Adenosine A_{2A} receptor activation is determinant for BDNF actions upon GABA and glutamate release from rat hippocampal synaptosomes

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Abstract Adenosine, through A_{2A} receptor (A_{2A}R) activation, can act as a metamodulator, controlling the actions of other modulators, as brain-derived neurotrophic factor (BDNF). Most of the metamodulatory actions of adenosine in the hippocampus have been evaluated in excitatory synapses. However, adenosine and BDNF can also influence GABAergic transmission. We thus evaluated the role of A_{2A}R on the modulatory effect of BDNF upon glutamate and GABA release from isolated hippocampal nerve terminals (synaptosomes). BDNF (30 ng/ml) enhanced K⁺-evoked [³H]glutamate release and inhibited the K⁺-evoked [³H]GABA release from synaptosomes. The effect of BDNF on both glutamate and GABA release requires tonic activation of adenosine A_{2A}R since for both neurotransmitters, the BDNF action was blocked by the A_{2A}R antagonist SCH 58261 (50 nM). In the presence of the A_{2A}R agonist, CGS21680 (30 nM), the effect of BDNF on either glutamate or GABA release was, however, not potentiated. It is concluded that both the inhibitory actions of BDNF on GABA release as well as the facilitatory action of the neurotrophin on glutamate release are dependent on the activation of adenosine A_{2A}R by endogenous adenosine. However, these actions could not be further enhanced by exogenous activation of A_{2A}R.

Keywords Brain-derived neurotrophic factor · Adenosine A_{2A} receptors · GABA release · Glutamate release · Synaptosomes · Hippocampus

Introduction

Adenosine is a neuromodulator that affects the activity of synapses at different levels—pre-, post-, and non-synaptically—through the activation of G-protein-coupled receptors [1]. Adenosine can also act as a metamodulator, controlling the actions of other modulators involved in synaptic activity such as brain-derived neurotrophic factor (BDNF) [1, 2].

BDNF is a neurotrophin that promotes neuronal survival, differentiation, and synaptic plasticity [3]. A considerable amount of data has provided molecular and functional evidence for a crosstalk between BDNF and adenosine A_{2A} receptors (A_{2A}R) [4]. Accordingly, there is evidence that activation of A_{2A}R induces the transactivation of TrkB-FL receptors [5, 6], TrkB-FL translocation to lipid rafts [7], and regulates BDNF [8] and TrkB-FL levels [9]. Regarding functional evidence, it is clear that the facilitatory actions of BDNF upon CA1 hippocampal synaptic transmission [8, 10–12] and synaptic plasticity, both long-term potentiation [9, 13, 14] and long-term depression [15], are dependent on A_{2A}R activation. CA1 hippocampal synaptic activity relies, in part, on the dynamics of the most relevant neurotransmitters in this brain region, glutamate and GABA. Previous work has demonstrated that BDNF facilitates glutamate release from hippocampal synaptosomes [16] in a mechanism dependent on endogenous adenosine, since it is prevented upon removal of extracellular adenosine with adenosine deaminase [7]. However, the adenosine receptor involved remained unknown. Regarding GABA release from hippocampal synaptosomes, BDNF has an inhibitory effect [16] but the influence of the adenosine

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receptors was totally unexplored. Therefore in the present study, the role of A_{2A}R on the modulatory effect of BDNF upon glutamate and GABA release from rat hippocampal synaptosomes was evaluated.

Methods

Drugs [³H]Glutamate (L-[G-³H]glutamic acid, specific activity 45 Ci/mmol) and [³H]GABA (4-amino-n[2,3-³H]butyric acid, specific activity 100 Ci/mmol) were from Amersham. Stock solutions of BDNF (kindly supplied by Regeneron) were prepared in phosphate-buffered saline (PBS), at a final concentration of 1 mg/ml. 2-[p-(2-carboxyethyl)phenethylamino]-5- ϵ -N-ethylcarboxamido adenosine (CGS 21680), 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261), and aminooxyacetic acid (AOAA) were from Sigma. Percoll, EDTA, KCl, MgSO₄·7H₂O, NaCl, and NaH₂PO₄·H₂O were from Sigma; glucose, sucrose, HEPES, bovine serum albumin (BSA), NaH₂PO₄·H₂O, NaOH, and HCl were from Merck. CGS 21680 and SCH 58261 were made up into a 5 mM stock solution in dimethylsulfoxide (DMSO). AOAA was made up to a 0.5 M aqueous stock solution. All these stock solutions were aliquoted and stored at -20 °C. Aqueous dilutions of these stock solutions were made daily.

Isolation of hippocampal synaptosomes The synaptosomes from Wistar rat hippocampus (two animals for experiment, 6–8 weeks old) were prepared as routinely in our laboratory [9]. Briefly, hippocampi were dissected and homogenized in ice-cold isosmotic sucrose solution (containing 0.32 M sucrose, 1 mM EDTA, 1 mg/ml BSA, and 10 mM HEPES, pH 7.4) and centrifuged at 3000g for 10 min; the supernatant was centrifuged again at 14,000g for 12 min. The pellet was resuspended in 45 % Percoll in KHR solution consisting of (in mM) NaCl 140, EDTA 1, HEPES 10, KCl 5, and glucose 5 and was centrifuged at 14,000g for 2 min. The synaptosomal fraction corresponds to the top buoyant layer and was collected from the tube. Percoll was removed by two washes with a KHR solution; the whole procedure was conducted at 4 °C, and synaptosomes were then kept on ice and used within 3 h as previously described [9].

[³H] neurotransmitter release from hippocampal synaptosomes [³H]GABA and [³H]glutamate release experiments were performed as previously described [9]. Synaptosomes (protein concentration 1–2 mg/ml) were resuspended in oxygenated Krebs medium (in mM: NaCl 125, KCl 3, NaH₂PO₄ 1, glucose 10 NaHCO₃ 25, CaCl₂ 1.5, and MgSO₄ 1.2) and allowed to equilibrate for 5 min at 37 °C. From this time

onward, all solutions applied to the synaptosomes were kept at 37 °C and continuously gassed with O₂/CO₂ (95 %/5 %). AOAA (0.1 mM) was present in all solutions used in [³H]GABA release experiments to prevent GABA catabolism by inhibition of GABA transaminase. The synaptosomes were loaded for 20 min at 37 °C, with [³H]GABA (1.5 μ Ci/ml, 18.5 nM), together with 0.625 μ M unlabeled GABA to decrease specific activity of the [³H]GABA solutions to 2.3 μ Ci/nmol and equally layered onto perfusion chambers over Whatman GF/C filters (flow rate, 0.8 ml/min; chamber volume, 90 μ l). For [³H] glutamate release experiments, synaptosomes were loaded with 0.2 μ M [³H]glutamate (specific activity was 30–60 Ci/mmol) for 5 min and equally layered onto perfusion chambers over Whatman GF/C filters (flow rate, 0.6 ml/min; chamber volume, 90 μ l). After a 20-min washout period, the effluent was collected for 40 min in 2-min intervals. The GABA or glutamate release from synaptosomes was stimulated during 2 min with a high-K⁺ solution (15 mM, isomolar substitution of Na⁺ with K⁺ in the perfusion buffer) at the 5th (first stimulation period [S1]) and 29th (second stimulation period [S2]) minute after starting sample collection. BDNF (30 ng/ml) was added to the superfusion medium at the ninth minute, therefore before S2, and remained in the bath up to the end of the experiments. To evaluate the ability of a drug to modify the effect of BDNF, the modifier drug was present during S1 and S2 in all chambers, and BDNF was added to test chambers before S2. Doing so, any effect that a modifier drug may have on neurotransmitter release per se is not expected to reflect in changes in the S2/S1 ratios. The effect of BDNF in the absence of the modifier drug was always assessed in parallel chambers from the same synaptosomal batch.

Calculation of drug effects on GABA and glutamate release At the end of each experiment, aliquots (500 μ l) of each sample as well as the filters from each superfusion chamber were analyzed by liquid scintillation counting. The fractional release was expressed in terms of the percentage of total radioactivity present in the preparation at the beginning of the collection of each sample. The amount of radioactivity released by each pulse of K⁺ (S1 and S2) was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium release. In each experiment, two synaptosome-loaded chambers were used as control chambers, the others being used as test chambers, test and control chamber having exactly the same drug compositions except for the presence of BDNF, which was added only to the test chambers and before S2. In all cases, the effect of BDNF on the K⁺-evoked tritium release was expressed as percentage of change of the S2/S1 ratios in test conditions (presence of BDNF) compared with the S2/S1 ratios in control conditions in the same pool of synaptosomes. The S2/S1 ratios in glutamate release experiments are usually relatively low [cf. 7, 17]

most probably due to intracellular metabolism of glutamate, since glutamate is easily incorporated in the Krebs cycle; indeed, shorter intervals between S2 and S1 may result in S2/S1 ratios closer to the unit [16]. A disadvantage of the short interstimulus interval is the restraint in the incubation times with test drugs, which are added between S1 and S2. In the present work, the glutamate and GABA release protocols were designed with a similar time interval between S2 and S1 to allow a better comparison between the effects of BDNF upon the release of both neurotransmitters.

Statistical analysis Results are presented as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and Student's *t* test were used to compare groups. Statistical significance was set at a $p < 0.05$.

Results

Glutamate release When synaptosomes were not exposed to any drug, the S2/S1 ratio was 0.75 ± 0.10 . In the same synaptosomal batches but in the presence of BDNF (30 ng/ml, during S2), the S2/S1 ratio increased to 0.95 ± 0.18 ($n=3$), corresponding to a significant increase of $32.6 \pm 5.5\%$ ($p < 0.05$) in evoked glutamate release (Fig. 1a, d). To study the influence of $A_{2A}R$ on this effect of BDNF, experiments were performed using either a selective $A_{2A}R$ agonist (CGS21680) [18] or a selective $A_{2A}R$ antagonist (SCH 58261) [19] throughout the release period (both S1 and S2) in all chambers (either test or control chambers), and BDNF being added before S2 in test chambers. In the absence of BDNF, the S2/S1 ratio was 0.78 ± 0.10 ($n=3$) in experiments where SCH58261 (50 nM) was used (Fig. 1c) and 0.77 ± 0.09 ($n=3$) in the experiments where CGS21680 (30 nM) was used (Fig. 1b); this values being not

Fig. 1 Modulation of BDNF effects by $A_{2A}R$ on K^+ -evoked $[^3H]$ glutamate release from rat hippocampal synaptosomes. Average fractional release of $[^3H]$ glutamate, showing the effect of BDNF 30 ng/ml in control conditions (a), in the presence of 30 mM CGS 21680 (b), and in the presence of 50 mM SCH 58261 (c). Synaptosomes were incubated with $[^3H]$ glutamate, and neurotransmitter release was evoked by two 15 mM K^+ stimuli of 2 min duration, as indicated (S1 and S2). All points in a–c represent the mean $\pm$ SEM of 3–9 experiments performed in duplicate, each of which using the same batch of synaptosomes. **d** Histogram representing the effects from the experiments in a–c

as percentage potentiation of $[^3H]$ glutamate release induced by BDNF in the absence or in presence of CGS 21680, or SCH 58261 (as indicated below each bar). Note that SCH 58261 fully blocked the effect of BDNF. The S2/S1 values from corresponding controls were taken as 100 % within each experiment. CGS 21680 or SCH 58261 were applied 10 min before the start of sample collection and were continuously superfused throughout the experiment, therefore being present during S1 and S2, BDNF was added before S2 (indicated by the horizontal bars). * $p < 0.05$; NS $p \geq 0.05$ (one-way ANOVA followed by Bonferroni's multiple comparison test)

significantly different ($p>0.05$) from the S2/S1 ratio in the absence of any drug. As shown in Fig. 1d, the effect of BDNF (added before S2) was completely absent in the experiments where SCH58261 was present ($n=3$, $p>0.05$ as compared with absence of BDNF in the same experiments). This suggests that $A_{2A}R$ receptors activation is required for the effect of BDNF. The levels of endogenous adenosine may, however, be enough to trigger the action of BDNF since in the experiments where CGS21680 was present throughout the experiment, the effect of BDNF was not significantly greater than that observed in the absence of the $A_{2A}R$ agonist (Fig. 1d), thus indicating that a further activation of $A_{2A}R$ by an exogenous agonist does not cause any further potentiation of the effect of BDNF.

GABA release In GABA release experiments, in control conditions (synaptosomes not exposed to any drug), the S2/S1

ratio was 1.16 ± 0.08 ($n=9$). When BDNF was added before the second stimulation period (S2), a reduction of the S2/S1 ratio to 0.69 ± 0.11 ($n=9$; $p<0.05$) was observed, corresponding to a significant decrease of $40.3\pm8.3\%$ in the evoked GABA release (Fig. 2a, d), consistent with what has been previously reported [16, 20]. When present during S1 and S2, neither CGS21680 (30 nM) nor SCH58261 (50 nM) significantly affected the S2/S1 ratio when compared to control condition (S2/S1_{CGS21680}: 0.94 ± 0.06 , $n=6$; S2/S1_{SCH58261}: 1.09 ± 0.06 , $n=4$; S2/S1_{control}: 1.16 ± 0.08 , $n=9$, Fig. 2b–d). However, the presence of SCH58261 fully prevented the inhibitory action of BDNF upon GABA release (S2/S1_{SCH58261+BDNF}: 1.17 ± 0.08 , $n=4$, $p>0.05$, Fig. 2c). Again, and as it occurred with the facilitatory action of BDNF upon glutamate release, the inhibitory action of BDNF upon GABA release was not further potentiated by the presence of CGS21680 (S2/S1_{CGS21680+BDNF}: 0.72 ± 0.03 , $n=6$; $p<0.05$

Fig. 2 Modulation of BDNF effects by $A_{2A}R$ on K^+ -evoked [3H]GABA release from rat hippocampal synaptosomes. Average fractional release of [3H]GABA, showing the effect of BDNF 30 ng/ml in control conditions (a), in the presence of 30 mM CGS 21680 (b), and in the presence of 50 mM SCH 58261 (c). Synaptosomes were incubated with [3H]GABA, and neurotransmitter release was evoked by two 15 mM K^+ stimuli of 2-min duration, as indicated (S1 and S2). All points in a–c represent the mean $\pm$ SEM of 3–9 experiments performed in duplicate, each of which using the same batch of synaptosomes. **d** Histogram representing the effects from the experiments in a–c as percentage potentiation of

[3H]GABA release induced by BDNF in the absence or in presence of CGS 21680, or SCH 58261 (as indicated below each bar). Note that SCH 58261 fully blocked the effect of BDNF. The S2/S1 values from corresponding controls were taken as 100 % within each experiment. CGS 21680 or SCH 58261 were applied 10 min before the start of sample collection and were continuously superfused throughout the experiment, therefore being present during S1 and S2, BDNF was added before S2 (indicated by the horizontal bars). * $p<0.05$; NS $p\geq0.05$ (one-way ANOVA followed by Bonferroni's multiple comparison test)

as compared with the absence of BDNF in the same experiments; $p > 0.05$ when compared with BDNF effect in the absence of CGS 21680, Fig. 2c, d).

Discussion

The main findings of the present study are that both the facilitatory action of BDNF on the release of glutamate and the inhibitory action of BDNF on the release of GABA from rat hippocampal synaptosomes are dependent on the activation of $A_{2A}R$ by endogenous adenosine, though those BDNF actions could not be further enhanced by exogenous activation of $A_{2A}R$.

Considering glutamate release from hippocampal synaptosomes, it has been previously shown that BDNF affects the release pool that requires calcium entry through voltage-dependent calcium channels, thus the exocytotic pool [16]. In contrast, the inhibitory influence of BDNF upon potassium-evoked GABA release from hippocampal synaptosomes has been shown to be independent of calcium entry into synaptosomes through voltage-sensitive calcium channels, but dependent of the activity of GABA transporters [16], thus suggesting that BDNF acts in the release pool that results from transport reversal. Accordingly, it has been shown that BDNF inhibits GABA transport into synaptosomes [21]. Interestingly, while evaluating GABA transport, the inhibitory effect of BDNF was not blocked by the presence of the $A_{2A}R$ antagonist but was potentiated when simultaneously $A_{2A}R$ s were exogenously activated by CGS21680 and adenosine was removed by adenosine deaminase [21]. We now report that the inhibitory action of BDNF is blocked by an $A_{2A}R$ antagonist but not exacerbated by an $A_{2A}R$ agonist. One may thus conclude that in both cases, the effect of BDNF is affected by $A_{2A}R$ activation, the main difference being, most probably, the levels of endogenous adenosine that are attained in each experimental condition, higher while evaluating GABA transport reversal induced by K^+ depolarization and lower while evaluating inward GABA transport. Indeed, adenosine is released by depolarization [22], and therefore the present experimental conditions favor an increase in the extracellular concentrations of adenosine.

$A_{2A}R$ activation leads to enhancement of GABA release from hippocampal synaptosomes [23]. Glutamate release from hippocampal synaptosomes of young animals is also enhanced by $A_{2A}R$ activation, an action that results from attenuation of adenosine A_{1R} -mediated inhibition [17]. It is worthwhile to note that in those studies, the $A_{2A}R$ agonist was used as the test drug, being therefore added before S2. The present experimental conditions were designed to test the influence of the $A_{2A}R$ ligands upon the effect of BDNF. Thus in our case, the $A_{2A}R$ ligands were used as modifier drugs being present throughout the release period (during both S1 and S2), thus not affecting the S2/S1 ratios. Interestingly, the

influence of $A_{2A}R$ s on GABA release at the hippocampus is synapse- and cell type-selective [24]. As we now show, $A_{2A}R$ also contributes to BDNF-mediated inhibition of GABA release; this action of BDNF being known to result from GABA transporters modulation [16]. Considering the astrocytic component of neuronal communication, $A_{2A}R$ s also contribute BDNF-mediated modulation of GABA transport [25]. The different actions of $A_{2A}R$ s in different synapses and their ability to affect different actions of BDNF at specific sites of the tripartite synapse may allow to finely shape excitatory and inhibitory signals, thus to fine tune neuronal function. This probably has a greater impact upon synaptic plasticity rather than upon neuroprotection since BDNF actions on plasticity are known to be controlled by $A_{2A}R$ [7, 13, 14], whereas neuroprotective actions of BDNF may not be affected by $A_{2A}R$ s [26].

In conclusion, we herein show that both the facilitatory action of BDNF upon glutamate release as well as the inhibitory action of BDNF upon GABA release from synaptosomes is dependent upon the co-activation of $A_{2A}R$, suggesting that adenosine receptors modulate both inhibitory and facilitatory fast signaling actions of BDNF in nerve terminals.

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