Therapeutic Potential of Canine Glial Restricted Progenitors Transplanted in Mouse Model of Demyelinating Disease

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Research

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Abstract

Background: Dysfunction of glia contributes to the deterioration of the central nervous system in a wide array of neurological disorders, thus global replacement strategies of glia are very attractive. Human glial restricted precursors (hGRPs) transplanted intraventricularly into neonatal mice extensively migrated and rescued lifespan in half of studied mice, while mouse GRPs (mGRPs) presented no therapeutic benefit. We hypothesized that the intrinsic developmental program (IDP) might be one of the main drivers of cell behaviour after grafting, with long migration distance and late myelination for hGRPs, compared to limited migration and early myelination for mGRPs. We studied in the same experimental setting canine GRPs (cGRP) to determine whether their migration, myelination and subsequent therapeutic potential falls between hGRPs and mGRPs. Additional motivation for selection of cGRPs was a potential for use in veterinary medicine due to growing population of dogs as companion animals. Methods: cGRPs were extracted from the brain of dog foetuses. The cells transplanted (4x10^5 cells) into anterior or posterior aspect of the lateral ventricle (LV) of neonatal, immunodeficient, dysmyelinated mice (shiverer, MBP^shi/shi, rag2^-/-). Outcome measures included early cell biodistribution, animal survival and myelination assessed with MRI, immunohistochemistry and electron microscopy. Results: Grafting of cGRP into posterior LV significantly extended animal survival, while no benefit was observed after anterior LV transplantation. In contrast, myelination of the corpus callosum was more prominent in anteriorly transplanted animals. Conclusions: The extended survival of animals after transplantation of cGRPs could be explained by the vicinity of transplant near the brain stem.

Introduction

CNS diseases are becoming a major burden to the society [1–3]. Glia is a major component of the central nervous system [4], and contributes to a wide array of disorders including neurological [5, 6] and psychiatric conditions [7, 8]. Moreover, transplantation of glial progenitors has been shown therapeutic in small animal models of a variety of brain diseases such as Pelizeaus-Merzbacher disease (PMD) [9], amyotrophic lateral sclerosis (ALS) [10], Huntington disease [11], spinal cord injury [12] as well as in vitro models including neonatal hypoxia-ischemia [13]. However, the transplantation of myelin-producing progenitor cells in patients with PMD was largely ineffective. The closer analysis of clinical data revealed a possible myelination only at a narrow area of implantation site [14, 15]. Indeed, we have recently shown in a mouse model of demyelination (shiverer mouse; MBP^shi/shi, which were also immunodeficient to avoid graft rejection; rag2^-/-), that a robust therapeutic effect of human glial restricted precursors (hGRPs) correlates well with their distribution throughout the entire mouse brain, while mouse GRPs (mGRPs) characterized by limited migration, failed to provide any therapeutic effect [16]. Interestingly, the mGRPs differentiated towards mature oligodendrocytes and myelinated much faster than hGRPs thus overall, we have observed a radically different behavior of GRPs depending on the species they come from. We hypothesized there are at least two reasons for such behaviours: 1) the GRPs execute their own intrinsic developmental program (IDP), which is closely linked with the size of the brain and species-appropriate
dynamics of myelination, more particularly hGRPs migrate far and myelinate late, and oppositely mGRPs migrate short and myelinate early and 2) due to a human-mouse mismatch xenografted hGRPs cannot fully respond to the host cues driving maturation of oligodendrocytes, allowing them to remain longer in highly migratory undifferentiated state and extensively migrate.

To test our hypotheses, we selected dog as a species with large brain to derive GRPs. If IDP would be a major driver of hGRP performance, we would expect that GRPs derived from dogs will perform somewhat between mGRP and hGRP in terms of migration and myelination. In contrast, if environmental factors such as capability of transplanted cells to read the host clues would have a dominant role, then GRPs derived from large animals would migrate equally well as hGRP after transplantation to dysmyelinated mice, which would subsequently translate to their widespread benefit equal to observed in case of hGRPs. Even though allografting of mGRPs in our previous study was disappointing, it was important to study performance of GRPs derived from other large brain, to fully understand the mechanisms governing engraftment and myelination. Such data will help maximize the success and predictability of clinical translation. Here, we used again shiverer mice due to the robust readouts they offer such as life-span at about six months, perfectly suited to reveal therapeutic effects of GRPs, as well as MBP mutation rendering endogenous myelin undetectable by antibodies to MBP, which guarantees ascribing any positive immune staining against MBP to the effect of activity of transplanted cells. The lack of compact myelin in shiverer mice is another advantage as it allows to determine the axon myelination by transplanted cells using MRI and electron microscopy.

Selection of dog as a large animal GRP donor has another very practical advantage. There is growing population of dogs as companion animals, with neurological health issues requiring attention. This creates solid market and demand for application of canine GRPs (cGRPs) in veterinary medicine. These therapeutic indications in veterinary medicine have important translational implication as many canine diseases closely resemble human diseases with an example of degenerative myelopathy being an excellent model of ALS [17, 18]. cGRPs can also be relatively easily obtained in large quantities. In summary, transplantation of cGRP in a mouse model of demyelination was performed to get a closer insight into a reason behind a radically different outcome of transplantation of hGRP vs. mGRP as well as their potential applicability in veterinary medicine.

**Methods**

**Experimental design**

Main goal of this project was assessment of therapeutic capacity of cGRPs transplanted into immunodeficient, dysmyelinated mice. Experimental design is presented on Fig. 1. The study is composed of one independent variable and five dependent variables (endpoints). The transplantation site is an independent variable and it is composed mainly of three levels: 1) anterior transplantation (A-transplanted at the level of bregma focused on targeting of forebrain), 2) posterior transplantation (P-transplanted at the level of lambda focused on targeting of midbrain and hindbrain), 3) negative control
(mutant mice without transplantation). Some endpoints included fourth level: positive control composed of wild type mice without defect in myelin. The dependent variables include 1) animal life span, 2) myelination detected by magnetic resonance imaging (MRI), 3) myelination detected by immunohistochemistry (IHC), 4) myelination detected by electron microscopy (EM), 5) early biodistribution of cGRPs.

Animal life span was measured by recording the time animals died. Shiverer mice die unexpectedly, therefore it is difficult to collect good quality tissue. Furthermore, each end point measure requires different tissue preservation thus separate cohorts of animals had to be used for assessment of post mortem endpoints such as IHC, EM and assessment of biodistribution.

The animals were sacrificed at three time points: 18, 31 and 40 weeks after neonatal transplantation. Post-mortem assessment of myelination was performed only in anteriorly transplanted animals, as MRI reported no myelination in posteriorly transplanted animals.

Animals for longitudinal in vivo MRI were randomly recruited from the cohort devoted to assessment of life span and IHC.

For assessment of early biodistribution cGRPs were fluorescently labelled with Dil and followed post mortem at three time points: 1, 3 and 28 days. In total there were 139 mice used in the study and the animal allocation to experimental and control groups is presented in Table 1.

All the procedures were conducted with appropriate approval from the Ethical Committee (IV Local Committee in Warsaw, 48/2013, 240/2017, 259/2017).
Table 1
Animal allocation to experimental and control groups.

|                          | Anteriorly transplanted  | Posteriorly transplanted  | Negative Control  | Positive control  |
|--------------------------|--------------------------|---------------------------|--------------------|-------------------|
|                          | (rag2, shiv)             | (rag2, shiv)              | (rag2, shiv)       | (rag2)            |
| Survival                 | N = 38, MRI = 5          | N = 12, for MRI:5         | N = 11             |                   |
| IHC                      |                          |                           |                    |                   |
| 18 weeks                 | N = 7(5)                 |                           | N = 9, for MRI:4   |                   |
| > 26 weeks               | N = 7(3)                 |                           |                    |                   |
| EM                       |                          |                           | 28.5 weeks         | 28.5 weeks        |
| 18 weeks                 | N = 5                    |                           | N = 5              | N = 5             |
| 31 weeks                 | N = 5                    |                           |                    |                   |
| Biodistribution          |                          |                           |                    |                   |
| 1 day                    | N = 6                    | N = 5                     |                    |                   |
| 3 days                   | N = 5                    | N = 5                     |                    |                   |
| 28 days                  | N = 7                    | N = 5                     | N = 2              |                   |

Procurement of cGRPs and preparation for transplantation

The procedure of cGRP isolation followed methodology for mGRP derivation [19]. Briefly, cGRPs were derived from mid-gestation (E 32–37) dogs. Isolated brains and spinal cords from canine foetuses were sectioned into small fragments and incubated with TrypLE Express (Gibco) with addition of 10 mg/ml of DNase-1 (Sigma) in RT for 10–12 min then the tissues were triturated and incubated for 10 min in 37 °C. TrypLe was inactivated through addition of 5 ml of complete GRP medium and the cell suspension was spun down (300 x g RT, 5 min), the supernatant was discarded, the pellet was suspended in GRP medium with 10 mg/ml of DNase and incubated for another 10 min in 37 °C followed by cell trituration and spinning down (300 x g, T, 5 min). Then the pellet was re-suspended in GRP medium with addition of 20 ng/ml bFGF-2 (Takara), obtained cells were plated on poly-L-lysine (Sigma) and laminin (Thermo Fisher Scientific) coated flasks and cultured in standard cell culture conditions (37 °C, 5% CO₂ concentration) for two passages. GRPs were then harvested, cryopreserved in ATCC medium (Takara) and stored in liquid nitrogen. Before transplantation cGRPs were thawed, counted and suspended in sterile saline. Labelling of cGRPs with lipophilic dye – DiL (Thermo Fisher Scientific) has been performed according to manufacturer’s instructions for their post mortem identification in short term experiments to assess their early intracerebral biodistribution.

Shiverer/rag2 mice
In order to investigate therapeutic activity of cGRPs we used double mutant immunodeficient, dysmyelinated shiverer mice (MBP<sup>shi/shi</sup>, rag2<sup>-/-</sup>) both males and females as previously described by us [16]. Animals were obtained through cross-breeding of shiverer mouse (C3HeB/FeJ-shiverer, The Jackson Laboratory) and rag2 mouse (B6(Cg)-<em>Rag2</em>tm1.1Cgn/J, The Jackson Laboratory). For clarity we are using “shiv/rag2” as simplified nomenclature for double mutants. Shiverer mice are characterized by recessive autosomal mutation in both alleles of MBP (myelin basic protein) gene resulting in protein truncation and subsequent demyelination. Characteristic feature of shiverer mice is trembling during locomotion, occasional seizures and lifespan shortened to around 200 days. In turn rag2 knockout results in defects in T and B cell development and thus impaired adaptive immunological response. Mice are genetically stable and some of the randomly chosen animals were genotyped in order to confirm the mutation in rag2 gene. Homozygous mutation of MBP gene is phenotypically visible through trembling. Due to immunodeficiency mice were bred and maintained in special pathogen free (SPF) environment in Laboratory of Genetically Modified Animals at MMRC, PAS.

**cGRP transplantation**

Mouse pups (P 2–3) were cryo-anesthetized and placed on ice in stereotaxic apparatus equipped with a mouse adaptor. cGRPs (total 2 × 10<sup>5</sup> cells in 4 ul of saline) were transplanted intraventricularly into each hemisphere using stereotaxic coordinates: AP:0.6, ML:1.0/-1.0 and DV:0.8 from bregma (anteriorly (A)-transplanted) or AP:0.8, ML:1.0/-1.0, DV:0.8 from lambda (Posteriorly (P)-transplanted). Control mice did not receive cGRP transplantation. After transplantation the pups were removed from stereotaxic apparatus, their vital functions were restored and they were returned to the cage with their mother.

**Immunohistochemistry**

For immunohistochemical studies the experimental and control mice were sacrificed after deep anaesthesia with a mixture of 100 mg/kg ketamine and 1 mg/kg medetomidine i.p. injection. Then the animals were perfused intracardially with 4% paraformaldehyde (PFA). The brains and spinal cords were removed, post-fixed overnight in 4% PFA and incubated in 20% saccharose until fully saturated and cryopreserved with dry ice and stored in -80 °C until cryo-cut into 20 µm sections for fluorescent immunohistochemistry. Briefly, tissue sections were rinsed in PBS and then incubated in 10% goat serum in PBS containing 0.25% Triton X-100 and 0.1% BSA for 60 min in room temperature (RT). Next, the sections were washed with PBS and incubated with primary antibodies (60 min, RT). The following antibodies (final dilution and source) were used for brain and spinal cord tissue staining: rat anti-MBP (myelin basic protein) monoclonal antibody (1:200, Millipore) to detect myelin protein; anti-GFAP (glial fibrillary acidic protein, 1:500, Dako) polyclonal antibody to display astrocytes and neural progenitors. Then tissue sections underwent the washing procedure, and the primary antibodies were visualized by applying appropriate secondary antibodies: goat anti-rat or goat anti-rabbit Alexa Fluor 488 or 546 (1:500, Thermo Fisher Scientific) for 60 min at room temperature and in the dark. Nuclei were counterstained with the fluorescent dye 5 µM Hoechst 33258 (Life Technologies). Images of immunostained tissues were acquired using a confocal laser scanning microscope (LSM 780, Carl Zeiss, Germany) and Cell Observer SD (Carl Zeiss, Germany) using a 20 x or 40 x objectives. A helium-neon laser (543 nm) was
utilized in the excitation of Alexa Fluor 546, while an argon laser (488 nm) was applied in the excitation of FITC. ZEN software was used for quantitative analysis of immunoreactivity in all sections. Five animals per group were analysed. Images from two sections per animal were acquired, and the number of positively stained cells was counted as well as fluorescence intensity was measured. The analysis was performed in Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, PAS.

**Transmission electron microscopy (TEM) analysis**

The myelin visualization in the brain and spinal cord of A-transplanted animals was performed in mice sacrificed at two points: 18 weeks and 31 weeks post cGRP transplantation. Non-transplanted, dysmyelinated rag2 animals served as a negative control and healthy rag2 mice served as a positive control. Before isolation of regions of interests, the experimental and control animals were anesthetized with a mixture of 100 mg/kg ketamine and 1 mg/kg medetomidine and perfused intracardially with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer (pH 7.4), followed by 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) infusion. Tissue samples collected from the corpus callosum and white matter of spinal cords were fixed in the above mentioned ice-cold fixative solution and post-fixed in 1% OsO$_4$ solution. After dehydration in the ethanol gradient, the tissue samples were embedded in epoxy resin (Epon 812). Thin sections of 50 nm were stained with 9% uranyl acetate and lead nitrate. Images were acquired using JEM-1200 EX (Jeol, Japan) transmission electron microscope equipped with MORADA camera and iTEM 1233 software. TEM analysis was performed in the Electron Microscopy Platform, Mossakowski Medical Research Centre, PAS.

**MRI analysis**

MR imaging was performed in Small Animal Magnetic Resonance Laboratory, Mossakowski Medical Research Centre, PAS. Imaging was performed 18, 32 or 40 weeks after cell transplantation. Mice were anesthetized with isoflurane (1.5-2% in oxygen) and positioned head first, prone in the MR-compatible water-heated bed. Body temperature and respiration rate were monitored throughout the study with MR-compatible probes (SA Instruments, Stony Brook, NY, USA). 7T MRI scanner (BioSpec 70/30 USR, Bruker, Ettlingen, Germany) equipped with transmit cylindrical radiofrequency coil (8.6 cm inner diameter, Bruker) and a mouse brain dedicated receive-only array surface coil (2 × 2 elements, Bruker) were used. The structural imaging protocol was performed as we described previously [20]. Briefly, we used T2-weighted TurboRARE sequence (TR = 7000 ms; TEff = 15 ms; RARE factor = 4; NA = 4; field of view, FOV = 22 mm × 22 mm; spatial resolution = 86 µm × 86 µm × 350 µm; 42 slices, no gap; scan time ~ 23 min). Measurements of signal intensity in the corpus callosum were performed in Fiji software. Briefly, the same size of corpus callosum midsections were outlined on T2 MRI images of similar coronal slice. Intensity of signal was measured and normalized to the signal intensity of the cortex for identical size ROI.

**Statistical analysis**
The least means squares incorporated into PROC MIXED (SAS 9.4) have been used to determine the differences in continuous variables between the groups for MRI endpoint. The dot plot was employed for their graphical presentation. The chi square test has been used to calculate significance in frequency distribution table, which served to quantify differences in cell biodistribution endpoint. The log-rank (Mantel-Cox) test was used to calculate differences in life span between groups. Kaplan-Meier curves to present animals’ survival were drawn using Graph Pad Prism 7.04 software.

Results

Survival analysis of shiv/rag2 mice transplanted with cGRPs

The median survival of control mice was 197.5 days, and the median survival of A-transplanted mice was extended only to 202 days (p = NS), while mice, which received P-transplants revealed significant extension of median survival to 253 days (p = 0.0005) (Fig. 2). More descriptively, there were only 10% of A-transplanted mice, which survived longer than 10% of the time of longest surviving non-transplanted mouse (around 20 days), while the same was observed in 50% of P-transplanted mice.

Analysis of early distribution of cGRP within the brain after their transplantation into two different transplantation sites

The injection site in A-transplantation group corresponds to anterior horns of the lateral ventricle, while for P-transplantation group the cells were infused to the area of ventricle lining hippocampus and neighbouring midbrain (Fig. 3A, B). In A-transplantation group cGRPs were detected in 6 out of 15 animals, while for P-transplantation group in 14 out of 15 animals (chi square = 9.6, p < 0.01). Mapping biodistribution of cGRPs confirmed their presence across the entire ventricular system in both groups of animals, although as expected more cells were present around their transplantation sites (Fig. 3C, D).

MRI analysis of myelination after cGRPs transplantation in shiv/rag2 mice

MRI analysis revealed strong T2 hypointensity in the corpus callosum in rag2 animals (Fig. 4A), and no hypointensity in this area in shiv/rag2 mice (Fig. 4E), confirming it as an excellent surrogate imaging marker of myelination. T2 hypointensity in corpus callosum was also observed in both experimental (transplanted) groups (Fig. 4B-D, F-G), though was much weaker than in positive controls (rag2). Interestingly, the T2 hypointensity was more prominent in A-transplanted mice and the area of myelination for both groups was rather limited and there was no expansion of that area over time beyond the earliest MRI time point at 18 weeks (Fig. 4B, C, D). As expected, there was a variability of T2 hypointensity among all animals. The analysis of MRI signal hypointensity revealed that myelination was pronounced in 18 weeks and 32 week animals transplanted in the anterior site in comparison to non-transplanted group (p = 0.0486; p = 0.0262 respectively). Myelination of corpus callosum of posteriorly transplanted animals was not significantly different from control animals (Fig. 4H)
Immunohistochemical analysis of myelin after cGRPs transplantation in shiv/rag2 mice

Immunohistochemical analysis performed in our studies confirmed that there was no MBP staining in control, non-transplanted shiverer/rag2 mice (Fig. 5A-C). In contrast, positive MBP staining was observed in corpus callosum of examined transplanted shiverer/rag2 mice at 18 weeks (Fig. 5E) and 32-week time point (Fig. 5H) while typically no positive staining was observed in the spinal cord (Fig. 5F), except one A-transplanted animal in which a robust myelination was also observed in the spinal cord (Fig. 5I).

TEM analysis of axon myelination in cGRP transplanted shiv/rag2 mice

In rag2 mice (positive control) as expected there was a compact myelin structure in the corpus callosum and spinal cord (Fig. 6A, E). There was no compact myelin observed in the brain and spinal cord of shiv/rag2 mice, except for single axons (Fig. 6B, F). cGRP transplanted shiv/rag2 mice revealed groups of axons surrounded by compact myelin in corpus callosum at 18 weeks (Fig. 6C) and 32 weeks (Fig. 6D). Moreover, actively myelinating oligodendrocytes were visible in the brains and spinal cords of transplanted mice. Proper, compact myelin around groups of axons (in contrast to single axons in non-transplanted group) was visible in all animals of transplanted group, however there were visible differences in numbers of properly myelinated axons in one experimental group. Compact myelin was also observed in spinal cord of some transplanted shiv/rag2 mice (Fig. 6G, H).

Discussion

Our study revealed several surprising findings, which require extensive discussion in the context of existing literature. First of all, we have shown that myelin repair of corpus callosum does not go in pair with benefit in animal survival. The mice transplanted to the anterior part of ventricular system did not show any meaningful extension of survival despite the robust myelination of corpus callosum. In contrast, the animals transplanted to the posterior part of ventricular system survived significantly longer, while the myelination of corpus callosum was negligible. Moreover, the analysis of early cGRP biodistribution detected the cells in nearly all posteriorly transplanted animals but cGRPs were found in only small number of anteriorly transplanted animals. While, assessment of myelination revealed compact myelin in all transplanted animals and none of the non-transplanted animals, there was high inter-animal variability in the level of compact myelin formation.

The cGRPs myelinated early and robustly as shown by MRI and post mortem analysis but anteriorly transplanted animals did not show any survival benefit, which puts cGRPs in pair with mGRPs rather, and not with hGRPs [16]. Interestingly, the same cGRPs transplanted more posteriorly significantly extended animal survival, which may be linked to the closer vicinity of brain stem, which might be more essential brain structure for animal survival. However, the extension of animal survival by cGRP posterior
transplantation was still less pronounced than by hGRPs in our previous study [16]. Therefore, we hypothesize that behaviour of transplanted GRPs rather depends on IDP than is driven by a species mismatch between donor and host.

The differences between transplantation sites seem to be of great importance. Cells transplanted anteriorly presented better myelination than animals with cells transplanted posteriorly. Localization of transplant near the anterior parts of ventricles and in the same time close to corpus callosum in case of anterior transplantation might be favourable for induction of myelination. Glial progenitors transplanted in such environment might be stimulated to differentiate into oligodendrocytes rather than remain as progenitors or become astrocytes and thus myelination is more pronounced. On the other hand, cGRPs transplanted posteriorly might migrate toward two important structures in terms of neurogenesis (hippocampus) or vital functions (brain stem). These are likely more essential structures from therapeutic perspective than the corpus callosum. Myelination of the corpus callosum was negligible in group of animals, which received more posterior cGRP transplantation, which is not surprising taking into account a distance between transplantation site and this brain structure, but despite weaker myelination the animals survived significantly longer. In our previous study we have shown that at the moment of death of control animals (~ 200 days), there was still no production of compact myelin by hGRPs [16]. Therefore, our current study further support hypothesis that myelination of corpus callosum is probably not essential to extend survival of dysmyelinated mice and it might not be the best target for cell implantation in clinical translation as well as a biomarker of outcome. The studies on optimal cell destination are certainly warranted as we have previously shown that the survival of transplanted GRPs also strongly depends on their implantation site in the brain [21]. More extensive migratory capacity of hGRPs was sufficient for colonization of brain stem area even after transplantation into anterior aspect of lateral ventricle, while cGRPs do not have such a quality. It would be also interesting in future to perform posterior transplantation of mGRPs (low migratory capacity) as transplanted at this location could be more therapeutic than previously transplanted anteriorly [16].

The early assessment of cGRP biodistribution revealed another surprising result. We could not detect any cGRPs in a majority of anteriorly transplanted animals, while the same was true for only one mouse transplanted posteriorly. The reason for this apparent discrepancy might be that cGRPs transplanted to the larger CSF compartment such as anterior lateral ventricle are less prone to the adherence to the ventricular wall so can remain as floating fraction and are washed out during cryo-processing. In contrast, cells injected into smaller fluidic compartment after posterior transplantation may facilitate firmer cell contact with ventricular wall and persistence in the brain after tissue processing procedure. Moreover, we detected graft-derived myelin in the spinal cord of only one of the investigated mice, and it was anteriorly transplanted mice. It seems that intraventricularly transplanted GRPs traveled with cerebrospinal fluid (CSF) toward the spinal cord, settled down in a new niche and after differentiation became functional. While neonatally transplanted GRPs into the CSF migrate well into brain parenchyma, cells injected at later stages of development usually sediment and/or accumulate on the border of brain or spinal cord parenchyma and demonstrate limited migration into the tissue [22, 23].
The variability of cell distribution and homing is a major feature of all neonatal GRP transplantations, which translates to the inconsistency of therapeutic outcomes. Imaging of the transplantation procedure in real-time with tracking labelled cells might be a solution, as it was shown effective for guiding intra-arterial [24], and intraparenchymal transplantation [25]. Since, we transplant cells to the fluid compartment, post mortem assessment of cell bio-distribution seems to be inadequate as the cells suspended in the fluid are lost during tissue processing. Majority of cell labelling studies uses MRI of iron oxide-labelled cells, however modalities based on nuclear medicine approaches are very compelling [26–28]. Radioactivity is quantitative in nature, it does not interfere with important imaging outcome measure techniques such as DTI or fMRI and is not subject to the numerous artefacts plaguing detection of iron oxide-labelled cells, which are especially pronounced in the spinal area.

Direct detection of transplanted canine cells is not possible due to the paucity of canine-specific antibodies and this certainly is a limitation of our study. However, we were able to conclude on successful engraftment based on the functional effects of transplanted cells namely myelin production. We have used two complementary methods: IHC detecting MBP, which is a protein, frequently preceding the process of myelination, and EM, which is a gold standard in detecting ultrastructure of myelin. Importantly, no MBP staining or compact myelin beyond single axons was observed in non-transplanted mice, so all positive findings can certainly be ascribed to transplanted cells. We can see formation of compact myelin, however not all of the axons were myelinated and the number of myelin layers was lower in comparison to control wild type animals. The diversity in migration patterns between mouse and human cells was also noticed by Windrem and colleagues [29]. Indeed, as shown by Kim and colleagues, intraventricularly transplanted GRPs in experimental autoimmune encephalomyelitis (EAE) model were mostly localized in ventricular system and were viable only until day 10th post-transplantation [30]. Since, we have not observed robust myelination of corpus callosum by MRI in P-transplanted mice, we have not dedicated additional groups specifically for IHC and EM in this groups of animals.

Our study has shown that cGRP are functional and therapeutic in a mouse model of dysmyelination; however, there is more development needed ahead of clinical translation of this approach in both human and veterinary medicine. It seems that migration of transplanted cGRPs is insufficient, therefore the methods warranting better brain coverage are needed, such as multipoint transplantations, cell engineering to increase their migration or cell delivery approach supporting more widespread biodistribution such as intra-arterial injection. Obviously, each transplantation approach has its own challenges such as risk of haemorrhage due to multiple brain puncture, deterioration of therapeutic potential due to cell engineering or need for cell diapedesis from arteries to the brain parenchyma. Cell labelling and imaging seems to be essential to guide future clinical application, as conclusive early assessment of cell biodistribution using post-mortem techniques is obviously not possible.

Conclusions

The intraventricularly injected cGRPs engraft into the brain of dysmyelinated mice and become functional after their transplantation. The survival benefit is independent from the myelination of corpus callosum
and depends on the transplantation location. Interestingly, the animals transplanted more posteriorly and in closer proximity to the brain stem lived significantly longer, while positive effect was not observed after more anterior transplantation. In summary, there is more research needed on the GRP delivery prior to the clinical translation of this concept.

**Abbreviations**

PD: Parkinson’s Disease; AD: Alzheimer’s Disease; HD: Huntington’s Disease; SMA: Spinal Muscle Atrophy; ALS: Amyotrophic Lateral Sclerosis; ER: endoplasmic reticulum; SOD1: superoxide dismutase 1; MNs: motor-neurons; MCT-1: monocarboxylate transporter; GRPs: glial restricted progenitors; DM: degenerative myelopathy; cGRPs: canine glial progenitors; bFGF-2: basic fibroblast growth factor; PFA: paraformaldehyde; PBS: phosphate buffered saline; FBS: foetal bovine serum; BSA: bovine serum albumin; MBP: myelin basic protein; GFAP: glial fibrillary protein; TEM: transmission electron microscopy; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid; PMD: Pelizaeus-Merzbacher Diseases; EAE: experimental autoimmune encephalomyelitis;

**Declarations**

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**Authors’ contributions**

LS planned, analysed and interpreted the results, wrote the manuscript

MM planned and performed immunohistochemical experiments

KD performed animal genotyping, planned and interpreted the results

PR performed immunohistochemical analysis

JS isolated and provided canine glial restricted progenitors

MF performed MRI experiments

MG performed and analysed electron microscopy experiments

MFB performed and analysed electron microscopy experiments
PW planned and interpreted the results
BL analysed and interpreted the results, contributed to writing of the manuscript
MJ planned and interpreted the results, wrote the manuscript.
All authors read and approved the final manuscript.

**Ethics approval**

All the procedures with animals were conducted with appropriate approval from the Ethical Committee (IV Local Committee in Warsaw, 48/2013, 240/2017, 259/2017).

**Disclosure of Potential Conflicts of Interest**

JS is an owner of for-profit Vetregen Laboratory and Stem Cell Bank for Animals, Warsaw, Poland, which provided cells for the current study within the governmentally funded project. PW and MJ are co-owners of IntraART, LLC and Ti-com, LLC, which however are not directly related to the current study.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

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