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Research Report

Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampal nerve terminals

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ABSTRACT

The lifespan of the predominant inhibitory neurotransmitter in the central nervous system, γ-aminobutyric acid (GABA), is determined by its uptake into neurons and glia, through high-affinity Na^+/Cl^- dependent transporters (GATs). We now evaluated how the uptake of GABA by nerve endings, which is mostly mediated by the GAT-1 subtype, is modulated by brain-derived neurotrophic factor (BDNF). BDNF (10–200 ng/ml) decreased GAT-1-mediated GABA uptake by isolated hippocampal rat nerve terminals (synaptosomes), an effect that occurred within 1 min of incubation with BDNF and blocked by the tyrosine kinase inhibitor K252a (100 nM) as well as by the PLC inhibitor, U73122 (3 μM). Maximum inhibition was attained with 100 ng/ml BDNF. In contrast with what has been observed for other synaptic actions of BDNF, the inhibition of GABA transport by BDNF does not require tonic activation of adenosine A_{2A} receptors since it was not blocked by the A_{2A} receptor antagonist SCH 58261 (50 nM). However, in synaptosomes previously depleted of extracellular endogenous adenosine by incubation with adenosine deaminase (1 U/ml), activation of A_{2A} receptors with the A_{2A} receptor agonist, CGS 21680 (30 nM), enhanced the inhibitory effect of BDNF upon GABA transport, an action prevented by the A_{2A} receptor antagonist, SCH 58261 (50 nM). It is concluded that BDNF, through TrkB and PLCγ signalling inhibits GAT-1-mediated GABA transport by nerve endings and that this action is not dependent on, but can be enhanced by, TrkB/A_{2A} receptor cross talk.

1. Introduction

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in adult central nervous system and plays a pivotal role in the balance between neuronal excitation and inhibition. GABA activity at synapses is terminated by rapid re-uptake through specific high-affinity Na^+/Cl^- dependent transporters (GATs), which are located on the plasma membrane of neurons and astrocytes, in close apposition to the synapse. The activity of GATs regulates GABA levels at synapses and therefore influences neuronal excitability. Changes in GAT activity are relevant both in pathology and therapy (see e.g. Allen et al., 2004;

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Abbreviations: ADA, Adenosine deaminase; AOOA, Aminooxyacetic acid; BDNF, Brain-derived neurotrophic factor; CGS 21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamido adenosine; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolol[1,5-c]pyrimidine; SKF 89976a, 1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; DMSO, Dimethyl sulfoxide

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Gether et al., 2006), an example being the GABA transporter inhibitor tiagabine, which is currently used as an antiepileptic drug (Jarvis et al., 1989).

The most copiously expressed GABA transporter in the brain is GAT-1 (see Conti et al., 2004) and it is regulated by several signalling cascades that include kinases and phosphatases as well as direct interaction with synaptic proteins (Beckman et al., 1998). The number of functional GAT-1 in cultured neurons is increased by direct tyrosine phosphorylation, an action mimicked by brain-derived neurotrophic factor (BDNF) making it a plausible candidate as the physiological trigger of the tyrosine phosphorylation signalling cascade (Law et al., 2000).

BDNF is a neurotrophic factor with functions in growth, survival and differentiation of neurons, causing long-term structural and molecular changes at synapses, which are crucial not only to development but also to synaptic function and plasticity in the adult (Vicario-Abejon et al., 2002). Most of the trophic and plastic actions of BDNF are mediated by TrkB receptors which trigger a complex signalling cascade leading to changes in transcription and in synthesis of key molecules involved in synapse formation and stabilization (Vicario-Abejon et al., 2002). BDNF also has fast actions on synapses that occur in a time scale of less than 1 h and lead to facilitation of synaptic transmission (Kang and Schuman, 1995; Diogenes et al., 2004). These relatively fast synaptic actions of BDNF may also require *de novo* protein synthesis, as it has been shown to occur in relation to the BDNF-induced increases in AMPA (Caldeira et al., 2007a) and NMDA (Caldeira et al., 2007b) receptor levels in the cytoplasmic membrane of cultured neurons. However, some synaptic BDNF actions result from a local and very fast action at synapses; indeed, this neurotrophin is able to facilitate glutamate release from synaptosomes (Sala et al., 1998; Canas et al., 2004; Pereira et al., 2006), which are isolated nerve endings and therefore lack the somatic machinery for modulation at the gene transcription level. Interestingly, BDNF inhibits carrier-mediated release of GABA from hippocampal synaptosomes (Canas et al., 2004), suggesting an inhibitory action upon GABA transporters. Since GABA transporters are physiologically designed to take up GABA, and since nerve endings are a relevant compartment to inactivate synaptic GABA, we now directly evaluated the influence of BDNF on GAT-1-mediated GABA uptake by isolated nerve endings.

2. Results

2.1. *BDNF decreases GABA uptake from rat hippocampal synaptosomes*

An initial experiment was performed to determine the Km value of GAT-1 for GABA in our experimental conditions. The concentrations of GABA ranged from 0.3 to 10 μM and the Km obtained was 5.05 μM (95% confidence intervals from 3.04 to 7.04 μM), a value similar to that already reported by others (Wood and Sidhu, 1986). The remaining experiments were therefore performed with 5 μM GABA. The uptake of GABA was almost completely blocked (less than 10% remained) by the selective GAT-1 inhibitor, SKF89976a (20 μM), an evidence that GAT-1 transporter is by far the predominant GABA transporter in the synaptosomal preparation. This also indicates that in the present experimental conditions, any contamination with astrocytic membranes, which predominantly possess GAT-3 (Schousboe et al., 2004), does not appreciably contribute to GABA transport values. In the experiments reported in this paper 20.6±2.07 pmol GABA per mg protein per second were transported in a GAT-1 dependent way (total minus residual uptake in the presence of the GAT-1 inhibitor) under control conditions (absence of any drug).

BDNF (10–200 ng/ml) caused a concentration dependent decrease in GAT-1-mediated GABA uptake, maximum effects being already observed with 100 ng/ml (Fig. 1A.a). An intermediate concentration of BDNF (30 ng/ml) was used in the remaining experiments. The effect of BDNF was very fast since a full effect could be observed after 1 min incubation and it was kept constant up to 20 min incubation (Fig. 1A.b). In the remaining experiments, a 5 min incubation period with BDNF was used.

BDNF operates the high-affinity receptor tyrosine kinase, TrkB, and the p75 receptor, which lacks catalytic activity (see e.g. Chao, 2003). The presently described inhibitory effect of BDNF can be attributed to activation of a receptor tyrosine kinase (Trk), since the tyrosine kinase inhibitor, K252a at a concentration (100 nM) that by itself was devoid of effect upon GABA uptake, prevented the inhibitory action of BDNF (30 ng/ml) upon GABA uptake (Fig. 1B).

BDNF, through tyrosine kinase B receptors, induces activation of different signalling pathways, namely extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3-K) and phospholipase Cγ (PLC-γ) (Chao, 2003; Huang and Reichardt, 2003), the last one being frequently involved in synaptic actions of BDNF (e.g. Pousinha et al., 2006). In rat hippocampal synaptosomes, BDNF induces PLC-γ activation but does not affect ERK or PI3-K/Akt signalling pathways (Pereira et al., 2006). Therefore, we only evaluated the requirement of the PLC-γ signalling pathway. The synaptosomes were previously incubated for 20 min with the PLC inhibitor, U73122, which was used at a supramaximal concentration (3 μM) (Bleasdale et al., 1990). As shown in Fig. 1C, U73122 (3 μM) fully prevented the inhibitory action of BDNF (30 ng/ml) upon GABA uptake by the synaptosomes, indicating that this effect of BDNF requires PLC activity.

2.2. *Endogenous activation of A_{2A} receptors is not required for the inhibitory action of BDNF upon GABA uptake*

We previously observed that blockade of adenosine A_{2A} receptors fully prevents the facilitatory action of BDNF upon synaptic transmission and plasticity at the hippocampus (Diogenes et al., 2004; Diogenes et al., 2007; Fontinha et al., 2008) as well as at the neuromuscular junction (Pousinha et al., 2006). Therefore, the influence of A_{2A} receptors upon the inhibitory action of BDNF on GABA transport was evaluated. The A_{2A} receptor antagonist, SCH 58261, at a concentration (50 nM) nearly 70 times its Ki value for A_{2A} receptors (Zocchi et al., 1996) and below its Ki value for other rat adenosine receptor subtypes, was incubated with the synaptosomes for 15 min before addition of BDNF (30 ng/ml) and the amount of GABA transported under these conditions was taken as 100%. As illustrated in Fig. 2, the inhibition of GABA uptake induced by BDNF (30 ng/ml) in the presence of SCH 58261 was not significantly different

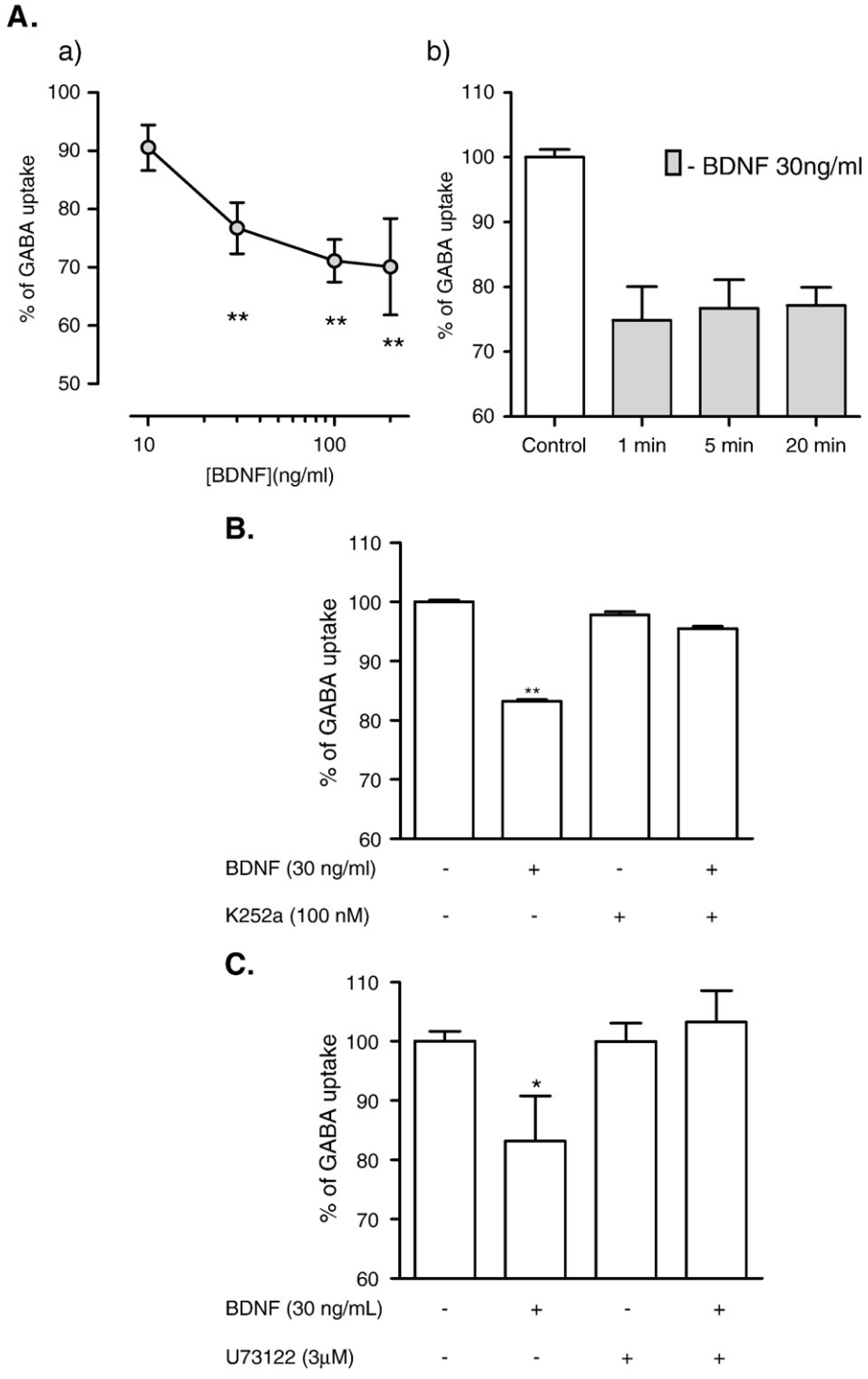

Fig. 1 – Brain-derived neurotrophic factor inhibits GABA uptake in hippocampal synaptosomes, through a TrkB and PLCγ-dependent mechanism. In panel A the concentration-response curve for the effect of BDNF ($n=6$) (a) and the time-course curve of BDNF effect (30 ng/ml, $n=4$) (b) are shown. Blockade of the effect of BDNF by the inhibitor of tyrosine kinase autophosphorylation, K252a ($n=3$) is shown in panel B, and the ability of the PLC inhibitor, U73122 ($n=5$) to prevent the effect of BDNF is illustrated in panel C. In each experiment, the effect of BDNF in the absence and in the presence of the enzyme inhibitors was always tested using the same synaptosomal batch. BDNF was incubated with the synaptosomes for 5 min, except for the time-course experiments (A.b), where the BDNF incubation times are indicated below each filled column. All other drugs were added 15 min before BDNF and their presence is indicated below each bar in B and C. The ordinates represent [^{3}H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. The results are mean ± S.E.M.; $^*p<0.05$, $^{**}p<0.01$, as compared with absence of BDNF in the same conditions.

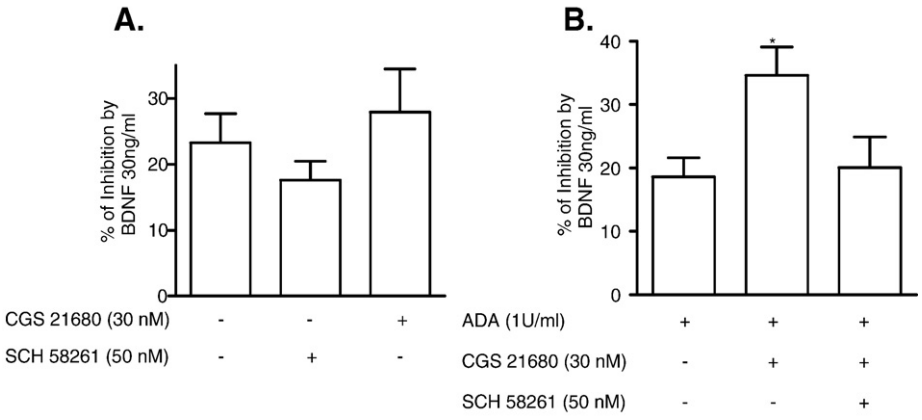

Fig. 2 – Modulation of the effect of BDNF upon synaptosomal GABA uptake by adenosine A_{2A} receptors. The A_{2A} receptor ligands were added to the synaptosomes either in the absence (A, $n=6$) or in the presence (B, $n=5$) of adenosine deaminase (ADA, 1 U/ml) to remove external endogenous adenosine. The ordinates represent the inhibition caused by BDNF (30 ng/ml), where 0% corresponds to absence of effect and 100% to a complete inhibition of GAT-1-mediated GABA transport. In each experiment, the effects of BDNF under the drug conditions indicated within each panel were always tested using the same synaptosomal batch. BDNF was incubated with the synaptosomes for 5 min. All other drugs were added 15 min before BDNF and their presence is indicated below each column. ADA (1 U/ml) was added 30 min before BDNF. The results are mean ± S.E.M.; $^*p<0.05$ as compared with the effect of BDNF in the same experiments but absence of CGS 21680 (first column).

($p>0.05$) from that observed with the same synaptosomal batch in the absence of SCH 58261. This indicates that tonic activation of adenosine A_{2A} receptors is not required to trigger a BDNF effect upon GABA uptake. A_{2A} receptor activation with a selective agonist, CGS 21680 (30 nM) (Jarvis et al., 1989) also did not significantly ($p>0.05$) influence the inhibitory effect of BDNF upon GABA transport (Fig. 2A).

By itself SCH 58261 (50 nM) inhibited GAT-1-mediated GABA uptake by 26±4.5% ($n=5$, $p<0.05$), suggesting that the adenosine released by synaptosomes is tonically activating A_{2A} receptors, to enhance GAT-1-mediated GABA transport. It is therefore possible that membrane A_{2A} receptors were already occupied with the endogenous ligand, which would occlude any further action of an exogenously added agonist, such as CGS 21680. To evaluate the influence of A_{2A} receptor activation in the absence of endogenous adenosine, synaptosomes were incubated with adenosine deaminase (ADA, 1 U/ml, added 30 min before GABA uptake assay) to inactivate external adenosine, and the effect of BDNF in the absence or presence of the A_{2A} receptor agonist was compared. As illustrated in Fig. 2B, activation of A_{2A} receptors with CGS 21680 (30 nM) in an external adenosine depleted background caused a significant ($p<0.05$) potentiation of the inhibitory effect of BDNF (30 ng/ml) upon of GAT-1-mediated GABA transport. This potentiation of the BDNF action by the adenosine A_{2A} receptor agonist was prevented by the selective A_{2A} receptor antagonist, SCH 58261 (50 nM), since in the simultaneous presence of the A_{2A} receptor agonist and antagonist BDNF (30 ng/ml) inhibited GABA uptake by a similar magnitude as in the absence of any A_{2A} receptor ligand (Fig. 2B).

3. Discussion

The main findings in the present study are that BDNF, through TrkB and PLCγ signalling inhibits GAT-1-mediated GABA transport by nerve endings, and that this action of BDNF is not dependent on, but can be enhanced by, cross talk with adenosine A_{2A} receptors.

The present results contrast with a previous report that BDNF enhances GAT-1-mediated GABA transport in serum deprived cultured neurons (Law et al., 2000). This discrepancy cannot be attributed to changes in the concentration of BDNF used since Law et al. (2000) used a BDNF concentration (100 ng/ml) also used in the present work. In isolated nerve endings (present work) as in neuronal cultures (Law et al., 2000) the effect of BDNF might result from TrkB receptor autophosphorylation since it is prevented by a tyrosine kinase inhibitor, K252a. The difference may reside in the use of intact cells (Law et al, 2000) versus a subcellular fraction specialized in synaptic signalling; the isolated nerve endings (present work). Indeed, the intact cells may allow BDNF-induced changes in gene expression and protein synthesis, whereas at the nerve endings most of the machinery for protein synthesis is lacking. The time course of the BDNF actions at the nerve endings (present work) and in intact neurons (Law et al., 2000) may also be different. The inhibition of GABA transport at the nerve endings is very fast, being already seen after 1 min incubation with BDNF. Law et al. (2000) used a 30 min incubation time, which in a whole neuron may be enough to allow BDNF-induced changes in protein synthesis (see Caldeira et al., 2007a,b for BDNF-induced changes in receptor expression). Interestingly, the two opposite actions of BDNF upon GAT-1, potentiation of GABA uptake by hippocampal neurons (Law et al., 2000) and fast inhibition of GABA uptake demonstrated in this paper, may have a coherent physiological goal. Thus, at a nerve ending, an inhibition of GABA transport may lead to an increase in the amounts of synaptic GABA, therefore to an increase in GABAergic signalling. On the other hand, an increase in GABA transport in other neuronal membrane

compartments may rescue GABA to replenish the releasable pool.

It is also of interest that the BDNF-induced inhibition of GABA transport at nerve endings appears to be similar when transport is either in the inward direction (present work) or is reversed, releasing GABA (Canas et al., 2004). This suggests a common mechanism used by BDNF to inhibit GAT-1 at nerve endings, independently of the concentration gradient of GABA across the cell membrane.

The molecular mechanisms that underlie the different ways BDNF uses to differentially modulate GAT-1 at nerve endings or entire neurons require further studies, but the evidence so far available points towards the involvement of different signalling cascades (Osawa et al., 1994; Law et al., 2000). Indeed, TrkB receptor signalling includes the activation of the Akt pathway, the activation of MAP kinases and the activation of PLCγ (e.g. Chao, 2003). TrkB receptor activation by BDNF in hippocampal nerve endings enhances phospholipase Cγ (PLC-γ) activity, leaving the extracellular signal-regulated kinase (ERK) and Akt phosphorylation pathways unaffected (Pereira et al., 2006). We observed that the inhibitor of PLC, U73122, prevented the effect of BDNF upon GABA transport in isolated nerve endings, but the effect of BDNF on GABA transport in the cultured neurons was not affected by PKC inhibition (Law et al., 2000). Activation of PLC leads to diacylglycerol formation and subsequent PKC activation, which is known to decrease GABA transport (Osawa et al., 1994) due to phosphorylation of GAT-1 serine residues (Quick et al., 2004). It is therefore not surprising that BDNF, through PLCγ-mediated signalling, decreases GAT-1-mediated GABA transport. However, through promotion of phosphorylation of tyrosine residues, BDNF may enhance GABA transport (Law et al., 2000). To reconcile these two observations Quick et al. (2004) proposed that the relative abundance of the two mutually exclusive phosphorylation states of GAT-1, one phosphorylated in serine residues and another phosphorylated in tyrosine residues, determines the relative subcellular distribution of the transporter. However, it is hard to anticipate how the action of BDNF, which induces phosphorylation of tyrosine and serine residues, will ultimately influence GAT-1. The present data showing that BDNF decreases GAT-1-mediated GABA transport at the nerve endings in a PLC-dependent way, together with the previous report that BDNF increases GAT-1-mediated transport in cultured neurons in a PLC independent way (Law et al., 2000) suggest that the subcellular distribution of the transducing systems operated by TrkB receptors may decide the fate of BDNF action upon GABA transport.

The effect of BDNF on synaptosomal GABA uptake was not appreciably affected by A_{2A} receptor blockade or removal of endogenous adenosine with adenosine deaminase, suggesting that adenosine A_{2A} receptor co-activation is not an essential step for this action of BDNF. This contrasts with what has been observed by us in what concerns the facilitatory action of BDNF on excitatory synaptic transmission (Diogenes et al., 2004; Pousinha et al., 2006) where A_{2A} receptor blockade fully prevents the action of BDNF. However, A_{2A} receptors, in spite of not being essential, may influence the action of BDNF upon GAT-1 at nerve endings since activation of adenosine A_{2A} receptors when they were not already occupied by the endogenous ligand, enhanced the BDNF-induced inhibition of GABA transport, an enhancement that was antagonized by the A_{2A} receptor antagonist. On the light of these results, one could expect that when A_{2A} receptors were occupied by the endogenous ligand, i.e., in the absence of adenosine deaminase, the A_{2A} antagonist *per se* would induce at least a slight attenuation of the effect of BDNF. The lack of appreciable effect of A_{2A} receptor blockade under these conditions may suggest partial A_{2A} receptor desensitization by endogenous adenosine that adds to a low efficacy of A_{2A} receptors to modulate the influence of BDNF upon GABA transport.

BDNF has been shown to quickly modulate GABAergic transmission in the hippocampus through pre- and postsynaptic mechanisms. Postsynaptically, BDNF decreases GABAergic transmission to pyramidal neurones (Tanaka et al., 1997), inducing a rapid downregulation of $GABA_A$ receptor surface expression (Brunig et al., 2001). Presynaptically, a decrease in GABAergic input to glutamatergic neurones has been reported (Frerking et al., 1998). A detailed analysis of the action of BDNF at different types of GABAergic synapses revealed that they are synapse specific (Wardle and Poo, 2003) but the trend is towards an inhibition of GABAergic transmission. The present results showing that BDNF inhibits GABA uptake by nerve endings add a new role of BDNF at synapses, which may lead to an increase in the lifespan of GABA at GABAergic synapses, counteracting the inhibition of GABAergic transmission caused by the neurotrophin. Interestingly, in immature neurons BDNF enhances, rather than inhibits, GABA release, and this is part of a positive feedback loop between GABA and BDNF expression (Obrietan et al., 2002). Since GAT-1-mediated transport in nerve terminals appears to contribute to the maturation of point-to-point GABAergic synapses (see Conti et al., 2004), a fine control of GABAergic transmission that simultaneously involves BDNF and GAT-1 may be particularly relevant in the shaping GABAergic synapses under maturation. Further studies are, therefore, required to evaluate how BDNF influences GAT-1 during development.

4. Experimental procedures

4.1. *Drugs used*

4-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA, specific activity 92.0 Ci/mmol) was from Amersham (Buckinghamshire, UK) and 4-amino-n-butyric acid (GABA) was from Sigma (St. Louis, USA). Stock solutions of BDNF (kindly supplied by Regeneron Pharmaceuticals, Tarrytown, New York) were in phosphate-buffer saline (PBS) at a final concentration of 1 mg/ml. 2-[*p*-(2-carboxethyl)phenethylamino]-5¢-*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 (AOOA) were from Sigma (St.Louis, USA). K252a was from Calbiochem (Darmstadt, Germany). 1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF 89976a) and 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U 73122) were from Tocris (Avonmouth, UK). Percoll, sucrose, EDTA, KCl, $MgSO_4.7H_2O$, NaCl and $NaH_2PO_4.H_2O$ were from Sigma, HEPES, BSA (initial fraction by cold alcohol

precipitation 97–99%), $NaH_2PO_4.H_2O$, NaOH and HCl were from Merck, $CaCl_2$ and HCl were from BDH and glucose was from Fluka.

CGS 21680, ZM 241385 and U 73122 were made up into a 5 mM stock solution in dimethylsulfoxide (DMSO). SCH 58261 was made up into a 10 mM stock solution in DMSO. K252a was made up to a 1 mM stock solution in DMSO. SKF 89976a was made up into a 10 mM aqueous stock solution. Aminooxyacetic acid (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. DMSO applied to the synaptosomes ranged from 0.0006% v/v to 0.06%; the highest DMSO concentration was virtually devoid of effect upon GABA uptake.

4.2. *Nerve terminal preparation*

The synaptosomal fraction from Wistar rat hippocampus (4 animals for experiment, 3–4 weeks old) was prepared as routinely in our laboratory (Pereira et al., 2006), according to the European guidelines (86/609/EEC). Briefly, the animals were anaesthetized with halothane before decapitation, the brain was quickly removed, the hippocampi were dissected out and added to 5 ml of a chilled sucrose solution (sucrose 0.32 M, EDTA 1 mM, HEPES 10 mM, 1 mg/ml BSA, pH 7.40); after homogenization at 4 °C, the volume was completed to 15 ml with ice-cold sucrose solution. After a first centrifugation at 3000 ×*g* for 10 min (Heraeus sepatech – Biofuge 28RS centrifuge, refrigerated at 4 °C), the supernatant was collected, centrifuged at 14,000 ×*g* for 12 min and the pellet was resuspended in 3 ml Percoll solution, which contained Percoll 45% (v/v) in KHR solution (NaCl 140 mM, EDTA 1 mM, HEPES 10 mM, KCl 5 mM e glucose 5 mM, pH 7.40), adjusted to pH 7.4 with NaOH. The mixture was centrifuged again at 14,000 ×*g* for 2 min and the top layer, which corresponds to the synaptosomal fraction, was removed, washed with 2 ml KHR solution and centrifuged again at 14,000 ×*g* for 2 min.

The synaptosomal fraction was resuspended in 1 ml of chilled Krebs-HEPES solution (glucose 10 mM, NaCl 125 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1 mM, $CaCl_2$ 1.5 mM, A.O.A.A. 0.1 mM and HEPES 10 mM, pH 7.40) and kept at 4 °C until use. The synaptosomal protein concentration was assayed according to the Bradford (1976) method using bovine serum albumin as standard.

4.3. *[^{3}H]GABA uptake*

The protocol for [^{3}H]GABA uptake was adapted from Santos et al. (1990). Briefly, the synaptosomal suspension (0.5 mg protein ml^{-1}) in Krebs-HEPES solution was preincubated at 37 °C for 20–35 min in a total volume of 300 μl, in the presence or absence of testing drugs, and the transport was initiated by addition of 5 μM [^{3}H]GABA (specific activity 0.133 Ci/mmol), unless otherwise specified. Transport was terminated after 40 s by the addition of 5 ml ice-cold Krebs-HEPES solution followed by low-pressure filtration through 1.2 μm filters (Millipore, Glass Fibre Prefilters) and a second wash with 10 ml of the same solution. The 40 second incubation time was chosen since we observed a linear correlation between the uptake of GABA vs. incubation times ranging from 0 to 80 s (correlation coefficient = 0.95). The filters were analysed by liquid scintillation counting for determination of tritium retained by synaptosomes after addition of 5 ml of scintillation cocktail (OptiPhase "HiSafe" 2, Perkin-Elmer, Foster City, CA, USA). GAT-1-mediated GABA uptake was calculated as the difference between the total radioactivity retained in the filters and the non GAT-1-mediated component of [^{3}H]GABA uptake, which was determined by preincubation with SKF89976a (20 μM), an inhibitor of GAT-1 transporter (Borden et al., 1994).

BDNF was added to the synaptosomes 5 min before addition of [^{3}H]GABA and the effect of BDNF was calculated taking as 100% the uptake of GABA in the absence of BDNF in the same experiments and under the same experimental conditions. Whenever the influence of any drug over the effect of BDNF was tested, that drug was incubated with the synaptosomes for at least 15 min before addition of BDNF; the effect of BDNF in the presence of these drugs was calculated taking as 100% the uptake of GABA in the absence of BDNF but in the presence of the same drugs. Whenever removal of endogenous adenosine was required, adenosine deaminase (ADA, 1 U/ml) was added 30 min before BDNF.

4.4. *Statistical analyses*

The values presented are mean ± S.E.M. of *n* experiments. To evaluate multiple comparisons, statistical significance was assessed by one-way ANOVA followed by Bonferroni correction using GraphPad Software (Prism, version 4.02 for Windows). Values of $p < 0.05$ were considered statistically significant.

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REFERENCES

Allen, N.J., Karadottir, R., Attwell, D., 2004. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. Pflugers Arch. 449, 132–142.

Beckman, M.L., Bernstein, E.M., Quick, M.W., 1998. Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. J. Neurosci. 18, 6103–6112.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., Bunting, S., 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J. Pharmacol. Exp. Ther. 255, 756–768.

Borden, L.A., Murali Dhar, T.G., Smith, K.E., Weinshank, R.L., Branchek, T.A., Gluchowski, C., 1994. Tiagabine, SK&F 89976-A, CI-966, and NNC-711 are selective for the cloned GABA transporter GAT-1. Eur. J. Pharmacol. 269, 219–224.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brunig, I., Penschuck, S., Berninger, B., Benson, J., Fritschy, J.M., 2001. BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of GABA(A) receptor surface expression. Eur. J. Neurosci. 13, 1320–1328.

Caldeira, M.V., Melo, C.V., Pereira, D.B., Carvalho, R., Correia, S.S., Backos, D.S., Carvalho, A.L., Esteban, J.A., Duarte, C.B., 2007a. Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. J. Biol. Chem. 282, 12619–12628.

Caldeira, M.V., Melo, C.V., Pereira, D.B., Carvalho, R.F., Carvalho, A.L., Duarte, C.B., 2007b. BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol. Cell. Neurosci. 35, 208–219.

Canas, N., Pereira, I.T., Ribeiro, J.A., Sebastiao, A.M., 2004. Brain-derived neurotrophic factor facilitates glutamate and inhibits GABA release from hippocampal synaptosomes through different mechanisms. Brain Res. 1016, 72–78.

Chao, M.V., 2003. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. 4, 299–309.

Conti, F., Minelli, A., Melone, M., 2004. GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. Brain Res. Brain Res. Rev. 45, 196–212.

Diogenes, M.J., Fernandes, C.C., Sebastiao, A.M., Ribeiro, J.A., 2004. Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. J. Neurosci. 24, 2905–2913.

Diogenes, M.J., Assaife-Lopes, N., Pinto-Duarte, A., Ribeiro, J.A., Sebastiao, A.M., 2007. Influence of age on BDNF modulation of hippocampal synaptic transmission: interplay with adenosine A2A receptors. Hippocampus 17, 577–585.

Fontinha, B.M., Diogenes, M.J., Ribeiro, J.A., Sebastiao, A.M., 2008. Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A(2A) receptor activation by endogenous adenosine. Neuropharmacology 54, 924–933.

Frerking, M., Malenka, R.C., Nicoll, R.A., 1998. Brain-derived neurotrophic factor (BDNF) modulates inhibitory, but not excitatory, transmission in the CA1 region of the hippocampus. J. Neurophysiol. 80, 3383–3386.

Gether, U., Andersen, P.H., Larsson, O.M., Schousboe, A., 2006. Neurotransmitter transporters: molecular function of important drug targets. Trends Pharmacol. Sci. 27, 375–383.

Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642.

Jarvis, M.F., Schulz, R., Hutchison, A.J., Do, U.H., Sills, M.A., Williams, M., 1989. [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. J. Pharmacol. Exp. Ther. 251, 888–893.

Kang, H., Schuman, E.M., 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267, 1658–1662.

Law, R.M., Stafford, A., Quick, M.W., 2000. Functional regulation of gamma-aminobutyric acid transporters by direct tyrosine phosphorylation. J. Biol. Chem. 275, 23986–23991.

Obrietan, K., Gao, X.B., Van Den Pol, A.N., 2002. Excitatory actions of GABA increase BDNF expression via a MAPK-CREB-dependent mechanism — a positive feedback circuit in developing neurons. J. Neurophysiol. 88, 1005–1015.

Osawa, I., Saito, N., Koga, T., Tanaka, C., 1994. Phorbol ester-induced inhibition of GABA uptake by synaptosomes and by *Xenopus* oocytes expressing GABA transporter (GAT1). Neurosci. Res. 19, 287–293.

Pereira, D.B., Rebola, N., Rodrigues, R.J., Cunha, R.A., Carvalho, A.P., Duarte, C.B., 2006. Trkb receptors modulation of glutamate release is limited to a subset of nerve terminals in the adult rat hippocampus. J. Neurosci. Res. 83, 832–844.

Pousinha, P.A., Diogenes, M.J., Ribeiro, J.A., Sebastiao, A.M., 2006. Triggering of BDNF facilitatory action on neuromuscular transmission by adenosine A2A receptors. Neurosci. Lett. 404, 143–147.

Quick, M.W., Hu, J., Wang, D., Zhang, H.Y., 2004. Regulation of a gamma-aminobutyric acid transporter by reciprocal tyrosine and serine phosphorylation. J. Biol. Chem. 279, 15961–15967.

Sala, R., Viegi, A., Rossi, F.M., Pizzorusso, T., Bonanno, G., Raiteri, M., Maffei, L., 1998. Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex. Eur. J. Neurosci. 10, 2185–2191.

Santos, M.S., Goncalves, P.P., Carvalho, A.P., 1990. Effect of ouabain on the gamma-[3H]aminobutyric acid uptake and release in the absence of Ca(+)+ and K(+)-depolarization. J. Pharmacol. Exp. Ther. 253, 620–627.

Schousboe, A., Sarup, A., Bak, L.K., Waagepetersen, H.S., Larsson, O.M., 2004. Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. Neurochem. Int. 45, 521–527.

Tanaka, T., Saito, H., Matsuki, N., 1997. Inhibition of GABAA synaptic responses by brain-derived neurotrophic factor (BDNF) in rat hippocampus. J. Neurosci. 17, 2959–2966.

Vicario-Abejon, C., Owens, D., McKay, R., Segal, M., 2002. Role of neurotrophins in central synapse formation and stabilization. Nat. Rev. Neurosci. 3, 965–974.

Wardle, R.A., Poo, M.M., 2003. Brain-derived neurotrophic factor modulation of GABAergic synapses by postsynaptic regulation of chloride transport. J. Neurosci. 23, 8722–8732.

Wood, J.D., Sidhu, H.S., 1986. Uptake of gamma-aminobutyric acid by brain tissue preparations: a reevaluation. J. Neurochem. 46, 739–744.

Zocchi, C., Ongini, E., Ferrara, S., Baraldi, P.G., Dionisotti, S., 1996. Binding of the radioligand [3H]-SCH 58261, a new non-xanthine A2A adenosine receptor antagonist, to rat striatal membranes. Br. J. Pharmacol. 117, 1381–1386.