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Selective activation of metabotropic glutamate receptor 7 induces inhibition of cellular proliferation and promotes astrocyte differentiation of ventral mesencephalon human neural stem/progenitor cells

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ABSTRACT

Expression of group III metabotropic glutamate receptors (mGluR) was established by RT-PCR and immunocytochemistry on a cultured clonal human neural stem/progenitor cell (hNSPC) line derived from fetal ventral mesencephalon (VM). Selective activation of these receptors by the group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) prevented increases in cAMP levels following forskolin stimulation, suggesting these receptors are coupled to their canonical G-protein coupled signal transduction pathway. Tonic exposure of undifferentiated cultures to L-AP4 resulted in a decrease in cellular metabolism and proliferation in the absence of toxicity, as measured by MTT and LDH assays, in a dose-dependent manner. This was confirmed by a reduction in BrdU incorporation into nuclear DNA, suggestive of an anti-proliferative effect of L-AP4. This effect was rescued by co-addition of the broad-spectrum group III mGluR competitive antagonist (RS)-a-cyclopropyl-4-phosphonophenylglycine (CPPG), demonstrating a receptor-mediated mechanism, but not mimicked by application of the cell permeable cAMP analogue dibutyrl cAMP (db-cAMP). The potency of these effects of L-AP4 indicates that this is an mGlu7 subtype-mediated effect. Tonic exposure of undifferentiated cultures to the mGlu7 selective allosteric agonist N,N′-bis(diphenylmethyl)-1,2-ethanediamine dihydrochloride (AMN082), but not the mGlu4 selective allosteric agonist (±)-cis-2-(3,5-dicholorphenylcarbamoyl)cyclohexanecarboxylic acid (VU0155041), or the mGlu8 selective agonist (S)-3,4-dicarboxyphenylglycine ((S)-3,4-DCPG) resulted in an identical anti-proliferative effect to L-AP4, confirming the involvement of the mGlu7 subtype. In differentiating cultures, tonic exposure to L-AP4 or AMN082 resulted in a significant shift towards an astrocyte cell fate. The mGlu7 receptor therefore provides a new opportunity to influence the proliferation and differentiation of ventral mesencephalon-derived hNSPC.

1. Introduction

A major challenge in developmental neurobiology is the elucidation of extracellular signals that regulate the proliferation of neural stem/progenitor cells (NSPCs) and drive their differentiation towards specific phenotypes (Canudas et al., 2004). Identification of these signals may permit pharmacological manipulation of isolated NSPCs from the brain towards specific cell fates. This is significant as cell replacement therapy (CRT) using fetal NSPCs represents a potentially promising strategy for the treatment of neurodegenerative disorders, including Parkinson's disease (PD) (Arenas, 2010).

Glutamate, the major excitatory neurotransmitter in the brain influences proliferation, differentiation and survival of both embryonic and NSPCs (Schlett, 2006; Melchiorri et al., 2007). The action of glutamate in the central nervous system (CNS) is mediated through two classes of membrane bound receptors: (1) the ionotropic glutamate receptors (iGluR) which form ligand-gated cation channels and (2) metabotropic glutamate receptors (mGluR) which are coupled to second messenger signaling pathways through G-proteins (Conn and Pin, 1997). Substantial evidence exists for a role of iGluR in regulating the proliferation of both rodent hippocampal and cortical NSPCs, particularly N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors (Cameron et al., 1995; LoTurco et al., 1995; Kitayama et al., 2003, 2004; Gandhi et al., 2008).

Interestingly, emerging evidence demonstrates roles for mGluR beyond their normal synaptic functions in the CNS (Nicoletti et al., 2007). These receptors, which form part of the class C heptahelix G-

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protein-coupled receptor (GPCR) family are divided into three major subtypes: group I (mGluR1 and mGluR 5), group II (mGluR2 and mGluR 3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) based on their amino acid sequence homology, signal transduction pathway and pharmacology (Conn and Pin, 1997; Schoepp et al., 1999). Specifically, of the eight known mGluR subtypes, expression of group I mGluR (mGluR1 and 5), group II mGluR (mGluR3) and group III mGluR (mGluR4 and 8) have been reported in cultured NSPCs from various brain regions (Canudas et al., 2004; Di Giorgi Gerevini et al., 2004, 2005; Suzuki et al., 2006; Saxe et al., 2007; Gandhi et al., 2008; Nakamichi et al., 2008). Of these, selective activation of mGluR3 and 5 supports proliferation and survival of NSPCs isolated from the subventricular zone (SVZ) of embryonic mouse forebrain (Di Giorgi Gerevini et al., 2004, 2005) and the neocortex (Gandhi et al., 2008), whilst a similar role has also recently been proposed for group II mGluR (Brazel et al., 2005).

In the context of group III mGluR, selective activation of mGluR4 negatively regulates proliferation of immature rat cerebellar granule cells and may play a key role in cerebellar development (Canudas et al., 2004). Nakamichi et al. (2008) have also reported that selective activation of group III mGluR by the broad-spectrum group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) negatively regulates proliferation of fetal mouse neocortical progenitors and promotes differentiation towards a glial cell fate *in vitro* (Nakamichi et al., 2008).

Analyses of glutamate receptor functions in human NSPCs (hNSPC) are very limited, but activation of NMDA receptors (NMDAR) is reported to increase both proliferation and neurogenesis in cortical precursors isolated from the fetal human brain (Suzuki et al., 2006), in contrast to the effects reported for group III mGluR activation in rodent cells (Nakamichi et al., 2008). Intriguingly, activation of NMDAR during proliferation and differentiation of fetal midbrain-derived hNSPC enhances dopaminergic neurogenesis *in vitro* (Wegner et al., 2009). Thus, pharmacological manipulation of NMDAR signaling may facilitate production of dopamine (DA) neurons from fetal hNSPCs.

Conversely, it may hence be hypothesised that group III mGluR suppress the capacity of hNSPCs to self-renew and direct cells towards a glial cell fate, in apposition to glutamate signaling through NMDAR (Nakamichi et al., 2008). Consistent with this hypothesis, expression of group III mGluR has been reported in hNSPCs derived from the ventral midbrain (Wegner et al., 2009). However, to date no reports have examined the role of these receptors in either proliferation or differentiation of NSPCs derived from the human fetal midbrain. Thus, the aim of the current study was to confirm the expression of mGluR subtypes and evaluate the effect of mGluR-mediated signal inputs on the proliferation and differentiation of mesencephalic hNSPCs.

2. Materials and methods

2.1. Pharmacological agents

Forskolin (FSK) and the selective group III mGluR antagonist (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG) were purchased from Tocris Bioscience (Bristol, UK). The broad spectrum group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), the selective mGlu4 allosteric agonist (±)-cis-2-(3,5-dichlorphenylcarbamoyl) cyclohexanecarboxylic acid (VU0155041), the selective mGluR 8 agonist (S)-3,4-dicarboxyphenylglycine ((S)-3,4-DCPG) and the selective mGlu7 allosteric agonist N,N′-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), were all purchased from Ascent Scientific (Bristol, UK). Dibutyrl cyclic adenosine monophosphate (db-cAMP) was purchased from Calbiochem (Merck, UK). Master stock solutions for each drug were prepared in 100% dimethylsulfoxide (DMSO) according to their solubility and manufacturer's instructions. Working stock drug solutions were then prepared from these by serial dilution in growth media. Master and working stocks were split into aliquots and stored at −20 °C. For each drug addition to cultures, fresh drug working stocks were thawed and diluted to the required final concentration in growth media on the day of use. The final concentration of DMSO in all solutions was 0.1%. Drug vehicle-treated cultures thus received culture media containing 0.1% DMSO. All other reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Cell culture

A v-myc conditionally immortalised clonal hNSPC line was used for all experiments (ReNcell VM, Millipore, Watford, UK). The derivation and properties of this cell line have been described previously (Donato et al., 2007). Briefly, primary cells were isolated from the ventral midbrain region of 10-week old fetal brains and expanded on laminin-coated culture dishes. Transfection was performed using the MMLV type retrovirus encoding the v-myc gene driven under the CMV promoter. Transfected cell colonies were isolated using antibiotic selection (gentamycin 150 μg/ml) before expansion into a cell line (Donato et al., 2007).

Expansion and maintenance of ReNcell VM cells were performed in laminin-coated tissue culture flasks (mouse, 10 μg/ml; Sigma–Aldrich, Poole, UK), in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM: F12; Gibco, Renfrew, UK) at 37 °C in a humidified atmosphere of 95% air/5% CO_2 (Donato et al., 2007). During normal expansion, ReNcell VM cells proliferated with a doubling time of 48 h (70–80% confluence). Care was taken to ensure that cells did not exceed 80% confluence (Donato et al., 2007). Cells were cultured in a chemically defined media consisting of DMEM: F12 supplemented with a range of components (Supplementary Table 1). The growth factors fibroblast growth factor-2 (FGF2, 10 ng/ml; Peprotech, London, UK) and epidermal growth factor (EGF, 20 ng/ml, Invitrogen, Renfrew, UK) were added to the media to stimulate proliferation, henceforth referred to as growth media. Under these routine growth conditions, ReNcell VM displayed an immature neural morphology: small polygonal cells with few processes, which developed into a tight cobblestone pattern when confluent. Previous studies using G-banded karyotype analysis of this cell line have shown a normal human male diploid (46, XY) karyotype (Donato et al., 2007). Following testing on multiple passages (26 and 36) a normal karyotype was consistently observed, supporting the role of myc in generating karyotypically stable cell lines (Donato et al., 2007). Therefore, for these practical reasons, all experiments were carried out on ReNcell VM between passages 15 and 35.

2.3. RT-PCR

To investigate the expression of mGluR in ReNcell VM cells, total RNA was extracted from undifferentiated cells using Trizol (Invitrogen, Renfrew, UK); DNAse treated (Turbo DNAse, Ambion, Huntingdon, UK) and reverse transcribed using random decamers and Accuscript (Stratagene, Aligent technologies, Stockport, UK). Individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, PCR buffer, dNTPs, relevant forward and reverse primers (Table 1) and Taq DNA polymerase (Ambion, Huntingdon, UK). Reactions were initiated by hot-start incubation at 95 °C for 15 min and PCR (mGluR1-8: denaturation at 95 °C for 40 s, annealing at 58 °C for 30 s and extension at 72 °C for 40 s) was performed for 35 cycles with a final extension at 72 °C for 10 min. Human genomic DNA was used a positive control. PCR products were visualised on a 2.5% agarose gel and images captured using the GelDoc-It® TS Imaging System (UVP, Cambridge, UK). All PCR primers were designed in house using Primer3 software (http://frodo.wi.mit.edu/primer3/).

2.4. Intracellular cyclic AMP measurement

Intracellular cAMP was measured in undifferentiated ReNcell VM cells seeded at 1×10^6 cells/well in laminin-coated 6-well plates. Twenty-four hours later, cells were pre-incubated for 20 min at 37 °C/5% CO_2 in Loake's buffer (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4) containing 1 mM isobutyl-1-methylxanthine (IBMX: Sigma–Aldrich, Poole, UK), to inhibit cAMP hydrolysis. The cells were then incubated with Locke's buffer containing 1 mM IBMX and 10 μM forskolin in the presence or absence of test compounds for an additional 20 min at 37 °C/5% CO_2. The

Table 1
Primers used for detection of mRNA expression of metabotropic glutamate receptors (mGluR) in undifferentiated ReNcell VMhNSPC.

Gene	Forward (5′–3′)	Reverse (3′–5′)
mGluR		
Group I		
GRM1	CTAGCTGGCATCTTCCTTGG	TGAGGCAATGATCACCTGAG
GRM5	TGATCCCCAAGAGATCCAG	GTCCACCGAGTCTCTGAAGG
Group II		
GRM2	CTGCACATCATCCTCTTCCA	GCAAACAGTGGGGACAAACT
GRM3	GAAGTCCCTCGACTCCACCAC	CATGTTCTGGGGGGAATTGTT
Group III		
GRM4	TCTGTCTCCAGCCCTGTCTT	ACAGGAAAGAAAACGGCAGA
GRM6	ATTGGGCAGTGTGGAAGAAAC	CCCAGTTCCTCAGCTCACTC
GRM7	CGGCGCTATGACTTCTTCTC	CTGCCTCTTTGGAAATCTGC
GRM8	GAGGATATTGGAAGCAGCAAA	TCAATTGATGCTCGTTTGG