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Combined Extrinsic and Intrinsic Manipulations Exert Complementary Neuronal Enrichment in Embryonic Rat Neural Precursor Cultures: an *in vitro* and *in vivo* analysis

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Abstract

Numerous CNS disorders share a common pathology in dysregulation of γ -amino butyric acid inhibitory signaling. Transplantation of GABA-releasing cells at the site of disinhibition holds promise for alleviating disease symptoms with fewer side effects than traditional drug therapies. We manipulated FGF-2 deprivation and MASH1 transcription factor levels in an attempt to amplify the default GABAergic neuronal fate in cultured rat embryonic neural precursor cells (NPCs) for use in transplantation studies. Naïve and MASH1 lentivirus-transduced NPCs were maintained in FGF-2 or deprived of FGF-2 for varying lengths of time. Immunostaining and quantitative analysis showed that GABA- and β -III-tubulin-immunoreactive cells generally decreased through successive passages, suggesting a loss of neurogenic potential in rat neurospheres expanded *in vitro*. However, FGF-2 deprivation resulted in a small, but significantly increased population of GABAergic cells derived from passaged neurospheres. In contrast to naïve and GFP lentivirus-transduced clones, MASH1 transduction resulted in increased BrdU incorporation and clonal colony size. Western blotting showed that MASH1 overexpression and FGF-2 deprivation additively increased β -III-tubulin and decreased CNPase expression, while FGF-2 deprivation alone attenuated GFAP expression. These results suggest that low FGF-2 signaling and MASH1 activity can operate in concert to enrich NPC cultures for a GABA neuronal phenotype. When transplanted into the adult rat spinal cord, this combination also yielded GABAergic neurons. These findings indicate that, even for successful utilization of the default GABAergic neuronal precursor fate, a combination of both extrinsic and intrinsic manipulations will likely be necessary to realize the full potential of NSC grafts in restoring function.

Indexing Terms

GABA; FGF-2; MASH1; differentiation; transplantation; clonal analysis

Dysregulation of GABAergic signaling resulting in hyperexcitability is implicated in a number of CNS disorders, including epilepsy, basal ganglia movement disorders and neuropathic pain

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(Galvan and Wichmann, 2007; Kleppner and Tobin, 2001; Scholz et al., 2005). Most current therapeutic strategies for treating these disorders involve the systemic administration of drugs that compensate for the loss of inhibitory signaling, sometimes producing undesirable cognitive and behavioral side effects. Cell transplantation could potentially supplement deficient CNS cell populations and deliver focal, physiologically specific therapy. The transplantation of GABAergic cells has been shown to produce behavioral recovery in animal models of neurological disorders (Bosch et al., 2004; Carlson et al., 2003; Eaton et al., 2007; Gernert et al., 2002).

Endogenous spinal dorsal horn GABAergic cells play a major role in inhibiting incoming nociceptive signals from the periphery. The number of endogenous GABAergic cells, as well as GAD expression, has been reported to decrease in animals with peripheral and central injuries (Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002). Reversal of mechanical allodynia after intrathecal injections of GABA receptor agonists (Hao et al., 1992; Hwang and Yaksh, 1997; von Heijne et al., 2001) or transplantation of GABAergic cells into the lumbar spinal cord (Eaton et al., 1999; Eaton et al., 2007) suggests there may be a close relationship between GABAergic cells and the presence of nociceptive behaviors.

Prospectively, cell replacement therapy can be used to treat CNS disorders caused by loss of endogenous neurons after injury (Bosch et al., 2004; Carlson et al., 2003). However, therapeutic cell transplantation requires a considerable number of cells per graft, and often several graft sites per animal (Wolfe et al., 2007). A renewable source of cells such as neural stem or precursor cells could provide adequate numbers for numerous transplants over time (Caldwell et al., 2001), and immature neuronal cells exhibit enhanced viability and integration with host tissue after transplantation (Alvarez-Dolado et al., 2006). A current limitation in this strategy is that the majority of multipotent rat neural precursor cells (NPC) that survive transplantation tend to differentiate into astrocytes (Cao et al., 2001), particularly in the traumatically- or ischemically-injured spinal cord (Benton et al., 2005; Cao et al., 2002). This may be species-related to some extent, as human neurosphere grafts have been shown to express neuronal phenotypes in the injured rat spinal cord (Akesson et al., 2007). Astrocytes are thought to be undesirable as they may play a role in exacerbating chronic pain symptoms following nerve injury (Ji et al., 2006) and in creating an impediment to axonal regrowth after spinal cord injury (McKeon et al., 1991). Transplantation of undifferentiated neural stem cells has been reported to produce allodynia in spinal cord injury models (Hofstetter et al., 2005; Macias et al., 2006). Promising outcomes have been reported using more fully differentiated neural precursors (Lee et al., 2001), pre-differentiated embryonic stem cells (Hendricks et al., 2006), or directed differentiation using neurogenin-2 transduction to suppress astrocytic differentiation (Hofstetter et al., 2005).

Extracellular signals bring about changes in gene expression that control NPCs decision whether or not to differentiate, and subsequently to take on neuronal or glial fate. Since differentiation to GABAergic phenotypes is thought to be a default fate of neuronal precursors, manipulating the extrinsic and intrinsic mechanisms that lead to neuronal differentiation could yield an enriched, self-renewing source of GABAergic precursors amenable to *in vivo* therapeutic use. Thus, the goal of this work is to take advantage of known molecular and environmental mechanisms to enrich inhibitory neuronal precursor populations for cell replacement strategies in the treatment of injury-induced pain.

FGF-2 promotes proliferation in NPCs (Gritti et al., 1996) and represses differentiation through upregulation of Notch signaling (Faux et al., 2001; Yoon et al., 2004). Downregulating antineurogenic effectors of FGF receptor signaling by depriving NPCs of exogenous FGF-2 appears to play an important role in promoting neurogenesis over gliogenesis in multipotent NPCs *in vitro*. It has been shown that NPCs can be influenced to undergo neurogenesis at the

expense of gliogenesis *in vitro* when FGF-2 concentration is diminished (Qian et al., 1997). Indeed, many *in vitro* neuronal differentiation protocols employ FGF-2 withdrawal in their methodologies (Caldwell et al., 2001; Ito et al., 2003; Su et al., 2007).

In transgenic animals with kinase-deficient FGF receptors, the loss of FGF signaling in developing nervous system results in upregulation of the proneuronal basic helix-loop-helix (bHLH) transcription factor MATH1 (Jukkola et al., 2006; Shin et al., 2004). A related member of the family, mammalian achaete-scute homolog 1 (MASH1), plays a role during early neuronal differentiation. MASH1 was first discovered in developing CNS and neural crest precursor cells (Guillemot et al., 1993; Lo et al., 1991) and was subsequently found to be involved in forebrain interneuron development (Reviewed in (Schuurmans and Guillemot, 2002). MASH1 expression is held in check by the Notch effector HES1 through multiple mechanisms (Kageyama et al., 1997; Sriuranpong et al., 2002) until conditions allow differentiation to proceed. The neurogenic potential of MASH1 overexpression has been demonstrated in cultured stem cells, neural precursor cells and even postnatal astrocytes (Berninger et al., 2007a; Berninger et al., 2007b; Farah et al., 2000; Hamada et al., 2006; Ikeda et al., 2004).

Thus, propagating MASH1 overexpressing precursor cells through successive passages has the potential to supply an enriched population of transplantable cells of a therapeutically desirable neuronal phenotype. This study examined the effects of extrinsic environmental manipulation (FGF-2 deprivation) and intrinsic genetic manipulation (MASH1 overexpression), either independently or in concert, on rat NPCs cultured through successive passages *in vitro*. It also examined survival and differentiation of such cells following transplantation into the lumbar spinal cord of adult rats. Preliminary findings from this work have been presented in abstract form (Furmanski et al., 2008; Lee et al., 2001).

MATERIALS AND METHODS

Animals

All animal procedures were performed in accordance with NIH and University of Miami Institutional Animal Care and Use Committee (IACUC) guidelines. Rats were housed two/cage, under a 12/12 hour light-dark cycle (lights off at 6 P.M.), with food and water *ad libitum*. Embryonic precursor cells were harvested from timed pregnant females to generate *in vitro* cultures and cells for transplantation. Male Sprague-Dawley rats (250–300g, Charles River Laboratories, Wilmington, MA) served as graft recipients.

In vitro evaluation of embryonic precursor cells following FGF-2 deprivation and MASH1 overexpression

Neural precursor cell harvest—NPC harvest and standard growth conditions were similar to procedures used previously in our laboratory and others (Schumm et al., 2002; Smith et al., 2003). Embryos were harvested at 14 days *in utero* (E14) from pregnant dams. Cortices and underlying lateral ganglionic eminences were collected in sterile Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA). Tissues were gently triturated mechanically and the resulting single-cell suspension was collected. Neural precursor cells were then resuspended in normal growth medium consisting of DMEM-F12 (Invitrogen), 1% Penicillin-Streptomycin (Invitrogen), 1% N2 Supplement (Invitrogen) and 10 ng/mL FGF-2 (R&D Systems, Minneapolis, MN). Neural precursor cells (NPCs) were seeded in T75 tissue culture flasks at approximately 1×10^5 cells/cm², and maintained at 5% CO₂ in 37° C. FGF-2 (10 ng/mL) was added into the culture medium daily. For studies examining MASH1 overexpression, cells were transduced with lentiviral vectors immediately after seeding cultures.

Overall experimental design—Experiments were designed to evaluate the effects of extrinsic (environmental) and intrinsic (genetic) manipulation on NPC fate over time. For evaluation of extrinsic influences, NPCs were switched to low (0.1 or 1.0 ng/ml) or no FGF-2 levels as compared with the normal mitogenic growth levels of FGF-2 (10ng/ml) or standard serum-induced differentiation conditions. This was done at 5 days following initial harvest (Passage 0, P0) and at P1, P2, and P3 for comparison in differentiation potential over continued passaging. For intrinsic manipulation, overexpression of MASH1 was used in order to enhance neuronal versus glial fate. Combination of MASH1 overexpression with FGF-2 manipulations was also done over time. Both quantitative immunocytochemical and neurochemical assays were done to compare NPC fate and GABAergic differentiation potential under the various conditions.

Lentiviral vector construction and NPC transduction—The pRRLsinPPT plasmid (pRRL) was constructed by the Miami Project to Cure Paralysis Viral Vector Core Lab based on the lentiviral transducing plasmid developed by Naldini et al. (Naldini et al., 1996). Genes of interest were cloned into the multiple cloning site (MCS) downstream from a CMV promoter and upstream from a Woodchuck posttranslational regulatory element (WPRE; (Zufferey et al., 1999). GFP control vectors express the *Aequoria victoria* enhanced GFP gene (pRRL-eGFP). Mouse MASH1 cDNA was obtained as a gift from Dr. David Anderson (California Institute of Technology; Pasadena, CA). An XbaI restriction site and human Myc epitope tag were added to the MASH1 cDNA 5' end via overlap extension polymerase chain reaction (sense primer: 5'-ATCTAGAATGGAACAGAACTTATTCTGAAGAAGATCTC-3'; WPRE antisense primer: 5'-GGCATTAAGCAGCGTATCC-3'). The XbaI-Myc-MASH1 PCR fragment was gel purified and cloned into pRRL (pRRL-MM) between the MCS XbaI and SalI sites. Cells transduced with a previously described lentivirus encoding modified monomeric red fluorescent protein (mRFP_{KDEL}) were used for control transplantation experiments (Gajavelli et al., 2008).

HEK 293T cells were cotransfected with three lentiviral helper plasmids (Addgene, Cambridge, MA) and either pRRL-eGFP or pRRL-MM. Viral particles were purified from transfected cell media via ultracentrifugation. Viral titer was determined by p24 ELISA (Perkin Elmer) and estimating titer units (TU) at 10–100/pg p24. Acutely dissociated rat NPCs were transduced with lentiviral particles at approximately 5 TU/cell.

Immunocytochemical analysis—In order to maintain NPCs potential, neurospheres (NS) were passaged to single-cells every 5 days as described earlier (Mammolenti et al., 2004). For passaging, NS were pelleted and resuspended in HBSS, 0.25% Trypsin-EDTA (Invitrogen) was added, and the suspension was gently agitated for 30 minute to ensure thorough digestion. Digestion was terminated with turkey egg trypsin inhibitor (Sigma) in HBSS and digested NS were gently dissociated by trituration through a fire-polished Pasteur pipette. Single-cell NPC suspensions were centrifuged and resuspended in growth medium for seeding new cultures. NPC cultures were propagated through three passages in this manner.

Five days after harvest and after each of the three passages, cohorts of naïve and MASH1 lentivirus-transduced NS were plated in varying trophic conditions on adhesive cover slips for GABA/BrdU immunocytochemistry and quantitative analysis. Glass cover slips (18 mm; Carolina Biological Supply Co.) were placed in 12 well plates, coated with poly-L-ornithine (PLO, Sigma) and fibronectin (Sigma). NS were plated in normal growth medium, low FGF-2 (1 ng/mL) medium, or medium containing 10% fetal bovine serum (FBS) without additional FGF-2. Adherent NS were maintained for two days with daily supplements of FGF-2 (as needed) and the thymidine analog 5-bromo-2-deoxyuridine (BrdU) for assessment of proliferation. In some cases (as noted), cohort naïve or MASH1 lentivirus-transduced NS cultures were completely withdrawn from FGF-2 for 3–7 days prior to fixation (Fig 2A).

For immunocytochemistry, adherent NPCs were fixed for 20 minutes with cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), then rinsed 3 times with 0.1 M phosphate-buffered saline (PBS). Cover slips were incubated in a blocking solution of PBS containing 0.4% Triton-X 100 and 5% goat serum. Antibodies used for immunolabeling were rabbit anti- β -III-tubulin (Sigma; 1:1000), rabbit anti-GABA (Sigma; 1:200), guinea pig anti-GABA (Chemicon; 1:2000), mouse anti nestin and mouse anti-BrdU supernatants (Developmental Studies Hybridoma Bank; both 1:10). Secondary antibodies were goat anti-mouse IgG Alexa 594, goat anti-guinea pig Alexa 594 and goat anti-rabbit IgG Alexa 488 conjugates (Invitrogen). Nuclei were counterstained with 1 μ M Hoechst dye (Sigma) in PBS for 30 minutes at room temperature. Cover slips were mounted on slides with Vectashield mounting medium for fluorescence (Vector Labs, CA) and sealed with Elmer's blue school glue gel. Imaging was carried out on an Olympus IX70 inverted microscope as described earlier (Castellanos et al., 2002).

Quantification of GABA-BrdU double immunolabeling was performed on a Zeiss Axiovert microscope with a motorized stage driven by Stereo Investigator software (Micro Bright Field) by an observer blinded to treatment group. Cover slips (3–4 per treatment condition/passage number) were outlined as contours under 5x magnification, and each contour was fractionated into 100 sampling sites. Each sampling site was visited by a 2500 μ m² counting frame at 40x dry magnification. Markers were tallied for NPCs labeled for Hoechst, GABA, BrdU and GABA-BrdU double immunoreactivity. Marker tallies for GABA, BrdU and GABA-BrdU double-IR were divided by the corresponding Hoechst counts to produce percentages. Effects of the various treatments during successive passaging were evaluated using ANOVA and the Newman-Keuls test for multiple post-hoc comparisons (Prism v.4, Graphpad; La Jolla, CA).

Clonal analysis—Immediately after harvest, NPCs were maintained naïve or transduced with eGFP- or MASH1 lentivirus as described above. Following each passage, NPCs were seeded in PLO-fibronectin coated 6-well plates at 1×10^3 cells/well. Individual cells were circumscribed using a plate stamp attached to an inverted microscope. NPCs were maintained for 3 days in normal growth medium with daily supplements of FGF-2 to a concentration of 10 ng/ml. NPCs were then fixed in 4% paraformaldehyde in 0.1 M PBS for 20 minutes and nuclei were counterstained with 10 μ M Hoechst dye in water for 20 minutes. NPC colonies were imaged and counted by hand. Colonies over 32 cells were excluded from analysis as such colonies could not have risen from cell divisions within the duration of the experiment. Counts were pooled and data analyzed for significant differences between colonies of the same passage number using one-way ANOVA with Student-Newman-Keuls post-test. Skewness of population distribution data was analyzed using the D'Agostino and Pearson omnibus normality test (Prism v.4, Graphpad).

Western blotting analysis—Cohort naïve or MASH1 lentivirus-transduced NS cultures were divided into three groups after each passage (P0 – P3): mitogenic/normal FGF-2, 3 days and 7 days FGF-2 deprived. Normal FGF-2 cultures were supplemented daily with 10 ng FGF-2 per ml culture medium, and were harvested for protein five days after each passage. FGF-2 deprived cultures were maintained as normal for two days, then resuspended in medium containing no FGF-2 and maintained in suspension culture for the allotted time prior to protein harvest.

Neurospheres were pelleted by centrifugation and lysed using a nuclear fractionation kit (Sigma). Protein concentrations from cytosolic and nuclear fractions were determined by BCA assay (Pierce, IL). Lysates were normalized and reduced with 5x SDS-PAGE prior to resolving on 10% polyacrylamide gels. Gels were transferred to PVDF membranes (Millipore) at 70 mV for 45 to 60 minutes. Membranes were blocked in Tris-buffered saline (TBS, pH 7.2) with 2% bovine serum albumin (BSA). Membranes were then transferred into blocking solutions

consisting of TBS with 2% BSA and 0.2% Tween-20 (TBST-BSA) for probing with one of the following primary antibodies: rabbit anti-cyclophilin A (1:2000; Upstate, NY), mouse anti-TATA-binding protein (TBP, 1:10,000; Sigma) in order to normalize loading of cytoplasmic and nuclear fractions respectively, rabbit anti- β -III-tubulin (1:50,000; Sigma) as a marker for early neuronal differentiation, mouse anti-cyclic nucleotide phosphodiesterase (CNPase, 1:2000; Sigma) as a marker for oligodendrocyte differentiation, rabbit anti-GFAP (1:5000; Sigma) as a marker for astrocytic differentiation, mouse anti-MASH1 (1:500; BD Pharmingen; San Jose, CA), and mouse anti-protein disulfide isomerase (PDI, 1:1000; Novus; Littleton, CO). Membranes were rinsed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in TBS with 0.2% Tween 20 and 2% BSA: goat anti-rabbit IgG (1:2000; Santa Cruz, CA) or goat anti-mouse IgG (1:2000; Chemicon, CA). Immunoreactivity was revealed by chemiluminescent reaction (Perkin Elmer) and detected on photographic film (Kodak; Rochester, NY). Exposed films were scanned using a Bio-Rad Fluor-S Multi-imager. Relative intensities of cytosolic and nuclear protein signals were normalized against cyclophilin A and TBP signals, respectively.

Transplantation of MASH1 lentivirus-transduced or naive precursor cells into the lumbar spinal cord of adult rats

In order to evaluate whether mitogen deprived MASH1 overexpressing passaged rat neurospheres could generate GABAergic cells following transplantation in the spinal cord, preliminary studies were done in comparison with previously successful primary neurospheres (Lee et al., 2001). Freshly harvested (P0) naïve precursor cells were grown in normal FGF-2 (10ng/ml for 7 days) and switched to no FGF-2 for 1 day as described previously (Johe et al., 1996). For comparison, passage 2 MASH1 transduced cells were used (2 days in 10 ng/ml and 1 day in 0 ng/ml FGF-2 prior to transplant). mRFP_{KDEL} transduced passage 1 NPCs served as control for lentivirus transduction. Cells from different treatments were transplanted intraspinally in male Sprague Dawley rats (250–300 g). Rats were anesthetized with 1% isoflurane/O₂, and a midline incision was made on dorsal skin to expose the lumbar vertebrae. Laminectomy was performed aseptically on L1–L2 vertebrae (Lee et al., 2008), and cells were transplanted as described earlier (Castellanos et al., 2002). Briefly, cells were loaded onto a glass micropipette attached to a Hamilton syringe, and injected into either the right or left lumbar gray matter using a stereotaxic stage (Stoelting, Wood Dale, IL). The glass pipette was placed at a depth of 1mm from the dorsal lumbar spinal surface, and 3 μ l of cells (~100,000 cells/ μ l) were injected at 1 μ l/min. Upon completion of injection, the glass pipette was left in place for 1 min. Following transplantation, muscles were sutured (Vicryl 4-0, Ethicon, NJ) and skin closed with wound clips. All transplanted rats received cyclosporine A (q.d. IP, 10 mg/kg; Bedford Labs; Bedford, OH) from –1D until sacrifice (+7D post-transplantation).

Perfusion, immunohistochemistry, and microscopy—Transplanted rats were perfused transcardially 7D post-injection with cold saline and 4% paraformaldehyde in PB. Lumbar spinal segments were dissected, post-fixed overnight, and transferred to 30% sucrose-PB for cryoprotection. Lumbar spinal cords were sectioned at 40 μ m using cryostat (Leica), and incubated overnight in: anti-GABA (1:200, Sigma), anti-Neu-N (1:200, Millipore, CA), anti-GFAP (1:200, Sigma), anti- β -III-tubulin (1:200, Sigma), or anti-nestin (1:10, DSHB, IA). Sections were washed 3 times in PB, then incubated for 2 hr in secondary antibody solutions (Alexa Fluor 488 or 594; Molecular Probe, CA). Sections were washed 3 times in PB, and counterstained for nuclei with TO-PRO-3 iodide (Invitrogen) as indicated, coverslipped with anti-fluorescent mounting media (Vectashield, Vector Labs). Images were acquired using a Zeiss Confocal microscope equipped with LSM510. Image acquisition setting were as described previously (Gajavelli et al., 2008).

Antibody characterization—Descriptions for Western blotting lysate preparation and fixation of cells for immunostaining are found in the corresponding methods sections above. Brief descriptions of all antibodies used in these studies can be found in Table 1. Detailed antibody information is as follows: The anti-GABA polyclonal antibody (Sigma A2052; 067K4769) was raised in rabbits against GABA conjugated to bovine serum albumin (BSA). The Sigma A2052 antibody bound specifically to GABA and GABA conjugated to keyhole limpet hemocyanin (KLH) on dotblots, but did not bind to BSA alone. The guinea pig anti-GABA antibody (Chemicon AB175) immunogen is GABA-KLH conjugate prepared using glutaraldehyde. The staining with this antibody could be blocked with 100 μ M glutaraldehyde conjugated GABA but not similarly conjugated glutamic acid, glutamate or taurine. The monoclonal anti-bromo-deoxyuridine (BrdU) was raised against 5-bromo-2'-deoxyuridine-BSA and supplied by DSHB, and used at 1:10 in immunocytochemistry and the staining pattern was similar to previous reports (George-Weinstein et al., 1993); (Swanson et al., 2005). We did not observe staining with the antibody in spinal sections containing NPC transplants that were not labeled with BrdU. Full-length recombinant rat MASH1 was used to generate the mouse anti-MASH1 IgG1 Clone: 24B72D11.1 (BD Pharmingen 556604, 73996). No staining with the antibody could be observed in adult rat spinal sections where the MASH1 expression is known to be absent. The size of the MASH1 band (~34Kda) reported in this study is similar to previous published data generated using the same antibody (Sriuranpong et al., 2002; Uchida et al., 2007). The monoclonal anti-TATA binding protein (TBP) Clone 58C9 (Sigma T1827; 087K4811) produces IgG2b. The mouse myeloma cells and splenocytes from Swiss Webster mice immunized with Drosophila TFIID complex (Gene ID: 37476) were fused to produce hybridoma 58C9. The size of the band identified in this study is identical to that identified using the same antibody by other studies (Quadt et al., 2002)

The anti- β -actin monoclonal antibody (Sigma A5441; 055K4854) belongs to the IgG1 class and was raised against synthetic β -actin peptide DDDIAALVIDNGSGK conjugated to KLH (North et al., 1993). The size of the single β -actin band is consistent with previous reports. The anti- β -III-tubulin monoclonal antibody (Clone SDL.3D10 Sigma T8660) belongs to the IgG1 class and was raised against synthetic β -III-tubulin peptide with the following sequence CESESQGPK conjugated to bovine serum albumin in BALB/c mice (Banerjee et al., 1990). The anti- β -III-tubulin (Sigma T2200, 127K4815) was developed in rabbit using as immunogen a synthetic peptide corresponding to amino acid residues 441–450 of human β -III-tubulin (Ala 446 to Ser446 substitution), conserved in mammals with N-terminal added cysteine, conjugated to KLH. The antibody was affinity-purified using the immunizing peptide immobilized on agarose. The size of the single β -III-tubulin band is consistent with that reported earlier (Joshi and Cleveland, 1989). Recombinant human Cyclophilin A full-length protein was used to generate the polyclonal rabbit anti-cyclophilin A (Upstate 07-313; 31643). Using the same antibody a previous publication reported a band of identical size for cyclophilin A (Xu et al., 2005). Full length purified native PDI from rat was used to generate the monoclonal IgG2a anti-protein disulfide isomerase clone RL90 (NB300-517; 106–129) purchased from Novus Biologicals. The band identified in this study using the antibody is identical to that reported in (Kaetzel et al., 1987). The IgG1 monoclonal antibody that specifically recognizes vertebrate neuronal nuclei (Millipore clone A60, MAB377) was generated against purified nuclei from mouse brain. A similar staining pattern was observed by other investigators (Fricker-Gates et al., 2004). No staining was observed in the nuclei of non-neuronal cells. Clone 11-5B (Sigma, C5922; 017K4801) anti-CNPase antibody IgG1 was generated using purified human 2', 3'-cyclic nucleotide 3'phosphodiesterase as immunogen. The band identified as CNPase is consistent with that identified by previous publications (Sprinkle, 1989). The band could not be seen cultures that did not contain oligodendrocytes. The anti-glial fibrillary acidic protein (GFAP) polyclonal (Sigma G9269; 127K4807) was developed in rabbits using GFAP purified from human brain as the immunogen. The IgG fraction is essentially free of other rabbit serum proteins. The immunostaining pattern and band size are consistent with those reported earlier

using the same antibody (Bertelli et al., 2000). The mouse anti-nestin antibody was generated using homogenized Sprague-Dawley rat spinal cord. The monoclonal anti-Nestin antibody (Rat-401) was purchased from Developmental Studies Hybridoma Bank, Iowa, to whom the Rat-401 clone was donated by Susan Hockfield. The immunostaining pattern observed with the antibody is similar to other publications since the antibody was made available (Cattaneo and McKay, 1990; Gajavelli et al., 2004).

RESULTS

Effects of FGF-2 concentration and MASH1 overexpression on neural precursor cells: Immunocytochemical evaluation

GABA-immunoreactive cells were abundant in cultured embryonic rat primary (P0) neurospheres (Fig. 1). *In vitro* immunohistochemical analysis revealed that cultures grown in low concentrations of FGF-2 (0.1 ng/ml; Fig 1A) generally contained a higher proportion of GABA-IR cells than those with normal concentration (10 ng/ml; Fig. 1B).

These initial observations were explored further during neurosphere expansion and passaging using stereological analysis (below). The overall experimental design is shown in Fig. 2A (BrdU in italics and for P3). After harvest and after each of three passages, cohorts of naïve and MASH1 lentivirus-transduced NS were plated on adhesive cover slips in varying trophic conditions and times as indicated in the figure for GABA/BrdU immunocytochemistry and quantitative analysis. BrdU-labeling was used as an indicator of neural precursor cell proliferation under the various treatment conditions. Cells were also imaged in phase to show morphological features (Fig. 2B). In naïve NPCs cultured in 10ng/ml of FGF-2, BrdU-immunoreactivity was observed both in GABA-IR as well as GABA-negative cells (Fig. 2B). Figures 3A and A' show naïve rat neurosphere cultures expanded in mitogenic FGF-2 concentrations. GABA-IR cells were densely concentrated in neurospheres cultured in low FGF-2 (1 ng/ml, Fig. 3B, 3B'). When NPCs were cultured in 10% FBS-containing differentiation medium, neurospheres flattened out into colonies as NPCs elongated and migrated away from the initial point of adhesion. GABA-IR was sparse in naïve NPCs, particularly in FBS cultures (Figures 3C, 3C'). In MASH1-transduced cultures, GABAergic cells appeared to migrate away from the spheres (Fig. 3D, 3D'). An overall increase in the cell population, particularly the BrdU-positive proliferating population, appeared to be the primary effect of MASH1 overexpression. This was especially noticeable when comparisons were made between NPCs cultured in ostensibly proliferative conditions, such as naïve vs. MASH1 transduced cultures in low FGF-2 or standard serum differentiation (Figs. 3B and E, 3C and F, respectively). Naïve cultures contained relatively few GABA-BrdU-double positive cells, irrespective of FGF-2 concentration or serum treatment (Fig. 3A–C), in comparison with MASH1 overexpressing cells (Fig. 3D–F).

Unbiased stereological quantitation of neural precursor cells (physical dissector and fractionation) revealed changes in the GABAergic cell population over successive passages in the various treatment groups (Fig. 4A). The proportion of GABAergic cells was influenced by both treatment and passage number (2-way ANOVA, $F(d.o.f. 5,3) = 10.4$ and 14.8 , respectively for treatment and passage; $P < 0.001$). In all cases, regardless of treatment, GABA-immunoreactive cell populations decreased through successive passages ($P < 0.05$ between each successive passage). The highest number and percent of GABA-IR cells was seen following 2-day differentiation of newly harvested cultures ($P0 = 15.27 \pm 3.86\%$) or from early passages ($P1; 11.98 \pm 4.12\%$), but this potential for GABAergic differentiation declines over successive passages ($P2$ and $P3; 10.16 \pm 3.38\%$ and $4.73 \pm 1.27\%$ respectively). While the percentage of differentiating GABAergic cells in proliferating precursor populations (those maintained in normal mitogenic FGF-2 levels) may be expected to be low ($P0 = 5.27 \pm 1.57\%$), these findings revealed that even standard differentiation in 10% FBS without FGF-2

resulted in extremely diminished induction of GABAergic differentiation by passage 3 ($0.75 \pm 0.29\%$). Nevertheless, at both early and later passages, exposure to low FGF-2 increased the GABAergic differentiation compared with parallel maintenance in normal FGF-2 ($P < 0.05$).

Since the aim of this study is to identify means of generating increased populations of GABAergic precursors by successive expansion *in vitro* prior to differentiation, effects of interventional treatments at later passages are the most critical. Therefore P3 populations were analyzed separately in order to identify effects in spite of reduced overall differentiation potential compared with newly harvested cells (Fig. 4B). This evaluation revealed maintenance of significantly higher GABAergic differentiation potential in P3 cultures exposed to low FGF-2 levels for 2 days prior to fixation ($4.73 \pm 1.27\%$; one-way ANOVA, $P < 0.001$ for both low FGF-2 treatment groups compared with normal FGF-2 levels). Although MASH1 transduction alone did not significantly alter the percent GABA-IR cells in the cultures ($P > 0.05$), it did appear to enhance GABAergic differentiation in serum ($P < 0.05$) compared with unmodified precursors.

Fractionation analysis for BrdU labeling (Fig. 4C) revealed significant differences between treatment groups (F (d.o.f. 5,3) = 19.9, $P < 0.001$). As would be expected, cultures treated with normal mitogenic levels of FGF-2 also exhibited the highest levels of BrdU incorporation ($67.80 \pm 6.17\%$), compared with reduced % BrdU labeling under differentiation conditions (either addition of serum without FGF-2 [$9.95 \pm 2.32\%$] or following placement in low FGF-2 [$26.10 \pm 9.35\%$]; $P < 0.05$). However, MASH1 transduced cultures under all three extrinsic manipulations had increased %BrdU compared with their naïve cohorts (normal = $85.45 \pm 1.86\%$, low = $53.63 \pm 4.10\%$, serum = $30.30 \pm 13.30\%$). In separate evaluations (not shown), markedly greater numbers of both proliferating BrdU-labeled cells and overall cell population as assessed by DAPI were observed in MASH1-transduced cultures compared with all other conditions (e.g. at P3 in normal FGF-2, 1051.75 ± 65.00 compared with 416.00 ± 82.40 BrdU-labeled cells/cover slip in MASH1 vs. naïve cultures, respectively; $P < 0.001$; and 1229.30 ± 63.0 vs. 633.50 ± 131.9 DAPI-labeled cells/field in MASH1 vs. naïve cultures, respectively; $P < 0.001$).

Quantification of GABA-BrdU double-immunostaining (Fig. 4D) showed that only two or three percent of any culture stained positive for both markers. There were no substantial differences between any of the culture conditions, suggesting that most of the ongoing proliferation was in non-GABAergic cells, perhaps reflecting the cessation of proliferation with GABA expression and differentiation. The proportion of GABA-IR cells decreased from passage 0 to passage 3 irrespective of FGF-2 or MASH1 conditions.

Similar trends were observed using β -III-tubulin as a marker for early neuronal differentiation. Since changes in β -III-tubulin expression were evaluated using Western blot analysis, in depth quantification was not performed. Nevertheless, observations suggested that the combined effects of MASH1 overexpression and FGF-2 deprivation in both naïve and MASH1 transduced cells resulted in increased proportions of β -III-tubulin-expressing cells compared with normal FGF-2 cultures (ranging from 10–14% in normal FGF-2 to 17–22% with FGF-2 deprivation at P3). In addition, double immunostaining for GABA and β -III-tubulin showed that, regardless of treatment, approximately 15% of the β -III-tubulin-IR cells are also GABAergic at P3, suggesting the presence of other GABAergic neuronal precursors in the cultures. GABA-IR and β -III-tubulin-IR cells were less abundant in naïve compared to MASH1-transduced NPCs when cultured in normal FGF-2 (Fig. 5A and B, respectively). However, the proportions of these immunolabeled cells with respect to total cell numbers did not differ substantially. Approximately 80% of the GABA-IR cells also stained positively for β -III-tubulin under most conditions, except for MASH1 transduced cells undergoing FGF-2 deprivation, which exhibited only 50% co-localization.

Nestin-IR was examined in order to further characterize the expanding cell population in MASH1-transduced cultures. In comparison with naïve neural precursor cultures (Fig. 5C), MASH1-transduced cultures showed increased nestin-IR cells (Fig. 5D). This MASH1-induced increase in nestin-positive cells was also seen when NPCs were deprived of FGF-2 for three days (data not shown). As in Fig. 5B, increased β -III-tubulin in MASH1-transduced cultures in comparison with naïve cultures (Fig. 5A) is readily apparent in 5D. However, β -III-tubulin did not colocalize with nestin these in cells.

Clonal analysis of naïve and MASH1-transduced NPC proliferation

Quantification of naïve and GFP-transduced clonal cultures revealed population distributions that skewed significantly toward smaller colony size (D&P normality test $P < 0.05$ at the time points) centered around 9 to 11 cells per colony (Fig. 6A and B, respectively). The majority of naïve and GFP colonies at all passage ages contained 6 to 8 cells. There were no significant differences in naïve and GFP colony population means at any passage age. MASH1 clonal colonies exhibited a greater variety of sizes, with passage 1 and 3 population distributions passing normality tests (D&P normality test $P = 0.2856, 0.0607$ respectively, Fig. 6C). Passage 1 MASH1 distribution was centered around 12 to 14 cells per colony, while passage 2 and passage 3 distributions centered around 9 to 11 cells per colony. Colony size in MASH1 NPCs was significantly greater than GFP NPCs at all passage ages and naïve NPCs after passages 1 and 3.

Effects of FGF-2 concentration and MASH1 overexpression on neural precursor cells: Western blotting

The experimental design was similar to that presented in Fig. 2A except that instead of low FGF-2 and FBS, complete FGF-2 deprivation was used for promoting differentiation (no FGF-2 condition). Western blots (Fig. 7A) for TBP in nuclear extracts from naïve and MASH1-transduced NPC suspension cultures confirmed equivalent protein loading across lanes. MASH1 protein was present in low levels in nuclear extracts from naïve NPCs. MASH1-transduced NPCs showed substantial overexpression of MASH1 that was sustained through passage 3. Depriving naïve and MASH1-transduced NPCs of FGF-2 for 3 days did not alter MASH1 expression.

Expression of β -III-tubulin was high in passage 0 and 1 NPCs, diminishing through passage 2 and 3 in naïve NS cultured in normal FGF-2 (Fig. 7A) as judged by comparison to cyclophilin A as a loading control (Fig. 7A bottom panel). In contrast, there was markedly increased β -III-tubulin expression at all time points when NPCs were deprived of FGF-2 for three days (Fig. 7B) compared with normal FGF-2 (Fig. 7A). β -III-tubulin declines in all cases by P3, but is still higher in the FGF-deprived condition compared with normal FGF-2 maintenance at P3 when it is barely detectable. MASH1 transduced cells, in both normal FGF-2 and FGF-2 deprived conditions, appeared to modestly increase β -III-tubulin levels, particularly during later passages. Further, FGF-2 deprivation and MASH1 overexpression together resulted in increased β -III-tubulin production at later passages. These results were consistent with the immunostaining in Fig. 5A and B.

The expression of CNPase, an oligodendrocytic marker, was low prior passage 1 in naïve NS cultured in normal FGF-2 was low prior to passage 1, but then increased considerably and was sustained through passage 3 (Fig. 7A). MASH1 overexpression markedly attenuated CNPase expression in P2 and P3 NPCs (Fig. 7A). Depriving NS cultures of FGF-2 for 3 days had little effect on CNPase expression in P0 and P1 cultures (Fig. 7B). However, at later passages, FGF-2 deprived cultures showed a modestly decreased CNPase levels (Fig. 7B). In addition, FGF-2 deprivation further potentiated the effects of MASH1 overexpression in reducing CNPase levels.

GFAP expression was low in Passage 0 neurospheres grown in normal FGF-2 cultures, but tended to increase after passage 1. This increase was sustained through passage 3 in both naïve and MASH1 cultures (Fig. 7A). In contrast, depriving NPCs of FGF-2 for three days attenuated GFAP expression up until passage 3 (Fig. 7B). However, GFAP expression increased considerably after passage 3 irrespective of trophic manipulation.

Since FGF-2 deprivation appeared to enhance neuronal and reduce glial differentiation, longer periods of FGF-2 deprivation were explored in preliminary studies (Fig. 7C-D). Results of 7-day FGF-2 deprivation indicate that β -III-tubulin expression was further increased over normal FGF-2 and 3-day FGF-2 deprivation (Fig. 7C upper panel), and that GFAP expression was further attenuated during longer FGF-2 deprivation (Fig. 7C middle panel). MASH1 overexpression also appeared to promote a slight increase in β -III-tubulin expression that was additive with the effects of 7-day FGF-2 deprivation. The endoplasmic reticulum enzyme protein disulfide isomerase (PDI), a marker for cellular stress, was upregulated by FGF-2 deprivation in a time-dependent manner in naïve NPC cultures. MASH1 overexpression appeared to attenuate mitogen deprivation-induced upregulation of PDI (Fig. 7B lower panel).

An example of MASH1-transduced cultures maintained for 7 days FGF-2 deprivation conditions is shown in Fig. 7D. Although not quantified due to the difficulty in maintaining long-term cultures under stressed conditions, these cultures appeared enriched in GABA-IR cells.

Transplantation of manipulated neural precursor cells in the lumbar spinal cord

Fig. 8 shows examples of intraspinal transplants from primary P0 neurospheres; fig. 8A–C) and P2 MASH1-transduced cultures mitogen deprived for 7 days (Fig. 8E–F). An example of passaged (P1) control cell transplants labeled with mRFP_{KDEL} is shown in Fig. 8D. Grafts of P0 cultures contain numerous GABA-IR cells and regions containing dense NeuN-IR cells can be found (Fig. 8A – C). At higher magnification, GABA-NeuN double positive cells (yellow) are found adjacent to NeuN-IR (green) in host grey matter (Fig. 8B). The P0-derived grafts were positive for GABA, but not for GFAP, which was only found in the host spinal parenchyma bordering the graft (Fig. 8C). In contrast, control P1 grafts contained numerous GFAP-IR cells (Fig. 8D). Cells in these control grafts were transduced with mRFP_{KDEL} which has a punctate appearance as observed previously in our laboratory (Gajavelli et al., 2008). Punctate mRFP_{KDEL} labeling is observed in GFAP-IR cells, due to the KDEL tag that restricts the label to endoplasmic ER (Gajavelli et al., 2008). In grafts of MASH1 transduced cells, NeuN and GABA-IR were also observed, although these appeared to be more sparsely distributed (Fig. 8E). Similar to the P0 grafts, the MASH1 transduced cells did not colocalize with GFAP in intraparenchymal graft regions (Fig. 8E, inset). However, a portion of the graft found outside of the spinal cord contained GABA-positive as well GFAP- positive cells (Fig. 8F).

DISCUSSION

The goal of these experiments was to enrich cultured rat NPCs for an inhibitory neuronal phenotype for use in transplantation into rodent models of CNS disorders. Although GABAergic neurons and committed precursors have been shown to ameliorate CNS disorders, this approach is limited by short supply of appropriate cells and potentially incomplete host-graft integration using mature neurons. In order to improve outcomes and enrich for desired inhibitory neuronal phenotypes, a combination of selected environmental and molecular manipulations was evaluated. The principal findings of this study are: 1) Despite expected ability to maintain proliferating NS cultures in a multipotential capacity, rat neurosphere cultures lose neurogenic potential during early passages as they are expanded in vitro; 2) MASH1-transduction results in increased proliferation and expanded populations of neural

precursors; 3) FGF-2 deprivation uncovers a small, but significant subpopulation of NPCs which sustain the capacity for GABAergic differentiation over passaging; 4) The combination of MASH1-overexpression and FGF-2 deprivation can increase neurogenesis and reduce gliogenesis in passaged rat neurosphere cultures; 5) While transplantation indicates that GABAergic cells can be generated using these combined manipulations, further interventions will be needed in order to maintain sufficient populations for practical application.

Rat neurospheres lose neurogenic potential

Induced neurogenesis in NPC cultures following mitogen withdrawal has been fairly well accepted in the literature (Johe et al., 1996; Shin et al., 2004), as is the role of MASH1 in specifying GABAergic neurons during development [reviewed in (Schuurmans and Guillemot, 2002)]. Thus, an objective of the current study was to determine how long this default GABAergic potential could be extended using these promising extrinsic and intrinsic manipulations in concert. In spite of expected maintenance in the GABAergic population, GABAergic cell numbers tended to decrease through successive passages irrespective of manipulations. One possible explanation is that early passages contain higher relative numbers of primary and early-born committed neuronal precursors, which do not get MASH1-transduced and are winnowed away with each passage. However, even if this were the case, much of the literature suggests that passaged neurospheres can maintain their multipotentiality and newly-born neurons can be derived from proliferating neural stem cells when subjected to differentiation conditions. Nevertheless, results from the current study indicated not only reduced GABAergic differentiation, but also decreased expression of early neuronal differentiation marker β -III-tubulin over successive passaging, even under standard differentiation protocols using serum and removal of mitogens. This loss in neurogenic potential was particularly apparent in unmanipulated cultures. Trends toward reduction in neuronal emergence with time (passage) in culture have been noted by others (Ostenfeld et al., 2002). Thus, it is likely that prolonged exposure to proliferation-promoting levels of FGF-2 (10ng/ml) actually decreases the neurogenic potential of NPCs, while promoting gliogenesis (Qian et al., 1997; Reimers et al., 2001; Tsai and Kim, 2005).

MASH1 transduction results in increased proliferation and expanded populations of neural precursors

Results from the current study indicated that MASH1-overexpression results in increased expression of β -III-tubulin and reduced expression of CNPase in passaged neurospheres, both under normal and FGF-2 deprived conditions. This may be due to promotion of neurogenesis which overrides the decline in neurogenic potential to some extent. Interestingly, it was also observed that MASH1-transduced cultures undergo more rapid expansion and contained increased cell numbers overall. This was further supported by observations indicating increased nestin-IR in MASH1-overexpressing cultures. Using BrdU as a marker for proliferation, overall numbers of BrdU-labeled cells were markedly increased in MASH1-transduced cultures particularly notable under serum differentiation conditions, which normally induce cessation of NS proliferation. In support for this, clonal analysis of naïve and GFP-transduced control cells skewed towards smaller colony size and tended to divide roughly once per day (~16–18 h) as expected (von Waechter and Jaensch, 1972), while MASH1 cells showed increased incidence of larger colonies and an overall significant increase in cells per colony. These findings suggest that MASH1 overexpression enhances the expansion of cultured rat neuronal precursors. Previously, deficiencies in cell proliferation have been documented in MASH1 knockout mice (Parras et al., 2004); however, this is perhaps the first observation of MASH1-stimulated increases in cell division rate.

Hypothetically, FGF-2 degrades MASH1 through downstream effects Notch and HES1 (Sriuranpong et al., 2002). Interestingly, MASH1 was robustly expressed through successive

passages, suggesting that expression can be maintained and propagated through undifferentiated precursors. Thus, effects of MASH1 transduction on cell proliferation and differentiation appear to be sustainable and may be particularly useful in the long-term expansion of rat neurosphere cultures.

FGF-2 deprivation uncovers a small, but significant subpopulation of NPCs which sustains the capacity for GABAergic differentiation after passaging

Although the GABAergic population generally declined over successive passages, exposure to low- or no-FGF-2 for several days appeared to maintain GABAergic differentiation potential in a small subpopulation, even at later passages. In contrast to standard differentiation in serum, differentiation under FGF-2 withdrawal alone resulted in higher yields of GABA-IR cells throughout all passages examined. In addition, FGF-2 withdrawal consistently increased β -III-tubulin levels and decreased CNPase and GFAP, suggesting promotion towards neuronal differentiation at the expense of glial differentiation. These findings were even more marked in cultures deprived of FGF-2 for more prolonged periods (7 days), and appeared to also increase maturation towards a GABAergic phenotype. While this harsh treatment may stress the cultures, these findings nevertheless suggest that a subpopulation of cells retains the ability to differentiate to GABAergic neuronal precursors under the appropriate conditions.

The combination of MASH1-overexpression and FGF-2 deprivation can increase neurogenesis and reduce gliogenesis in passaged rat neurosphere cultures

Although MASH1-overexpression alone did not appear to promote neuronal differentiation, increased β -III-tubulin expression and reduced CNPase expression were observed when cultures were differentiated by FGF-2 deprivation, and MASH1-overexpression further enhanced the effects of FGF-2 deprivation. In particular, CNPase, an indicator of mature oligodendrocytes, was attenuated by FGF deprivation, strongly attenuated by MASH1 overexpression, and additively downregulated in the context of both after passage 2.

In addition, the upregulation of PDI, an endoplasmic reticulum (ER) protein, was attenuated by MASH1 overexpression in mitogen deprived cultures. Upregulation of PDI is an adaptive response to misfolded protein accumulation in the ER under conditions of cell stress. MASH1 is implicated in cell survival; loss of MASH1 is known to result in apoptosis in a variety of cell types (Elmi et al., 2007; Guillemot et al., 1993; Hu et al., 2004; Ohsawa et al., 2005). The effect of MASH1 on PDI expression suggests a potential link between downstream effects of MASH1 and reduced ER stress in cell survival (Uehara, 2007). Thus, the use of MASH1 overexpression, which can stimulate neurosphere expansion and reduce cellular stress, in conjunction with differentiation under FGF-2 deprivation to promote neuronal and reduce glial differentiation, may together increase the yield of neuronal precursors available for transplantation.

Transplantation indicates that GABAergic cells can be generated using these combined manipulations

Preliminary transplantation studies in the adult rat spinal cord indicated that GABA-IR cells can be obtained following the transplantation of MASH1-transduced NPCs pre-differentiated under FGF-2 deprivation. Although primary neurosphere grafts appeared more robust and fully differentiated in these pilot studies, these findings are nevertheless encouraging, particularly since undifferentiated rat NPC grafts in the adult spinal cord primarily differentiate to astrocytes (Cao et al., 2001). Similarly, grafts of P1 NPCs (mRFP-labeled controls) in the current study were densely GFAP-positive, in contrast to the markedly reduced presence of GFAP-expressing cells in the intraspinal grafts of MASH1-transduced FGF-2 deprived cells. It is possible that these combined manipulations can initiate differentiation towards neuronal/GABAergic phenotypes which continues towards maturation following transplantation.

Longer term follow-up studies as well as evaluations in the lesioned spinal cord will be important to pursue in follow-up studies. The injury environment appears to be particularly detrimental to neuronal differentiation of NSC grafts, as even neuronal-restricted precursors, which can differentiate into β -tubulin-positive neurons in the intact spinal cord, fail to do so in the traumatically injured spinal cord (Cao et al., 2002). Active autolysis, ischemia, and focal pathological processes may contribute to restrictive molecular cues in the graft microenvironment (Benton et al., 2005). Nevertheless, in contrast to traumatic spinal cord injury, chronic pain syndromes such as those following peripheral nerve injury or inflammation often occur in the absence of any overt damage or ischemia in the spinal cord, and appear to involve much more subtle losses in neuronal subpopulations such as vulnerable GABAergic interneurons. Thus retention of a GABAergic fate in a relatively intact spinal parenchyma is relevant in determining potential applications for pain therapies.

In conclusion, despite the presumed default GABAergic fate of neuronal precursors, the ability to obtain substantial numbers of these cells for transplantation becomes progressively limited in expanding rat neurosphere cultures over successive passaging. Brief FGF-2 withdrawal and MASH1 overexpression, separately or in concert, are not conducive to maximizing GABAergic differentiation once rat NPCs are grown in proliferative elevated FGF-2 conditions long-term. However, MASH1 overexpression stimulated an increase in cell proliferation and also appeared to be neuroprotective, as cells exhibited decreased ER stress when deprived of FGF-2. In addition, the concerted effects of MASH1 and FGF-2 deprivation were able to keep gliogenesis in check while maximizing enrichment of immature neurons for up to 3 weeks in culture. These findings reveal limitations to current methodologies and point towards potential steps for enriching long-term NPC cultures for neuronal populations.

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Figure 1.

Low magnification photomicrographs showing *in vitro* GABAergic cells cultured under different FGF-2 concentrations. The proportion of GABAergic cells was higher under low FGF-2 culture condition (**A**) compared to mitogenic (10 ng/ml or normal) FGF-2 culture condition (**B**). Bar is 50 μ m.

Figure 2.

Serial passage methodology assesses temporal characteristics of cell proliferation and GABA neurogenic potential of embryonic rat NPCs under differing culture conditions. A: Schematic summarizing the experimental design. B: Representative micrograph of adherent naïve passage 2 cells cultured in mitogenic FGF-2 concentration (10 ng/ml) and stained for GABA and 5-bromo-2-deoxyuridine (BrdU). GABA- immunoreactive (IR) but BrdU-negative cells appear green (arrows), while double-IR cells appear yellow (arrow heads). Phase contrast reveals total cell numbers and morphology. Scale bar = 100 μ m.

Figure 3.

Immunofluorescent labeling for GABA (green) and BrdU (red) in naïve and MASH1-transduced Passage 2 NPCs. Naïve NPCs (A-C) and MASH1-transduced NPCs (D-F) were plated in media containing 10 ng FGF-2/ml (A, A', D, D'), 1 ng FGF-2/ml (B, B', E, E') or 10% FBS (C, C', F, F'). Panels labeled X' show neurospheres imaged at 40x magnification for detail. Elevated FGF-2 increased cell numbers and promoted BrdU incorporation (A, D), while conditions that favor differentiation resulted in lower BrdU incorporation (B and E, C and F). GABA-immunoreactive cells were most enriched in cultures plated in neurogenic differentiation conditions (B, E), and tended to remain within adherent neurospheres in naïve cultures. MASH1-transduced cultures exhibited greater cell numbers and incorporated more BrdU than naïve counterparts, particularly those plated in FBS (F). GABA-immunoreactive cells showed a greater propensity to migrate away from parent neurospheres in MASH1-transduced cultures compared to naïve counterparts. Scale bar in X = 150 μ m and scale bar in X' = 50 μ m.

Figure 4.

Quantitative analysis of GABA and BrdU immunostaining in rat embryonic NPCs propagated through successive passages in vitro. A. The proportion of GABA-IR cells decreased over passages irrespective of FGF-2 concentrations or MASH1-overexpression. B. GABA-IR cells were most enriched in naïve and MASH1-overexpressing (OE) FGF-2-deprived cultures. C. BrdU-IR cells were most numerous in cultures exposed only to normal FGF-2. Naïve NPC cultures in 10% FBS produced high proportions of BrdU-IR cells in early passages, but produced diminishing numbers through time. MASH1-OE cultures maintained higher proportions of BrdU-IR cells through time. D: BrdU and GABA double-IR were most abundant in FGF-2 deprived NPCs. Double-IR NPCs comprised higher proportions of total GABA-IR cells at later passages after primary neurons are culled off. MASH-OE cultures produced more double-IR cells than naïve at later passages.

Figure 5.

Immunofluorescent staining for markers of neurons and undifferentiated precursors in Passage 2 NPCs cultured in mitogenic FGF-2 concentrations (10 ng/ml). MASH1-transduced NPCs (B, D) had elevated numbers of β -III-tubulin- and nestin-IR cells compared to naïve cultures (A, C). Overall proportions of GABA-IR neurons, β -III-tubulin-IR cells, and nestin-IR precursors to total cell numbers were similar between naïve and MASH1-transduced cultures. Scale bar = 100 μ m for A-D.

Figure 6.

Clonal analysis of naïve and lentivirus-transduced NPCs. A-C: Population histograms of naïve, GFP-expressing and MASH1-transduced NPCs, respectively. Control NPCs tended to produce smaller clonal colonies, while MASH1 NPCs produced a broader range of colony sizes. D: Mean MASH1 colony size significantly larger than for naïve controls after passages 1 and 3 and GFP controls at all times.

Figure 7.

Western blot analysis of NPC cultures. A. Naïve (N) and MASH1-overexpressing (M) NPCs were cultured through 3 passages (P). Upper blot images were obtained from nuclear lysate fractions and lower images from cytosolic fractions. MASH1 was substantially upregulated in lysates from MASH1-transduced cultures. Expression levels of β -III-tubulin were high in early cultures and diminish with time. B. FGF-2 deprivation rescued β -III-tubulin expression in later passages (P2 and P3). MASH1-forced expression and FGF-2 deprivation each downregulated CNPase at later passages, and the two conditions additively downregulated CNPase. GFAP expression rose after passage 1 and was attenuated by FGF-2 deprivation. C. Expanded Western blot analysis of NPCs grown continuously in normal FGF-2 (N) and NPCs deprived of FGF-2 for 3 or 7 days. Blots stained for β -III-tubulin showed further increased expression with increasing duration of FGF-2 deprivation (upper panel). GFAP was further attenuated by 7 day FGF-2 deprivation until passage 2. Neither MASH1 nor FGF-2 deprivation attenuated GFAP after passage 3 (middle panel). PDI was upregulated time-dependently during FGF-2 deprivation but attenuated by MASH1 overexpression (lower panel). D. Numerous GABA cells (red) can be seen in P2-MASH1 OE culture deprived of mitogen for 7 days and immunostained for GABA.

Figure 8.

Images of rat embryonic cells seven days after intraspinal transplantation were processed for GABA, NeuN, Nestin and GFAP immunohistochemistry. The dotted line delineates the transplantation site. A. The P0 naïve transplant was double positive for Neu-N (green) and GABA (red) and appears yellow (due to combined red and green fluorescence). B. A boxed region in A is shown at higher magnification, cross-sections of neurospheres double positive for GABA and NeuN (yellow) can be seen. Cell bodies are adjacent to host grey matter (green). C. The P0 culture containing sections (dotted line and box) were triple stained for GFAP (green), GABA (red) and NeuN (blue). The boxed region is shown at higher magnification in the inset. The endogenous astrocytes appear green and flank the GFAP-negative transplant that appears purple due to the combined fluorescence of NeuN (blue) and GABA (red). D. For comparison, P1 mRFPKDEL control-labeled cells (punctate red staining) were stained for GFAP (green) 1 week after intraspinal transplant. E. Spinal cord sections containing the MASH1 overexpressing P2 transplant (dotted line) were triple stained for GFAP (green), GABA (red) and NeuN (blue). The transplant region in the white matter is double positive for NeuN (blue) and GABA (red) and appears purple. In the inset the transplanted cells are shown at a higher magnification, GFAP channel is off for clarity. F. A portion of the graft found outside of the spinal cord contained transplant derived GFAP-IR cells. A high magnification image of the extruded transplant shows distinct GFAP-IR and GABA-IR cell bodies. NeuN (blue) was off for clarity. The outlined square encloses a cross section of a neurosphere with distinct GABA-IR (red) and GFAP-IR (green) cells along the periphery of the neurosphere.

Table 1

Primary Antibodies used in this study

Antigen	Immunogen	Antibody details	Dilution	Citation/Control
anti-GABA	GABA conjugated to bovine serum albumin (BSA) bound GABA and GABA-KLH on dotblots but not BSA alone.	rabbit Sigma A2052; 067K4769	1:200	See Antibody characterization below
anti-GABA	GABA-KLH conjugate prepared using glutaraldehyde	guinea pig Chemicon AB175	1:2000	See Antibody characterization below
anti-BrdU	5-bromo-2'-deoxyuridine-BSA	DSHB, mouse	1:10	(George-Weinstein et al., 1993); (Swanson et al., 2005)
anti-TATA-binding protein (TBP)	immunized with <i>Drosophila</i> TFIID complex	mouse Clone 58C9 (Sigma T1827; 087K4811)	1:10000	(Quadt et al., 2002)
Anti-MASH	full-length recombinant rat MASH-1	BD Pharmingen mouse Clone: 24B72D11.1 556604, 73996	1:500	(Uchida, 2007 #100) (Sriuranpong et al., 2002)
anti-cyclophilin A	Recombinant human Cyclophilin A full-length protein	Rabbit, Upstate, NY 07-313; 31643	1:2000	(Xu et al., 2005)
anti-protein disulfide isomerase	rat full length purified native PDI	mouse Novus, CO clone RL90 (NB300-517; 106-129)	1:1000	(Kaetzel et al., 1987)
anti- β -actin	Synthetic β -actin peptide DDDIAALVIDNGSGK conjugated to keyhole limpet hemocyanin (KLH)	monoclonal Sigma A5441; 055K4854	1:500000	(Gajavelli et al., 2004); (North et al., 1993)
anti- β -III-tubulin	a synthetic peptide corresponding to amino acid residues 441-450 of human β -tubulin III (Ala 446 to Ser446 substitution) conserved in mammals with N-terminal added cysteine, conjugated to key hole limpet (KLH).	rabbit Sigma T2200, 127K4815	1:1000	(Joshi and Cleveland, 1989)
anti- β -III-tubulin	CESESQGPk conjugated to bovine serum albumin in BALB/c mice.	Mouse Clone SDL3D10 Sigma T8660	1:200	(Banerjee et al., 1990)
anti-Neu-N	purified nuclei from mouse brain	Millipore clone A60, MAB377	1:200	(Fricker-Gates et al., 2004)
anti-GFAP	GFAP purified from human brain	Rabbit, Sigma G9269; 127K4807	1:1000	(Bertelli et al., 2000)
anti-cyclic nucleotide phosphodiesterase (CNPase)	purified human 2', 3'-cyclic nucleotide 3'phosphodiesterase	mouse clone 11-5B Sigma, C5922; 017K4801	1:2000	(Sprinkle, 1989)
anti-nestin	homogenized Sprague-Dawley rat spinal cord	Developmental Studies Hybridoma Bank (DSHB), monoclonal, Rat-401	1:20	(Cattaneo and McKay, 1990)