

Group I Metabotropic Glutamate Receptors: A Potential Target for Regulation of Proliferation and Differentiation of an Immortalized Human Neural Stem Cell Line

Julie Ladeby Erichsen¹, Morten Blaabjerg², Helle Bogetofte¹, Alberto Martinez Serrano³ and Morten Meyer¹

¹Department of Neurobiology Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark, ²Department of Neurology, Odense University Hospital, Odense, Denmark and ³Department of Molecular Biology and Center of Molecular Biology Severo Ochoa, University Autonoma Madrid-C.S.I.C. Campus Cantoblanco, Madrid, Spain

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Abstract: Human neural stem cells (NSCs) from the developing embryo or the subventricular zone of the adult brain can potentially elicit brain repair after injury or disease, either via endogenous cell proliferation or by cell transplantation. Profound knowledge of the diverse signals affecting these cells is, however, needed to realize their therapeutic potential. Glutamate and group I metabotropic glutamate receptors (mGluRs) affect proliferation and survival of rodent NSCs both during embryonic and post-natal development. To investigate the role of group I mGluRs (mGluR1 and mGluR5) on human NSCs, we differentiated an immortalized, forebrain-derived stem cell line in the presence or absence of glutamate and with addition of either the group I mGluR agonist DHPG or the selective antagonists, MPEP (mGluR5) and LY367385 (mGluR1). Characterization of differentiated cells revealed that both mGluR1 and mGluR5 were present on the cells. Addition of glutamate to the growth medium significantly increased cell proliferation and reduced cell death, resulting in increased cell numbers. In the presence of glutamate, selective activation of group I mGluRs reduced gliogenesis, whereas selective inhibition of group I mGluRs reduced neurogenesis. Our results substantiate the importance of glutamate signalling in the regulation of human NSCs and may as such be applied to promote proliferation and neuronal differentiation.

In the adult brain, multi-potent neural stem cells (NSCs) are mainly found in the forebrain subventricular zone (SVZ) and in the dentate gyrus of the hippocampus, where they continuously proliferate and differentiate into neurons, astrocytes and oligodendrocytes [1–3]. Human NSC lines are highly valuable as a source of cells for differentiation studies and genetic manipulations [4], and they can be maintained in long-term culture in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF) [5–7]. *In vivo* and *in vitro* studies have shown that stem cells can recapitulate embryonic and adult tissue development and therefore can be used for repair of injured or defective tissue [8–10].

Given that glutamate is the primary excitatory neurotransmitter in the central nervous system (CNS), extensive research has been focused on the importance of this particular neurotransmitter. Today, it is well accepted that glutamate plays an important role in developmental synaptogenesis, plasticity, learning and memory processes [11,12]. Other studies document the importance of glutamate for proliferation, differentiation and survival of both embryonic stem cells (ESCs) and NSCs [13–15].

In the CNS, glutamate exerts its effects through two different groups of glutamate receptors: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors

(mGluRs). iGluRs are fast-acting ligand-gated ion channels and control the flow of cations leading to depolarization of neurons [16]. In contrast, mGluRs are slow acting and modulate the response of the iGluRs [12]. mGluRs are members of the super family C of G-protein-coupled receptors (GPCRs) through which they activate different signal transduction pathways [17]. They can be subdivided into three different groups based on signal transduction pathways, sequence homology and pharmacological properties. Group I (mGluR1,5) is positively coupled to phospholipase C (PLC), and group II (mGluR2,3) and group III (mGluR4,6,7,8) are negatively coupled to the adenylate cyclase (AC) [18–21]. Group I mGluRs are mainly localized on the post-synaptic glutamatergic neuron [22], where activation enhances post-synaptic glutamatergic excitability via interactions with other post-synaptic processes such as iGluRs and ion channels [23,24]. Group I mGluRs activate a positive-feedback system that creates calcium oscillations and prolonged neurotransmission [18,25,26]. mGluR subtype expression has been detected during embryonic development [27–30] and also demonstrated in cultured neural progenitor cells [15,31–34]. Activation of mGluR3 and mGluR5 has been shown to support proliferation and survival of mouse NSCs and ESCs [35,36]. Furthermore, activation of mGluR3 can inhibit astrocyte differentiation by maintaining an undifferentiated, proliferative state of NSCs [14]. In addition, mGluR5 activation plays an important role in neuroblast proliferation in the ventral telencephalon and in the regulation of human hippocampal development [29,37]. mGluR1 is less well described in the context of embryonic development and NSCs.

Author for correspondence: Morten Meyer, Department of Neurobiology Research, Institute of Molecular Medicine, University of Southern Denmark, J.B. Winsløws Vej 21, DK-5000 Odense C, Denmark (fax +4565906321, e-mail mmeyer@health.sdu.dk).

Expression of mGluR1 has been detected during human prenatal development and post-natally in cortical areas, but not in the SVZ [30]. However, a study has demonstrated mGluR1 expression on neural progenitors from adult mouse SVZ and a negative effect of mGluR1 inhibition on their proliferation [31].

The aim of this study was to investigate the effects of glutamate and group I mGluRs on proliferation, differentiation and survival of human NSCs derived from the developing forebrain. We establish for the first time the presence of mGluR1 and mGluR5 on differentiating NSCs of human forebrain origin and prove a positive effect of glutamate on their proliferation, survival and neuronal differentiation.

Materials and Methods

Human neural stem cells. Collection of foetal tissue, cell isolation and immortalization are described elsewhere [38]. Briefly, human foetal tissue (striatum and adjacent SVZ; gestational week 10–10.5, crown-rump length 30 mm) was donated for research after written informed consent was obtained from the women seeking abortion. Tissue procurement was performed in accordance with the Declaration of Helsinki and in agreement with the ethical guidelines of the Network of European CNS Transplantation and Restoration (NECTAR). Approval to use these tissues for research was granted by the Lund University Hospital Ethical Committee, and their use was in compliance with Spanish law 35/1988 on Assisted Reproduction.

Immortalization of telencephalic cells was carried out by infection with a retroviral vector (LTR-vmc-SV40p-Neo-LTR; replication defective) coding for *v-myc*, a p100 gag-myc fusion protein isolated from the avian virus genome. The resulting human neural stem cell line (hNS1) divides approximately every 40 hr and have proven to be stable even after long-term culturing (4 years; stable telomerase activity) [38].

hNS1 cells were stored at -80°C in 1-ml Eppendorf tubes with 0.5 ml HNSC100 medium (see below) containing pen-strep (Gibco Invitrogen, Taastrup, Denmark), 10% bovine serum albumin (BSA, Sigma Brøndby, Denmark) and 10% dimethyl sulfoxide (DMSO, Sigma Brøndby, Denmark).

Maintenance and passaging of stem cells. Cells were thawed in 37°C water, centrifuged at $130 \times g$ (5 min.; 4°C), resuspended in culture medium and counted using a Bürker-Türk counting chamber. Propagation of cells took place in poly L-lysine (PLL, 10 $\mu\text{g}/\text{ml}$; Sigma)-coated culture flasks (Nunc T-25) containing 15 ml HNSC100 medium (DMEM/F12 w. Glutamax (Gibco), 2% (v/v) 30% glucose (Sigma), 0.5% (v/v) 1M Hepes (Gibco), 2.5% (v/v) AlbuMAX-I (Gibco), 1% (v/v) N2 supplement (Gibco), 1% (v/v) NEAA (Sigma) and 1% penicillin/streptomycin (Gibco) supplemented with 20 ng/ml EGF (R&D Systems Oxon, UK) and 20 ng/ml bFGF (R&D Systems) at 36°C in controlled atmospheric condition of 5% CO_2 and 95% humidified air. Medium was changed every 4 days, and cells passaged at 70% confluence.

For cell passaging, medium was removed, adherent cells washed with phosphate-buffered saline without calcium and magnesium (D-PBS; pH 7.4; Gibco) and cells detached by trypsin-EDTA (Gibco) diluted 1:10 in D-PBS for 5 min. at 36°C . Trypsinized cells were separated using a sterile Pasteur pipette, and the resulting cell suspension centrifuged for 5 min. at 4°C at $130 \times g$. The supernatant was removed, and the cells counted and resuspended in new T75 culture flasks.

Differentiation of stem cells. To investigate the effect of glutamate on differentiation of hNS1 cells, standard serum-free synthetic medium

(HNSC100 medium, see above) containing L-glutamate (4 μM , Sigma) or medium without glutamate was used.

The group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 10 μM , Tocris R&D Systems, Oxon, UK), the selective mGluR1a antagonist (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385; 10 μM , Tocris) or the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP; 1 μM , Tocris) was added to the medium with and without glutamate as described in the following to investigate the influence of group I mGluRs on differentiation of hNS1 cells. Previous work has proven that such low doses of glutamate or DHPG are not excitotoxic [39,40].

For differentiation, 20,000 cells/well were seeded into PLL-coated 24-well plates (Costar) with 0.5 ml hNS1 medium/well – with or without glutamate and the group I mGluR ligands mentioned above ($n = 12$ –24/condition; 4 independent experiments). Cultures were grown for 12 days *in vitro* (DIV), and the medium was changed every fourth day. Samples for analysis of lactate dehydrogenase (LDH) were collected at days 4 and 12 (see below).

Cell fixation and immunocytochemistry. Cells were fixed in 4% paraformaldehyde in 0.15 M phosphate buffer, pH 7.4, for 20 min. and then rinsed for 3×15 min. with 0.05 M Tris buffered saline (TBS, pH 7.4)/0.1% Triton X-100 (Sigma). Cultures were then pre-incubated for 30 min. in 0.05 M TBS/10% FBS (Gibco) before incubation with one of the following primary antibodies in 0.05 M TBS/10% FBS for 24 hr at 4°C : human nuclei (HN) 1:2000 (monoclonal mouse; Chemicon Millipore, Hellerup, Denmark), β -tubulin III (β -tub III) 1:1000 (monoclonal mouse; Sigma), glial fibrillary acidic protein (GFAP) 1:5000 (polyclonal rabbit; DAKO Glostrup, Denmark) and Ki67 1:800 (polyclonal rabbit; Abcam Cambridge, UK). Control staining was performed by excluding primary antibodies or using IgG rabbit 1:20,000 (DAKO) and IgG mouse 1:200 (DAKO). Non-bound antibody was washed off by rinsing for 3×15 min. in TBS/0.1% Triton-X-100. The cells were then incubated for 1 hr with one of the following biotinylated secondary antibodies in 0.05 M TBS/10% FBS: anti-mouse antibody 1:200 (Amersham VWR-Bie and Berntsen, Denmark) and anti-rabbit antibody 1:200 (Amersham VWR-Bie and Berntsen, Denmark). Cells were washed for 3×15 min. with TBS/0.1% Triton X-100 and incubated for 1 hr in horse radish peroxidase (HRP)-conjugated streptavidin (DAKO) diluted 1:200 in 0.05 M TBS/10% FBS, then rinsed for 15 min. in TBS before visualization of the immunocytochemical complexes with 50 mg 3,3'-diaminobenzidine (DAB; Sigma) and 33 μl hydrogen peroxide, H_2O_2 , per 100 ml TBS for 15 min. After rinsing for 15 min. with TBS and briefly with distilled water, cultures were cover-slipped with Aquatex (MerckVWR-Bie and Berntsen, Denmark). Photos of the stainings were taken using a bright field microscope attached to a Leica DC camera Ballerup, Denmark.

Double immunofluorescence staining. Double immunofluorescence staining was made using primary antibodies: anti- β -tub III 1:1000 (monoclonal mouse; Sigma), anti-mGluR1 1:500 (polyclonal rabbit; Upstate)/anti-mGluR5 1:500 (monoclonal rabbit; R&D systems), anti-GFAP-CY3 1:800 (monoclonal mouse; Sigma), anti-mGluR1/anti-mGluR5, anti-HN 1:2000 (monoclonal mouse; Chemicon) and anti-mGluR1/anti-mGluR5 to visualize the localization of group I mGluRs on differentiated hNS1 cells. The cells were rinsed for 3×15 min. with TBS/0.1% Triton-X-100. Non-specific binding sites were blocked by incubation with TBS containing 5% goat serum for 30 min. The cells were then incubated for 48 hr at 4°C with primary antibodies (see above) diluted in 5% goat serum in TBS. Non-bound antibody was washed out by rinsing for 3×15 min. with TBS/0.1% Triton-X-100 followed by incubation for 2 hr with the following fluorophore-conjugated secondary antibodies: anti-rabbit Alexa Fluor 488 1:200 (Invitrogen) and anti-mouse Alexa Fluor 555 1:200 (Invitrogen)

diluted in 5% goat serum in TBS, no light to preserve fluorescence. Excess non-bound antibody was washed off by rinsing for 3×15 min. with TBS and briefly with distilled water. Cells were cover-slipped with Fluoromount® (DAKO). Omission of the primary or secondary antibody served as negative controls. Images were recorded using a Zeiss photomicroscope, with an Alexa 488 and a CY3 filter kit, attached to a Leica DC camera (Leica Microsystems).

Cell counting. Quantification of cells was performed using bright field microscopy (Olympus Ballerup, Denmark). Numbers of Ki67-positive cells, HN-positive cells, β -tub III-positive cells and GFAP-positive cells were calculated based on analysis of six randomly selected areas per well. Cell counting was performed using an ocular counting grid with 100 equally sized squares at $\times 100$ magnification (Olympus; counting area, 1 mm^2), guided by pre-defined fix points in a computer-designed grid placed underneath the culture trays. Only cells displaying an intense immunostaining, well-preserved cellular structure and a distinct nucleus were counted. All cell counts were performed by experimenter blind to sample identity.

Lactate dehydrogenase assay. As a measure of cell death, the content of released LDH was determined at 4 and 12 days *in vitro* using 90- μl medium samples. 10 μl of Tris/NaCl buffer (0.813M Tris, 2.033M NaCl; pH 7.2) was added to each sample, and samples were stored at -20°C until analysis. The rate of conversion of NADH to NAD^+ as an indicator of LDH activity was measured by a fully automatic spectrophotometer (Cobas Mira, Hoffmann LaRoche). 20 μl pyruvate and 240 μl NADH (both Sigma) were added to each 20 μl media sample. The absorbance at 340 nm (37°C) was used as an index of NADH concentration. Before each set of measurements, changes in absorbance of LDH standard solutions (Boehringer Mannheim) were measured for calculation of a standard curve.

Statistical analysis. Statistical comparisons were performed using InStat, Graph Pad software (www.graphpad.com). The number of Ki67-positive cells, HN-positive cells, β -tub III-positive cells and GFAP-positive cells in the different experimental groups was compared by one-way analysis of variance (ANOVA) followed by the Bonferroni test. All values in text and graphs are presented as mean \pm standard error of mean (SEM).

Results

Expression of group I metabotropic glutamate receptors.

To establish the presence of group I mGluRs on our human NSCs (hNS1 cells) after *in vitro* differentiation, we performed double immunocytochemistry using antibodies against mGluR1/mGluR5 and the pan-neuronal marker β -tubulin III (β -tub III) or the astroglial marker glial fibrillary acidic protein (GFAP). A distinct co-existence of both receptor types was detected on both β -tub III-positive neurons and GFAP-positive astrocytes (fig. 1C, F, I and L).

Effect of glutamate on hNS1 cells.

To investigate the effects of glutamate in standard growth medium (see Materials and Methods), we differentiated hNS1 cells in standard medium *versus* glutamate-free medium. When grown in standard glutamate-containing medium, a significant increase in the numbers of human nuclei (HN)-positive cells (total cells) (fig. 2A,D) and Ki-67-positive proliferative cells (fig. 2B,D) was seen. To investigate whether the increased

number of HN-positive cells was due to enhanced proliferation exclusively, LDH measurements were performed in samples of conditioned culture medium collected at days 4 and 12 *in vitro*. At both time-points, significantly less LDH was detected for cultures grown in glutamate-containing medium, compared to cultures grown in medium without glutamate supplements (fig. 2C), suggesting less cell death in cultures exposed to low-dose glutamate.

Phenotypic characterization of the cultures showed that cells differentiated for 12 days in standard growth medium (4 μM glutamate) contained significantly (1.3-fold; $p < 0.05$) more β -tub III-positive neurons than cells grown in glutamate-free culture medium. Analysis of GFAP-positive cells revealed a non-significant (1.1-fold; $p > 0.05$) tendency for more astroglia.

Effect of glutamate and group I mGluRs on stem cell differentiation.

To eliminate the possibility that low doses of glutamate present in standard cell culture medium would activate other glutamate receptor subtypes than group I mGluRs, further cell differentiation experiments were performed using both standard medium (low-dose glutamate) and glutamate-free medium. Cells were differentiated in the presence or absence of the group I mGluR agonist DHPG or the selective antagonists LY367385 (mGluR1) or MPEP (mGluR5). In glutamate-containing medium, activation of group I mGluRs with DHPG significantly reduced the number of GFAP-positive astroglial cells, whereas no significant change was detected for β -tub III-positive neuronal cells. In contrast, selective inhibition of mGluR1 (LY367385) or mGluR5 (MPEP) significantly reduced the number of β -tubulin III-positive cells (fig. 3A,B) but had no significant effect on GFAP-positive cells (fig. 3C,D).

When glutamate was omitted from the growth medium, we found no effect of DHPG-mediated group I mGluR activation on either β -tub III-positive cells (fig. 4A,B) or GFAP-positive cells (fig. 4C,D). When adding the selective antagonists LY367385 or MPEP to the cells, only a non-significant tendency for reduced number of β -tub III-positive cells was seen (fig. 4A). In addition, no effect of LY367385 and MPEP on numbers of GFAP-positive astroglial cells was found.

Discussion

Over the recent years, *in vivo* studies have characterized the expression of mGluRs in the developing brains of rats and mice, where group I mGluRs (mGluR1 and mGluR5) are differentially regulated [22,27,28]. Moreover, cultured mouse forebrain precursor cells have been reported to express functional mGluR3 (group II mGluR subtype) and mGluR5 [32].

In rats, the level of mGluR1 expression increases post-natally [41], whilst mGluR5 expression increases perinatally with the highest expression level around second post-natal week, before decreasing to adult levels [23,42,43]. mGluR5 has been found in neurogenic zones, such as the rat external granule layer (EGL) of the cerebellar cortex and in the SVZs [32].

Similar results have been shown for differentiating mouse neural precursor cells, where mGluR5 is present at the highest

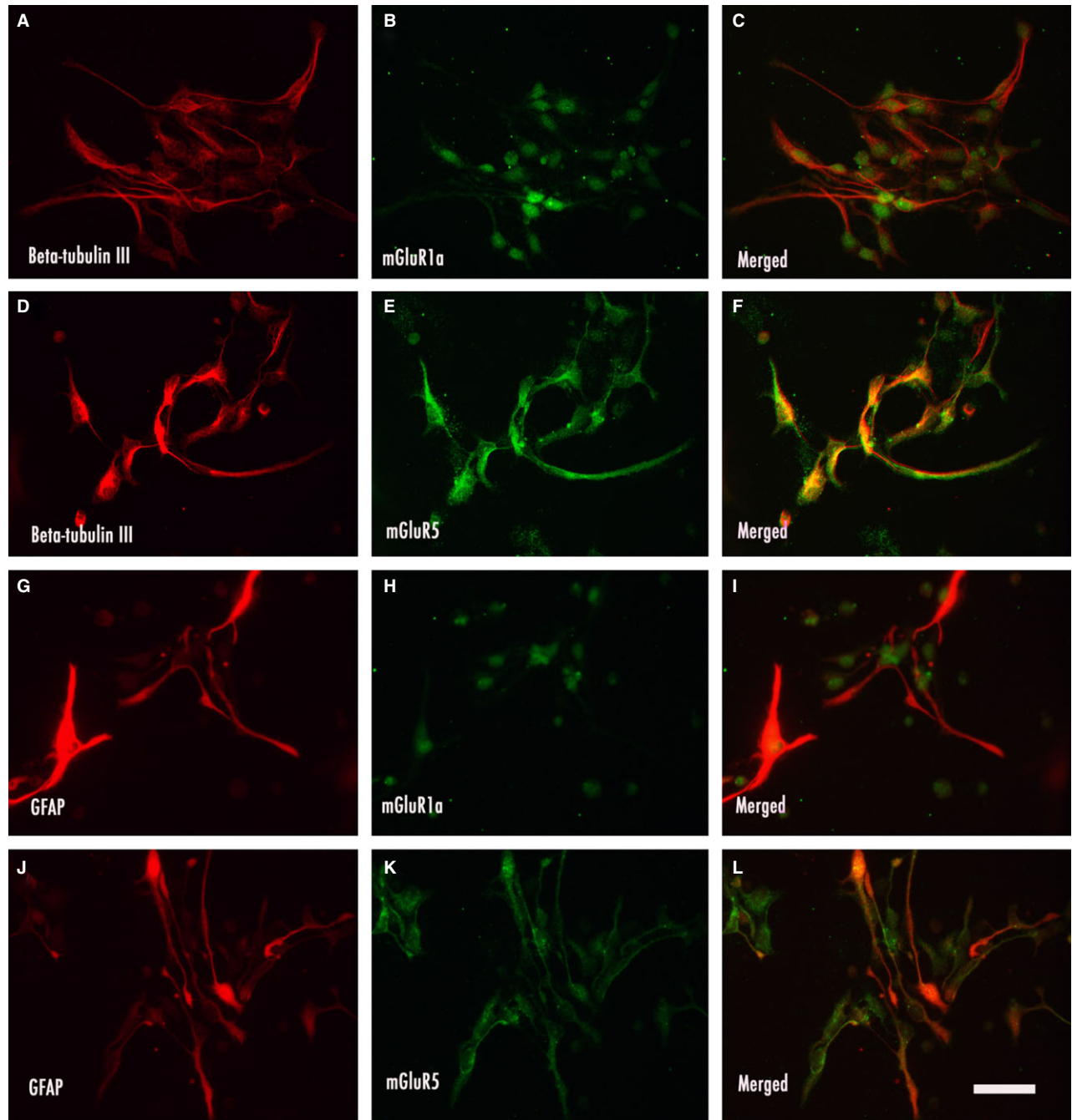


Fig. 1. Double immunofluorescence staining of differentiating forebrain-derived human neural stem cells (NSCs). To establish the presence of group I metabotropic glutamate receptors (mGluRs), human NSCs were differentiated for 12 days in standard cell culture medium without mitogens. Combined immunofluorescence staining for the neuronal marker β -tubulin III (A) and mGluR1a (B) revealed a significant co-localization of these markers (C). Similarly, β -tubulin III (D) and mGluR5 (E) were co-expressed in the cells (F). Also double immunofluorescence staining for the astroglial marker GFAP (G) and mGluR1a (H) showed co-localization (I). In addition, GFAP (J) and mGluR5 (K) were co-expressed in some cells (L). Scale bar = 50 μ m.

level around day 1–3 and then gradually decreases [33]. mGluR5 is also expressed in astrocytes and oligodendroglial progenitor cells [15,44].

During human prenatal development, mGluR5 expression can be detected in cortical areas from gestational week 9 and possibly earlier on, with strong expression in the ventricular/SVZ. mGluR1 has been observed in the cortical plate from

gestational week 13, persisting throughout prenatal development [30]. A recent study has shown mGluR5 to be present prenatally in the human hippocampus on NSCs, neuroblasts and mature neurons [29].

In this study, we report that differentiating NSCs, derived from the human forebrain SVZ, express both mGluR1 and mGluR5. We also provide evidence that regulation of

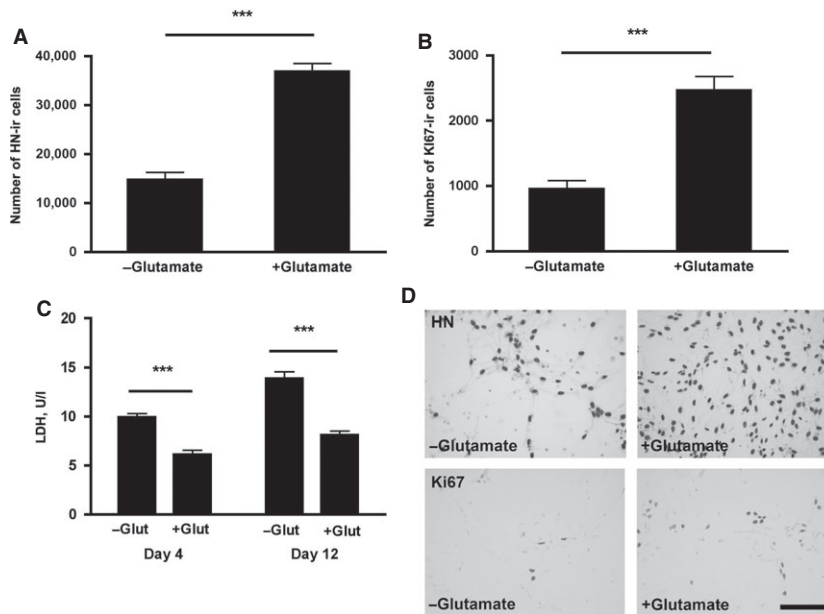


Fig. 2. Quantification of human neural stem cells (NSCs) differentiated with or without low-dose glutamate supplements in the culture medium. To investigate the effects of glutamate-containing supplements in the growth medium, NSCs were differentiated for 12 days in medium with or without glutamate. When grown in glutamate-containing medium, a significant increase in the numbers of human nuclei (HN)-positive cells (total cells) (A) and Ki-67-positive proliferative cells (B) was seen. To investigate whether the increased number of HN-positive cells was due to enhanced proliferation exclusively, lactate dehydrogenase (LDH) measurements were performed in samples of conditioned culture medium collected at days 4 and 12 *in vitro* (C). At both time-points, significantly less LDH was detected for cultures grown in glutamate-containing medium, compared to cultures grown in medium without glutamate supplements, suggesting less cell death in cultures exposed to low-dose glutamate. Images of HN-positive and Ki67-positive cells in differentiating cultures of human NSCs (D). Data are shown as mean \pm SEM; $n = 12$ –24/condition; 4 independent experiments. *** $p < 0.001$. Scale bar = 100 μm .

glutamate signalling may serve as a tool to promote proliferation and neuronal differentiation of human NSCs.

Exposure to low-dose glutamate stimulates cell proliferation and reduces cell death.

It has previously been reported that exposure to low-dose glutamate or the group I mGluR agonist DHPG significantly increases the proliferation rates of cultured human neural progenitor cells of neocortical origin [45,46]. In addition, glutamate treatment results in significantly more neurons after differentiation compared to untreated controls [45]. Moreover, glutamate has been found to promote both proliferation and survival of cultured neural precursor cells derived from the rat forebrain SVZ [13].

In accordance with this, we found that differentiation of our multi-potent human NSCs in low-dose glutamate-containing medium resulted in a significantly higher total cell number compared to cultures grown without glutamate supplements. Furthermore, the number of ki67-positive cells was significantly enhanced for cells exposed to glutamate, providing further evidence that glutamate, at certain concentrations, stimulates cell proliferation.

Interestingly, analysis of LDH in conditioned culture medium collected at days 4 and 12 revealed a significantly lower concentration of LDH for glutamate-treated cultures compared to corresponding cultures grown under glutamate-free conditions. This finding suggests that low-dose glutamate has a

positive effect on cell viability, which is consistent with a study showing decreased apoptosis of human neural progenitors in the presence of the group I mGluR agonist DHPG [46]. The observed beneficial effect of glutamate on cell survival may be mediated by mGluR5, as inhibition of mGluR5 has been described to increase apoptosis in neural progenitors [31,36,46]. However, it could also be explained by the presence of mGluR3 on the hNS1 cells, as its activation has been reported to reduce apoptosis in rodent neural precursor cells [13].

Immunocytochemical analysis of cultures differentiated in standard growth medium (4 μM glutamate) revealed increased numbers of β -tub III-positive neurons and a non-significant tendency for more GFAP-positive astroglial cells. Combined with our observation of increased proliferative activity, these data may suggest that low-dose glutamate mainly stimulates the division of precursor cells with neurogenic potential. This hypothesis is supported by a previous study on the influence of glutamate on proliferation and neurogenesis of human neural progenitor cells derived from the foetal cortex [45].

Effects of glutamate and group I mGluRs on neurogenesis and gliogenesis.

Previous studies have shown that inhibition of mGluR5 leads to apoptosis in mouse neuronal progenitor cells undergoing differentiation into neurons [31,36]. Mice treated with a selective antagonist of mGluR5, MPEP, show decreased

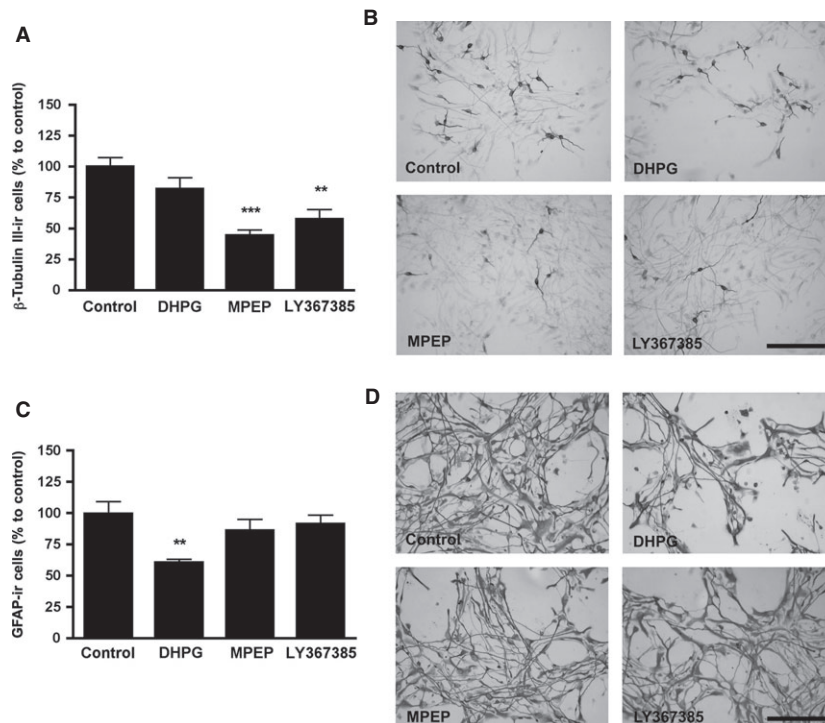


Fig. 3. Quantification of differentiating human neural stem cells (NSCs) cultured in medium with low-dose glutamate and group I metabotropic glutamate receptor (mGluR) agonists or antagonists. To investigate the effects of glutamate and group I mGluRs on differentiation of human NSCs, cells were differentiated for 12 days in low-dose glutamate (4 μ M)-containing culture medium in the presence or absence of the group I mGluR agonist DHPG (10 μ M) or the selective antagonists LY367385 (mGluR1; 10 μ M) and MPEP (mGluR5; 1 μ M). Activation of group I mGluRs with DHPG significantly reduced the number of GFAP-positive astroglial cells, whereas no such significant change was detected for β -tubulin III-positive neuronal cells. In contrast, selective inhibition of mGluR1 (LY367385) or mGluR5 (MPEP) significantly reduced the number of β -tubulin III-positive cells (AB) but had no significant effect on GFAP-positive cells (CD). Data are shown as mean \pm SEM; n = 12–24/condition; 4 independent experiments. ** p < 0.01, *** p < 0.001. Scale bars = 200 μ m.

proliferation of progenitor cells in the hippocampus at early post-natal stages [47]. Also, the mGluR1 antagonist, LY367385, has been reported to reduce the number of putative neuronal progenitors in organotypic hippocampal slice cultures [48]. In accordance with this, our study showed a significant reduction in β -tub III-positive cells in cultures exposed to both the selective mGluR5 antagonist MPEP and the selective mGluR1 antagonist LY367385. This could indicate that group I mGluRs are involved in the modulation of neuronal differentiation. However, the observed robust effect was only present for cultures grown in glutamate-containing medium (fig. 3). Cultures grown under similar culture conditions but without glutamate supplements displayed only a tendency towards this effect (fig. 4). This tendency may reflect that group I mGluRs are constitutively active on hNS1 cells even without glutamate stimulation and that further activation with low-dose glutamate in the medium increases the effects of group I mGluRs. Interestingly, we did, however, not observe an increased number of β -tubulin III-positive cells when applying the group I mGluR agonist DHPG. One explanation could be that the effects were due to the interaction between mGluRs and the ionotropic glutamate receptor NMDA. Other studies have indicated that activation of group I mGluRs causes internalization and thereby functional hypoactivity of NMDA receptors [39]. In line with

this hypothesis, it has been shown that activation of NMDA receptors is important for neurogenesis. Further studies combining pharmacological manipulation of both NMDA and mGluRs are needed to clarify these mechanisms [49].

Similar mechanisms may explain our observation of a significant reduction in the number of GFAP-positive cells (fig. 3C,D) after exposure to DHPG, which may hint to a decrease in astroglial differentiation. Activation of mGluR3 has been shown to impair astrocyte differentiation, but such results have not been reported previously for group I mGluR agonists [14]. Interestingly, the reduction in GFAP-positive cells was not present in differentiating hNS1 cells grown under glutamate-free conditions.

The general lack of effect of group I mGluR ligands on HSN1 cells differentiated without glutamate might be explained by the lower proliferation rates and increased cell death in these cultures blurring any effects. Glutamate signaling through other mGluRs, like mGluR3, might be needed for the effect of group I mGluR ligands to become apparent. Further studies are, however, needed to address this issue.

In conclusion, we have shown that both mGluR1 and mGluR5 are present on differentiating human NSCs of fore-brain origin. Furthermore, low-dose glutamate in standard growth medium increases cell proliferation and reduces cell

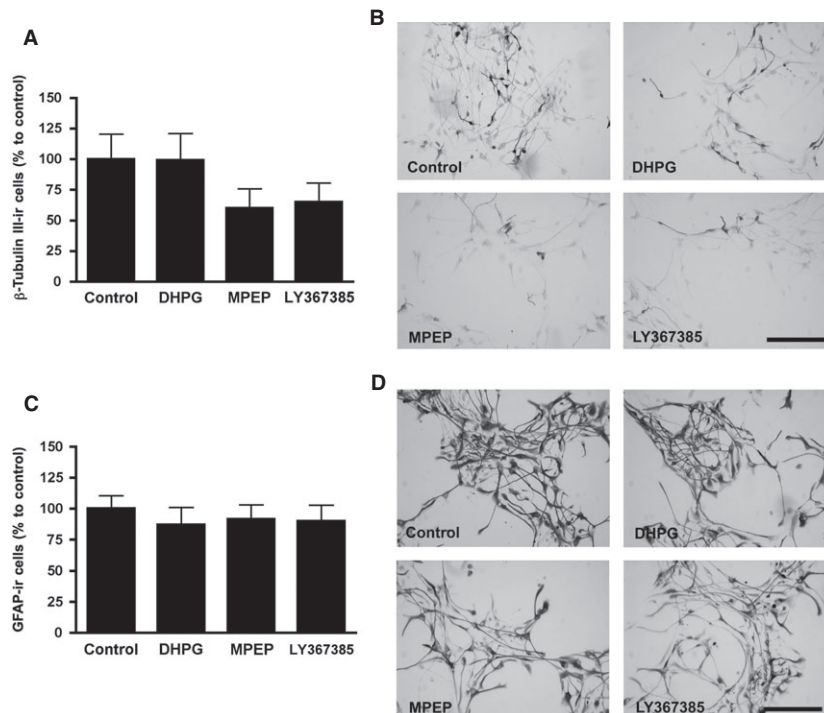


Fig. 4. Quantification of differentiating human neural stem cells (NSCs) treated with group I metabotropic glutamate receptor (mGluR) agonists or antagonists under glutamate-free conditions. To investigate the effects of group I mGluRs on differentiation of human NSCs at glutamate-free conditions, we differentiated cells for 12 days in culture medium with or without the group I mGluR agonist DHPG (10 μ M) or the selective antagonists LY367385 (mGluR1; 10 μ M) and MPEP (mGluR5; 1 μ M). After omission of glutamate in the growth medium, no effect of DHPG-mediated group I mGluR activation was observed on either β -tubulin III-positive cells (AB) or GFAP-positive cells (CD). When adding the selective antagonists LY367385 or MPEP, only a non-significant tendency for reduced number of β -tub III-positive cells was seen (AB). In addition, no effect of LY367385 and MPEP on numbers of GFAP-positive astroglial cells was found (CD). Data are shown as mean \pm SEM; n = 12–24/condition; 4 independent experiments; p > 0.05. Scale bars = 200 μ m.

death in these cultures. Selective activation of group I mGluRs in the presence of low-dose glutamate reduces gliogenesis, whereas selective inhibition of group I mGluRs reduces neurogenesis. Our results substantiate the importance of glutamate signalling in the regulation of NSCs and may as such be applied to promote proliferation and neuronal differentiation of human NSCs.

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