Glial-Restricted Precursors Protect Neonatal Brain Slices from Hypoxic-Ischemic Cell Death Without Direct Tissue Contact

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Glial-Restricted Precursors (GRPs) are tripotential progenitors that have been shown to exhibit beneficial effects in several preclinical models of neurological disorders, including neonatal brain injury. The mechanisms of action of these cells, however, require further study, as do clinically relevant questions such as timing and route of cell administration. Here, we explored the effects of GRPs on neonatal hypoxia-ischemia during acute and subacute stages, using an *in vitro* transwell co-culture system with organotypic brain slices exposed to oxygen-glucose deprivation (OGD). OGD-exposed slices that were then co-cultured with GRPs without direct cell contact had decreased tissue injury and cortical cell death, as evaluated by lactate dehydrogenase (LDH) release and propidium iodide (PI) staining. This effect was more pronounced when cells were added during the subacute phase of the injury. Furthermore, GRPs reduced the amount of glutamate in the slice supernatant and changed the proliferation pattern of endogenous progenitor cells in brain slices. In summary, we show that GRPs exert a neuroprotective effect on neonatal hypoxia-ischemia without the need for direct cell–cell contact, thus confirming the rising view that beneficial actions of stem cells are more likely attributable to trophic or immunomodulatory support rather than to long-term integration.

Introduction

HYPOXIC-ISCHEMIC ENCEPHALOPATHY (HIE) affects 1–3 of 1,000 newborns in the United States [1,2]. Fifteen to twenty percent of these children die during the neonatal period. Of the surviving infants, 25% develop neurologic sequelae such as cerebral palsy, seizures, intellectual disability, and sensory or learning impairment [3]. Factors known to play a role in perinatal HIE are energy failure, glutamate release, overactivation of excitatory receptors, oxidative stress, and upregulation of proinflammatory cytokines [4,5]. Excitotoxicity is believed to play a major role in HIE, since the neonatal brain exhibits maturation-dependent expression of glutamate receptors, the topography of which correlates with regions of hypoxic-ischemic cell death [4,6].

The only treatment option for infants with HIE to this date is mild hypothermia, which has been shown to reduce mortality and morbidity if initiated within 6 h after birth [7–10]. Aside from this narrow therapeutic window, hypothermia is only partially protective and there is a dramatic need for other therapeutic options. Although cell-based therapy is widely debated as a strategy for neonatal brain injury, little is

known about the mechanisms of action through which these cells confer their beneficial effect. Furthermore, the route and timing of administration are major determinants of whether cell-based therapy has any promise in this field, since there are significant ethical and logistical challenges associated with delivery of cells in a newborn.

Glial-restricted precursor (GRPs) are tripotential progenitors that can differentiate into oligodendrocytes and at least two types of astrocytes [11,12]. These cells have been proposed as a potential therapeutic approach for a series of neurological disorders, including neonatal brain injury, which is characterized by disturbances in both white and gray matter of the central nervous system [13–17]. We have previously shown that GRPs improve neurobehavioral and neuropathological outcomes after neonatal ischemia-induced white matter injury in mice [18]. Interestingly, an overwhelming fraction of the transplanted GRPs could not be detected after 2 months; however, animals showed improved outcomes, suggesting that GRPs exert a beneficial effect without long-term integration and differentiation in the host.

In this study, we used an *in vitro* model of neonatal hypoxia-ischemia to test the hypothesis that GRPs do not require direct

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cell contact to be neuroprotective and to determine whether any beneficial effect is time dependent. To gain insight into possible mechanisms of action of these cells, we also evaluated whether GRPs enhance endogenous cell proliferation or alter extracellular levels of the excitatory neurotransmitter glutamate in our experimental paradigm.

Materials and Methods

Figure 1 shows an outline of the experimental design. All animal experiments were approved by the Johns Hopkins Animal Care and Use Committee (Protocol No. MO12M428). The day of birth was defined as P1.

Cell derivation

GRPs were derived as previously described [19]. Briefly, time-pregnant C57BL/6-Tg(UBC-GFP)30Scha/J dams (Jackson Laboratories) were euthanized at day E13.5 and the embryos were extracted from the uterine sacks. Under a dissection microscope, spinal cords were isolated from each embryo and the meninges were removed. Cell suspensions were derived by cutting the spinal cords into small pieces followed by gentle trituration and digestion with pre-warmed trypsin supplemented with 1 mg/mL DNase (Sigma-Aldrich). After derivation, the GRP population was purified by magnetic sorting using the Miltenyi Biotec MACS system with Anti-A2B5 MicroBeads and, subsequently, plated into PDL (50 µg/mL)-Laminin (15 µg/mL)-coated dishes. A2B5+GRPs were maintained in a chemically defined medium, consisting of Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12) 1:1 (Invitrogen), B27 (50×; Invitrogen), bovine serum albumin (0.5%), fibroblast growth factor 2 (FGF-2; 20 ng/mL; Invitrogen), and heparin (1 µg/mL; Sigma), which was changed the day after derivation and every 3 days thereafter. After 1–2 weeks in culture, cells were used for the setup of co-cultures with organotypic brain slices.

Organotypic brain slice culture

Organotypic brain slices were obtained from neonatal mice on postnatal day (PND) 5 and cultured according to the Interface method as first described by Stoppini et al. in 1991 with some modifications [20]. Pups were rapidly decapitated, and brains were extracted and glued onto the stage of an Integraslice Microtome after cerebella were removed. The stage was submerged in a water bath filled with derivation medium (MEM+20 mM Hepes+1% P/S) that was cooled to 3°C during the whole procedure. The brains were cut into 350 µm-thick sections with a frequency of 80 Hz, and the central five slices were used. Each slice was placed on top of a Millicell Cell Culture Insert (diameter 30 mm, pore size 0.4 µm) and the insert was transferred to a six-well plate, where every well contained 1 mL of tissue culture medium consisting of 50% MEM, 25% HBSS, 25% heat-inactivated horse serum, supplemented with additional 12.5 mM Glucose, and 1% P/S.

After derivation, slices were washed twice and the six-well plates were placed in a humidified incubator with 5% CO₂ at 37°C. The medium was changed the day after derivation and then every 2 days with a gradual change to GRP medium so that from the seventh day onward all slices received GRP medium.

Oxygen-glucose deprivation and co-culture of organotypic brain slices with cell cultures

Seven days after derivation, slices were divided into two groups, where one group was left untreated and subsequently served as a control, whereas the other group was exposed to 60 min of combined oxygen-glucose deprivation (OGD) to mimic a hypoxic-ischemic insult. For the procedure, slices were washed once and transferred to six-well plates containing 1 mL of OGD medium (DMEM, no Glucose; Invitrogen), which had been previously kept in an anoxic environment for at least 3 h. The plates were placed

FIG. 1. Experimental design. After derivation, slices were allowed to recover for 1 week, before they were exposed to 60 min of combined OGD. The day of OGD was thereafter counted as d0. Twenty-four hours after OGD, a subset of slices on their culture inserts was transferred to dishes with previously plated and well-attached glial progenitor cells and co-cultured with them for the remaining period (=GRP24 group). Similarly at d3, after OGD, another set of slices was transferred to dishes with previously plated and well-attached GRPs (=GRP72 group) and co-cultured with them for the remaining time. Slices that were kept in native culture conditions for the whole time served as controls. At d1, d3, d5, and d7, PI intensity was measured and culture medium for quantification of LDH and glutamate was collected. Fresh medium that was added to replace the collected medium contained BrdU, which was used to assess the amount of cell proliferation 7 days after OGD. GRP, glial-restricted precursor; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; PI, propidium iodide.

in an anaerobic chamber, which was then closed and flushed with a gas mixture consisting of 95% N/5% CO₂ at 37°C. After 60 min of exposure to this environment, the OGD-treated slices were returned to their previous culture conditions. At either 24 or 72 h after completion of OGD, the slices were transferred to the six-well plates with previously plated and well-attached GRPs and co-cultured with them for the remaining period in a common medium but separated by the insert. This setup allowed them to interact through soluble factors but not through any direct cell–cell contact.

Evaluation of cell death based on propidium iodide intensity

Cell death was quantified as previously validated by numerous studies. The red-fluorescent molecule propidium iodide (PI) was added to the culture medium of organotypic brain slices in a final concentration of 5 µg/mL on 1, 3, 5, and 7 days after completion of OGD, 2 h before pictures were taken. Pictures were taken on an Olympus IX70 inverted microscope with a Qimaging Retiga 1350B camera and its associated software. Four images were taken in cortical regions of each slice (two/hemisphere), and fluorescence intensity was measured in selected regions using ImageJ's Integrated Density function. To compare results from multiple experiments, outcomes were normalized to the mean intensity of the control group and expressed as fold changes of this value.

Lactate dehydrogenase and protein quantification

Conditioned medium was collected at 24 h, 3 days, 5 days, and 7 days after completion of OGD, snap frozen, and stored at –80°C. Lactate dehydrogenase (LDH) and protein concentration were measured using Roche's Cytotoxicity Detection Kit (LDH) and Pierce BCA Protein Assay, respectively, according to the protocols. LDH values were divided by the amount of protein in each sample, and the results were expressed relative to control slices in each experiment.

Glutamate measurement

Glutamate concentration in collected slice supernatant was measured with the Amplex Red Glutamic Acid Kit (Invitrogen) by following the protocol, and results were expressed relative to the amount of glutamate in supernatant of control slices.

Cell proliferation and immunocytochemistry

Measurement of cell proliferation was based on quantification of BrdU incorporation into newly synthesized DNA of proliferating cells. BrdU was added to the medium 24 h after completion of OGD and with every medium change thereafter (d3, d5) at a final concentration of 3 µg/mL. At the end of the cultivation period (7 days after OGD), slices were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 1 h at room temperature. To label BrdU-positive cells, slices were permeabilized by successive incubation in 95% methanol, 2 N HCl, and 0.1% Triton X-100 and subsequently processed for immunohistochemical detection of BrdU using a monoclonal Anti-BrdU antibody (Sigma-Aldrich; mouse, 1:1,000). Evaluation of whether newly generated cells were of

glial or neural lineage was done by co-staining slices with primary antibodies against NG2 (Millipore; rabbit, 1:400) and Doublecortin (Santa Cruz; goat, 1:200). After overnight incubation at 4°C, fluorescent secondary antibodies were added for 1 h at 37°C and after nuclear staining with Hoechst, slices were mounted onto glass slides using Dako's Fluorescent Mounting Medium. For every slice, 20 pictures distributed across the cortex were taken using an Olympus Fluoview 500 confocal laser scanning microscope. BrdU-stained cells were manually counted three times and assessed for co-localization of NG2 or DCX, and the average of the three counts was used for quantification.

Statistical analysis

Data are expressed as the mean ± standard error of mean for each group of slices. Graphs were plotted and statistically analyzed using the software GraphPad Prism 5. To compare differences between groups, unpaired *t*-tests or one-way analyses of variance (ANOVAs) followed by post-hoc Tukey's Multiple-Comparison Tests were performed. Correlation between glutamate concentration and PI intensity was assessed by Pearson's correlation coefficient. For all figures, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

GRP characteristics

Our derivation protocol resulted in a highly viable population of GRPs that was characterized by a uniformly high expression of A2B5 after 1–2 weeks in culture, when the cells were used for experiments (Fig. 2). At this stage, 40%–70% of the cells expressed the progenitor cell markers Nestin and Sox2 and were still rapidly proliferating. Further characterization by immunocytochemistry revealed that almost all of the cells were also PDGFRα positive, about 50% were Olig2 positive, and few started expressing NG2, indicating the beginning of differentiation into an oligodendrocytic direction. About 20% of the cells were positive for the astrocyte marker GFAP, and <1% stained for the neuronal marker TUJ1 (not shown). The culture medium did not contain any factors directing the cells into a specific lineage; instead, the presence of the growth factor basic FGF (bFGF) ensured their maintenance in a rather immature state.

OGD induces massive cell death in the cortex and hippocampus of organotypic brain slices

After derivation, brain slices were allowed to recover for 7 days from cutting trauma. On day 7, they were exposed to 1 h of combined oxygen-glucose deprivation (OGD), which led to massive cell death as determined by LDH release and PI uptake. Figures 3 and 4, respectively, represent the quantification of both cell death markers from different experiments with *n* = 25–30 slices each. LDH values represent the amount of cell death in intervals of 1 day (24 h after OGD) or 2 days (days 1–3, 3–5, 5–7), whereas PI intensity indicates the cumulative degree of tissue injury. OGD-exposed slices showed an increase in both variables compared with control slices. LDH release peaked at 24 h after the injury and decreased during the culture period, whereas PI intensity showed a steady increase in OGD-treated as well as in control slices.

FIG. 2. Characterization of GRPs 1 week after derivation. After derivation, cells were purified by magnetic sorting with Anti-A2B5 magnetic beads, plated onto PDL/Laminin-coated dishes, and maintained in a chemically defined medium. This procedure led to an evenly attached monolayer of cells, which, when examined between the first and second week in culture, had a GRP-typical morphology (**a**) and were characterized by the almost 100% expression of A2B5 (**b**). At this stage, about 40%–70% of cells were positive for the progenitor cell markers Nestin and Sox2, respectively (**c**). Further assessment revealed beginning differentiation into an oligodendrocytic direction, as a high number of cells was PDGFR α positive (**d**, red), with few cells beginning to express NG2 (**d**) and about 50% of cells expressing Olig2 (**e**). About 20% of cells stained positive for the astrocyte marker GFAP (**f**). Color images available online at www.liebertpub.com/scd

FIG. 3. Amount of LDH/protein released into the medium of organotypic brain slices on d1 (**a**), d3 (**b**), d5 (**c**), and d7 (**d**) after OGD, relative to control slices. One hour of OGD induced severe tissue damage as could be seen by the almost 6 $\times$ higher amount of LDH in the medium of OGD-treated slices compared with controls 24 h after the injury. Slices that were co-cultured with GRPs beginning at this time point (OGD+GRP24, $n=5$) had only a slighter reduced amount of LDH in their culture medium on the following measurements. On d3 (**b**), OGD-exposed slices still released significantly more LDH than control slices, although to a much lower degree than on the first day. GRPs that were added to OGD-treated slices at this time point (OGD+GRP72, $n=5$) were able to significantly reduce the amount of LDH release compared with OGD-only slices on all following measurements. Furthermore, GRPs also improved the viability of control slices, as they prevented the gradual increase of cell death and thus LDH release that was seen in control slices without any cells. On d5 (**c**), control+GRP24 slices ($n=5$) contained $\sim 50\%$ of the LDH amount of control-only slices ($n=5$) and this value even decreased on d7 (**d**). (**e**) Shows the progression of LDH/protein release over time expressed as percentage of the maximal LDH/protein amount measured. Significance was tested by ANOVA followed by Tukey's Multiple-Comparison Test (**b–d**) or unpaired t -test (**a**). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ANOVA, analysis of variance.

FIG. 4. Cortical PI intensity in organotypic brain slices on d1 (a), d3 (b), d5 (c), and d7 (d) after OGD, relative to control slices. Cell death induced by OGD was the most pronounced in the cortex and the hippocampus 24 h later (e). For quantification of cell death, areas in the cortex were selected (f) and PI intensity was measured. (g) Shows the progression of cell death over time, expressed as percentage of maximal PI intensity in each experiment. $n=15/\text{group}$; significance was tested by ANOVA followed by Tukey's Multiple-Comparison Test (b–d) or unpaired t -test (a). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Twenty-four hours after OGD, injured slices released on average six times more LDH than control slices ($P<0.0001$) and had a fourfold higher uptake of PI in the cortex ($P<0.0001$). These values decreased over time, and OGD slices reached LDH levels comparable to control slices on the fifth day after the insult. PI intensity remained elevated for a prolonged period and lost significance only at the last measurement, 7 days after OGD. PI uptake was predominantly seen in the cortex and hippocampus, suggesting that most dying cells were neurons (Fig. 4e).

Effect of GRP-brain slice co-culturing

Initially, a set of control slices without OGD exposure were co-cultured with GRPs (control+GRP24) to exclude any possibility of noxious effects. Control slices without GRP cells showed a steady increase in LDH release and PI uptake over time (Figs. 3e and 4g, respectively). Co-culture with GRPs prevented this increase, with differences starting to show after 4 days of co-culture. After 4 and 6 days, control+GRP24 slices had about half the cell death of control-only slices.

The major interest, however, was the effect of GRP co-culturing on injury induced by OGD. Two time points were chosen to treat OGD-exposed brain slices with GRPs—at either 24 h (“OGD+GRP24”) or 72 h (“OGD+GRP72”) after completion of OGD. These time points were believed to roughly correspond to phases of acute and subacute injury. In the OGD+GRP24 group, co-cultured slices had about 50% less cell death compared with OGD-only slices,

based on PI intensity 3 days after OGD ($P<0.05$) (Fig. 4b). The amount of LDH in the culture supernatant on this day, however, was comparable between OGD-only and OGD+GRP24 groups (Fig. 3b) and at all following measurements, there was no difference between OGD-only and OGD+GRP24 groups. On the other hand, slices that were treated with GRPs at 72 h after OGD (OGD+GRP72) showed a persistent reduction in cell death with a significantly lower amount of PI intensity and LDH release on both d5 and d7 after OGD.

Of interest might also be the notion that, based on LDH release on d5 and d7, both control and OGD-injured slices had a significantly higher amount of cell death compared with control slices that were co-cultured with GRPs; whereas no statistical significance was found between this group and the OGD+GRP24/72 group. Thus, co-culture with GRPs improved the final outcome of control as well as OGD-injured brain slices, and—in the latter case—was especially effective when added during the subacute phase of the injury.

Glutamate concentration in supernatant of organotypic brain slices correlates with PI intensity

Figure 5 shows the amount of glutamate in the culture supernatant of organotypic brain slices. OGD led to a profound increase of glutamate in the medium of OGD-exposed slices. Twenty-four hours after completion of OGD, there was an about $1.5\times$ higher glutamate concentration compared with control slices ($P<0.0001$). The amount decreased over time to levels comparable to those of control slices on d5

FIG. 5. Amount of glutamate released into the medium of organotypic brain slices on d1 (a), d3 (b), d5 (c), and d7 (d) after OGD, relative to control slices. Slices that were exposed to OGD had a significantly higher concentration of glutamate in the medium than control slices 24 h after the injury. Co-cultivation with GRPs starting either at this time point (ODG+GRP24) or at the later time point (ODG+GRP72) markedly lowered the amount of glutamate in the cultivation medium on every following measurement. On all days, slices that were co-cultured with cells had significantly lower concentrations of glutamate than slices without cells. (e) Shows the time course of glutamate concentration expressed as percentage of the maximal amount measured. Significance was tested by ANOVA followed by Tukey's Multiple-Comparison Test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

after OGD. Slices co-cultured with GRPs had significantly less glutamate in the supernatant at every time point measured, compared with OGD-only as well as with control slices. The time course depicted in Fig. 5e shows that addition of GRPs 72 h after OGD led to a steady decrease in glutamate levels, whereas GRP administration already 24 h after the insult resulted in an initial steep decline, which, however, was followed by a partial rebound between d3 and d5. Thereafter, the concentration decreased again and on d7, slices that were co-cultured with GRPs had approximately half the amount of glutamate in their supernatant compared with slice cultures without cells, irrespective of OGD (one-way ANOVA $P < 0.0001$) (Fig. 5d).

To assess whether there was any association between glutamate concentration in the culture medium and cell death, Pearson's correlations were performed. Interestingly, the amount of glutamate on d1 after OGD showed a strong positive correlation with the degree of cell death (PI intensity) on this day ($R = 0.5299$, $P < 0.002$, $n = 34$) (Fig. 6a) as well as with that on d7 after OGD ($R = 0.5711$, $P < 0.001$, $n = 33$) (Fig. 6b). Other combinations (d3–d7, d5–d7) did not show significant correlations and are not displayed.

BrdU-NG2-DCX staining

BrdU incorporation as a measure of cell proliferation did not show any significant difference between OGD and control slice groups. Seven days after OGD, there was an equal number of BrdU-positive cells in cortices of control slices, OGD-exposed slices without cells, and OGD-exposed slices co-cultured with GRPs (Fig. 7b). To assess the fate of these cells, the glial precursor marker NG2 and the neural

precursor marker DCX were used. In all five groups, the majority of newly generated cells were NG2 positive ($84.96\% \pm 2.15\%$) and thus of glial lineage (Fig. 7c). Although the differences were very subtle, in OGD-treated slices, the percentage of (NG2⁺BrdU⁺)/BrdU⁺ cells was lower compared with controls ($P < 0.01$) and although co-cultivation with GRPs beginning at 72 h after OGD prevented this decrease, co-cultivation with GRPs beginning at 24 h after OGD even increased the amount compared with OGD-only slices ($P < 0.001$) (Fig. 7c). A small amount of the newly generated cells co-labeled with DCX, which is expressed in neuronal progenitors. This proportion was significantly higher in slices that had been co-cultured with GRPs (Fig. 7d).

For all measurements, we assessed whether there was a difference between male- and female-derived slices; however, gender had no effect either on the extent of tissue injury after OGD or on the degree of protection exerted by co-cultivated GRPs (data not shown).

Discussion

Transplantation of glial progenitor and glial progenitor-derived cells has been shown to have beneficial effects in a number of conditions, such as amyotrophic lateral sclerosis, transverse myelitis, and multiple sclerosis, and also enhances regeneration after spinal cord injury [13–17, 21–26]. Additionally, our group recently showed that GRPs are able to improve structural as well as functional outcomes in a mouse model of ischemic neonatal white matter injury [18]. Despite these encouraging results, the mechanisms by which transplanted GRPs ameliorate outcome remain poorly understood

FIG. 6. Correlation of glutamate concentration in the medium of organotypic brain slices with cell death, evaluated by cortical uptake of PI. To test whether there was any association between glutamate levels and cell death, Pearson's correlations were performed. The amount of glutamate in culture supernatant 24 h after OGD showed a strong positive correlation with cell death on this day ($R=0.5299$, $P<0.002$, $n=34$) (a) and also with cell death on d7 after OGD ($R=0.5711$, $P<0.0001$, $n=33$) (b), irrespective of co-cultivation with GRPs.

and clinically relevant questions such as timing and route of administration still remain to be answered. Furthermore, there has been some disagreement in regard to the optimal candidate for cell transplantation and conflicting results from various groups have spurred a discussion that remains yet to be resolved. The promising outlook created by the results of

some of the aforementioned studies was a little dampened by an important series of papers from Davies et al. [27–29], who found not only that undifferentiated GRPs were ineffective in alleviating disability after spinal cord injury, but also that transplantation of these cells as well as of astrocytes that were derived from them by treatment with CNTF caused

FIG. 7. Evaluation of cell proliferation in organotypic brain slices after OGD. BrdU was present in the culture medium from d1 up to d7, at which day slices were fixed and BrdU incorporation was assessed. Most of the newly generated cells co-expressed the glial marker NG2 (green), whereas a smaller percentage was also DCX positive (red) (a). All BrdU⁺ DCX⁺ cells were also NG2⁺. There was no difference in the amount of BrdU⁺ cells between all groups (b), and also the number of BrdU⁺ NG2⁺ cells was comparable (c). However, co-culturing slices with cells significantly increased the amount of BrdU⁺ DCX⁺ cells (d). This effect was the most pronounced in the OGD+GRP24 group, which had a number of BrdU⁺ DCX⁺ cells that was significantly higher than that of the OGD-only group as well as that of the control group. Co-culturing slices with GRPs beginning at 72 h after OGD also increased the number of BrdU⁺ DCX⁺ cells but was only compared with OGD-only slices. $n=5$ /group; values considered significant where * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Color images available online at www.liebertpub.com/scd

mechanical allodynia, thermal hyperalgesia, and neuropathic pain. In the same studies, transplantation of BMP-4-pre-differentiated GRPs, on the other hand, resulted in significant functional as well as morphological recovery and thus significantly better outcomes.

In contrast to these findings, Jin et al. [16] found no difference between GRPs and GRP-derived BMP-4-astrocytes in a similar model of spinal cord injury, where delayed transplantation of both cell types resulted in an improved lesion environment, although without functional recovery. Importantly, in their study, no increased sensibility or pain was observed. Also, Haas et al. compared acute transplantation of multiple preparations of GRPs and GRP-derived astrocytes and found them to be equally effective in promoting axon regeneration after spinal cord injury [30]. The seeming contradiction of these findings not only indicates shortcomings of our current understanding but also highlights the fact that there is plenty of room for improvement of current cell-based approaches. Notably, none of the described studies was conducted in neonates, whose brain displays its own characteristic environment that is barely comparable to that of the adult. Thus, although we used native GRPs in our previous [18] as well as in the current study, future investigations comparing different preparations of transplantable cells in the context of a newborn, developing brain will be highly relevant in the transition process from pre-clinical to clinical studies. Importantly, even the slightest possibility of pain or a similar adversity has to be excluded before the step from bench to bedside can safely be made. As the *in vitro* nature of the present study did not allow us to examine features such as subjective sensation, we focused rather on technical aspects of the matter and cede functional observations to succeeding projects.

Here, we show that GRPs exert a neuroprotective effect on OGD-injured brain slices of neonatal mice, an *in vitro* model of neonatal hypoxia-ischemia. The observed effect was not dependent on direct cell–cell contact, which confirms the rising view that beneficial actions of stem cells are more likely attributable to trophic or immunomodulatory support rather than to cell replacement or long-term integration. OGD-treated organotypic brain slices that were co-cultured with GRPs without any contact had a decreased amount of cortical cell death, as evaluated by LDH release and PI staining and this effect was especially pronounced when cells were added during the subacute phase of injury. Although GRPs that were administered 24 h after OGD, that is, at a time point when cell death peaks, initially reduced the amount of injury in cortices of OGD-exposed slices, they failed to elicit a statistically significant improvement on the last day of measurement. GRPs that were added 72 h after OGD, on the other hand, showed a strong neuroprotective effect that lasted at least up to the seventh day after OGD.

Although the extent of the therapeutic window remains to be determined, the fact that in regard to tissue protection, delayed administered GRPs were superior to those given in the acute phase might be especially useful in HIE, where the exact timing of the insult is often not known, but where the brain experiences a long-lasting damaging response that substantially contributes to the disturbances of structure and function seen in children with neonatal hypoxia-ischemia. One reason for the inferiority of early administered GRPs might be the detrimental effect of the injured tissue on the cells. A certain limitation of the present investigation is that

GRPs were characterized only at the beginning of the study, because of which it is not clear what population of cells was lastly responsible for the observed effects.

There is no doubt that OGD-injured slices release an abundance of factors that influence the composition of co-cultured cells. Future experiments focusing on the identification of these factors and further characterization and classification of GRPs after exposure to the injured environment will provide important clues about processes involved in the observed results. In such experiments, it would also be highly interesting to compare the co-culture system with approaches using conditioned media of different preparations of GRPs. If the addition of conditioned media should result in similar outcomes, it would provide further and firm evidence for the relevance of soluble factors in the neuroprotective and neuroregenerative potential of GRPs. Besides facilitating the identification of these factors, this strategy would moreover allow a focused analysis of distinct cell types in regard to their neuroprotective efficacy and thus aid in the identification of the most promising candidates for further investigations.

The excitatory neurotransmitter glutamate plays a crucial role in neonatal HIE, as NMDA receptors show a particularly high density and an increased excitability in the neonatal period [31,32]. In addition to energy failure and consequent influx of Ca^{2+} with neurotransmitter release [33], hypoxia-ischemia also leads to a focal disruption of glutamate receptors [34] and decreases striatal high-affinity uptake of glutamate into synaptosomes [35]. The resulting accumulation of extracellular glutamate together with the increased susceptibility of infants to its cytotoxic effects play a central role in the pathophysiology of HIE. Glutamate antagonists have been shown to potently reduce neuronal cell death *in vitro* as well as *in vivo* by various mechanisms, including TNF α inhibition by NF κ B, reduction of reactive oxygen species, and regulation of energy metabolism [36–42]. In our study, OGD led to a drastic increase of glutamate levels in the supernatant of brain slices, which stayed elevated for days after the injury.

In addition, we found that the glutamate concentration 24 h after the injury, that is, before any GRPs were added, was positively correlated to the amount of cell death not only on this day but also on the seventh day after the injury. This observation highlights the long-lasting cytotoxic role of excess glutamate after neonatal hypoxia-ischemia and underscores the potential of the model to recapitulate hypoxic-ischemic injury. Interestingly, non-contact co-cultivation with GRPs at either the 24 or 72 h time points remarkably decreased glutamate levels. Notably, delayed (72 h post-OGD) administration of GRPs resulted in a steady decline, whereas the profound reduction in glutamate concentration that was observed when GRPs were added during the acute phase was followed by a partial rebound before levels decreased again. One straightforward explanation for this pattern might be the demise of GRPs after acute exposure to the toxic environment created by injured slices, as this might also explain the shorter and lower degree of improvement in this group. It is likely that the potent ability of GRPs to reduce glutamate concentration is—besides other factors—co-responsible for their neuroprotective effects. Indeed, Maragakis et al. have shown that glial-restricted precursors overexpressing glutamate transporters enhance the survival

of motor neurons in spinal cord cultures by increasing glutamate transporter expression and function [43] and in a study by Lepore et al. in a rat model of amyotrophic lateral sclerosis, the neuroprotective effects of transplanted GRP-derived astrocytes were partly mediated by the astrocytic glutamate transporter GLT1 [22].

In a study using mesenchymal stem cells, on the other hand, glutamate excitotoxicity and neuronal cell death were reduced by mechanisms involving alteration of gene expression and decreased neuronal glutamate receptor expression and function, thus lowering their susceptibility to excess glutamate [41]. It remains undetermined what mechanism led to the decrease in glutamate levels in our study, and although uptake of glutamate by GRPs is an attractive hypothesis, future studies using gene silencing and competitive blocking of glutamate transporters in GRPs will hopefully shed light on this question.

It has been repeatedly documented that transplantation of various types of stem and progenitor cells leads to stimulation of endogenous cell proliferation, and it is acknowledged that this feature plays a prominent role in the therapeutic potential of this approach. For example, a recent study by Chen et al. showed that transplantation of oligodendrocyte precursor cells increased endogenous NSC proliferation and improved cognitive function in a rodent model of HIE [44]. Although this and similar studies focused on the hippocampus and subventricular zone, we assessed the situation in cortical regions of the brain. There, in our model, we could not find a difference in the total number of newly generated cells between slices that were exposed to OGD, co-cultured with cells, or served as controls. Interestingly, however, addition of GRPs led to an alteration in the profile of proliferating cells. In all groups, the majority of new cells were NG2-positive glial precursors, which is in accordance with previous studies that have shown that this cell type constitutes the main pool of proliferating cells in the brain after birth [45,46]. OGD-exposed slices had a marginally lower proportion of NG2-positive cells than control slices, which might be due to the increased presence of proinflammatory cytokines [47] after the hypoxic ischemic insult, whereas co-culture with GRPs either prevented (OGD+GRPs at 72 h after OGD) their decrease or even raised (OGD+GRPs at 24 h after OGD) their percentage. More striking than this rather small difference in the number of progenitors was the finding that in slices that were co-cultured with GRPs, the fraction of proliferating cells that expressed the neuronal precursor marker doublecortin was remarkably higher compared with OGD-only slices as well as with control slices.

It remains unclear whether this was a direct effect, that is, related to specific factors secreted by co-cultured GRPs, or whether it was contributable to the different milieu elicited by the presence of co-cultured cells. Previous investigations have shown that NG2 cells maintain considerable plasticity [48], and some studies indicate that aside from being precursors of myelinating oligodendrocytes [49], these cells are also able to generate functioning neurons [48,50] as well as astrocytes [51], depending on microenvironmental cues. A study by Sypecka et al. showed that injured tissue decreases the capacity of NG2 glia to form neurons, whereas a number of trophic factors, including IGF-1, GDNF, and BDNF, increase the percentage of NG2 cells with a neuronal fate [52].

Another factor that might be involved in the plasticity of these cells is the transcription factor Sox2, as Heinrich et al. exemplified in a recent study, where retrovirus-mediated expression of Sox2 was able to induce NG2 glia into DCX neurons in cerebral cortices of adult mice after stab wound injury [53]. It has been shown that oligodendrocyte progenitor cells are a rich source of growth factors and cytokines, including BDNF, SCF, CNTF, GDNF, NGR, IL-1b, and IL-10, among others [54], and although we did not perform growth factor measurements, it is likely that the observed effects in the present study are also, at least partly, attributable to release of trophic factors by co-cultured GRPs. Thus, future efforts to investigate the transcriptome of glial progenitor cells might help to identify bioactive molecules that are released by these cells and might be utilized for therapeutic approaches.

Conclusion

In summary, this study gives further evidence that GRP cells might be beneficial in neonatal hypoxia-ischemia due to their neuroprotective and neuroregenerative potential. Their effects did not require direct cell contact and were more pronounced when GRPs were applied during the subacute phase of injury. Although they remarkably lowered glutamate concentration, it is unclear whether this was the mechanism behind the observed findings. It is likely that several ways of action enable stem and progenitor cells, including GRPs, to substantially improve outcome after hypoxic-ischemic brain injury, where single-target approaches are often insufficient. Given the difficulty of translating cell-based therapy for newborn infants due to ethical and logistical challenges, it is pertinent that these mechanisms are further investigated to achieve optimal beneficial effects.

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