Cellular/Molecular

Activation of Adenosine A_{2A} Receptors Induces TrkB Translocation and Increases BDNF-Mediated Phospho-TrkB Localization in Lipid Rafts: Implications for Neuromodulation

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Brain-derived neurotrophic factor (BDNF) signaling is critical for neuronal development and transmission. Recruitment of TrkB receptors to lipid rafts has been shown to be necessary for the activation of specific signaling pathways and modulation of neurotransmitter release by BDNF. Since TrkB receptors are known to be modulated by adenosine A_{2A} receptor activation, we hypothesized that activation of A_{2A} receptors could influence TrkB receptor localization among different membrane microdomains. We found that adenosine A_{2A} receptor agonists increased the levels of TrkB receptors in the lipid raft fraction of cortical membranes and potentiated BDNF-induced augmentation of phosphorylated TrkB levels in lipid rafts. Blockade of the clathrin-mediated endocytosis with monodansylcadaverine (100 μM) did not modify the effects of the A_{2A} receptor agonists but significantly impaired BDNF effects on TrkB recruitment to lipid rafts. The effect of A_{2A} receptor activation in TrkB localization was mimicked by 5 μM forskolin, an adenylyl cyclase activator. Also, it was blocked by the PKA inhibitors Rp-cAMPs and PKI-(14–22), and by the Src-family kinase inhibitor PP2. Moreover, removal of endogenous adenosine or disruption of lipid rafts reduced BDNF stimulatory effects on glutamate release from cortical synaptosomes. Lipid raft integrity was also required for the effects of BDNF on hippocampal long-term potentiation at CA1 synapses. Our data demonstrate, for the first time, a BDNF-independent recruitment of TrkB receptors to lipid rafts induced by activation of adenosine A_{2A} receptors, with functional consequences for TrkB phosphorylation and BDNF-induced modulation of neurotransmitter release and hippocampal plasticity.

Introduction

The neurotrophin brain-derived neurotrophic factor (BDNF) is essential in the regulation of neuronal survival and differentiation. Abundant evidence has now established that BDNF is also involved in the modulation of synaptic transmission and plasticity (Poo, 2001; Chao, 2003). BDNF activates the TrkB tyrosine kinase receptor and the p75 receptor, which belongs to the tumor necrosis factor receptor family. We and others have reported that TrkB receptor function is modulated by activation of adenosine A_{2A} receptors (Lee and Chao, 2001; Diógenes et al., 2004; Mojsilovic-Petrovic et al., 2006; Fontinha et al., 2008; Tebano et al., 2008). This TrkB/A_{2A} receptor cross talk has two consequences, which may operate independently: (1) facilitation of BDNF-induced actions on synaptic transmission and plasticity by A_{2A} receptor agonists; and (2) direct phosphorylation and activation of TrkB receptors, in the absence of BDNF, a process called transactivation. Notably, transactivation of TrkB receptors usually requires longer exposure to A_{2A} agonists than facilitation of synaptic actions of BDNF.

Adenosine is an important modulator of the nervous system that acts via activation of G-protein-coupled receptors A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2003; Sebastiao and Ribeiro, 2009a). Adenosine receptors are distributed widely in the nervous system, where the high-affinity A_1 and A_{2A} receptors are responsible for the fine tuning of neurotransmitter release and modulation of other signaling molecules (Sebastiao and Ribeiro, 2009a).

Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains that concentrate specific proteins and lipids. Although protein affinity for these domains is not totally under-

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stood, it is known that palmitoylated, myristoylated, and glycosylphosphatidylinositol-anchored proteins are enriched in these domains (Simons and Toomre, 2000; Pike, 2003). Lipid rafts have been implicated in the regulation of signal transduction in multiple cell types, including neurons, by promoting close proximity or segregation of signaling molecules (Fielding and Fielding, 2004; Lim and Yin, 2005). There is now increasing evidence that lipid rafts are essential for BDNF signaling, and both TrkB and p75 receptors can be localized in these domains (Wu et al., 1997; Higuchi et al., 2003; Suzuki et al., 2004). Translocation of TrkB receptors to lipid rafts is regulated by BDNF, and is required for its effects on glutamate release and synaptic fatigue (Suzuki et al., 2004), and for activation of the phospholipase C (PLC) pathway (Pereira and Chao, 2007).

In this work, we investigated whether adenosine A_{2A} receptor activation affects TrkB receptor localization in lipid rafts and how BDNF actions on glutamate release and long-term potentiation (LTP) are affected by removal of endogenous adenosine and disruption of lipid rafts. We show that A_{2A} receptor activation induced TrkB translocation and increased BDNF-induced phospho-TrkB (pTrkB) receptors in lipid rafts. Moreover, our results suggest that the mechanisms used by A_{2A} receptor agonists to induce TrkB translocation are different from those used by BDNF, and involve cAMP and Src-family kinase activation. Finally, lipid raft disruption abolished the potentiating effects of BDNF on glutamate release and LTP.

Materials and Methods

Materials. Cell culture media, Alexa Fluor 488-coupled goat anti-rabbit antibody and Alexa Fluor 594-coupled cholera toxin subunit B were obtained from Invitrogen. BDNF was a kind gift of Regeneron Pharmaceuticals. 4-[2-[[6-Amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]-benzene propanoic acid hydrochloride (CGS 21680), 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385), forskolin, 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP2), protein kinase inhibitor-(14–22)-amide, myristoylated (PKI 14–22), and (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) were from Tocris Cookson. Adenosine deaminase (ADA; EC 3.5.4.4) was from Roche. Mouse anti-TrkB antibody was from BD Biosciences. Anti-phospho-Trk (pTyr-490) was from Cell Signaling Technology. Rabbit anti-TrkB antibody was from Millipore. The antibodies for Fyn and HRP-coupled anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology. [^{3}H]ZM 241385 and ECL plus reagent were obtained from GE Healthcare. Ultra Clear centrifuge tubes were from Beckman. Bradford reagent was from Bio-Rad. All other reagents were purchased from Sigma.

Neuronal cortical cultures. Cortical neurons were dissected from E18 Sprague Dawley embryos, obtained from Harlan Interfauna Iberica, as described previously (Pereira and Chao, 2007). Animals were handled according to the European Community guidelines and Portuguese law on animal care. Dissection was performed in cold HBSS medium supplemented with 0.37% glucose under sterile conditions. The cortices were trypsinized for 15 min, centrifuged, and resuspended in MEM supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 0.37% glucose, and 25 U/ml penicillin/streptomycin. Cells were dissociated, counted, and plated in poly-L-lysine-coated dishes at a density of 10^5 cells/cm^2. On the following day, medium was changed to Neurobasal medium supplemented with 0.5 mM glutamine, 2% B27, 25 U/ml penicillin/streptomycin, and 25 μM glutamate. On day *in vitro* (DIV) 4, half the medium was replaced by the above-mentioned solution (excepting glutamate) with 5-fluoro-2-deoxyuridine. On the day of the experiment, cells were starved for 4 h in MEM containing 0.37% glucose, 2 mM glutamine, and 10 μM MK-801. Twenty nanomolar CGS21680 and 50 nM ZM241385 were added for 30 min, followed by addition of 20 ng/ml BDNF for 5 or 40 min, as indicated. Inhibitors were added 15 min before CGS 21680 and/or BDNF incubation and remained present until cell lysis. Methyl-β-cyclodextrin (MβCD) was the only exception, being present only 15 min before incubation with CGS 21680.

Lipid raft isolation. Lipid rafts were isolated as described previously (Pereira and Chao, 2007). Briefly, cortical neurons (DIV 7–11) were lysed in TNE buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA) containing 0.5% Triton X-100, and supplemented with protease and phosphatase inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). After solubilization for at least 20 min at 4°C, the lysates were combined with a 60% Optiprep solution to yield a 35% Optiprep mixture. This solution was placed in the bottom of the ultracentrifuge tube and overlaid with 8 ml of a 30% Optiprep solution in lysis buffer followed by 3 ml of lysis buffer. Samples were centrifuged for 6 h at 36,400 rpm in a Beckman XL-90 ultracentrifuge, using an SW41Ti rotor, at 4°C. After discarding the first 1 ml, eight fractions (from top to bottom) were collected and equal volumes of each fraction were applied in an 8% SDS-PAGE gel.

Immunocytochemistry. The protocol used has been previously described by Harder et al. (1998), with minor modifications. DIV 6–7 cortical neurons were starved for 4 h before CGS 21680 (20 nM) treatment for 30 min. Cortical neurons were then incubated with a rabbit anti-TrkB antibody (1:500) and Alexa Fluor 594-coupled cholera toxin subunit B (2 μg/ml) for 1 h at 12°C in MEM with 50 mM HEPES and 2 mg/ml BSA, pH 7.3. Cells were washed and incubated with the Alexa Fluor 488-coupled anti-rabbit antibody for 45 min (1:300) under the same conditions. Fixation was done with 4% paraformaldehyde for 5 min followed by cold methanol for 5 min. Images were taken using a Zeiss LSM 510 confocal microscope and analyzed with the help of the ImageJ software. Copatching was quantified as the percentage of TrkB receptor clusters colocalized with GM1 patches. In each experiment, 10 cells per condition were analyzed.

Isolation of synaptosomes. The 3- to 5-week-old Wistar rats were decapitated under halothane anesthesia, and synaptosomes were prepared as described previously (Canas et al., 2004). Briefly, the cortices were dissected in an ice-cold Krebs' solution composed of (in mM) NaCl 124, KCl 3, NaH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1, $CaCl_2$ 2, and glucose 10, that had been previously gassed with 95% O_2 and 5% CO_2, pH 7.4. The cortices were homogenized in an ice-cold isosmotic sucrose solution (0.32 M, containing 1 mM EDTA, 1 mg/ml bovine serum albumin, and 10 mM HEPES, pH 7.4), and centrifuged at 3,000 $\times$ *g* for 10 min; the supernatant was centrifuged again at 14,000 $\times$ *g* for 12 min. The whole procedure was conducted at 4°C. 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 14,000 rpm 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; synaptosomes were then kept on ice and used within 3 h.

Glutamate release from synaptosomes. Synaptosomes were resuspended in 2 ml of oxygenated Krebs' medium 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 95% O_2 and 5% CO_2. Synaptosomes were loaded with 0.2 μM [^{3}H] 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 μl). In the cholesterol oxidase experiments, 2 U/ml enzyme were incubated with the synaptosomes for 1 h at 37°C in oxygenated Krebs' solution before glutamate incubation. After a 20 min washout period, samples were continuously collected for 40 min in 2 min intervals. A high-K^+ solution (15 mM, isomolar substitution of Na^+ by K^+ in the Krebs' solution) was added for 2 min in the 5th [first stimulation period (S_1)] and 29th [second stimulation period (S_2)] minutes to stimulate glutamate release. BDNF (20 ng/ml) was added from the ninth minute onward, and its effect was quantified as percentage changes of the S_2/S_1 ratio compared with the S_2/S_1 ratio in the absence of BDNF in the same synaptosomal batch and under similar drug conditions. The S_2/S_1 ratio was calculated as the area under the curve corresponding to the amount of tritium released due to S_2 over the amount of tritium released

due to S_1, after subtraction of basal release (averaged tritium content of the 2 samples before stimulation and 2 samples after stimulation on returning to basal levels). To evaluate the influence of a drug on the effect of BDNF, that drug was added 10 min after starting the washout period and remained present until the end of experiments, being therefore present during S_1 and S_2. In the case of exogenously added cholesterol, MβCD–cholesterol complexes were perfused only during the first 10 min of the washout period. None of the drugs affected the S_2/S_1 ratio, when compared with the S_2/S_1 ratio in the absence of any drug.

Acutely prepared hippocampal slices. The 3–5-week-old rats were decapitated under halothane anesthesia. Hippocampal dissection was performed in ice-cold Krebs' solution, previously gassed with 95% O_2 and 5% CO_2, as described above. Four hundred-micrometer-thick slices were cut perpendicularly to the long axis of hippocampus with a McIlwain tissue chopper, and allowed to recover functionally and energetically for at least 1 h in a resting chamber, filled with oxygenated Krebs' solution, at room temperature.

High-frequency stimulation of acutely prepared slices. Groups of four hippocampal slices were placed in 100 μl chambers and continuously perfused (0.5 ml/min) with oxygenated Krebs' solution at 30°C. After 1 h, slices were field stimulated using a high-frequency stimulation protocol. Trains of 100 Hz were applied for 50 ms, every 2 s, for 1 min (150 pulses). Thirty minutes after stimulation, slices were homogenized in detergent-free TNE buffer containing protease and phosphatase inhibitors (as above). Triton X-100 (final concentration 0.5%) was added to the homogenate, and after 1 h incubation at 4°C, lipid rafts were isolated in discontinuous Optiprep gradients, as described above. When 1 U/ml ADA was used, it was added to the perfusion solution 30 min before the high-frequency stimulation and remained present up to the end of the experiment. Using the same stimulation and perfusion conditions, it has been previously shown that considerable amounts of ATP and adenosine were released and detected in the bath after stimulation (Cunha et al., 1996a).

LTP. After a 1 h recovery period, as described above, one hippocampal slice was transferred to a recording chamber for submerged slices (1 ml plus 5 ml dead volume), where it was continuously superfused at a flow rate of 1.5 ml/min with Krebs' solution at 32°C; all drugs were added to this superfusion solution. Perfusion tubes were coated with 0.1 mg/ml BSA before experiments to avoid adsorption of BDNF to the tubes. Evoked field EPSPs (fEPSPs) were recorded extracellularly through a microelectrode filled with 4 M NaCl (2–4 MΩ resistance) placed in the stratum radiatum of the CA1 area. Two independent pathways of Schaffer collateral/commissural fibers were stimulated (rectangular pulses of 0.1 ms duration) alternately once every 20 s by two bipolar concentric wire electrodes placed on the Schaffer fibers in the stratum radiatum, in the CA1 area (see Fig. 10*A*). The initial intensity of the stimulus (80–150 mA) was adjusted to obtain a submaximal fEPSP slope with a minimum population spike contamination, of similar magnitude in both pathways. The averages of eight consecutive fEPSPs from each pathway were obtained and quantified as the slope of the initial phase of the potential. Recordings were obtained with an Axoclamp 2B amplifier (Molecular Devices), digitized, and continuously stored on a personal computer with the LTP program (Anderson and Collingridge, 2001). The independence of the two pathways was tested by evaluating paired-pulse facilitation (50 ms interval) across both pathways, <10% facilitation usually being observed. When a higher facilitation was observed, the slice was discarded. LTP was induced after obtaining a stable recording of fEPSP slope in the two pathways for at least 30 min, by theta burst stimulation, consisting of one train of 15 bursts (200 ms interburst interval), with four pulses (100 Hz) each (Fontinha et al., 2008). LTP was quantified as the percentage change in the average slope of the fEPSP taken from 50 to 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min that have preceded the induction of LTP. One hour after LTP induction in one of the pathways, BDNF (20 ng/ml) was added to the superfusion solution and was delivered continuously to the slices. LTP was induced in the second pathway no less than 30 min after BDNF perfusion, based on the stability of fEPSP slope values. The effect of BDNF on LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), and with the

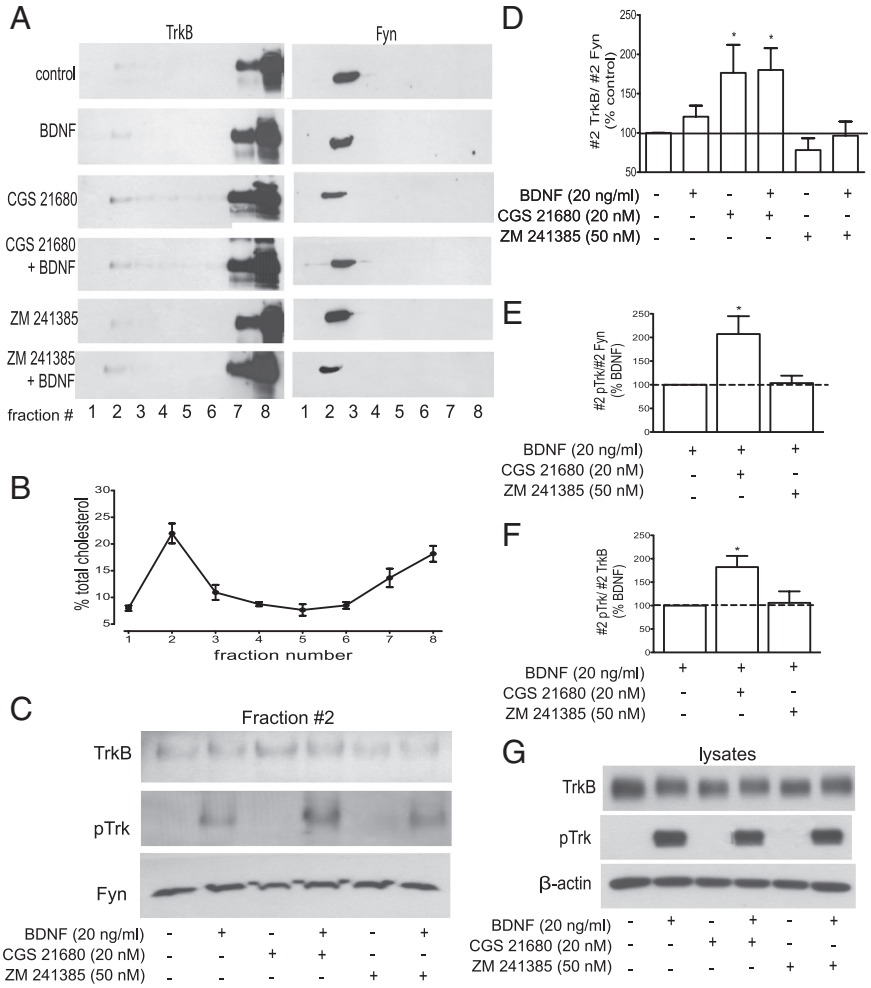


Figure 1. Activation of adenosine receptors enhances the levels of TrkB full length and potentiates BDNF-induced pTrkB localization in lipid rafts. On DIV 8–11, cortical neurons were starved for 4 h and incubated with/without 20 nM CGS 21680 or 50 nM ZM 241385 for 30 min before 5 min incubation with 20 ng/ml BDNF, as indicated. Lysates were prepared in 0.5% Triton X-100-containing buffer and fractioned in a discontinuous Optiprep gradient, as described in Materials and Methods. ***A***, Equal volumes of each gradient fraction were probed for TrkB (1:200) and Fyn (1:400). Note that Fyn, a lipid raft marker, was only detected in fraction 2, which therefore was considered the lipid raft-containing fraction. ***B***, Quantification of cholesterol content in each gradient fraction. Fraction 2 was highly enriched in cholesterol, containing ~22% of total cholesterol. ***C***, Representative Western blot analysis of the lipid raft fraction (fraction 2) obtained from the Optiprep density gradients. Antibodies used were TrkB (1:200), pTrk (pY490, 1:750), and Fyn (1:400). ***D***, Quantitative analysis of TrkB staining in fraction 2, normalized by Fyn staining in this fraction; 100% represents staining in the absence of any drug. ***E***, ***F***, Quantification analysis of BDNF-induced changes in fraction 2 pTrk, normalized by fraction 2 Fyn (***E***) or by total TrkB (***F***) in cells preincubated with CGS 21680 or ZM 241385, as indicated below each bar. One hundred percent correspond to the staining obtained in the presence of BDNF alone. ***G***, Analysis of TrkB and pTrk staining in total lysates of cells treated with/without CGS 21680, ZM 241385, or 20 ng/ml BDNF, as indicated. Results are expressed as mean ± SEM of six independent experiments. *$p < 0.05$, compared with 100%.

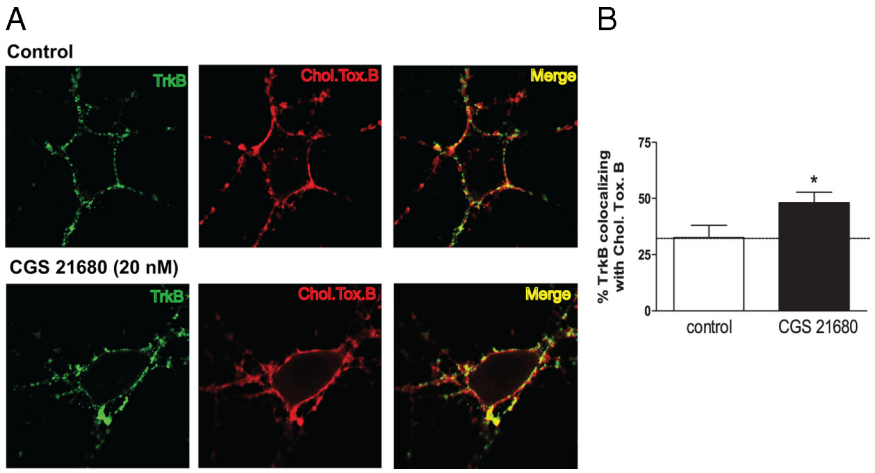


Figure 2. CGS 21680 treatment increases copatching between TrkB receptors and cholera toxin subunit B (Chol. Tox. B). ***A***, Alexa Fluor 594-coupled cholera toxin subunit B (2 μg/ml) and an anti-TrkB receptor antibody (1:500) raised against the extracellular domain of TrkB receptors were used in the copatching experiments of cultured cortical neurons. TrkB receptor patches were labeled with an Alexa Fluor 488-coupled goat anti-rabbit antibody (1:300). ***B***, Quantification of the percentage of TrkB receptors copatched with cholera toxin subunit B. Results are expressed as mean ± SEM of five independent experiments. *$p < 0.05$, compared with control.

magnitude of LTP in the second pathway in the presence of BDNF (test pathway); when BDNF was tested in the presence of 1 mM MβCD, MβCD was added at least 30 min before LTP induction in the first pathway and remained in the bathing solution until the end of the experiment. Each pathway was used as control or test on alternate days. This protocol allows the comparison between the effects of BDNF on LTP under different experimental conditions, keeping as an internal control the magnitude of LTP under the same drug condition and slice, but in the absence of BDNF.

Immunoblotting. Lysates were denatured with 5× sample buffer (350 mM Tris, 30% glycerol, 10% SDS, 600 mM dithiothreitol, and 0.012% bromophenol blue, pH 6.8), and equal volumes were loaded into gels. Proteins were run in SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and blocked for 1 h at room temperature with 5% nonfat milk in TBS with 0.1% Tween-20 (TBS-T). Incubations with the primary antibodies were performed overnight at 4°C, all of them diluted in 3% BSA in TBS-T and 0.02% sodium azide. HRP-coupled secondary antibodies were diluted in blocking buffer and incubated for 1 h at room temperature. Detection of proteins was made with ECL plus Western blotting detection.

Radioligand binding. [^{3}H] ZM 241385 binding was performed as described previously (Diógenes et al., 2007) with minor modifications. Briefly, 3–4-week-old rat cortices were dissected as described above and centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was centrifuged again at 14,000 × *g* for 12 min, and the pellet was resuspended in 50 mM Tris-HCl, pH 7.4, with 1 mM EDTA and 2 mM EGTA, and incubated with 2 U/ml ADA for 30 min at 37°C. Membranes were precipitated and resuspended in 50 mM Tris-HCl, pH 7.4, with 2 mM $MgCl_2$ and 4 U/ml ADA. A total of 110–200 μg of protein was incubated with 0.1–7 nM [^{3}H] ZM 241385 for 60 min at room temperature, in a final volume of 300 μl. Specific binding was calculated by subtraction of the nonspecific binding, defined in the presence of 2 μM xanthine amine congener. Reaction was stopped by addition of cold incubation buffer followed by vacuum filtration through glass fiber filters (FilterMAT for receptor binding, Skatron Instruments) using a semiautomatic cell harvester from Skatron Instruments. The samples were transferred to scintillation vials, and radioactivity was measured by a liquid scintillation analyzer (Tri Carb 2900TR, PerkinElmer). Membrane protein content was measured using the Bio-Rad protein assay (Bradford, 1976).

Cholesterol measurements. Cholesterol content from the gradient fractions was analyzed using a colorimetric assay (Sepúlveda et al., 2006) with minor modifications. Briefly, lipids were extracted by mixing 200 μl of samples from each gradient fraction with 1 ml of chloroform/methanol (2:1), transferred to a glass tube and dried under nitrogen. Reaction was started by adding 0.75 ml of acetic acid (glacial) and 0.5 ml of 2.5% ferric chloride in 85% phosphoric acid to the tube. After 30 min, absorbance was measured at 550 nm. Standard curves with cholesterol allowed direct quantification of cholesterol levels in each fraction.

Data analysis. All data are expressed as mean ± SEM from the indicated number of experiments. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction for multiple comparisons. When only two means were analyzed, a Student's *t* test analysis was performed. Values of $p < 0.05$ were considered to represent statistically significant differences.

Results

Adenosine A_{2A} receptor activation induces TrkB translocation to lipid rafts and potentiates BDNF-induced phospho-TrkB levels in this membrane microdomain

To study the role of adenosine A_{2A} receptors in TrkB receptor distribution in different membrane domains, isolation of lipid rafts from membranes of cultured cortical neurons was performed based on their relative insolubility in nonionic detergents (see Materials and Methods). The low density of Triton X-100-insoluble lipid rafts allowed their subsequent separation by density gradient fractionation. Eight gradient fractions were collected, and equal volumes of each fraction were analyzed by Western blotting, as shown in Figure 1*A*. Lipid rafts were localized in fraction 2 (from top to bottom), as demonstrated by the presence of the lipid raft marker Fyn in this fraction. The remaining membranes were found in the bottom fractions of the gradient (Fig. 1*A*, fractions 7 and 8). Because lipid rafts are domains enriched in cholesterol, its levels were determined for each fraction. Fraction 2 contained ~22% of the total cholesterol present in the gradient, being highly enriched in this lipid compared with its neighboring fractions (Fig. 1*B*). Cholesterol was also found in the bottom fractions (Fig. 1*B*, 13 and 18%, respectively, of total cholesterol in fractions 7 and 8), which possess the majority of cellular membranes, but no staining of the lipid raft marker Fyn (Fig. 1*A*).

The role of adenosine A_{2A} receptors on TrkB localization was studied by treating cortical neurons with the A_{2A} selective agonist CGS 21680 or with the A_{2A} receptor antagonist ZM 241385. For a better comparison, we analyzed in the same gel fraction 2 samples from cells incubated under different conditions, as shown in Figure 1*C*. When cells were treated with CGS 21680 (20 nM) for 30 min, there was a marked increase in TrkB staining in fraction 2 (176 ± 35% of the control, $p < 0.05$, $n = 6$) (Fig. 1*D*). This incubation time with the A_{2A} receptor agonist clearly induced maximal translocation of TrkB receptors to lipid rafts (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Therefore, a 30 min preincubation time with CGS 21680 before the addition of BDNF was always used while evaluating the influence of A_{2A} receptors on the effect of BDNF on TrkB receptor translocation.

The A_{2A} receptor antagonist ZM 241385 (50 nM) did not influence TrkB receptor sublocalization in lipid rafts (88 ± 15% of the control, $p > 0.05$, $n = 6$) (Fig. 1*D*). As expected, the effect of CGS 21680 was completely prevented by preincubation with ZM 241385 (88 ± 17% of the control, $p > 0.05$, $n = 3$). We then investigated the influence of A_{2A} agonists and antagonists on the effects of BDNF in TrkB receptor localization and phosphoryla-

tion after a short (5 min) incubation time with BDNF. Treatment with BDNF (20 ng/ml) for 5 min did not significantly change TrkB localization but induced the phosphorylation of TrkB receptors in the lipid raft fraction (Fig. 1*C*). While a preincubation for 30 min with CGS 21680 (20 nM) alone had no effect on lipid raft TrkB phosphorylation, it resulted in an increased phosphorylation of TrkB receptors in lipid rafts in response to BDNF treatment for 5 min (Fig. 1*C*). This increase was observed when the pTrkB signal was normalized either to Fyn (207 ± 38% of the BDNF condition, $p < 0.05$, $n = 5$) (Fig. 1*E*) or to total TrkB (182 ± 24% of the BDNF condition, $p < 0.05$, $n = 5$) (Fig. 1*F*). This suggests that CGS 21680 facilitates BDNF-induced increase of the proportion of phosphorylated, and hence active, TrkB receptors in the lipid rafts. The A_{2A} antagonist ZM 241385 (50 nM) did not modify BDNF-induced pTrkB staining in the lipid rafts (Fig. 1*C*).

Importantly, A_{2A} receptor activation with CGS 21680 or blockade with ZM 241385 did not modify (112 ± 13% and 114 ± 11% of the control, respectively, $p > 0.05$, $n = 3$) TrkB phosphorylation or expression in total lysates (Fig. 1*G*), suggesting that A_{2A} receptors act specifically as modulators of TrkB receptor localization in lipid rafts.

To image TrkB receptors in lipid rafts at membranes of cortical neurons, we performed copatching experiments between TrkB receptors and the subunit B of cholera toxin. This subunit of cholera toxin specifically binds to the GM1 gangliosides present in lipid rafts, so that the coalescence of cross-linked lipid microdomains induced by this toxin allows a specific imaging of lipid rafts (Harder et al., 1998; Guirland et al., 2004). As shown in Figure 2, a significant proportion (33 ± 5%, $n = 5$) of TrkB receptors copatch with cholera toxin B in cortical membranes. CGS 21680 (20 nM) treatment induced a significant increase in the degree of colocalization (48 ± 5%, $p < 0.05$, $n = 5$) (Fig. 2) between TrkB receptors and the subunit B of cholera toxin.

The results described above show that after a short (5 min) incubation time with BDNF the neurotrophin itself has only a minor influence on TrkB translocation to lipid rafts. Adenosine A_{2A} receptor activation relocates TrkB receptors in the membrane and facilitates TrkB receptor phosphorylation in lipid rafts. We next examined how CGS 21680 influences the kinetics of BDNF-mediated TrkB translocation and phosphorylation in lipid rafts. As shown in Figure 3, the proportion of pTrkB over total TrkB was already maximal after 5 min of BDNF incubation. Facilitation of pTrkB localization in lipid rafts by adenosine A_{2A} agonists was also maximal after a short (5 min) incubation time with BDNF. For longer incubation times, the ability of CGS 21680 to increase the pTrkB/TrkB ratio in lipid rafts markedly diminishes, being virtually null after BDNF incubation for 40 min (Fig. 3*B*). However, even at prolonged incubations with BDNF (40 min), total TrkB and pTrkB staining was more pronounced in the presence of CGS 21680 (Fig. 3*A*,*C*,*D*).

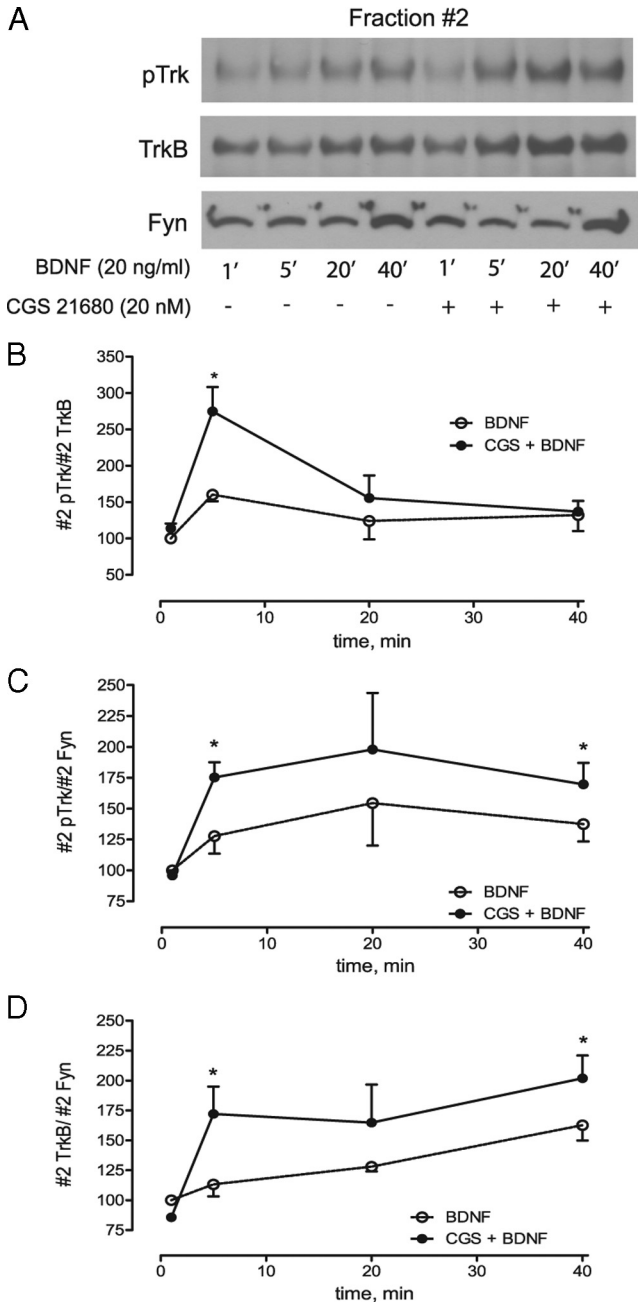


Figure 3. Time course of TrkB receptor phosphorylation in lipid rafts by BDNF. ***A***, Lipid raft (fraction 2) analysis of pTrk and TrkB staining in lipid rafts from cells incubated with BDNF for 1–40 min in the absence or presence of the A_{2A} receptor agonist CGS 21680 (20 nM), as indicated. Fraction 2 was probed for pTrk, TrkB, and Fyn. ***B***, ***C***, Densitometry analysis of fraction 2 pTrk staining, normalized by total fraction 2 TrkB (***B***) and fraction 2 Fyn (***C***). ***D***, Densitometry analysis of fraction 2 TrkB staining, normalized by fraction 2 Fyn. Results are expressed as mean ± SEM of three (1 and 20 min) or eight (5 and 40 min) experiments. *$p < 0.05$, compared with BDNF alone.

A_{2A} receptors are not required for BDNF-induced TrkB translocation to lipid rafts

To characterize in more detail the cross talk between adenosine A_{2A} and TrkB receptors, in the next series of experiments neurons were incubated with BDNF for 40 min, i.e., in conditions where BDNF per se has a marked effect on TrkB translocation to lipid rafts. Treatment of cortical neurons with BDNF (20 ng/ml) for 40 min significantly increased TrkB levels in fraction 2 (164 ± 17% of the control, $p < 0.01$, $n = 6$) (Fig. 4*B*,*C*). Activation of adenosine receptors also resulted in an increase up to 140 ± 6% of the control in TrkB staining in lipid rafts ($p < 0.05$, $n = 6$) (Fig. 4*B*,*C*). When BDNF was applied for 40 min in the presence of CGS 21680, TrkB staining was increased up to 224 ± 20% of the control (Fig. 4*C*), approximately the sum of the effect of each drug alone, suggesting the existence of two different pathways involved in TrkB translocation by BDNF and CGS 21680. Furthermore, incubation with an A_{2A} receptor antagonist, ZM 241385 (50 nM), for 30 min before BDNF addition did not modify ($p > 0.05$, $n = 5$) (Fig. 4*C*) BDNF-induced TrkB translocation to

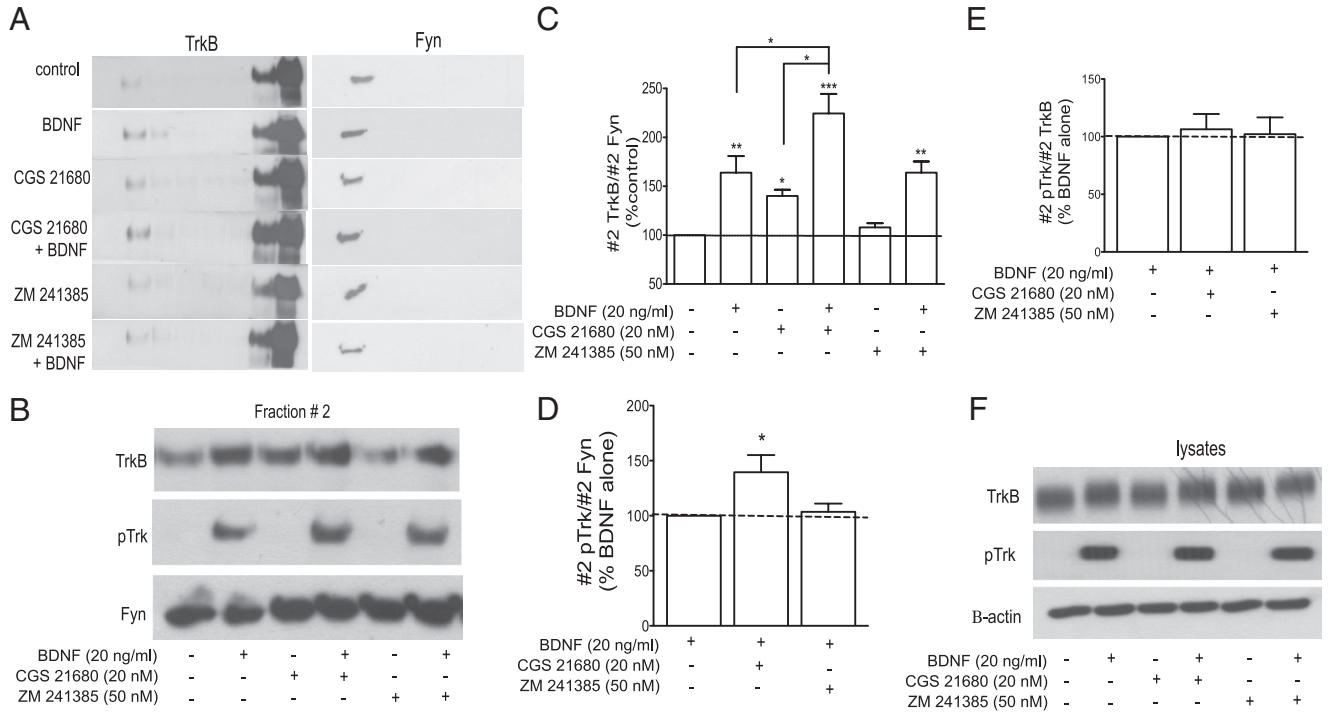


Figure 4. A_{2A} receptors are not required for maximal BDNF-induced TrkB translocation to lipid rafts. On DIV 7–11, cortical neurons were starved for 4 h before treatment with/without 20 nM CGS 21680, 50 nM ZM 241385, and/or 20 ng/ml BDNF (40 min), as indicated. ***A***, Equal volumes of each density gradient fraction were immunoblotted for TrkB and the lipid raft marker Fyn. ***B***, Staining of lipid raft fraction 2. Membranes were probed for TrkB, pTrk, and Fyn. ***C***, Densitometry analysis of TrkB staining in lipid rafts (fraction 2), normalized by fraction 2 Fyn. ***D***, ***E***, Densitometric analysis of the pTrk staining in fraction 2 normalized by Fyn (***D***) and total TrkB (***E***). One hundred percent correspond to pTrk staining in the presence of BDNF alone. ***F***, Total lysates were treated as described in Materials and Methods, lysed, and probed for TrkB, pTrk, and β-actin. In ***C***, ***D***, and ***E***, results are expressed as mean ± SEM of six independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with 100%, unless otherwise indicated.

lipid rafts, indicating that A_{2A} receptors are not required for this process.

When the levels of pTrkB in lipid rafts were analyzed, CGS 21680 pretreatment resulted in a 40% increase (Fig. 4*D*) in pTrkB staining in lipid rafts in response to BDNF (vs BDNF alone). However, this reflects the augmented number of TrkB receptors in these domains induced by CGS 21680 treatment, because the fraction of pTrkB/TrkB is not modified by CGS 21680 (Figs. 3*B*, 4*E*). Blockade of A_{2A} receptors with ZM 241385 (50 nM, 30 min) did not induce any significant change in BDNF-induced pTrkB levels in fraction 2 (Fig. 4*B*,*D*), further indicating that A_{2A} receptors potentiate, but are not required, for BDNF-induced TrkB phosphorylation in lipid rafts.

No effects of the A_{2A} receptor agonist CGS 21680 and antagonist ZM 241385 on TrkB expression or BDNF-induced (20 ng/ml, 40 min) TrkB phosphorylation were observed in the analysis of total lysates (95 ± 9% and 107 ± 7% of the control, respectively, $p > 0.05$, $n = 3$) (Fig. 4*F*).

CGS 21680 and BDNF use different mechanisms to recruit TrkB receptors to lipid rafts

BDNF-induced TrkB translocation to lipid rafts requires TrkB receptor phosphorylation and internalization (Suzuki et al., 2004; Pereira and Chao, 2007). It is also known that most of the activated Trk receptor internalization takes place through clathrin-coated pits (Grimes et al., 1996; Du et al., 2003; Zheng et al., 2008). Although a 30 min incubation with CGS 21680 did not induce TrkB phosphorylation on its own (Fig. 1*C*), we investigated the need of TrkB internalization for CGS 21680-induced TrkB translocation to lipid rafts, using the clathrin-dependent endocytosis inhibitor monodansylcadaverine (MDC) (Haigler et al., 1980; Ivanov, 2008). Alone, MDC did not significantly affect TrkB localization (Fig. 5). Interestingly, MDC did not prevent CGS 21680-induced recruitment of TrkB receptors to lipid rafts (Fig. 5), suggesting that the mechanism used by A_{2A} receptors to translocate TrkB receptors to lipid rafts dos not require internalization through clathrin-coated pits. For comparison and as a positive control, we evaluated the influence of MDC on BDNF-induced TrkB translocation. In the presence of MDC, BDNF-induced TrkB translocation to lipid rafts was significantly attenuated (Fig. 5). As expected, BDNF-induced enhancement of pTrkB levels in lipid rafts was markedly reduced in MDC-treated neurons (Fig. 5*A*,*C*).

The effects of CGS 21680 on TrkB translocation involve cAMP and Src-family tyrosine kinase activation

Since A_{2A} receptor-induced translocation of TrkB receptors to lipid rafts is independent of TrkB phosphorylation and internalization (Fig. 5), occurring through a process different from that used by BDNF, we further evaluated the mechanisms involved in this process. Adenosine A_{2A} receptors are primarily coupled to G_s-proteins, and most of its actions involve activation of adenylyl cyclase. We first investigated the role of cAMP for CGS 21680-induced TrkB translocation by using forskolin, a known adenylyl cyclase activator (Awad et al., 1983). As shown in Figure 6*A*,*D*, forskolin (5 μM) mimicked the effect of CGS 21680 in inducing TrkB translocation to lipid rafts. In cells incubated in the presence of forskolin, CGS 21680 did not cause a further enhancement of TrkB staining in fraction 2 (Fig. 6*A*,*D*). We then investigated the role of protein kinase A, one of the main cAMP effectors, on CGS21680-induced TrkB translocation to lipid rafts. We used Rp-cAMPs, an inactive analog of cAMP that in-

hibits activation of PKA by substrate competition (Lochner and Moolman, 2006). In cells incubated with Rp-cAMPs (100 μM), the effect of CGS 21680 on TrkB receptor translocation to lipid rafts was fully prevented (Fig. 6*B*,*D*), supporting a role for PKA in this process. Moreover, the PKA inhibitor peptide PKI-(14–22) also prevented the effect of CGS 21680 in TrkB receptor recruitment to lipid rafts (Fig. 6*B*,*D*). Unexpectedly, incubation with H-89 (1 μM) did not block the effect of CGS21680 on TrkB localization (Fig. 6*B*,*D*). This discrepancy might result from an H-89-induced inhibition of other kinases (Leemhuis et al., 2002; Lochner and Moolman, 2006; Murray, 2008) that might counteract the consequences of PKA inhibition; alternatively, it may result from an inefficient inhibition of PKA by the concentration of H-89 used, since the IC_{50} for PKA inhibition by H-89 is highly dependent on the intracellular ATP concentration (Murray, 2008). Higher concentrations of H-89 were not tested due to lack of selectivity. Neither 100 μM Rp-cAMPs, 1 μM PKI-(14–22), nor 1 μM H-89 per se influenced the levels of TrkB on lipid rafts when added in the absence of CGS 21680 (Fig. 6*D*).

The possible involvement of other transduction pathways that can also be activated by A_{2A} agonists, such as PLC (see Fredholm et al., 2001) and Src-family tyrosine kinases (Rajagopal and Chao, 2006), was also investigated. Figure 6*C* shows the influence of the PLC inhibitor U73122 (4 μM) (Bleasdale et al., 1990) and the Src-family tyrosine kinase inhibitor PP2 (0.5 μM) (Bain et al., 2003) on CGS 21680-induced TrkB translocation to lipid rafts. The PLC inhibitor did not prevent the CGS 21680 effect on TrkB staining in fraction 2. Importantly, PP2 blocked the effect of CGS 21680 on TrkB localization to lipid rafts, suggesting an involvement of Src-family tyrosine kinases (Fig. 6*C*,*D*).

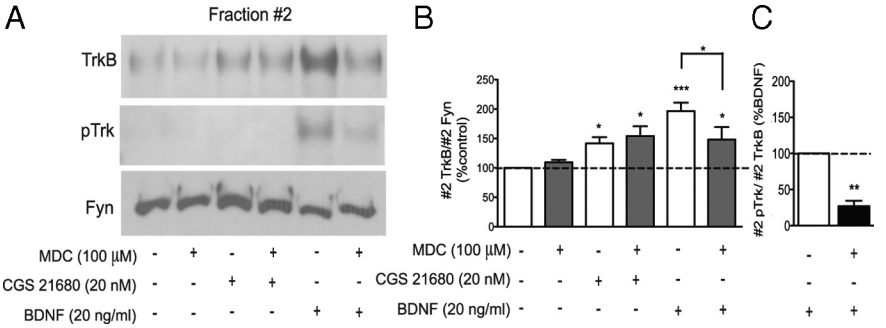


Figure 5. Influence of clathrin-dependent endocytosis on CGS 21680-induced and BDNF-induced TrkB recruitment and phosphorylation to lipid rafts. On DIV 7–11, cortical neurons were treated with 20 nM CGS 21680 for 30 min or 20 ng/ml BDNF for 40 min in the presence or absence of the clathrin-dependent endocytosis inhibitor monodansylcadaverine (100 μM), where indicated. ***A***, Density gradient fraction 2 was immunoblotted and probed for TrkB, pTrk, and Fyn. ***B***, Quantification of fraction 2 TrkB/fraction 2 Fyn. ***C***, Quantification of fraction 2 pTrk/fraction 2 TrkB after BDNF treatment in the presence/absence of MDC, as indicated below each bar. Data are expressed as mean ± SEM of four to six independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared with 100%, unless otherwise indicated. Note that the clathrin-dependent endocytosis inhibitor, MDC, attenuated BDNF-induced TrkB translocation to lipid rafts but did not influence CGS 21680-induced TrkB recruitment to these membrane domains.

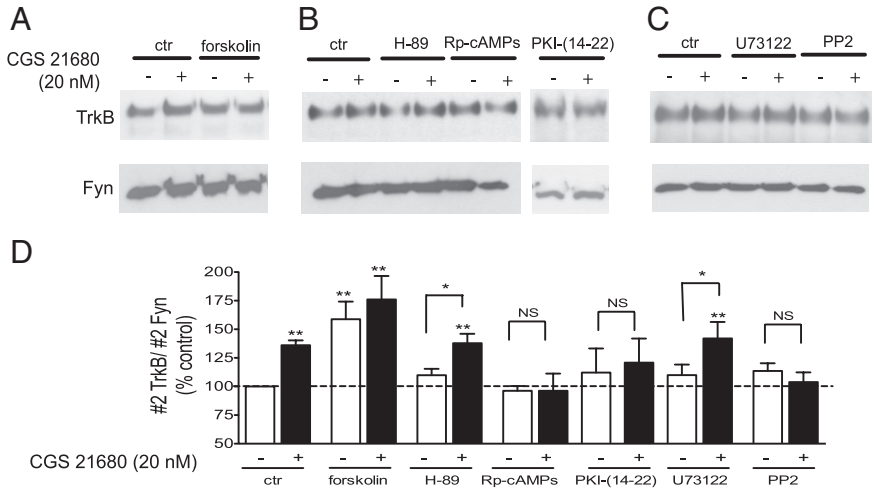


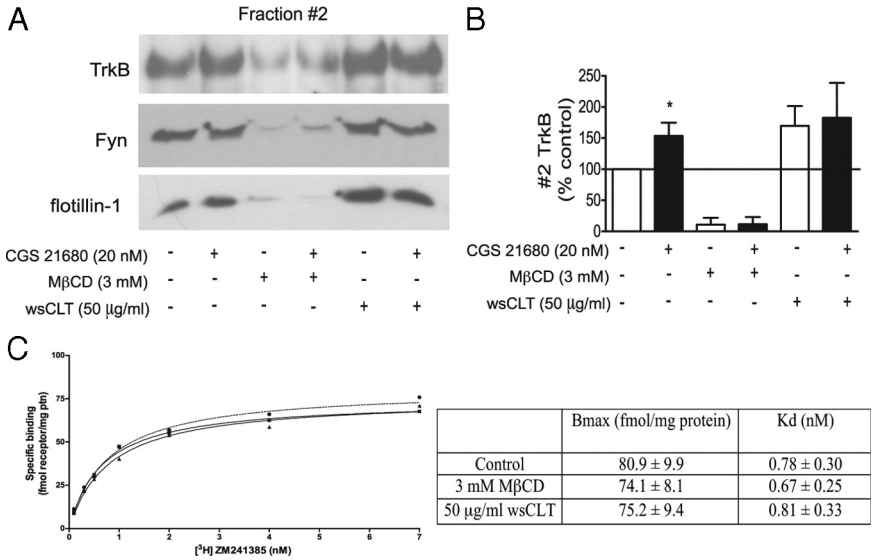
Figure 6. Signaling pathways involved in CGS 21680-induced TrkB translocation to lipid rafts. Cultured cortical neurons were incubated with/without 20 nM CGS 21680 for 30 min in the presence of the adenylate cyclase activator forskolin (5 μM), the PKA inhibitor H-89 (1 μM), the cAMP antagonist Rp-cAMPs (100 μM), the PKA inhibitor PKI-(14–22) (1 μM), the phospholipase C inhibitor U73122 (4 μM), or the Src-family kinase inhibitor PP2 (500 nM), as indicated. Cells were lysed and processed for lipid raft isolation. ***A–C***, Fraction 2 obtained from the density gradients of cells under different conditions was probed for total TrkB and Fyn, which was used as a loading control. ***B***, ***D***, Densitometry analysis of TrkB/Fyn staining obtained in ***A–C***. Data are expressed as mean ± SEM of four to six independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; NS, no statistical difference ($p > 0.05$), compared with 100%, except when otherwise indicated.

Influence of membrane cholesterol content on TrkB and A_{2A} receptors

To investigate the role of cholesterol and lipid raft integrity in CGS 21680-induced TrkB translocation to lipid rafts, we used the cholesterol-sequestering agent MβCD (Simons and Toomre, 2000). Incubation of cortical neurons with 3 mM MβCD alone decreased TrkB levels in the lipid rafts. This reduction in TrkB staining was accompanied by a decrease of the lipid raft marker proteins Fyn and flotillin-1 in fraction 2 (Fig. 7*A*). In the presence of MβCD, adenosine A_{2A} receptor activation with CGS 21680 did not induce any detectable increase in TrkB levels in fraction 2 (Fig. 7*A*,*B*). These results show that MβCD disturbed lipid raft integrity and that under these conditions A_{2A} receptor agonists were no longer able to induce TrkB translocation.

Another approach was to use MβCD–cholesterol complexes [50 μg/ml cholesterol, or water-soluble cholesterol (wsCLT)] to load cells with cholesterol. The MβCD/cholesterol ratio used was 6:1, which is considered optimal for cell loading (Christian et al., 1997). This treatment led to an increased TrkB partition to lipid rafts, indicating that there is a cholesterol-dependent regulation of TrkB receptor localization in membrane subdomains (Fig. 7*A*,*B*). Interestingly, in cholesterol-loaded cells, incubation with CGS 21680 (20 nM) did not cause a further enhancement of TrkB receptor staining in the lipid raft faction (Fig. 7*B*), suggesting that under conditions of high cholesterol and TrkB receptor localization in lipid rafts A_{2A} receptors are no longer able to promote further TrkB receptor translocation.

To elucidate whether adenosine A_{2A} receptor activation could directly influence the amount of cholesterol in lipid rafts, we measured cholesterol content in the lipid raft fraction (fraction 2) and the nonraft fractions (fractions 7 and 8) after CGS 21680 treatment. No



	Bmax (fmol/mg protein)	Kd (nM)
Control	80.9 ± 9.9	0.78 ± 0.30
3 mM MβCD	74.1 ± 8.1	0.67 ± 0.25
50 μg/ml wsCLT	75.2 ± 9.4	0.81 ± 0.33

Figure 7. Effects of cholesterol depletion and loading on the partition of TrkB receptors to lipid rafts and on adenosine A_{2A} receptor binding properties. Cultured cortical neurons were treated with/without 20 nM CGS 21680 for 30 min after incubation with 3 mM MβCD or MβCD–cholesterol complexes (50 μg/ml cholesterol, wsCLT). ***A***, Lipid raft (fraction 2) analysis of the effect of MβCD on TrkB sublocalization after CGS 21680 treatment. MβCD–cholesterol complexes were used to load cholesterol to membranes. ***B***, Quantification of fraction 2 TrkB. ***C***, Saturation curves for the specific binding of the A_{2A} receptor antagonist [^{3}H] ZM 241385 in cortical membranes from cells in control conditions (open circles), after incubation with MβCD (closed squares), or with wsCLT (closed triangles) treatment. Data are expressed as mean ± SEM of three to four independent experiments. *$p < 0.05$, compared with 100%.

changes were observed in the cholesterol levels present in the raft fraction 2 (11.0 ± 1.1 in control and 11.6 ± 0.9 μg/ml after CGS 21680, $p > 0.05$, $n = 5$) or in the nonraft fractions 7 and 8 (7.1 ± 0.4 in control and 7.8 ± 0.5 μg/ml after CGS 21680, $p > 0.05$, $n = 5$).

To examine the influence of lipid rafts on adenosine A_{2A} receptors, saturation binding experiments were performed using the selective A_{2A} antagonist [^{3}H] ZM 241385. Membrane samples were incubated in the presence of MβCD and MβCD–cholesterol complexes. As illustrated in Figure 7*C*, neither lipid raft disruption with 3 mM MβCD nor cholesterol enrichment with 50 μg/ml wsCLT resulted in changes in the parameters for [^{3}H] ZM 241385 binding ($p > 0.05$, $n = 3$). These results do not exclude the importance of lipid rafts for optimal A_{2A} receptor signaling, but strongly suggest that ligand affinity and density of this receptor are conserved while interfering with lipid raft integrity.

BDNF-induced facilitation of glutamate release depends on endogenous adenosine and lipid raft integrity

The functional relevance of A_{2A}-induced TrkB translocation to lipid rafts was first assessed by investigating the effect of BDNF on glutamate release after extracellular adenosine depletion and lipid raft disruption. Cortical synaptosomes were labeled with [^{3}H]glutamate as previously described (Canas et al., 2004), and neurotransmitter release was evoked twice (S_1 and S_2) by perfusion with 15 mM KCl for 2 min (see Materials and Methods). In control conditions, the S_2/S_1 ratio was 0.79 ± 0.02 ($n = 14$); when BDNF (20 ng/ml) was added before S_2, the ratio was increased to 1.05 ± 0.02 ($p < 0.01$, $n = 14$), corresponding to a 33 ± 3% enhancement in evoked glutamate release (Fig. 8). To investigate how endogenous extracellular adenosine influences this effect of BDNF, synaptosomes were incubated with the adenosine-degrading enzyme ADA, which was added before S_1 and remained in the perfusion up to the end of sample collection (see Materials and Methods). As shown in Figure 8*B*, the removal of endogenous adenosine with 1 U/ml ADA completely prevented the effect of BDNF on glutamate release. The presence of ADA (1 U/ml) during S_1 and S_2 did not affect the S_2/S_1 ratio, which in the control conditions was 0.81 ± 0.04 and in the presence of ADA was 0.84 ± 0.05 ($p > 0.05$, $n = 3$).

The relevance of lipid rafts was first studied by perfusing synaptosomes with MβCD. Per se, 1 mM MβCD treatment caused a slight increase (12 ± 3%, $n = 3$) in the basal release of tritium but did not influence S_2/S_1 ratios (2.2 ± 2.5% change, $n = 3$) or the amount of tritium released in response to K^+ stimulation (1.3 ± 4% change, $n = 3$), indicating that K^+-evoked glutamate release was not compromised at this concentration of MβCD. In synaptosomes incubated with MβCD, the BDNF-induced enhancement of glutamate release was significantly smaller than in control conditions (Fig. 8*C*), thus supporting a relevant role of lipid rafts in BDNF-induced modulation of glutamate release. However, BDNF was still able to induce a small but significant increase on release (Fig. 8*C*). This could be explained either by incomplete disruption of lipid rafts with 1 mM MβCD or by involvement of lipid raft-independent mechanisms in the effect of BDNF on glutamate release. Higher concentrations of MβCD (2–3 mM) caused a marked increase in basal release of tritium (data not shown), probably due to compromised synaptosomal integrity, precluding the possibility of testing higher concentrations of MβCD. Alternatively, we used cholesterol oxidase, an enzyme that converts cholesterol into 4-cholesten-3-one, altering the structure of rafts (Hérincs et al., 2005; Ivanov, 2008). As shown in Figure 8*C*, preincubation of synaptosomes with 2 U/ml cholesterol oxidase abolished the effect of BDNF on glutamate release ($p < 0.01$, $n = 4$). Together, these data suggest that BDNF enhances glutamate release through an adenosine- and lipid raft-dependent mechanism.

Interestingly, cholesterol addition (MβCD–cholesterol complexes, 50 μg/ml) did not modify ($p > 0.05$, $n = 6$) the facilitatory action of BDNF on glutamate release, suggesting that either the endogenous cholesterol levels were enough to trigger the maximal effect of BDNF and/or synaptosomes were not able to efficiently incorporate exogenously added cholesterol to their membranes.

The presence of MβCD (1 mM), cholesterol oxidase (2 U/ml), or wsCLT (50 μg/ml) during S_1 and S_2 did not affect the S_2/S_1 ratio. In the MβCD experiments, the S_2/S_1 ratio in the control was 0.75 ± 0.02, and in the presence of MβCD was 0.77 ± 0.02 ($p > 0.05$, $n = 6$). In the cholesterol oxidase experiments, the S_2/S_1 ratio in the control was 0.81 ± 0.03, and in the presence of cholesterol oxidase was 0.85 ± 0.04 ($p > 0.05$, $n = 5$). In the exogenous cholesterol experiments, the S_2/S_1 ratio in the control was 0.74 ± 0.03, and in the presence of excess cholesterol was 0.76 ± 0.03 ($p > 0.05$, $n = 6$).

High-frequency stimulation of hippocampal slices increases TrkB and pTrkB receptor localization in lipid rafts in an adenosine-dependent manner

BDNF release is stimulated after increased neuronal activity (Figurov et al., 1996), but it is still not understood how such a diffusible molecule preferentially potentiates active synapses. Neuronal activity and increased intracellular cAMP levels have been shown to gate synaptic actions of BDNF (Boulanger and Poo, 1999a,b; Nagappan and Lu, 2005). Adenosine concentration is also markedly increased under high-frequency neuronal firing due to catabolism of endogenously released ATP (Wieraszko et al., 1989; Cunha et al., 1996a), and this ATP-derived adenosine preferentially activates A_{2A} receptors (Cunha et al., 1996b). We have previously demonstrated a potentiating effect of BDNF on hippocampal LTP that requires the activation of A_{2A} receptors by endogenous adenosine (Fontinha et al., 2008), supporting a role for A_{2A}-induced gating of BDNF effects on synaptic plasticity. Therefore, we hypothesized that activity-derived activation of A_{2A} receptors by endogenous adenosine could induce TrkB translocation to lipid rafts. To test this hypothesis, hippocampal slices were stimulated for 1 min using a high-frequency paradigm that was shown to induce the release of ATP and adenosine (see Materials and Methods) (Cunha et al., 1996a). After 30 min, the lipid rafts were isolated from slice lysates and fraction 2 was analyzed by immunoblotting. As illustrated in Figure 9, field stimulation of hippocampal slices induced TrkB translocation to lipid rafts (121.5 ± 3% of the control, $p < 0.001$, $n = 6$) (Fig. 9). Field stimulation of hippocampal slices also induced a significant increase in the pTrkB staining and the pTrkB/TrkB ratio in fraction 2 ($p < 0.05$, $n = 6$) (Fig. 9). The role of endogenous adenosine in high-frequency stimulation-induced recruitment of TrkB receptors into lipid rafts was investigated by incubation of the slices with the adenosine-degrading enzyme ADA (1 U/ml) from 30 min before the high-frequency stimulation until the end of the experiment. Under these conditions, the effect of field stimulation on both TrkB and pTrkB receptor localization was completely prevented (Fig. 9*B*), supporting the hypothesis that adenosine released during intense synaptic activity plays a role in targeting TrkB receptors to lipid rafts.

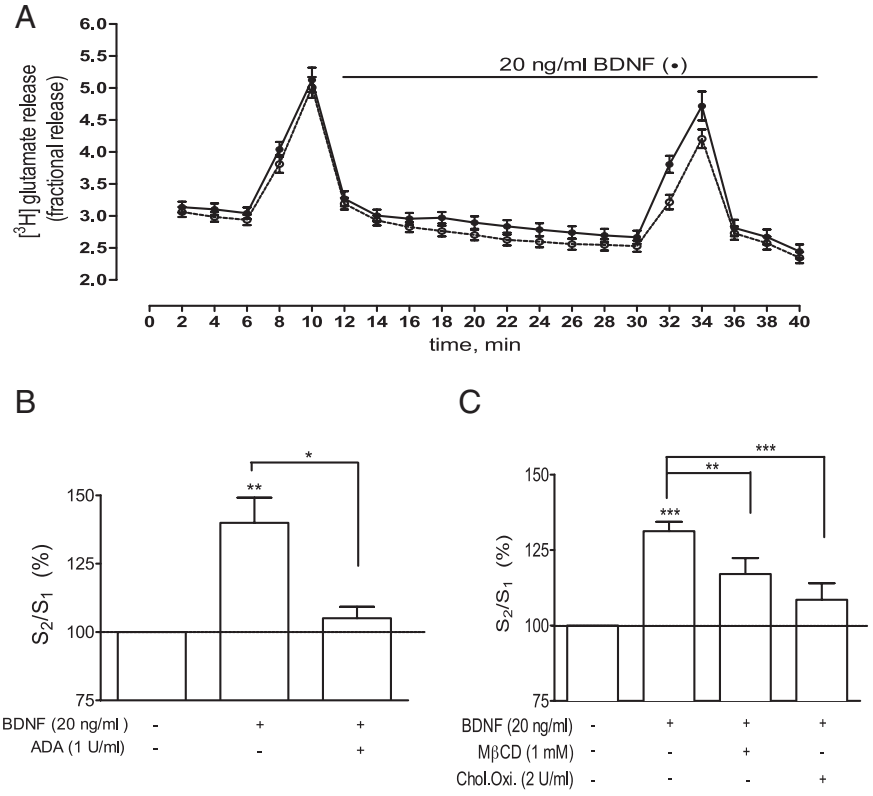


Figure 8. BDNF increases glutamate release in an adenosine- and lipid raft-dependent manner. ***A***, Averaged time course of [^{3}H] glutamate release from cortical synaptosomes. Synaptosomes were labeled with [^{3}H] glutamate, and stimulation of neurotransmitter release was induced twice, at 5–7 min (S_1) and 29–31 min (S_2), as described in Materials and Methods. Samples were collected every 2 min. BDNF (20 ng/ml) was added at 9 min and remained in the perfusion solution until the end of the experiments (closed circles). Control curves in the absence of any drug, performed in parallel with the same synaptosomal batch, are represented by the open circles. ***B***, ***C***, S_2/S_1 ratios, calculated in each experiment from the time course curves, as described in Materials and Methods. BDNF (20 ng/ml) was tested in the presence/absence of 1 U/ml ADA, 1 mM MβCD, or 2 U/ml cholesterol oxidase (Chol.Oxi.), as indicated below each bar. In each experiment, the S_2/S_1 ratio obtained while BDNF was present during S_2 was normalized, taking as 100% the S_2/S_1 ratio obtained in parallel chambers under the same drug conditions but in the absence of BDNF. Data are represented as mean ± SEM of three to six independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with 100%, except when otherwise indicated.

Lipid raft integrity is required for BDNF-induced facilitation of long-term potentiation

Since extracellular adenosine is required for BDNF-induced enhancement of LTP (Fontinha et al., 2008) and for high-frequency stimulation-induced TrkB translocation to lipid rafts (Fig. 9), we investigated whether lipid rafts were required for the facilitation of LTP induced by BDNF. The LTP-inducing protocol was similar to that previously used to detect facilitatory actions of endogenous or exogenous BDNF on LTP (Chen et al., 1999; Kramár et al., 2004; Fontinha et al., 2008). To evaluate the influence of lipid raft disruption on the facilitatory effect of BDNF on LTP, MβCD was used at a low concentration to avoid marked changes in synaptic integrity that could cause alterations in the synaptic plasticity phenomena. Hippocampal slices were incubated with MβCD (1 mM) for 30 min before and during the entire LTP experiment. At this concentration, MβCD had a very mild effect on basal synaptic transmission (−4.6 ± 0.4%), as shown in Figure 10*B*. Furthermore, in MβCD-treated slices, the magnitude of LTP was similar to what was observed in other slices in the absence of MβCD (Fig. 10*H*, open bars). Most importantly, the magnitude of LTP in two consecutive pathways on the same slice (see Materials and Methods) was similar in the presence of MβCD throughout the entire LTP-inducing protocol ($p > 0.05$, $n = 3$) (Fig. 10*C*). This allowed us to study the modulatory role of BDNF on LTP in slices that were perfused with MβCD throughout the entire recording period. To evaluate the effect of BDNF on LTP, we compared the magnitude of LTP in the first pathway (in the absence of BDNF) with that in the second pathway (in the presence of BDNF), in the same slice. The effect of BDNF on LTP in the absence or presence of MβCD was then compared.

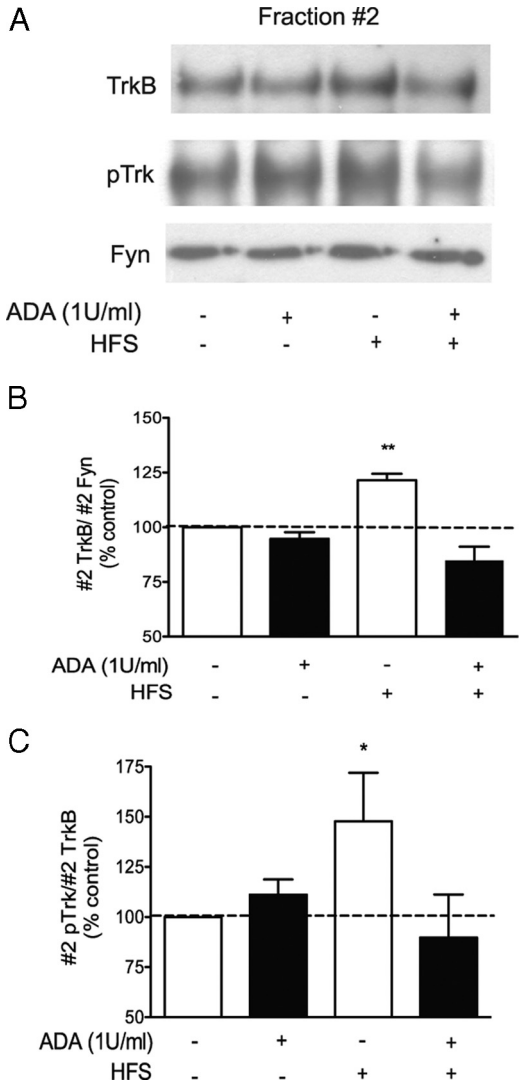


Figure 9. High-frequency stimulation of hippocampal slices induces TrkB translocation and increases pTrk staining in lipid rafts in an adenosine-dependent manner. Hippocampal slices were superfused with 1 U/ml ADA, where indicated, 30 min before the high-frequency stimulation (HFS). HFS was applied for 1 min as described in Materials and Methods, and after 30 min slices were homogenized and lipid rafts were isolated by discontinuous Optiprep gradients. ***A***, Fraction 2, containing lipid raft membranes, was immunoblotted for TrkB, pTrk, and Fyn, which was used as a loading control. ***B***, Quantifications of fraction 2 TrkB/fraction 2 Fyn. ***C***, Quantifications of fraction 2 pTrk/fraction 2 Fyn. Data are represented as mean ± SEM of six independent experiments. $*p < 0.05$, $**p < 0.01$, compared with 100%.

As expected from previous reports (Fontinha et al., 2008), BDNF (20 ng/ml) enhanced ($p < 0.05$, $n = 6$) (Fig. 10*D*,*H*) the magnitude of LTP in control slices (Fig. 10*D*). In contrast, in MβCD-treated slices, the facilitatory effect of BDNF on LTP was not observed ($p > 0.05$, $n = 5$) (Fig. 10*E*,*H*), indicating that lipid raft integrity is necessary for the facilitatory actions of BDNF on hippocampal synaptic plasticity.

Discussion

The main finding of the present work is that adenosine A_{2A} receptor activation increases the levels of TrkB receptors in lipid rafts and potentiates BDNF-induced TrkB phosphorylation in these membrane microdomains. Furthermore, relevant actions of BDNF at synapses, such as facilitation of glutamate release and synaptic plasticity, require both lipid raft integrity and A_{2A} receptor activation. Altogether, our data suggest that A_{2A} receptors contribute to the translocation of TrkB receptors toward specific membrane areas where TrkB activation and subsequent signaling occurs. Noteworthy, this is the first evidence for BDNF-independent TrkB translocation to lipid rafts.

Active A_{2A} receptors were not required for BDNF-induced recruitment of TrkB receptors, since the presence of an A_{2A} receptor antagonist did not influence BDNF-induced translocation to lipid rafts. Furthermore, the effects of the A_{2A} receptor agonist and of BDNF on TrkB translocation were additive. Inhibition of clathrin-dependent endocytosis with MDC did not influence the action of the A_{2A} receptor agonist, but significantly reduced BDNF-induced TrkB translocation to lipid rafts. Together, these results strongly suggest that A_{2A} agonists and BDNF act through different mechanisms to recruit TrkB receptors to lipid rafts.

We observed that incubation of cells with BDNF for 5 min did not recruit detectable amounts of TrkB receptors to lipid rafts; however, under the same conditions, we could detect pTrkB staining in these microdomains. This suggests that either BDNF phosphorylates a small amount of TrkB receptors already present in the lipid rafts and/or that BDNF-induced translocation of pTrkB was not detectable in the analysis of total TrkB receptors. Adenosine A_{2A} receptor activation markedly increased the levels of TrkB receptors in the lipid rafts and potentiated BDNF-induced pTrkB staining in lipid rafts from cells incubated for a short period (5 min) with BDNF. It is possible that the increased concentration of TrkB receptors in lipid rafts induced by CGS 21680 leads to increased proximity and autophosphorylation of TrkB receptors not fully phosphorylated by a short BDNF exposure. In contrast, after BDNF treatment for 40 min TrkB localization in lipid rafts is already high and probably maximally activated. Accordingly, a further increase in TrkB concentration in these membrane domains induced by A_{2A} receptor activation did not change the proportion of pTrkB (as a function of total TrkB receptors) in lipid rafts. However, the total amount of pTrkB receptors was higher in cells incubated for 40 min with BDNF in the presence of the A_{2A} agonist. Altogether, the data suggest that activation of A_{2A} receptors per se induces translocation to lipid rafts of TrkB receptors that are prone to be phosphorylated by BDNF. Additionally, A_{2A} receptor-induced clustering of TrkB receptors in lipid rafts may play a role in the facilitatory effects of A_{2A} agonists on TrkB receptor function at synapses, since BDNF-induced TrkB phosphorylation in lipid rafts may be facilitated as a consequence of increased receptor proximity.

It is unlikely that the influence of A_{2A} receptors on TrkB translocation is due to a facilitation of endogenous BDNF actions or TrkB transactivation. This conclusion is supported by the lack of detectable amounts of pTrkB receptors in lysates or in lipid rafts isolated from cells incubated with the A_{2A} receptor agonist (Fig. 1), whereas pTrkB receptors could be detected in lipid rafts isolated from cells incubated with BDNF. Furthermore, the inhibitor of clathrin-dependent endocytosis, MDC, differently affects BDNF-induced and CGS21680-induced TrkB translocation. Moreover, transactivation of TrkB receptors, i.e., phosphorylation of TrkB receptors in the absence of BDNF, requires prolonged (2–3 h) exposure to A_{2A} receptor agonists and mostly involves intracellular TrkB receptors (Lee and Chao, 2001; Rajagopal et al., 2004).

Although MDC treatment significantly attenuated BDNF-induced TrkB translocation to lipid rafts, some receptors were still recruited by BDNF in the presence of MDC. This may suggest that BDNF is able to recruit some TrkB receptors to lipid rafts independently of internalization through clathrin-coated pits.

Alternatively, MDC treatment may not have fully inhibited clathrin-dependent endocytosis and/or compensatory mechanisms of receptor internalization may account for the effect of BDNF observed in the presence of MDC.

Neither BDNF nor A_{2A} receptor activation induced translocation of the truncated TrkB receptors to lipid rafts, suggesting that A_{2A} receptors act on the intracellular domain of TrkB receptors to induce its recruitment to lipid rafts. The tyrosine kinase Fyn is possibly one of the mediators, as inhibition of Src-family kinases prevented the influence of A_{2A} receptors on TrkB translocation. Indeed, Fyn can be activated by A_{2A} agonists, and it is known that Fyn is required for TrkB localization in lipid rafts (Rajagopal and Chao, 2006; Pereira and Chao, 2007). The requirement of cAMP for the effect of CGS 21680 on TrkB translocation suggests that A_{2A} receptors are operating through the adenylyl cyclase/cAMP transduction pathway. Accordingly, the adenylyl cyclase activator forskolin mimicked the effect of the A_{2A} receptor agonist on TrkB translocation.

Modifications of the cholesterol content in the cells by treatment with a cholesterol-chelating compound, such as MβCD, or by the addition of cholesterol fully prevented the effects of A_{2A} receptor agonists on TrkB translocation to lipid rafts, indicating the need of optimal cholesterol levels for this process. When cells were loaded with excess cholesterol, TrkB localization in lipid rafts was increased, possibly due to alterations in size and/or properties of the lipid rafts. This may influence TrkB partition on different membrane domains, affecting the ability of A_{2A} receptors to modulate their translocation. Excess cholesterol, however, did not increase BDNF-induced glutamate release, which further supports the concept of a tightly regulated cholesterol concentration in the lipid rafts for optimal partition of proteins among these domains.

As previously observed in cultured neurons (Suzuki et al., 2004), the facilitatory effect of BDNF on glutamate release from acutely isolated nerve endings was affected by lipid raft disruption. Moreover, we showed that lipid raft-disturbing drugs also prevent the facilitatory action of BDNF on LTP. Additionally, removal of endogenous extracellular adenosine or blockade of A_{2A} receptors prevented BDNF actions on glutamate release (present work), synaptic transmission, and LTP (Diógenes et al., 2004, 2007; Fontinha et al., 2008). Together, this evidence strongly suggests that the facilitatory action of BDNF at glutamatergic synapses requires lipid raft integrity as well as the presence of extracellular adenosine and A_{2A} receptor activation.

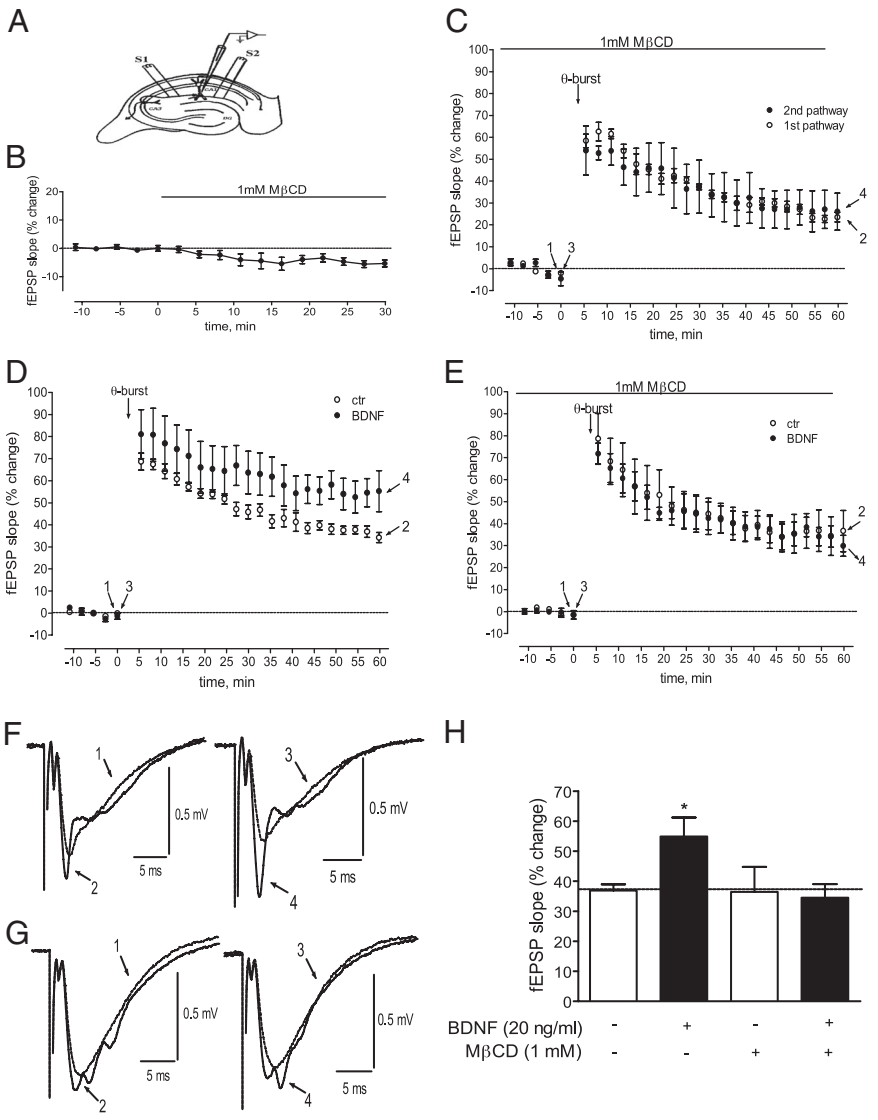


Figure 10. BDNF enhances long-term potentiation in a lipid raft-dependent manner. ***A***, Schematic representation of a transverse hippocampal slice with the electrode configuration used to record fEPSPs in the CA1 apical dendritic layer (stratum radiatum) evoked by electric stimulation of two independent pathways of the Schaffer fibers, S_0 and S_1. ***B–E***, Averaged time course changes in the fEPSP slope. The small inhibition of fEPSP caused by 1 mM MβCD is illustrated in ***B***. ***C–E***, Changes in the fEPSP slope induced by the theta burst (θ-burst) stimulation of slices (see Materials and Methods), as indicated by the arrow in each panel. Zero percent corresponds to the averaged slopes recorded for 10 min before MβCD (***B***: before MβCD, -0.55 ± 0.02 mV/ms; after MβCD, -0.51 ± 0.01 mV/ms; $n = 7$) or theta burst stimulation (***C***: ○, -0.49 ± 0.02 mV/ms; ●, -0.49 ± 0.02 mV/ms; $n = 3$; ***D***: ○, -0.52 ± 0.01 mV/ms; ●, -0.49 ± 0.04 mV/ms; $n = 4$; ***E***: ○, -0.49 ± 0.01 mV/ms; ●, -0.47 ± 0.01 mV/ms; $n = 4$). ***F***, ***G***, recordings from representative experiments, where each trace represents the average of eight consecutive responses obtained before and after LTP induction, in the absence (***F***, left) or presence of BDNF (***F***, right), or in the presence of MβCD (***G***, left) or MβCD + BDNF (***G***, right). Recordings under same-day conditions, but before and 60 min after LTP induction are superimposed; recordings obtained before LTP are indicated by the dotted lines. All recordings in ***F*** were obtained from a single slice at the points indicated in ***D***. All recordings in ***G*** were obtained from a single slice at the points indicated in ***E***. Each recording is composed of the stimulus artifact, followed by the presynaptic volley, and the fEPSP. ***H***, Comparison of the effect of BDNF on LTP in the absence or presence of MβCD, as indicated. $^*p < 0.05$, compared with the first column.

As occurs with A_{2A} receptor agonists (Diógenes et al., 2004; Fontinha et al., 2008) and cAMP (Boulanger and Poo, 1999b), high-frequency neuronal firing triggers facilitatory actions of BDNF at excitatory synapses (Figurov et al., 1996; Boulanger and Poo, 1999b; Nagappan and Lu, 2005; Matsumoto et al., 2006). We therefore investigated whether TrkB receptors could be targeted to lipid rafts as a consequence of intense synaptic activity. We observed that high-frequency stimulation of hippocampal slices results in a higher density of both TrkB and pTrkB receptors

in lipid rafts, an effect completely abolished when endogenous extracellular adenosine was removed. To our knowledge, this is the first demonstration of activity-dependent recruitment and activation of TrkB receptors in lipid rafts, and notably, this is fully dependent on the presence of extracellular adenosine.

The levels of extracellular adenosine at synapses are tightly regulated and fluctuate according to the rate of neuronal firing (for a review, see Sebastiao and Ribeiro, 2009b). On high-frequency neuronal firing, the release of the adenosine precursor ATP is increased (Wieraszko et al., 1989), A_{2A} receptor activation is favored (Correia-de-Sá et al., 1996), and adenosine inactivation systems are inhibited (Pinto-Duarte et al., 2005). We demonstrated that TrkB translocation to lipid rafts is facilitated by high-frequency neuronal firing, A_{2A} receptor activation, and adenylate cyclase/cAMP transduction pathway, the transducing system operated by A_{2A} receptors. Furthermore, the actions of BDNF on glutamate release and synaptic plasticity require both lipid raft integrity and endogenous extracellular adenosine. Altogether, the data reported here strongly suggest that A_{2A} receptor-induced TrkB translocation to lipid rafts plays an important part in the mechanism through which enhanced neuronal activity, A_{2A} receptor activation, and cAMP facilitate BDNF actions at active synapses.

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