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Characterization of the antagonist actions of 5-BDBD at the rat P2X4 receptor

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Abstract

P2X receptors (P2XRs) are a family of ATP-gated ionic channels that are expressed in numerous excitable and non-excitable cells. Despite the great advance on the structure and function of these receptors in the last decades, there is still lack of specific and potent antagonists for P2XRs subtypes, especially for the P2X4R. Here, we studied in detail the effect of the P2X4R antagonist 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD) on ATP-induced currents mediated by the rat P2X4R and compared its specificity among another rat P2XRs. We found that 5-BDBD is a potent P2X4R antagonist, with an IC₅₀ of 0.75 μ M when applied for 2 min prior and during ATP stimulation. Moreover, at 10 μ M concentration, 5-BDBD did not affect the ATP-induced P2X2aR, P2X2bR, and P2X7R current amplitude or the pattern of receptor desensitization. However, at 10 μ M concentration but not 0.75 μ M 5-BDBD inhibited the P2X1R and P2X3R-gated currents by 13 and 35% respectively. Moreover, we studied the effects of 5-BDBD in long-term potentiation experiments performed in rat hippocampal slices, finding this antagonist can partially decrease LTP, a response that is believed to be mediated in part by endogenous P2X4Rs. These results indicate that 5-BDBD could be used to study the endogenous effects of the P2X4R in the central nervous system and this antagonist can discriminate between P2X4R and other P2XRs, when they are co-expressed in the same tissue.

Keywords

Purinergic signaling; P2X4 receptor; 5-BDBD; long-term potentiation

Introduction

The family of ionotropic receptors activated by extracellular ATP, termed P2X receptors (P2XRs), is responsible of several physiological and pathological responses mediated by the

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release of ATP to the extracellular medium. These responses occur in a variety of cell types and include processes such as immune response, vascular contraction/dilatation pain signaling, and long-term potentiation [9, 32]. Crystallization of the zebrafish P2X4.1R has revitalized the interest in purinergic signaling; now with available crystals in both closed and open states [16, 21] several of the evidence obtained in the pre-crystal era have been validated and new hypotheses about the mechanism responsible for conformation and gating properties has been proposed [20]. Also, thanks to the recent advances in the field, new specific antagonists or modulators have been introduced [28], constituting a major advance to establish the specific contribution of P2XRs *in vivo* and with the certain possibility to develop specific therapeutics targeting these receptors. However, there is still a need for specific and potent antagonists for some of P2XR subtypes, although some specific antagonist for the P2X4R have been recently developed and described, including 5-BDBD, PSB-12054, PSB-12062, BX430 and NP-1815-PX [31].

Among P2XRs, the P2X4R subtype is one of the more extensively studied; this receptor is widely expressed through the central nervous system, contributing in events such as synaptic plasticity [25, 30] and microglia activation after *status epilepticus* [34] in the hippocampus. In spinal microglia, P2X4Rs are important for pain processing and constitutes a potential target for the treatment of neuropathic pain [18, 33]. In addition, there is abundant evidence that brain P2X4Rs plays a role in alcohol-related disorders being a potential therapeutic target for these pathologies [12]. One of the particularities of this receptor is its resistance to suramin and PPADS, a broad-range purinergic antagonists, and therefore it has been difficult to synthesize antagonist derived from these molecules, as it has been the case for other P2XRs [9]. The native channels could be recognized by application of ivermectin, an allosteric regulator, which enhances the peak amplitude of current, causes a leftward shift in the EC50 value for ATP and delays receptor deactivation [9]. However, in tissues expressing multiple types of P2XRs, contribution of P2X4R could not be estimated without a highly selective P2X4R antagonist.

In 2005, the benzodiazepine-derived 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD) was introduced as an specific P2X4R antagonist [11]. This finding opens the field for future studies with recombinant and native P2X4Rs. The compound was originally described as a competitive orthosteric antagonist [3], but more recent work using radioligand binding studies suggests an allosteric mechanism of action [1]. Although 5-BDBD has been used in numerous studies, there is no information about its specificity, i.e. its potential effect on other P2XR subtypes. In the present work, we characterized the effects of 5-BDBD on HEK293 cells expressing different rat P2XRs and measured the currents gated by ATP using electrophysiological techniques. We found that 5-BDBD is a potent P2X4R antagonist and that saturating concentrations does not inhibit P2X2aR and P2X2bR, two splice forms of P2X2Rs, which desensitize with similar kinetics as P2X4R [9], and does not affects gating of P2X7R. Besides, it has a very modest effect on the P2X1R and the P2X3R. Finally, we tested the effects of 5-BDBD on LTP from rat hippocampal slices and found that this antagonist can block P2X4R activity in this model. Thus, 5-BDBD could be used as a useful antagonist to study the effects mediated by the P2X4R in native tissues.

Materials and Methods

Chemicals.

5-BDBD, PPADS and AF-353 were obtained from Tocris (Minneapolis, MN). ATP, penicillin–streptomycin, and salts used to prepare the incubation media were purchased from Sigma-Aldrich (St. Louis, MO). LipofectAMINE 2000 reagent was from Invitrogen (Carlsbad, CA).

Receptor transfection and current measurements.

Experiments were done with rat P2XRs. HEK293 cells were routinely maintained in DMEM containing 10% (v/v) fetal bovine serum and 100 µg/ml gentamicin (Invitrogen, Carlsbad, CA). Cells were plated at a density of 500,000 cells per 35 mm culture dish. The transient transfection was conducted 24 h after plating the cells using 2 µg of DNA and 5 µl of LipofectAMINE 2000 reagent in 2 ml of serum-free Opti-MEM. After 4.5 h of incubation, the transfection mixture was replaced with normal culture medium. The experiments were performed 24–48 h after transfection. Before recordings, transfected cells were mechanically dispersed and re-cultured on 35-mm dishes for 2–10 h. Electrophysiological experiments were performed on cells at room temperature using whole-cell patch-clamp recording techniques. The currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) and were filtered at 2 kHz using a low-pass Bessel filter. Patch electrodes, fabricated from borosilicate glass (type 1B150F-3; World Precision Instruments, Sarasota, FL), using a Flaming Brown horizontal puller (P-87; Sutter Instruments, Novato, CA, USA), were heat polished to a final tip resistance of 2–4 MΩ. All current records were captured and stored using the pClamp 9 software packages in conjunction with the Digidata 1322A analog-to-digital converter (Molecular Devices, Sunnyvale, CA). Patch electrodes were filled with a solution containing the following (in mM): 142 NaCl, 1 MgCl₂, 10 EGTA, and 10 HEPES, pH adjusted to 7.35 with 10 M NaOH. The osmolarity of the internal solutions was 306 mOsm. The bath solution contained the following (in mM): 142 NaCl, 3 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, pH adjusted to 7.35 with 10 M NaOH. The osmolarity of this solution was 295–305 mOsm. ATP was daily prepared in bath buffer and applied using a rapid solution changer system (RSC-200; Biologic Science Instruments, Claix, France). Stock solutions of 5-BDBD were prepared in dimethylsulfoxide, and aliquots were stored at –20 °C. The current responses were recorded from single cells clamped at –60 mV. Concentration–response data were collected from recordings of a range of ATP concentration applied to single cells with a washout interval of 1–5 min between each application and normalized to the highest current amplitude.

Western blot.

Protein extracts from transfected and untransfected HEK293 cells, and rat brain were obtained in T-PER buffer (Pierce, Rockford, IL) with Complete Mini protease inhibitor cocktail tablets and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostic, Indianapolis, IN). Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Membranes were soaked in blocking buffer (5% nonfat dry milk in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBS-T)) for 1 h at

room temperature, and then incubated overnight at 4 °C, with a P2X4R primary antibody (Iomone Labs, Jerusalem, Israel) diluted in 1% nonfat dry milk blocking buffer. The membranes were then washed with TBS- and incubated for 1 h at room temperature with secondary antibodies diluted in 1% nonfat dry milk blocking buffer. Immunoreactivity was detected by using Super-Signal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Animals.

One-month-old male Sprague-Dawley rats were housed with food and water available *ad libitum* under 12:12 h light-dark cycle. Room temperature was maintained at 22–23 °C. Rats were anesthetized with halothane gas, decapitated and their brains rapidly removed for hippocampal slice preparation. All the protocols dealing with the maintenance and handling of the animals were followed as stated in the Bioethical Guidelines of the Universidad Católica del Norte and in the National Research Commission guidelines.

Hippocampal slice preparation.

After decapitation, brains were rapidly immersed in ice-cold dissection buffer containing (in mM) 212.7 sucrose; 5 KCl; 1.25 NaH₂PO₄; 3 MgSO₄; 1 CaCl₂; 26 NaHCO₃; and 10 glucose; pH 7.4. Hippocampi were dissected and transverse slices (400 µm thick) were obtained from the middle third portion using a vibratome (DSK microslicer DTK-1000, Ted Pella, Inc, Redding, California). Slices were transferred to an interface storage chamber containing artificial cerebrospinal fluid (aCSF) saturated with 95% O₂ / 5% CO₂ and were left at least 1 hour at 37 °C. After this procedure, slices were maintained in aCSF or incubated 1 hour with 5-BDBD, PPADS or AF-353 before recording. aCSF contained (in mM) 124 NaCl; 5 K⁺; 1.25 NaH₂PO₄; 1.0 MgCl₂; 2.0 CaCl₂; 26 NaHCO₃; and 10 glucose; pH 7.4. Single slices were then transferred to a recording chamber where they were kept completely submerged in ACSF and continually perfused (2 ml/min).

Long-term potentiation (LTP) experiments.

Field responses from control and 5-BDBD-incubated hippocampi were evoked by electrical stimulation (biphasic, constant current, 200 µs stimuli) delivered every 15 s at the Schaffer collateral pathway using bipolar electrodes connected to a stimulus isolator unit (A365, WPI, Sarasota, FL, USA) and recorded in the stratum radiatum, in order to visualize field Extracellular Postsynaptic Potentials (fEPSP) at the CA1 hippocampal area, using glass micropipettes (1–2 MΩ) filled with ACSF as recording electrodes. At the beginning of each experiment, stimulus/response curves were done by increasing the intensity of the stimulus in order to adjust it to elicit 50% of the maximum response. LTP was elicited after 20 min of a stable baseline by theta burst stimulation (TBS) consisting on 5 trains of stimulus with an inter-train interval of 10 s. Each train consisted of 10 bursts at 5 Hz, each burst having 4 pulses at 100 Hz. After TBS, data acquisition lasted for 1 h. Data were acquired using an extracellular amplifier (EX4–400, Dagan Corporation, Minneapolis, MN, USA) and a data acquisition board (National Instruments, Austin, TX) controlled through Igor Pro software (Wavemetrics Inc, USA). LTP values were constructed by normalizing the fEPSP using baseline as 100%. Magnitude of LTP was observed between 40 and 60 min following TBS.

Data analysis.

Concentration–response curves were performed applying ATP for 5 s in the 0.1– 1000 μ M dose range. Curves were normalized against the concentration of ATP that evoked the maximal response, in the absence and in the presence of 5-BDBD. To calculate 5-BDBD IC_{50} , increasing concentrations of the antagonist were pre and co-applied with 10 μ M ATP, the average of all the control currents was used as the normalized response (100%). The ATP EC_{50} and 5-BDBD IC_{50} were interpolated from each concentration–response curve. Likewise, the maximal ATP current (I_{max}) was obtained from each ATP concentration–response curve. Each experiment was repeated in at least four separate cells. Curve fitting, EC_{50} , IC_{50} , and I_{max} were obtained with GraphPad software. Statistical analyses included the nonparametric Mann–Whitney test (GraphPad software). Electrophysiological recordings on rat hippocampal slices were performed using slices from at least three different animals for each condition (control and incubated with 5-BDBD, PPADS or AF-353). Normalized fEPSP slopes were compared between experimental groups using Mann-Whitney tests and data were presented as mean $\pm$ S.E.

Results

We first characterized effects of 5-BDBD on HEK293 cells transiently expressing the rat P2X4R, and measured the currents induced by ATP using the whole-cell configuration. 5-BDBD inhibited 10 μ M ATP-induced currents of rP2X4R-expressing HEK293 cells in a concentration-dependent manner (Fig. 1). In our hands, the best conditions to achieve inhibition was a 2 min pre-application of 5-BDBD and then a co-application with ATP, so we used this protocol in all our subsequent experiments. After washout of 10 μ M 5-BDBD for 5 minutes, the currents were almost completely recovered (Fig. 1A). Detailed analysis of the concentration dependent effects of 5-BDBD on 10 μ M ATP-induced current revealed an IC_{50} of $0.75 \pm 0.27 \mu$ M (Fig. 1B, $n = 7$), in good agreement with two other studies that reported IC_{50} s between 0.5 and 1.2 μ M [3, 11].

Next, we examined effects of 1 μ M 5-BDBD on the ATP concentration response mediated by the P2X4R. 5-BDBD application for 2 min displaced rightward the ATP concentration–response curve, shifting the EC_{50} from 4.7 ± 1.8 to $15.9 \pm 3.9 \mu$ M ($p < 0.05$, Mann-Whitney test; Fig. 2, $n = 4–7$), without significantly altering the maximal response (controls = 2.5 ± 0.6 nA vs. 5-BDBD-treated = 2.0 ± 0.6 nA). Altogether these results suggest that 5-BDBD is a potent inhibitor of P2X4R with competitive characteristics.

The missing information about this compound is its specificity for P2X4R. Because P2X2R desensitizes during the prolonged agonist application with comparable kinetics as P2X4R [9], we initially tested effects of this compound on P2X2R using both functional splice forms in rats, termed P2X2aR and P2X2bR [22]. Application of 5-BDBD in 10 μ M concentration did not affect the peak amplitude of currents induced by 10 μ M ATP applied for 5 s (Fig. 3A and B). 5-BDBD was also ineffective in changing the pattern of P2X2aR and P2X2bR desensitization during sustained agonist application (data not shown). The P2X7R current triggered by 1 mM ATP was also not affected by 2-min application of 5-BDBD in 10 μ M concentration (Fig. 3A and B). For the P2X3R, we observed a modest but significant $35.9 \pm 10.1\%$ inhibition ($p < 0.05$) of the current amplitude with 5-BDBD (Fig. 3A

and B, $n = 4$) whereas in P2X1R, 5-BDBD induced inhibition was $12.7 \pm 5.6 \%$ (Fig. 3B, $n = 6$, non-significant). For comparison, inhibition of P2X4R under these conditions was $83.2 \pm 3.1 \%$ (Fig. 3B, $n = 7$, $p < 0.01$). These results indicate that 5-BDBD could be specifically used to discriminate between P2X1R, P2X2aR, P2X2bR, P2X3R, P2X4R, and P2X7R.

Finally, we tested the effects of 5-BDBD on endogenous P2X4R-mediated activity. For that aim, we performed LTP protocols in rat hippocampal slices. It has been showed before that the P2X4R participates in the consolidation of LTP [30]. We confirmed the expression of P2X4R but not P2X1R nor P2X3R in rat hippocampus, located in both synaptic and extrasynaptic regions (Fig. 4A and B). Then, we performed extracellular recordings and induced LTP in hippocampal slices. Incubation of slices with $10 \mu\text{M}$ 5-BDBD for 60 minutes decreased the late phase of LTP, that was reflected by the significant decrease of field excitatory postsynaptic potential slope values at 40–60 minutes after LTP-induction (Fig. 4C and E), indicating that 5-BDBD can antagonize endogenous P2X4R-mediated activity. As control, we performed experiments in the presence $3 \mu\text{M}$ PPADS, a non-specific antagonist that has no effect on P2X4R, and 100 nM AF-353, a P2X3R antagonist. In both cases we did not observed any significant change on field excitatory postsynaptic potential slope values at 40–60 minutes after LTP-induction (Fig. 4D and E).

Discussion

There is evidence about the physiological roles of P2XRs, especially in the central nervous system [32]. This family of receptors has gained attention from the scientific community because of its particular structural and gating properties and for the recent availability of crystal structures of both closed and open states of the zebrafish P2X4.1R. This has been traduced in a major advance and understanding of the structure-activity relationships that governs processes such as gating, ion permeation and allosteric regulation [9, 20]. These advances had also prompted the search for new antagonists with high selectivity and potency for P2XRs. For example, NF449 is a selective antagonist for the P2X1R [29], whereas AF-353, RO-4, RO-85 and RO-51 are good and selective P2X3R antagonists [5, 6, 14, 19]. For the P2X7R there are several specific antagonists available, including –438079 or A-70003, which are good candidates to be used as therapeutically agents [10]. Recently it was described the first P2X2R-specific antagonists, the anthraquinone derivatives termed PSB-10211 and PSB-1011 [4].

In contrast, it has been difficult to find good antagonists for the P2X4R. For example, this receptor is resistant to suramin and PPADS, which antagonizes other P2XR subtypes, and only TNP-ATP has been shown to behave as a good antagonist, but this compound also inhibits other P2XRs with high potency [9]. Paroxetine, an analog of monoamine uptake blockers, was shown to inhibit the P2X4R with micromolar affinity, in addition to its antidepressant actions [27]. new P2X4R antagonist, a phenoxazine derivative termed PSB-12062, was also reported to have good specificity for the P2X4R, acting through an allosteric mechanism [17]. Recently, a new antagonist for the P2X4R with interesting pharmacological properties, termed BX430, has been described [2]. However, this antagonist is selective for human and zebrafish receptors without any effect on rat and mouse receptors [2].

Although 5-BDBD seems to act as P2X4R competitive antagonist, it has been recently demonstrated by a radioligand study that its mechanism of action is allosteric [1]. This is not totally unexpected, there is evidence that compound that are apparently competitive antagonists they are in fact negative allosteric modulators [13, 37]. 5-BDBD could provide a starting point to develop more potent and selective antagonists for this receptor. In fact a compound derived from 5-BDBD, termed NP-815-PX has been successfully tested as an anti-allodynic drug in mice [26]. In our hands 5-BDBD inhibited rP2X4R-mediated currents with an estimated IC_{50} of 0.75 μ M, in good agreement with other studies that have used this compound in the hP2X4R [3, 11]. Moreover, radiolabeled analogs of 5-BDBD has been recently developed as a prototype of the first compound for PET studies targeted to the P2X4R, that could be useful to study neuroinflammatory processes associated with brain diseases [35]. Although the ability of 5-BDBD to permeate the blood-brain barrier has not been tested, we infer that being a benzodiazepine derivative, this compound should be able to penetrate in brain tissues [15].

A detailed description of 5-BDBD specificity for the P2X4R was lacking from previous studies to direct investigations of P2X4R function in native tissues and eventual development of more specific compounds based on the 5-BDBD structure as the base. Here, we tested the actions of 5-BDBD among several P2XRs, and found that it perfectly dissociated between P2X2aR, P2X2bR, P2X4R and P2X7R. However, it has an effect on the P2X3R and a small effect on the P2X1R, although at the ATP EC_{50} or the different effects of agonists such $\alpha\beta$ -me-ATP or antagonists such PPADS could be used in combination with 5-BDBD to dissociate between P2X3/P2X1R and P2X4R mediated responses. Rat P2X5R and P2X6R generate very low currents and could not be used to test effects of 5-BDBD.

There is also evidence that 5-BDBD can antagonize endogenous P2X4R-mediated responses; the NLRP3 inflammasome activation in human renal tubule epithelial cells [7]; the regulation of the capillary diameter at cochlear lateral wall of guinea pigs inner ear [36] and the P2X4R-mediated calcium influx in human monocytes and macrophages [24]. In all these cases the P2X4R was not the sole purinergic receptor but using antagonists for different P2XR subtypes clearly demonstrates that 5-B DBD is useful to determine the contribution of P2X4R in physiological responses. Interestingly, we have also shown in this work that 5-BDBD can dissect the contribution of the 2X4R in physiological models. In this context, we showed here that 5-BDBD decreases LTP, specially the late phase of the response. It has been proposed that P2X4Rs participates in the formation and consolidation of LTP. In fact, ivermectin, a P2X4R positive allosteric modulator increases the frequency of EPSPs, and the magnitude of LTP is decreased in KO mice lacking the P2X4R [30]. Furthermore, it appears that P2X4R down-regulates NMDA receptors via interaction with PSD-95 multi-protein complex, and dissociation of this interaction decreases the threshold to induce LTP but also decreases its net magnitude [23]. Other studies have addressed that allosteric P2X4R-modulators, such as zinc [8], can also regulate long-term potentiation through P2X4Rs in rat hippocampal slices [25]. Finally, in P2X4R-deficient mice it has been shown an altered hippocampal synaptic potentiation, and an obliteration of ivermectin potentiation.

In summary, we have characterized 5-BDBD as a specific P2X4R antagonist, the development of such molecules will help to clearly establish to contributions of P2XRs to physiological processes and will also help to design specific drugs to be used in pathologies in which these receptors are involved.

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Highlights

- We confirmed that 5-BDBD is a potent P2X4 receptor antagonist
- The selectivity of 5-BDBD allows to discriminate between P2X4 and other P2X subtypes.
- 5-BDBD can be used to determine endogenous P2X4-mediated functions in the central nervous system.

Figure 1. 5-BDBD inhibits rP2X4R-mediated currents.

A. Representative recording from a single HEK293 cell expressing the rP2X4R. Currents were gated by a 5 s application of 10 μ M ATP, voltage membrane was set to -60 mV. 5-BDBD was pre-applied for 2 min and then co-applied with ATP at the concentrations indicated; washout between ATP pulses was 4 minutes. **B.** Summary of the 5-BDBD concentration-response protocols performed on rP2X4R expressing cells and activated by 10 μ M ATP. Curves were normalized to the response obtained by ATP alone at the beginning of the experiments. Symbols represent the mean values, and bars indicate SE values from $n = 7$.

Figure 2. A rightward shift in the ATP EC_{50} by 5-BDBD.

A. Representative recordings from rP2X4R-HEK293 cells of ATP induced currents in the absence (upper panel) and in the presence of 1 μM 5-BDBD, pre-applied for 2 minutes and then co-applied with ATP (lower panel). Voltage membrane was held at -60 mV . **B.** ATP concentration-response curves in the absence (black circles) and presence (open squares) of 1 μM 5-BDBD. Curves were normalized against the maximal response obtained in each condition. Dotted lines represent the EC_{50} values, $4.7 \pm 1.8 \mu\text{M}$ ($n = 7$) in the absence and $15.9 \pm 3.9 \mu\text{M}$ ($n = 4$) in the presence of 1 μM 5-BDBD.

Figure 3. Specificity of 5-BDBD among rat P2XRs.

A. Representative recordings of HEK293 cells expressing the P2X2aR, P2X2bR, P2X3R or P2X7R in the absence or in the presence of 10 μ M 5-BDBD (pre-applied for 2 minutes and then co-applied with ATP). The ATP concentrations used were 1, 10 and 1000 μ M A P for the P2X3R, P2X2R and P2X7R, respectively. Voltage membrane was held at -60 mV. **B.** Summary of the results obtained with 5-BDBD. *, $p < 0.05$; **, $p < 0.01$, as compared with the response obtained with ATP alone (inside bars) or comparing between P2XRs (Mann-Whitney test).

Figure 4. Effects of 5-BDBD on LTP.

A and B. Representative Western blots of P2X4R (**A** and **B**) or P2X1R and P2X3R (**B**) in synaptic fractions obtained from rat hippocampus (right panel). H: Homogenate, S: supernatant, PSD: Postsynaptic densities. As P2X4R positive control, transfected HEK cells were used. **C.** LTP curves obtained from rat hippocampal slices incubated for 1 hour with 10 μ M 5-BDBD in aCSF (grey squares, $n=20$ slices, 6 rats) or aCSF alone (white circles, $n=16$ slices, 5 rats). **D.** LTP curves obtained from slices incubated by 1 hour with 3 μ M PPADS (dark grey squares, $n=8$ slices, 3 rats) or 100 nM AF-353 (light grey diamonds, $n=9$ slices, 6

rats) and the corresponding control experiments (white circles, n=18 slices, 6 rats). TBS was applied after 20 minutes of stable baseline. %fEPSP: % field Excitatory Postsynaptic Potential, TBS: Theta burst stimulation **E**. Late phase-LTP quantification of curves obtained from experiments shown in **C** and **D**. Mean %fEPSP were obtained from each curve taking points from 40 to 60 minutes and compared using t-student test. *** p < 0.001.