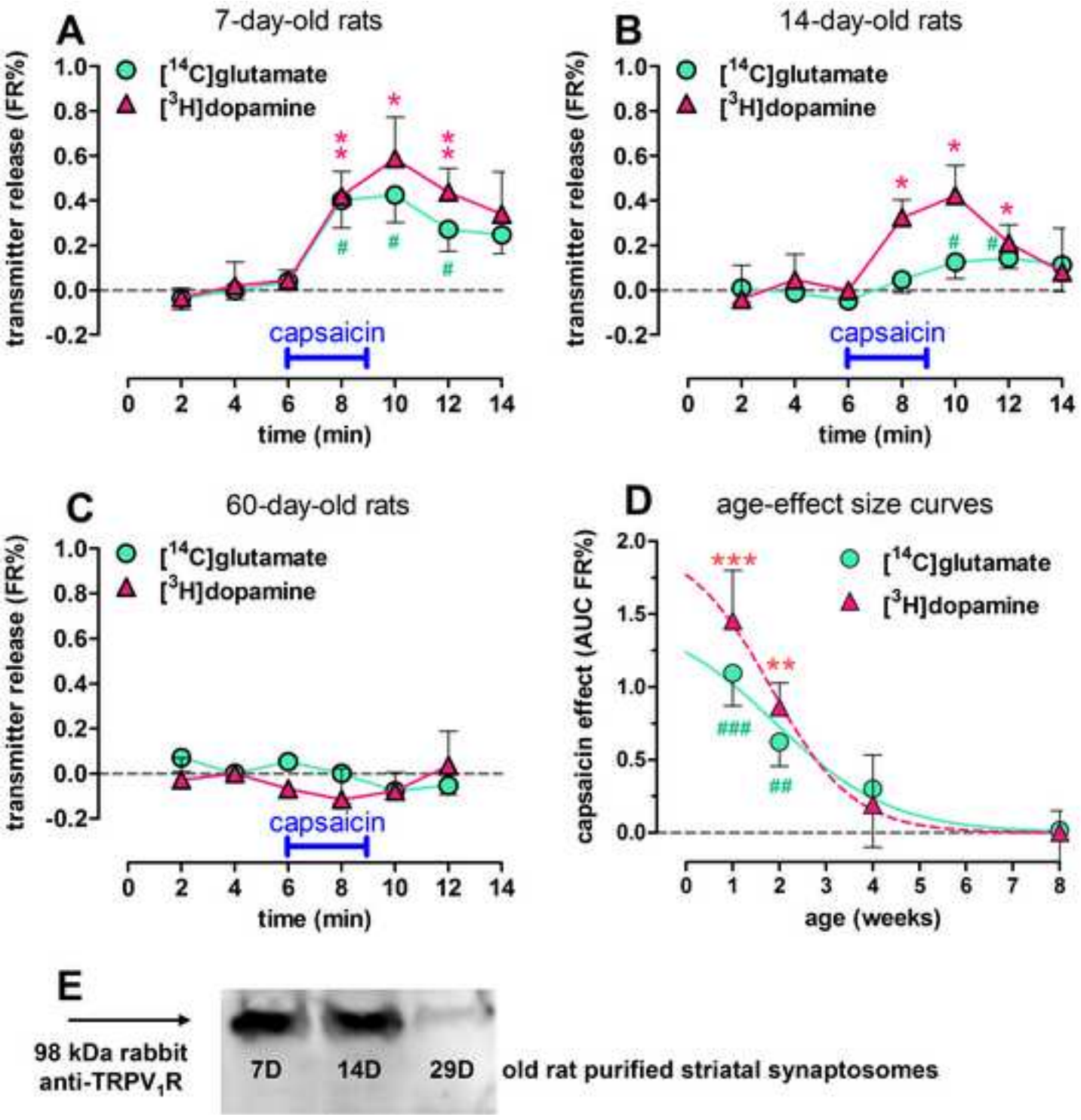

Figure 3

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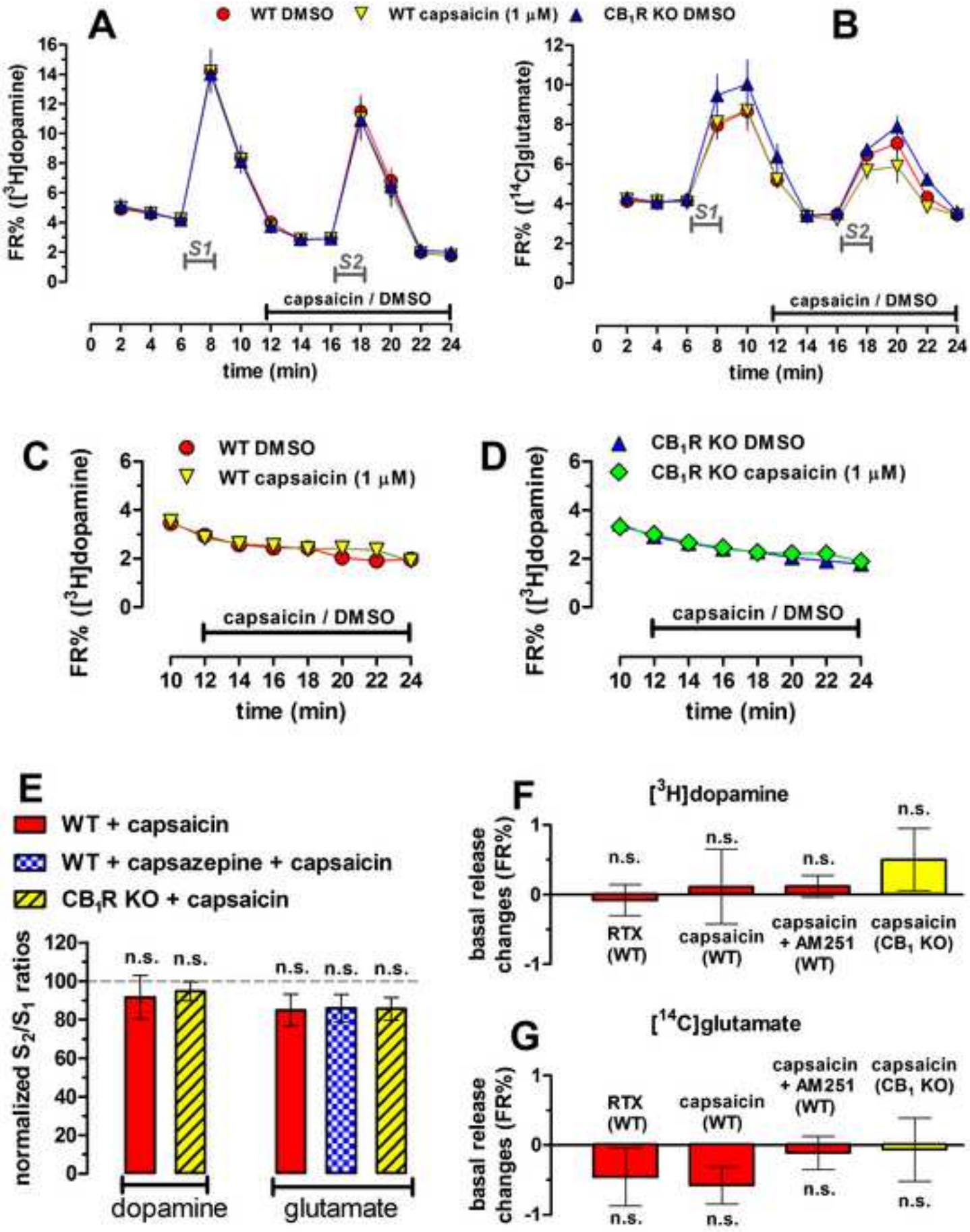

Figure 4

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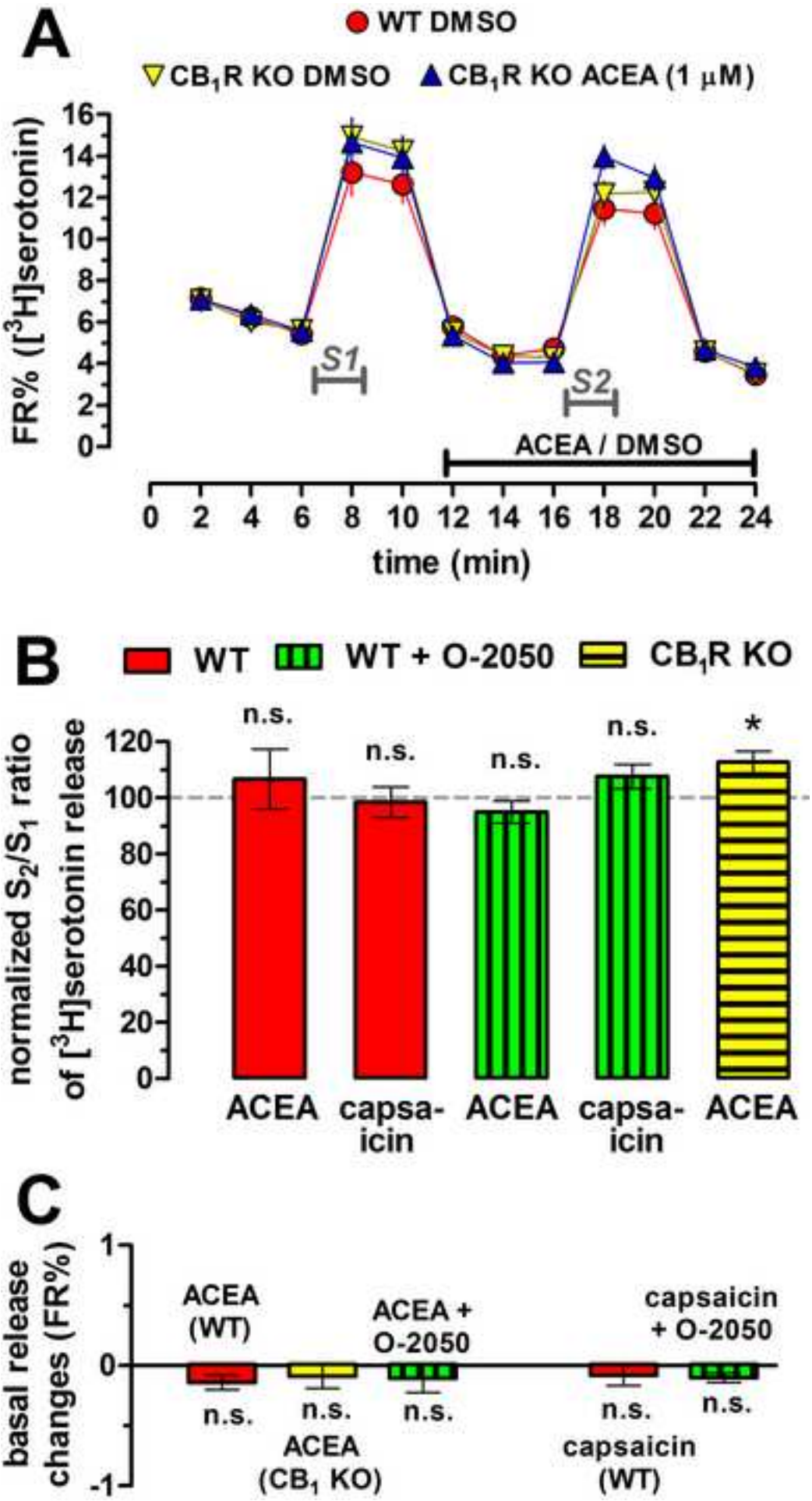

Table 1

animal	transmitter	age (D)	brain area	$TRPV_1$ effect?	reference
rat	D-aspartate	>32 ?	hippocampus	no	D'Amico et al., 2004
rat	D-aspartate	>32 ?	hippocampus	no	Cannizzaro et al., 2006
rat	GABA	>32 ?	hippocampus	no	D'Amico et al., 2004
rat	GABA	15-22	hippocampus	no	Hájos and Freund, 2002
rat	GABA	42-56	hippocampus	no	Köfalvi et al., 2006, 2007
rat	GABA	12-24	substantia nigra p.c.	no	Marinelli et al., 2003
rat	GABA	12-24	substantia nigra p.c.	no	Marinelli et al., 2007
rat	glutamate	35-56	cerebellum	no	Sasamura et al., 1998
rat	glutamate	28-42	hippocampus	yes	Al-Hayani et al., 2001
rat	glutamate	15-22	hippocampus	yes	Gibson et al., 2008
rat	glutamate	28-42	hippocampus	yes	Huang et al., 2002
rat	glutamate	15-22	hippocampus	yes	Hájos and Freund, 2002
rat	glutamate	15-27	hippocampus	yes	Jensen and Edwards, 2012
rat	glutamate	42-56	hippocampus	no	Köfalvi et al., 2007
rat	glutamate	35-56	hypothalamus	yes	Sasamura et al., 1998
rat	glutamate	12-22	locus coeruleus	yes	Marinelli et al., 2002
rat	glutamate	42-56	striatum	no	Ferreira et al., 2009
rat	glutamate	12-24	substantia nigra p.c.	yes	Marinelli et al., 2003
rat	glutamate	12-24	substantia nigra p.c.	yes	Marinelli et al., 2007
rat	glutamate	14-21	ventral tegmental a.	yes	Marinelli et al., 2005
rat	dopamine	42-56	striatum	no	Ferreira et al., 2009
mouse	GABA	42-49	striatum	no	Musella et al., 2009
mouse	glutamate	20-77	hippocampus	no	Benninger et al., 2008
mouse	glutamate	15-21	hippocampus	yes	Gibson et al., 2008
mouse	glutamate	70-84	striatum	no	Ferreira et al., 2009
mouse	glutamate	42-49	striatum	yes	Musella et al., 2009
mouse	glutamate	8-15	superior colliculus	yes	Maione et al., 2009
mouse	glutamate	35-70	superior colliculus	no	Maione et al., 2009
mouse	dopamine	70-84	striatum	no	Ferreira et al., 2009

Table 1. Summary of the literature reporting presynaptic $TRPV_1$R-mediated actions in forebrain preparation. D, days; p.c., pars compacta; a., area

Table 2

animal/tissue	transmitter	resting FR%	S_1 FR%	S_2/S_1	filter (DPM)
rat 7D striatum	[^{3}H]dopamine	4.03 ± 0.52	n.a.	n.a.	194593 ± 10367
rat 60D striatum	[^{3}H]dopamine	3.89 ± 0.36	n.a.	n.a.	172117 ± 20732
rat 7D striatum	[^{14}C]glutamate	4.85 ± 0.31	n.a.	n.a.	95695 ± 5427
rat 60D striatum	[^{14}C]glutamate	4.51 ± 0.35	n.a.	n.a.	102173 ± 7034
mouse >50D cortex	[^{3}H]serotonin	4.85 ± 0.31	5.8 ± 0.4	0.71 ± 0.06	57396 ± 3409
mouse >50D striatum	[^{3}H]dopamine	4.73 ± 0.63	12.0 ± 1.6	0.85 ± 0.05	115169 ± 9327
mouse >50D striatum	[^{14}C]glutamate	5.14 ± 0.54	8.0 ± 1.1	0.93 ± 0.06	99065 ± 8149