

Regulated release of BDNF by cortical oligodendrocytes is mediated through metabotropic glutamate receptors and the PLC pathway

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ABSTRACT

A number of studies suggest that OLGs (oligodendrocytes), the myelinating cells of the central nervous system, are also a source of trophic molecules, such as neurotrophins that may influence survival of proximate neurons. What is less clear is how the release of these molecules may be regulated. The present study investigated the effects of BDNF (brain-derived neurotrophic factor) derived from cortical OLGs on proximate neurons, as well as regulatory mechanisms mediating BDNF release. Initial work determined that BDNF derived from cortical OLGs increased the numbers of VGLUT1 (vesicular glutamate transporter 1)-positive glutamatergic cortical neurons. Furthermore, glutamate acting through metabotropic, and not AMPA/kainate or NMDA (*N*-methyl-*D*-aspartate), receptors increased BDNF release. The PLC (phospholipase C) pathway is a key mediator of metabotropic actions to release BDNF in astrocytes and neurons. Treatment of OLGs with the PLC activator *m*-3M3FBS [*N*-(3-trifluoromethylphenyl)-2,4,6-trimethylbenzenesulfonamide] induced robust release of BDNF. Moreover, release elicited by the metabotropic receptor agonist ACPD [*trans*-(1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid] was inhibited by the PLC antagonist U73122, the IP₃ (inositol triphosphate 3) receptor inhibitor 2-APB (2-aminoethoxydiphenylborane) and the intracellular calcium chelator BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-kis(acetoxymethyl ester)]. Taken together, these results

suggest that OLG lineage cells release BDNF, a molecule trophic for proximate neurons. BDNF release is regulated by glutamate acting through mGluRs (metabotropic glutamate receptors) and the PLC pathway. Thus glutamate and BDNF may be molecules that support neuron-OLG interactions in the cortex.

Key words: brain-derived neurotrophic factor (BDNF), metabotropic glutamate receptor, oligodendrocyte, phospholipase C.

INTRODUCTION

OLGs (oligodendrocytes) are known classically as the myelinating cells of the CNS (central nervous system). As such, they play an important role in increasing the conduction velocity of the action potential by forming myelin sheaths, and thereby promoting saltatory conduction down the axon. In recent years, however, OLG lineage cells have begun to be appreciated as an important source of trophic factors. For example, growth factors such as GDNF (glial cell line-derived neurotrophic factor), IGF-1 (insulin-like growth factor-1), TGF- β (transforming growth factor- β) and others are expressed by OLG lineage cells in culture (da Cunha et al., 1993; McKinnon et al., 1993; Shinar and McMorris, 1995; Raabe et al., 1997; Hayase et al., 1998; Strelau and Unsicker, 1999; Wilkins et al., 2001; Wilkins et al., 2003).

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Abbreviations: ACPD, *trans*-(1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; 2-APB, 2-aminoethoxydiphenylborane; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-kis(acetoxymethyl ester); BDNF, brain-derived neurotrophic factor; BF, basal forebrain; CC, corpus callosum; CNS, central nervous system; DCG-IV, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; GDNF, glial cell line-derived neurotrophic factor; IGF-1, insulin-like growth factor-1; IP₃, inositol triphosphate; MCPG, (*S*)- α -methyl-4-carboxyphenylglycine; MEM, minimal essential medium; mGluR, metabotropic glutamate receptor; *m*-3M3FBS, *N*-(3-trifluoromethylphenyl)-2,4,6-trimethylbenzenesulfonamide; NM-15, nutrient medium-15; NMDA, *N*-methyl-*D*-aspartate; NSFM, neuron serum-free medium; NT, neurotrophin; OCM, oligodendrocyte-derived conditioned medium; OLG, oligodendrocyte; OSFM, OLG serum-free medium; PLC, phospholipase C; VAMP2, vesicle-associated membrane protein 2; VGLUT1, vesicular glutamate transporter 1.

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Studies in our laboratory and others have revealed that NTs (neurotrophins) are also OLG lineage cell-derived (Byravan et al., 1994; Dai et al., 2001, 2003). We have found that NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor) and NT3 mRNAs are expressed by BF (basal forebrain) and CC (corpus callosal) OLGs, as well as OLGs in the frontal, cingulate and parietal cortical regions at postnatal ages *in vivo*. NT proteins and mRNAs are associated with BF OLGs in culture (Dai et al., 2001, 2003) and BDNF and NT3 produced by BF OLGs enhance the survival and function of BF cholinergic neurons (Dai et al., 2003). Thus the results suggest that OLG-derived NTs are trophic for nearby neurons in the CNS.

What is not as clear is how release of the NTs may be regulated. We suggest that neurotransmitters may play a role. OLGs express a number of neurotransmitter receptors and OLG function and development are influenced by neuronal signals (Patneau et al., 1994; Ghiani et al., 1999; Ragheb et al., 2001; Rogers et al., 2001; Stevens et al., 2002; Luyt et al., 2003; Deng et al., 2004; Karadottir and Attwell, 2007; Luyt et al., 2007). Of particular interest to us is that glutamate receptors are present on OLG lineage cells (Yoshioka et al., 1995; Matute et al., 1997; Luyt et al., 2003; Deng et al., 2004; Karadottir et al., 2005; Salter and Fern, 2005; Luyt et al., 2006; Micu et al., 2006). Studies on other cell types suggest that such receptors may regulate trophic content. For example, glutamate increases BDNF release in hippocampal neurons and in glial cells, such as astrocytes, Schwann cells and Muller cells (Canossa et al., 2001; Taylor et al., 2003; Verderio et al., 2006; Jean et al., 2009). BDNF is of interest because it is a factor that influences neuronal survival, proliferation and differentiation (Alderson et al., 1990; Hyman et al., 1991; Friedman et al., 1993; Bartkowska et al., 2007). Regulation of release of this factor by OLGs, therefore, may have important roles on proximate neurons.

In the present study, we investigated the possibility that glutamate signals may influence BDNF release from cortical OLGs. We report that OLG-derived BDNF increases numbers of VGLUT1(+) (where VGLUT1 is vesicular glutamate transporter 1) glutamatergic neurons. Moreover, glutamate, through the activation of metabotropic receptors and the PLC (phospholipase C) pathway, increases BDNF release by OLGs.

MATERIALS AND METHODS

Experimental animals

Time-mated Sprague–Dawley rats (Hilltop Laboratories) were housed in clear plastic cages with corncob bedding and nestlets. Food and water were available *ad libitum*. The animals were managed by the UMDNJ/Robert Wood Johnson Animal Facility, which is accredited by AAALAC (the Association for Assessment and Accreditation of Laboratory

Animal Care). Animal transportation, maintenance, husbandry and housing were in compliance with the Laboratory Animal Welfare Act (PL 89-544; PL-91-579). Use of animals was also in compliance with the National Institutes of Health guidelines (NIH Manual Chapter 4206).

OLG-enriched cultures

OLGs were purified using a modification (Gallo et al., 1996) of the method of (McCarthy and de Vellis, 1980). Postnatal day 1 rat pups were killed by hypothermia-induced anaesthesia followed by exsanguination. The frontal, parietal and cingulate cortices were dissected, mechanically dissociated and plated on to 75 cm² flasks precoated with poly-D-lysine (0.1 mg/ml). The dissociated cells were cultured in NM-15 (nutrient medium-15), containing MEM (minimal essential medium) with Earle's salts and L-glutamine (Cellgro), supplemented with 15% (v/v) heat-inactivated fetal bovine serum, penicillin/streptomycin (0.5 units/ml and 0.5 µg/ml respectively) and glucose (6 mg/ml). Cells were maintained at 37°C in a 95% air and 5% CO₂ humidified incubator for 12 days. Fresh NM-15 was added every 3–4 days. At the end of day 12, the mixed cultures were shaken overnight (14–16 h) at 250 rev./min in order to remove supernatant containing OLG lineage cells and microglia. To further purify the OLGs, the supernatant was plated on to uncoated 150 mm polystyrene dishes at room temperature (22°C) for 90 min for microglial attachment. OLG lineage cells remaining in the supernatant were collected, centrifuged at 1000 g at 22°C for 10 min, and plated on to 10 cm poly-D-lysine-coated dishes at 3.0 × 10⁶ cells per dish. Cells were expanded for 48 h in NM-15. NM-15 was then replaced with OSFM (OLG serum-free medium) for 5 days, comprising a 1:1 mix of Ham's F12 (Gibco) and BME (basal medium Eagle; Invitrogen), penicillin/streptomycin (0.5 units/ml and 0.5 µg/ml respectively), insulin (25 µg/ml), transferrin (100 µg/ml), putrescine (60 µM), progesterone (20 nM), selenium (30 nM) and glucose (6 mg/ml) plus thyroxine (0.5 µM), triiodothyronine (0.08 µg/ml) and glutamine (1.5 mM). Cultures were highly enriched consisting of 91% OLG lineage cells with the remaining cells comprising ED1-positive microglia and GFAP-positive astrocytes.

Neuronal cultures

E17 (embryonic day 17) fetuses were obtained from time-mated Sprague–Dawley rats. Once the rats were killed by CO₂ inhalation, the embryos were removed and the frontal, cingulate and parietal cortices were dissected, mechanically dissociated and plated on to 12-well poly-D-lysine-coated plates at 5.0 × 10⁵ cells per well. Cells were plated in NSFM (neuron serum-free medium) comprising a 1:1 mixture of Ham's F12 medium (Gibco) and Eagle's MEM, transferrin (100 µg/ml), insulin (25 µg/ml), selenium (30 nM), putrescine (60 µM), progesterone (20 nM), glucose (6 mg/ml) and penicillin/streptomycin (0.5 units/ml and 0.5 µg/ml respectively).

OCM (OLG-derived conditioned medium)

To prepare OCM, cortical OLG-enriched cultures were rinsed three times with PBS to remove the serum. The cultures were then incubated with OSFM for 5 days. To evaluate the effect of OCM on neuronal cultures, OSFM (1 part OSFM/1 part NSFM) or OCM (1 part OCM/1 part NSFM) was added to the cultures for 5 days. To specifically evaluate the role of BDNF in OCM, a neutralizing anti-BDNF antibody (Promega) was pre-incubated for 2 h (at 4°C) with OCM before adding the mixture to neuronal cultures.

Culture treatments

Before drug treatment of cultures, OLGs grown in OSFM for 5 days were washed twice with PBS. MEM (5 ml) was added to each of two dishes (10 cm diameter), which were combined for each control and treatment group. When cultures were treated with antagonist, the antagonist or vehicle was added in OSFM for the time specified. The agonist was then added alone or with antagonist in MEM for 10 min.

Treated and control media were collected and concentrated to 50 µl at the same time and in an identical manner, using Amicon Ultra Centrifugal Devices 5 kDa (Millipore) or Vivaspin 20, 5000 MWCO (Sartorius Biolab Products; MWCO is molecular weight cut-off). BDNF levels were evaluated by ELISA or Western blot analysis. Owing to the variability within the controls of individual experiments, each treatment condition was compared with its own control within the same experiment.

ELISA

ELISA was performed using the BDNF Emax ImmunoAssay System kit (Promega), according to the manufacturer's protocol.

Western blot analysis

BDNF release

Media from ACPD [*trans*-(1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid]- or vehicle-treated OLGs were collected and concentrated as indicated above. Equal volumes of concentrate (25 µl) were subjected to electrophoresis using NuPAGE 12% Bis-Tris gels (Invitrogen) and were transferred to an Immobilon-P transfer membrane (Millipore). BDNF in the membrane was detected using an anti-BDNF antibody (1:100; Santa Cruz Biotechnology) and ECL (enhanced chemiluminescence; PerkinElmer).

Activated caspase 3

Treatment medium was removed after 10 min, plates were washed three times in PBS and OSFM was added for an additional 24 h. OLGs were harvested and lysed in buffer containing EDTA (10 mM), EGTA (2 mM), SDS (0.1%), CHAPS

(1%), Nonidet P40 (0.5%), Triton X-100 (1%), Tris/HCl (50 mM), NaCl (150 mM), leupeptin (10 µg/ml), aprotinin (10 µg/ml), soyabean trypsin inhibitor (20 µg/ml), NaF (50 µM), PMSF (1 mM), microcystin (0.5 µM) and Na₃VO₄ (0.5 mM). The cell debris was centrifuged and the supernatant was collected. The protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Thermo Scientific). Proteins were subjected to electrophoresis using a 12% NuPAGE Bis-Tris gel and transferred as described above. The membrane was stained with an activated anti-(caspase 3) antibody (1:1000; Cell Signaling Technology) and protein was detected using ECL. To normalize for loading, the same blots were stripped and evaluated for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein levels using an anti-GAPDH antibody (1:1000; Biodesign). Western blot bands were quantified densitometrically using Quantity One 4.2.1 software and Universal Hood Gel Documentation Systems (Bio-Rad Laboratories).

Immunocytochemistry

OLGs were fixed in 4% (w/v) PFA (paraformaldehyde) for 1 h at room temperature. Glutamate receptors were evaluated using antibodies (1:200) against mGluR1 (where mGluR is metabotropic glutamate receptor), mGluR2/3, mGluR5, GluR6/7 (Upstate), GluR1–4 and NR3A (Santa Cruz Biotechnology). VGLUT1(+) neurons were detected using anti-VGLUT1 antibody (1:5000; Chemicon). Vesicular proteins were evaluated using anti-(secretogranin II) antibody [1:1000; a gift from Dr Tsuyoshi Watanabe (Department of Anatomy, Asahikawa Medical College, Asahikawa, Japan)], using the methods as described in Sakai et al. (2003), anti-(chromogranin A) (1:200; Dako) or anti-VAMP2 (vesicle-associated membrane protein 2, 1:200; Synaptic Systems) antibodies. Immunopositive cells were detected using the ABC (avidin–biotin complex) kit (Vector laboratories), and DAB (diaminobenzidine) precipitation (Sigma). Negative staining controls were treated identically, but received no primary antibody.

Quantification

VGLUT1(+) neurons

VGLUT1(+) cell numbers were determined by counting random fields within each well of 12-well plates under a phase-bright microscope. The region counted represented 6% of the well surface area or 20 mm². Since the number of VGLUT1 cells varied in controls from experiment to experiment, each treatment group was expressed as a percentage of its own control within the same experiment.

Glutamate- and ACPD-treated OLGs

Numbers were determined by counting OLG-like cells in random fields of the individual wells of 12-well plates under

a phase-bright microscope. The region counted represented 2.5% of the well surface area or 9 mm². Each treatment group was expressed as a percentage of its own control within the same experiment.

Treatment drugs used

The general mGluR agonist ACPD, the Group I mGluR agonist, DHPG [(*RS*)-3,5-dihydroxyphenylglycine], the Group II mGluR agonist, DCG-IV [(2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)-glycine], the general mGluR antagonist, MCPG [(*S*)- α -methyl-4-carboxyphenylglycine], the PLC inhibitor U73122 and the IP₃ (inositol trisphosphate) receptor antagonist 2-APB (2-aminoethoxydiphenylborane) were purchased from Tocris. Glutamate, kainate, NMDA (*N*-methyl-D-aspartate), the calcium chelator, BAPTA/AM [1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] and the PLC agonist m-3M3FBS [*N*-(3-trifluoromethylphenyl)-2,4,6-trimethylbenzenesulfonamide], were purchased from Sigma. U73122, 2-APB, BAPTA/AM and m-3M3FBS were dissolved in DMSO (dimethyl sulfoxide). Their respective control cultures were treated identically, with DMSO added to MEM as a vehicle. Doses for each drug and times of pretreatment were determined by dose-response and time of action experiments.

Statistical analysis

Statistical significance was determined using a paired Student's *t* test or ANOVA and a Fisher's test as appropriate using StatView 5.0.1 software.

RESULTS

Effects of OCM-derived BDNF on the numbers of VGLUT1(+) glutamatergic cortical neurons

BDNF influences the development and survival of cortical neurons (Barnabe-Heider and Miller, 2003; Liu et al., 2003; Bartkowska et al., 2007). Moreover, BDNF derived from BF OLGs enhances the survival of proximate neurons (Dai et al., 2003). To determine whether BDNF derived from cortical OLGs has similar actions, we evaluated the effects of OCM on the numbers of VGLUT1(+) neurons. These neurons represent one population of glutamate-producing neurons in the cortex (Fremeau et al., 2004; Liguz-Lecznar and Skangiel-Kramska, 2007) and are reported to express TrkB in culture (Swanwick et al., 2004; Gomes et al., 2006). OCM elicited an increase in VGLUT1(+) neurons, that was reduced in the presence of neutralizing anti-BDNF antibody, suggesting that BDNF in OCM is trophic to VGLUT1(+) glutamatergic neurons (Figure 1).

OLGs express vesicular proteins and respond to glutamate by releasing BDNF

Since BDNF secreted by OLGs is trophic to glutamate-producing cells, we considered whether glutamate may influence the release of BDNF from OLGs. Neurons and astrocytes, which release BDNF in response to glutamate, express vesicular proteins associated with regulated release (Hepp and Langley, 2001; Montana et al., 2006). Some of these proteins are reported in OLGs and are thought to be involved in myelin assembly (Madison et al., 1996, 1999; Larocca and Rodriguez-Gabin, 2002; Sloane and Vartanian, 2007; Feldmann et al., 2009; Schardt et al., 2009). Cortical OLGs in our cultures similarly exhibited VAMP2, in addition to chromogranin A and secretogranin II (Figure 2A), suggesting that cortical OLGs may possess the capacity for regulated release of BDNF. To evaluate whether regulated release occurs in OLGs, cortical OLGs were treated for 10 min with glutamate (100 μ M) and BDNF in the medium was assayed. Levels of BDNF were elevated in the medium (Figure 2B), indicating that glutamate may regulate the release of BDNF from OLGs.

mGluRs mediate BDNF release

Glutamate acting through ionotropic receptors mediates BDNF release from Schwann cells (Verderio et al., 2006) and, through mGluRs and ionotropic receptors, may mediate release from neurons and astrocytes (Canossa et al., 2001; Bergami et al., 2008; Jean et al., 2009). To determine which glutamate receptor may mediate release in OLGs, we first evaluated the receptors present. When evaluated immunocytochemically, OLGs were found to

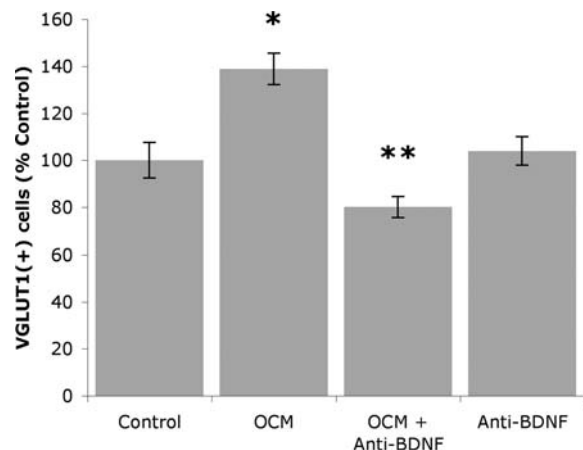


Figure 1 OCM increases the number of VGLUT1(+) neurons

VGLUT1(+) neurons/mm² are increased when grown in cortical OCM for 5 days. The OCM effect is blocked by an anti-BDNF neutralizing antibody. Values are the percentage of the control \pm S.E.M (*n*=4). In total, three cultures were assayed per condition in each experiment. **P*<0.05, significantly different compared with the control. ***P*<0.05, significantly different compared with OCM alone. Data were analysed by ANOVA and Fisher's test.

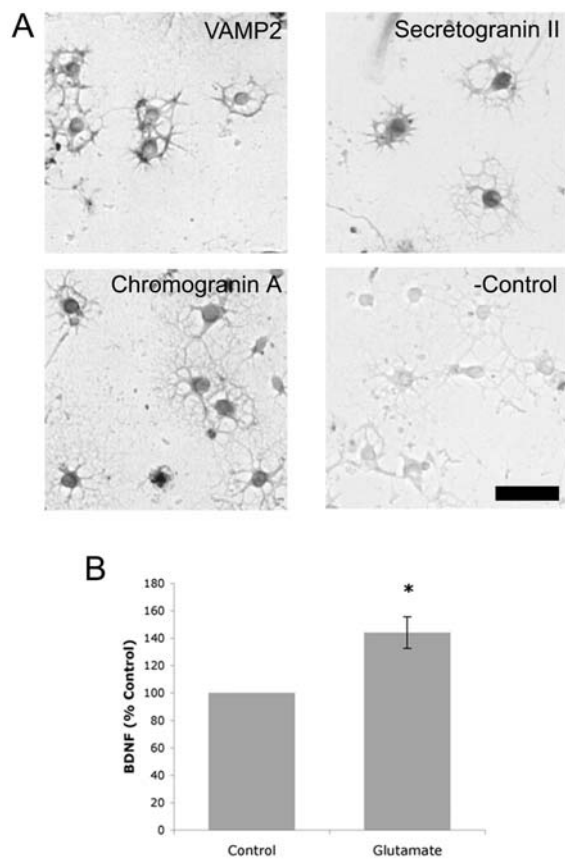


Figure 2 Cortical OLGs express vesicular proteins and respond to glutamate by the release of BDNF

(A) Cortical OLGs express vesicular proteins. Immunocytochemical analysis revealed the presence of VAMP2, secretogranin II and chromogranin A. The representative negative control did not receive primary antibody. Scale bar=50 μ m. (B) Glutamate (100 μ M) elicits the release of BDNF after 10 min stimulation as determined by ELISA ($n=6$). Values are the mean BDNF (pg/50 μ l) levels expressed as a percentage of the control \pm S.E.M. * $P<0.05$, significantly different compared with the control. Data were analysed using a paired Student's t test.

express ionotropic receptors. Receptor subunits of kainate and AMPA, as well as NMDA receptors, were detected (Figure 3A). Moreover, both Group I (mGluR1 and 5) and Group II (mGluR 2/3) mGluRs were identified on the cells (Figure 3B).

To determine which receptors may influence OLGs, cells grown for 5 days in OSFM were treated for 10 min with kainate (200 μ M), NMDA (100 μ M) or ACPD (10 μ M), an agonist that stimulates Group I and Group II mGluRs. Neither kainate nor NMDA had any effect on BDNF release as determined by ELISA (Figures 4A and 4B). However, ACPD increased the amount of BDNF secreted into the medium within 10 min of stimulation (Figure 4C). Similar results were obtained by Western blot analysis (Figure 4D), suggesting that in cortical OLGs, metabotropic, but not ionotropic, glutamate receptors mediate release.

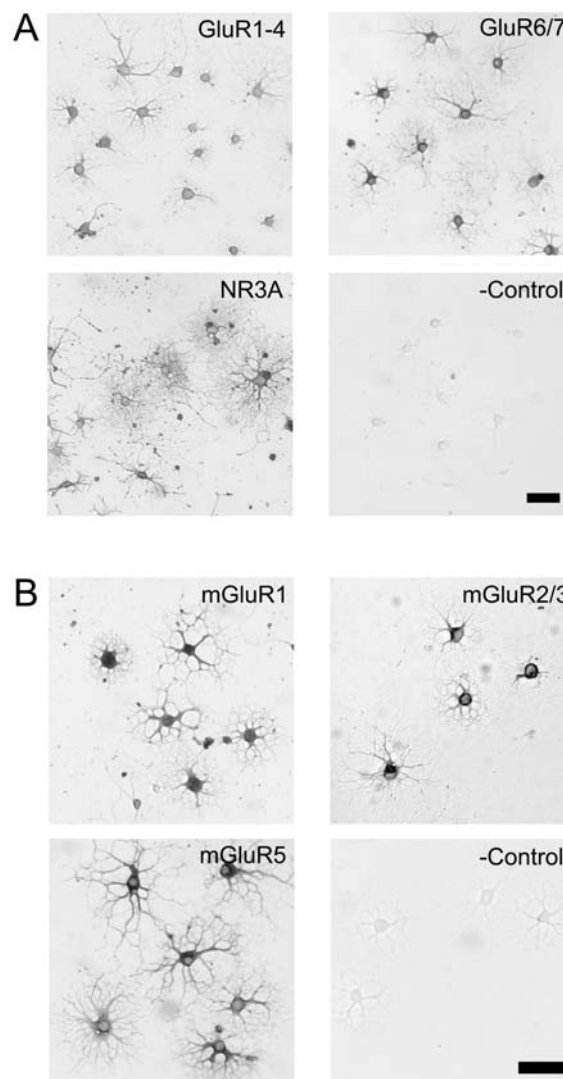


Figure 3 Cortical OLGs express glutamate receptors in culture

Immunocytochemical analysis revealed the presence of (A) ionotropic AMPA GluR1-4, kainate GluR6/7 and NMDA NR3A subunits and (B) mGluR1, mGluR2/3 and mGluR5 in OLGs. The representative negative control did not receive primary antibody. Scale bar=50 μ m.

To further confirm mediation by mGluRs, the effects of MCPG, a Group I and Group II metabotropic antagonist, was evaluated. MCPG (300 μ M) inhibited the actions of glutamate, confirming the involvement of mGluRs in elevating extracellular BDNF (Figure 4E).

ACPD is an agonist for both Group I and Group II mGluRs. To determine which group mediates release, we used the Group I-specific agonist DHPG and the Group II-specific agonist DCG-IV. Stimulation of OLGs for 10 min with DHPG (100 μ M), but not DCG-IV (10 μ M), increased extracellular BDNF (Figure 5), suggesting that the release of BDNF occurs through the activation of Group I mGluRs and not Group II mGluRs.

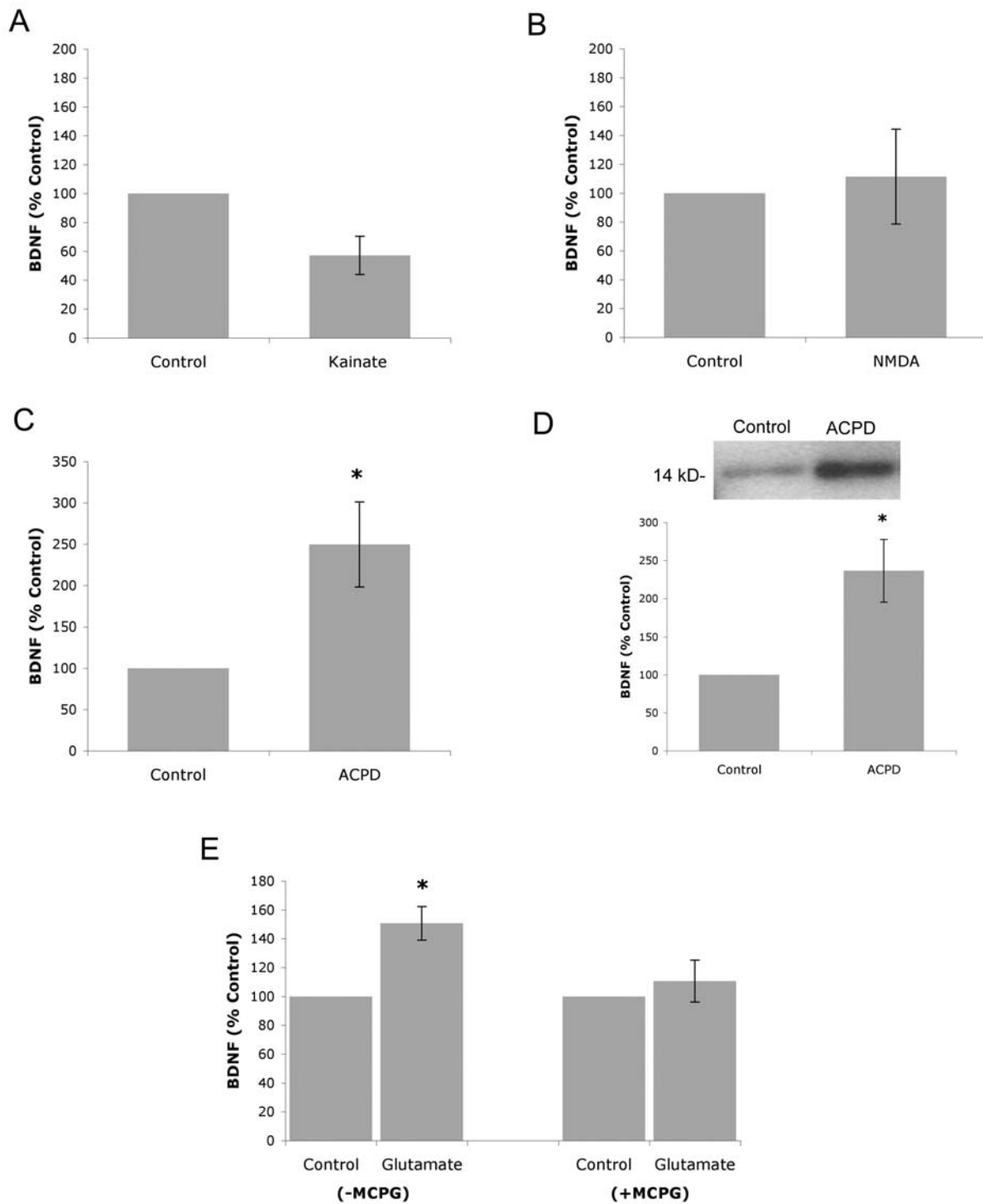


Figure 4 BDNF release by cortical OLGs in response to glutamate is mediated by metabotropic, and not ionotropic, receptors
 A 10 min stimulation with 200 μ M kainate (A) or 100 μ M NMDA (B) does not elicit BDNF release, as determined by ELISA ($n=5$ for A and $n=3$ for B). NMDA-treated cells and their vehicle control were co-treated with glycine (5 μ M). (C) ACPD (10 μ M) elicits BDNF release in the medium after 10 min stimulation, as determined by ELISA ($n=9$). (D) ACPD (10 μ M for 10 min) elicits an increase in BDNF release as determined by Western blot analysis. The histogram represents the densitometric analysis of three independent Western blot experiments. (E) Pretreatment with MCPG (300 μ M for 4 h), inhibits the glutamate-induced BDNF release as determined by ELISA ($n=5$). Glutamate (-MCPG) is compared with control (-MCPG), whereas glutamate (+MCPG) is compared with control (+MCPG) in the same experiment. Values represent BDNF (pg/50 μ l) levels expressed as a percentage of the control \pm S.E.M. * $P<0.05$, significantly different compared with the control. Data were analysed using a paired Student's t test.

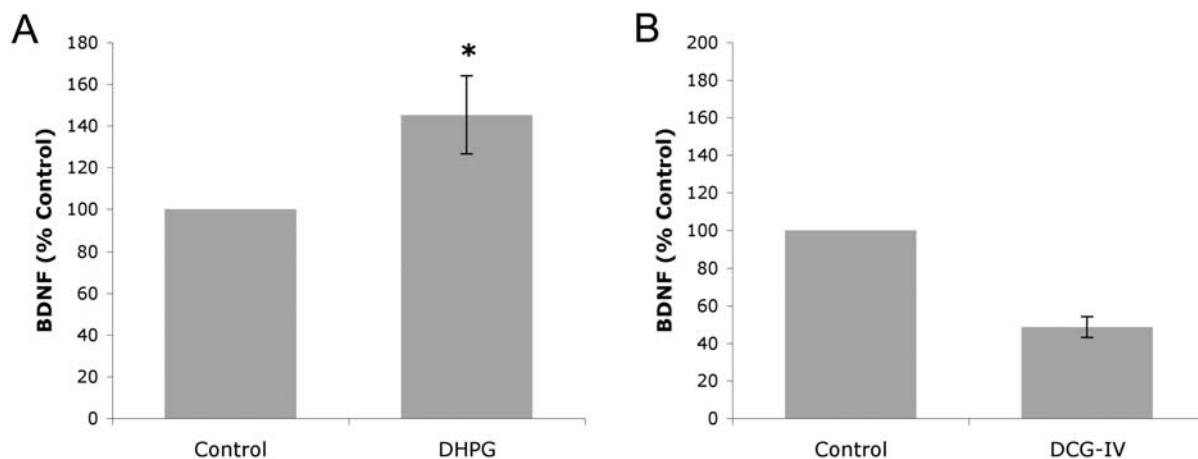


Figure 5 Group I, but not Group II, mGluRs mediate BDNF release (A) DHPG (100 μ M) induces BDNF release into the medium after 10 min stimulation, as determined by ELISA ($n=6$). * $P<0.05$, significantly different compared with the control. (B) DCG-IV (10 μ M) does not induce BDNF release after 10 min stimulation, as determined by ELISA ($n=3$). Values are BDNF (pg/50 μ l) levels expressed as a percentage of the control \pm S.E.M. Data were analysed using a paired Student's t test. * $P<0.05$, significantly different compared with the control.

Metabotropic-mediated release occurs through the PLC pathway

Group I mGluRs are coupled to the PLC pathway (Chuang et al., 2001; Hannan et al., 2001; Hermans and Challiss, 2001). Moreover, stimulation of metabotropic receptors through this pathway is responsible for the release of BDNF in hippocampal neurons (Canossa et al., 2001) and astrocytes (Jean et al., 2009). Activation of PLC induces the cell membrane hydrolysis of PIP₂ (phosphatidylinositol biphosphate) into IP₃ and DAG (diacylglycerol). IP₃ then binds to its receptors on the endoplasmic reticulum causing calcium release.

To evaluate whether the PLC pathway is involved in the mGluR-mediated effect on BDNF, we used the PLC pathway activator m-3M3FBS. Stimulation with m-3M3FBS (25 μ M for 20 min), induced a 2.5-fold increase in the amount of BDNF released compared with the control (Figure 6A). Moreover, in the presence of the PLC inhibitor U73122 (1 μ M), the IP₃ receptor inhibitor 2-APB (150 μ M), and the intracellular calcium chelator BAPTA/AM (80 μ M) ACPD-induced increases in extracellular BDNF were blocked (Figures 6B–6D), suggesting that the PLC pathway is a critical mediator.

ACPD or glutamate treatment does not activate caspase 3 or affect cell number

Glutamate has been reported to be toxic to OLGs. The degree of toxicity depends on the OLG population being evaluated, the length of glutamate exposure and the receptor subtype being activated. AMPA/kainate and NMDA receptors have been shown to mediate these toxic effects (Sanchez-Gomez and Matute, 1999; Fern and Moller, 2000; Deng et al., 2003; Rosenberg et al., 2003; Wosik et al., 2004; Karadottir et al., 2005; Salter and Fern, 2005; Deng et al., 2006; Micu et al., 2006).

To rule out the possibility that the release of BDNF is due to toxicity, OLGs were treated with glutamate, ACPD or vehicle control for 10 min and activated caspase 3 levels were evaluated at 24 h post-stimulation. Caspase 3 is a key executioner caspase activated during glutamate toxicity (Sanchez-Gomez et al., 2003). Caspase 3 was not activated in our cultures and the levels of caspase 3 were not different among glutamate, ACPD and vehicle control groups. In contrast, staurosporine, known to activate caspase 3 and used as a positive control, did activate caspase 3 (Figures 7A and 7B).

In other studies total cell numbers were determined 24 h post-stimulation. No changes were observed among the different treatment groups (Figure 7C), suggesting that the experimental conditions used in the present study do not kill the OLGs.

DISCUSSION

The present study indicates that cortical OLG-derived BDNF provides trophic support to proximate neurons in culture. As such, it complements previous work indicating that cortical, CC and BF OLGs contain BDNF mRNA *in vivo* and that BF OLGs release BDNF that supports proximate BF neurons (Dai et al., 2003). Moreover, the present study demonstrates that the release of BDNF is regulated by glutamate in a process mediated by metabotropic receptors and the PLC pathway. It is known that OLGs express a host of other growth factors *in vivo* and *in vitro* (da Cunha et al., 1993; McKinnon et al., 1993; Shinar and McMorris, 1995; Raabe et al., 1997; Hayase et al., 1998; Strelau and Unsicker, 1999; Dougherty et al., 2000; Wilkins et al., 2001; Dai et al., 2003; Wilkins et al.,

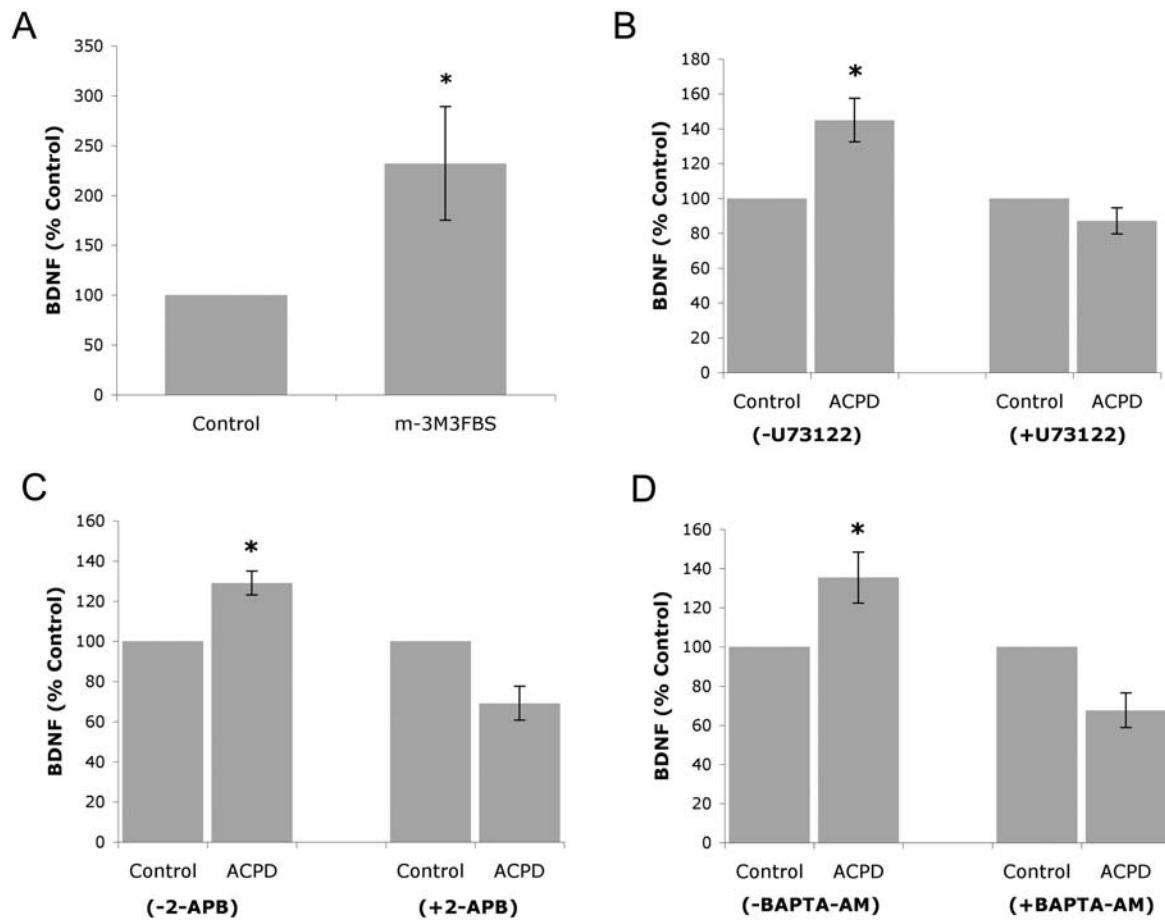


Figure 6 mGluRs mediate BDNF release through the PLC pathway (A) The PLC agonist m-3M3FBS (25 μ M) induces BDNF release after 20 min stimulation, as determined by ELISA ($n=8$). (B) The PLC inhibitor U73122 (1 μ M with 15 min pretreatment) inhibits the ACPD-induced BDNF release in OLGs, as determined by ELISA ($n=3$). (C) The IP₃ antagonist 2-APB (150 μ M with 30 min pretreatment) inhibits the ACPD-induced BDNF release in OLGs, as determined by ELISA ($n=3$). (D) The intracellular calcium chelator BAPTA-AM (80 μ M with 3 h pretreatment) inhibits the ACPD-induced BDNF release in OLGs, as determined by ELISA ($n=3$). Each treatment condition is compared with its own control, not treated with agonist. In all experiments the effects of ACPD in comparison with the control were run with or without inhibitor in the same experiment. Values represent the mean BDNF (pg/50 μ l) levels expressed as a percentage of the control \pm S.E.M. * $P < 0.05$, significantly different compared with the control. Data were analysed using a paired Student's t test.

2003) and that some of these, when released from OLG lineage cells, support neurons (Wilkins et al., 2001, 2003). However, to the best of our knowledge, this is the first report that release of BDNF, or any trophic factor released from OLGs, can be regulated within 10 min of stimulation. The studies suggest that OLGs may release such substances in a manner critically sensitive to glutamate and possibly neurostimulation.

OLGs are responsive to neuronal signals

That OLGs are responsive to neuronal signals is consistent with a rich literature which indicates that neurons can regulate OLG lineage cells throughout development. Thus it has been known for some time that optic nerve transection results in loss of OLG progenitors and mature OLGs (David et

al., 1984), and elimination of electrical activity of retinal ganglion cells or transection of the optic nerve results in decreases in progenitor proliferation (Barres and Raff, 1993).

Moreover, a variety of ion channels have been detected in OLG lineage cells (Barres et al., 1988, 1989, 1990; Kettenmann et al., 1991), as well as neurotransmitter receptors (Patneau et al., 1994; Ghiani et al., 1999; Ragheb et al., 2001; Rogers et al., 2001; Stevens et al., 2002; Luyt et al., 2003; Deng et al., 2004; Karadottir and Attwell, 2007; Luyt et al., 2007). Responses to transmitter substances are now being defined. For example, with respect to glutamate, OLG precursors lying in close proximity to synaptic specializations of hippocampal (Bergles et al., 2000) or callosal axons (Ziskin et al., 2007) are responsive to glutamate released from these sites. Culture

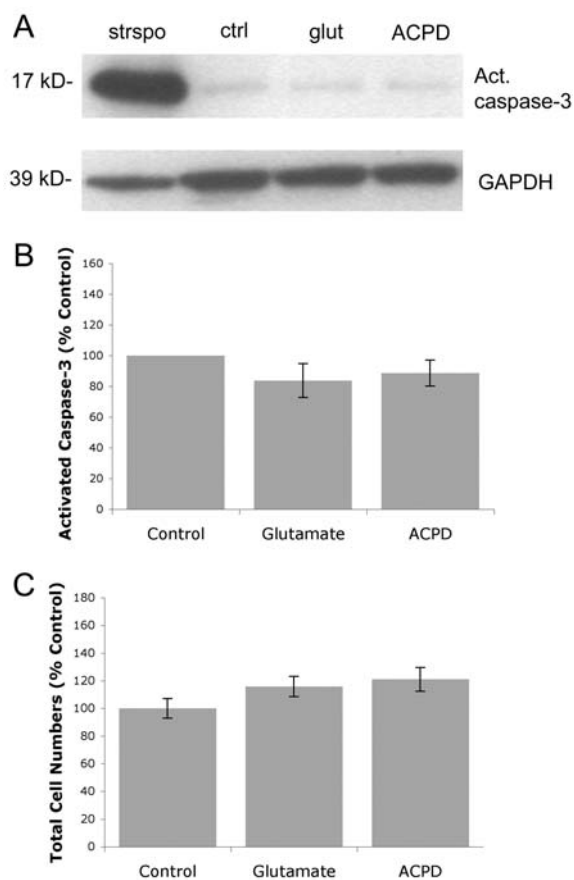


Figure 7 Treatment of OLGs with glutamate (100 μ M) or ACPD (10 μ M) for 10 min does not activate caspase 3 or affect cell number (A) Western blot analysis of cell lysates at 24 h post-stimulation reveals no change in activated (Act.) caspase 3 levels in control vehicle (ctrl), glutamate (glut)- or ACPD-treated OLGs. Staurosporine (300 nM for 4 h) is used as a positive control (strspo). (B) Densitometric analysis of three independent Western blot experiments. Values are the means \pm S.E.M. (C) Total cell numbers evaluated at 24 h post-stimulation reveal no difference among control vehicle, glutamate- and ACPD-treated groups. Values are the mean cells/mm² expressed as a percentage of the control \pm S.E.M. Results represent three independent experiments and each condition within an experiment was performed with three cultures. Data were analysed using ANOVA.

studies reveal that AMPA/kainate and metabotropic receptors mediate the influx of calcium (Butt and Tutton, 1992; Patneau et al., 1994; Liu et al., 1997; Luyt et al., 2006). Glutamate, through ionotropic receptors, inhibits proliferation and stimulates OLG progenitor migration (Liu and Almazan, 1995; Gallo et al., 1996; Gudz et al., 2006). It may also stimulate death (Yoshioka et al., 1995; Matute et al., 1997). Metabotropic receptors are found prominently on young OLGs (Deng et al., 2004; Luyt et al., 2006). Interestingly, with respect to the present study, stimulation of these metabotropic receptors limits AMPA/kainate receptor-mediated OLG cell death and excitotoxicity (Kelland and Toms, 2001; Deng et al., 2004; Luyt et al., 2006), suggesting that these receptors may mediate a protective role.

The release of BDNF in response to glutamate is consistent with responses of neurons and astrocytes

In the present study we report that the release of BDNF is dependent on metabotropic receptors and the PLC pathway, which results in the mobilization of calcium from intracellular stores. Although ionotropic receptors are present on the OLGs, they do not appear to mediate release since neither stimulation with kainate nor NMDA elicited release. In contrast, treatment with the mGluR agonist, ACPD, resulted in an increase in extracellular BDNF and the effects of glutamate were completely abolished by the mGluR antagonist, MCPG. These studies complement others using neurons and astrocytes which suggest that, in other cells, glutamate and mGluRs are involved in the release of several factors, including neurotrophins (Bruno et al., 1998; Ciccarelli et al., 1999; Canossa et al., 2001; Jean et al., 2009). Moreover, where it was studied, release of BDNF through mGluRs was dependent on the PLC pathway and the mobilization of calcium (Canossa et al., 2001; Jean et al., 2009).

Cortical OLGs are reported to express a variety of vesicular proteins (Madison et al., 1996, 1999; Sloane and Vartanian, 2007; Feldmann et al., 2009; Schardt et al., 2009), many of which are developmentally regulated and may play a role in the regulation of myelin assembly (Larocca and Rodriguez-Gabin, 2002; Feldmann et al., 2009; Schardt et al., 2009). However, the presence of these proteins in OLGs in the present study suggests that they may also be capable of controlling the regulated secretion of BDNF seen previously in neurons and glial cells (Canossa et al., 2001; Hartmann et al., 2001; Brigadski et al., 2005; Verderio et al., 2006; Bergami et al., 2008; Jean et al., 2009). Although it has yet to be determined whether BDNF is associated with these or any other vesicular proteins in OLGs, VAMP2, secretogranin II and chromogranin A are associated with BDNF storage, trafficking and release. For example, BDNF was found to co-localize with chromogranin A in transfected AtT-20 cells, a mouse pituitary cell line (Goodman et al., 1996), and with secretogranin II in hippocampal neurons (Egan et al., 2003; Chen et al., 2004). In Schwann cells (Verderio et al., 2006) and astrocytes (Bergami et al., 2008) BDNF release is inhibited by tetanus toxin, illustrating a VAMP2-dependent mechanism. Interestingly, however, in Schwann cells glutamate induces release in a process dependent on ionotropic glutamate receptors (Verderio et al., 2006), suggesting differences in BDNF release in peripheral compared with central glia, although the role of metabotropic receptors was not examined.

Significance for the neuron–glia interaction *in vivo*

Previous studies indicate that BF OLG-derived conditioned media applied to the BF cholinergic neurons enhances survival and function of these cells (Dai et al., 2003). The observed effects were partly due to BDNF, as neutralizing

antibodies blocked trophic activity (Dai et al., 2003). The present study extends this observation to the cortex, by showing that cortical OCM applied to embryonic cortical neurons increased the numbers of VGLUT1(+) glutamatergic neurons, a result also dependent on BDNF. Others have implicated OLG-derived GDNF and IGF-1 as having similar roles (Wilkins et al., 2001, 2003). The results suggest that OLGs from multiple brain regions may provide support to proximate neurons.

Although these studies have been performed in culture, they may have bearing on situations *in vivo*. OLGs and their associated neurons are affected in a number of degenerative diseases such as multiple sclerosis and Alzheimer's disease (Ferguson et al., 1997; Trapp et al., 1998; Jellinger and Stadelmann, 2001; Sjoberg et al., 2005). Signals that underlie the cross-talk between neurons and OLGs remain poorly understood. The roles of integrins, the Notch and Jagged cell-cell interacting pathway, and growth factors in mediating OLG-neuron interactions during normal development and injury, are actively being explored (Wilkins et al., 2001, 2003; Bozzali and Wrabetz, 2004; Baron et al., 2005; Laursen and Ffrench-Constant, 2007). Our finding that OLGs may provide trophic support to their nearby neurons through the actions of NTs and neurotransmitters suggest another mechanism that may underlie this interaction. BDNF is an important modulator of survival and development (Alderson et al., 1990; Hyman et al., 1991; Friedman et al., 1993; Bartkowska et al., 2007) and mGluRs are protective to both neurons and OLGs following injury (Koh et al., 1991; Schroder et al., 1999; Deng et al., 2004). Continued understanding of this glutamate-BDNF cross-talk may provide an approach with which to enhance the viability of both OLG and proximate neurons damaged in a host of degenerative diseases of the CNS.

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