

Transplantation of Human Stem Cell-Derived GABAergic Neurons into the Early Postnatal Mouse Hippocampus to Mitigate Neurodevelopmental Disorders

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Abstract

A reduced number or dysfunction of inhibitory interneurons is a common contributor to neurodevelopmental disorders. Therefore, cell therapy using interneurons to replace or mitigate the effects of altered neuronal circuits is an attractive therapeutic avenue. To this end, more knowledge is needed about how human stem cell-derived GABAergic interneuron-like cells (hdINs) mature, integrate, and function over time in the host circuitry. Of particular importance in neurodevelopmental disorders is a better understanding of whether these processes in transplanted cells are affected by an evolving and maturing host brain. The present protocol describes a fast and highly efficient generation of hdINs from human embryonic stem cells based on the transgenic expression of the transcription factors *Ascl1* and *Dlx2*. These neuronal precursors are transplanted unilaterally, after 7 days *in vitro*, to the hippocampus of neonatal 2-day-old mice. The transplanted neurons disperse in the ipsi- and contralateral hippocampus of a mouse model of cortical dysplasia-focal epilepsy syndrome and survive for up to 9 months after transplantation. This approach allows for investigating the cellular identity, integration, functionality, and therapeutic potential of transplanted interneurons over an extended time in developing healthy and diseased brains.

Introduction

The establishment, maturation, and refinement of neuronal networks occur during the perinatal and early postnatal period and represent crucial time windows for brain development¹. From an after-birth exuberance of connectivity, the brain

evolves to a fine-tuning of connections that extend until adolescence². Consequently, alterations in genes expressed during these periods, as well as external factors or insults, define an individual's predisposition to multiple

neurodevelopmental disorders. Impairments in cognition and motor function unfold over time, and pharmacological treatments are limited, the majority targeting symptoms with the risk of severe side effects.

Dysfunction in gamma-aminobutyric acid (GABA)ergic inhibition has been shown to be a major contributor to the underlying cause of various neurodevelopmental disorders³, such as fragile X syndrome, Angelman syndrome, epilepsy, schizophrenia, and autism. GABA is the major inhibitory transmitter of the central nervous system and is instrumental for maintaining excitatory/inhibitory (E/I) balance, the synchronization of neuronal firing, and computation. GABAergic interneurons are a heterogeneous population of neurons, with increasing functional intricacy in more complex brain regions⁴ and with evolution^{5,6}. Considering the limited endogenous regenerative capacity of the human brain and the implication of interneuron dysfunction in several neurological disorders, the transplantation of GABAergic interneurons may be a promising therapeutic avenue to explore. Along this line, human stem cell-derived GABAergic interneuron-like cells (hdINs) seem to be the most translational and viable source for this purpose compared to rodent allogenic neuronal precursors or other sources used elsewhere⁷. Protocols for generating GABAergic neurons from diverse cell sources are available^{8,9,10,11,12,13}, but more knowledge is required on how hdINs mature, integrate, and function over time in a developing pathologic brain. Several studies have identified alterations in genes active during cortical patterning, establishing neuronal connectivity¹⁴, and tuning physiological E/I balance¹⁵. Neonatal transplantation of hdINs into mouse models with corresponding genetic perturbations allows us to follow the interplay between host and graft, which is

knowledge necessary to determine potential therapeutic strategies.

Immunomodulation is commonly and successfully used in xenograft transplantations to avoid triggering a host immune response and rejection¹⁶. However, the administration of immunosuppressive drugs, such as cyclosporine A, causes renal toxicity after chronic administration, is labor-intensive because of the need for daily intraperitoneal injections to achieve stable systemic concentrations, causing animal stress¹⁷, and has off-target effects that can interact with pathology¹⁸. In addition, compromising the immune system has been shown to change behavioral phenotypes¹⁹, with alterations in the corresponding neuroanatomical regions²⁰. Transplantation in the first week of life has been shown to allow for adaptation to transplanted cells^{21,22}, while others have reported initial survival followed by rejection of the grafts within the first postnatal month^{23,24}.

This protocol describes procedures from hdIN generation to cell transplantation in neonatal mice resulting in long-term graft survival and allowing for the investigation of the neuronal specificity, synaptic integration, function, and therapeutic potential of transplanted human interneurons during physiological and pathological development.

Protocol

All experimental procedures were approved by the Malmö/Lund Animal Research Ethics Board, ethical permit number 12548-19, and conducted in agreement with the Swedish Animal Welfare Agency regulations and the EU Directive 2010/63/EU for animal experiments. C57BL6/J and *Contactin-associated protein-like 2 (Cntnap2)* knock-out (KO) mice, both males and females, were used at postnatal day (P) 2 for the present study. Human embryonic stem cells (hESCs)

were used. The animals and stem cells were obtained from commercial sources (see **Table of Materials**).

1. Generation of the hdIN precursors

NOTE: All the steps in this section are done in a cell culture hood. The hESCs were maintained as feeder-free cells on coated plates using a stem cell culture medium and passaged as colonies.

1. Perform forward programming of the hESCs to hdIN precursors (**Figure 1**).

NOTE: The forward programming approach is based on the procedure described in Gonzalez-Ramos et al.⁸ and Yang et al.⁹.

1. Transduce the hESCs with lentiviral vectors containing the Tet-On system in a fresh stem cell culture medium containing 10 μM of ROCK inhibitor (RI) (see **Table of Materials**). Keep at 37 °C in the incubator.
2. Change the medium to a fresh stem cell culture medium 16 h after transduction and maintain the transduced cells in the same way as the non-transduced hESCs.
3. When confluent, detach the hESCs as single cells using a cell detachment solution (see **Table of**

Materials) and plate them in coated 6-well plates at a density of 3×10^5 cells/well in stem cell culture medium containing 10 μM of RI on day *in vitro* (DIV) -1.

4. At 1 DIV, replace the culturing medium with N2 medium containing 2 g/L of doxycycline (DOX, see **Table of Materials**).

NOTE: DOX is used to induce transgene expression of *Ascl1* and *Dlx2*⁹, from 1 DIV to 7 DIV, and then continues *in vivo* by its addition to the drinking water²⁵.

5. At 2 DIV, an antibiotic resistance selection period starts that will last until 5 DIV. Add puromycin (puro) and hygromycin (hygro) to the fresh medium (see **Table of Materials**). Change the medium on 2 DIV, 4 DIV, and 5 DIV.

NOTE: Optimize the concentrations of puro and hygro before the differentiation protocol using transduced and non-transduced hESCs to ensure the survival of only the cells carrying the antibiotic resistance cassettes. In this protocol, 0.5 $\mu\text{g}/\text{mL}$ of puro and 750 $\mu\text{g}/\text{mL}$ of hygro were used.

6. At 5 DIV, the antibiotic selection period ends. Change to N2 medium supplemented with 2 g/L of DOX and 4 μM of cytosine β -D-arabinofuranoside (Ara-C, see **Table of Materials**).

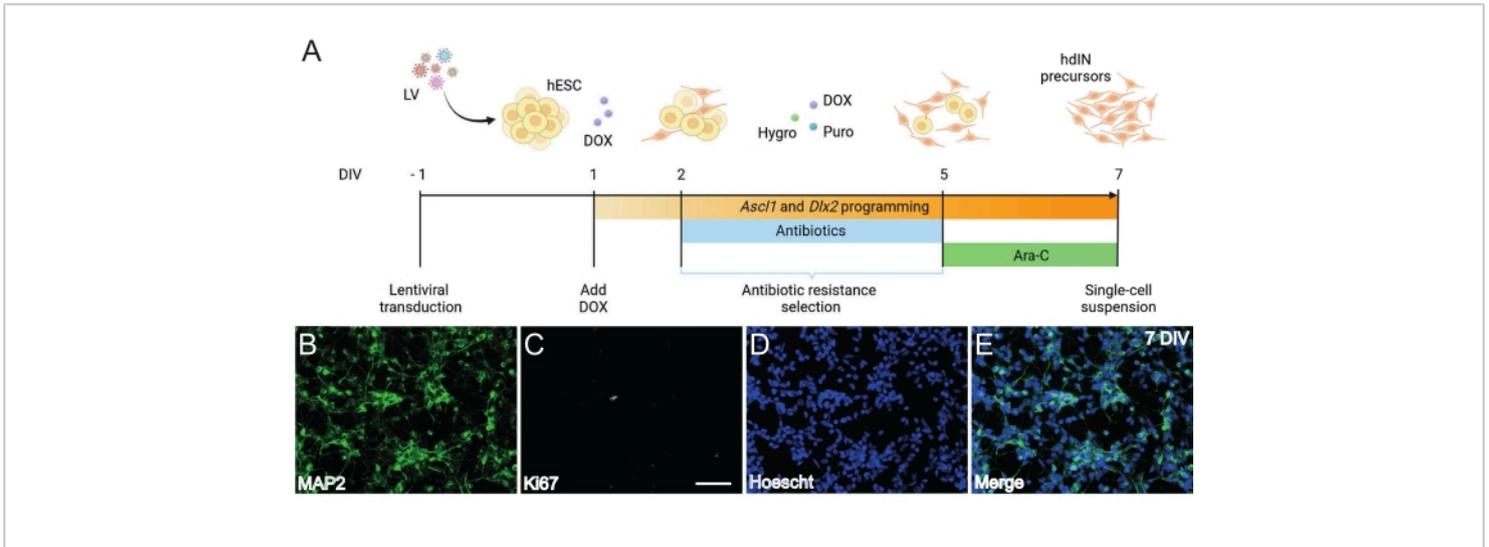


Figure 1: Generation of hdIN precursors from hESCs by overexpressing *Ascl1* and *Dlx2*. (A) Schematics of the differentiation protocol used for the generation of hdIN precursors. (B-E) Immunocytochemistry of hdIN precursors at 7 DIV for (B) the neuronal marker MAP2, (C) the proliferative marker Ki67, (D) general nuclear staining, and (E) a merge of the previous markers. Scale bar: 50 μ m. [Please click here to view a larger version of this figure.](#)

2. Preparation of the single-cell suspension for transplantation

NOTE: All the steps in this section are done in the cell culture hood. On 7 DIV, hdIN precursors are dissociated and used for transplantation.

1. Perform detachment of the cells following the steps below.
 1. Remove the medium and carefully rinse the cells with DPBS without calcium and magnesium.
 2. Add 400 μ L of the cell detachment solution (see **Table of Materials**) to each well of the 6-well plate.

NOTE: Ensure the coverage of the whole surface by the solution.
 3. Incubate for 2-3 min at 37 $^{\circ}$ C in the incubator until the border of the cells starts to look shiny, indicating

enzymatic degradation of cell surface proteins and detachment from the well surface.

NOTE: To increase cell survival, do not wait too long when all the cells are completely detached and floating around.

4. Then, add 600 μ L of fresh N2 medium to each well of the 6-well plate to stop the enzyme, and mechanically with the pipette, help to detach the cells to obtain a single-cell suspension.
5. Transfer the cell suspension to a plastic tube (15 mL) and centrifuge at 180 x g for 4 min at room temperature (RT).
2. Perform resuspension of the cells.
 1. Discard the supernatant using a vacuum system (carefully, without disturbing the pellet) and resuspend the pellet in the transplantation medium

containing 10 μM of RI, 1 $\mu\text{g}/\text{mL}$ of DNase, and 2 $\mu\text{g}/\mu\text{L}$ of DOX.

2. Count the total cells in the suspension using a counting chamber and a manual cell counter and adjust the volume to a final concentration of 100,000 cells/ μL .
3. Keep the cell suspension in a closed tube on ice until transplantation for a maximum of 4 h.

NOTE: The transplantation has to be performed on the same day as the preparation of the cell suspension (7 DIV).

3. Intrahippocampal cell transplantation

NOTE: All the steps in this section are performed outside the cell culture hood in the animal facility. Early postnatal transplantation of cells into the brain was performed on P2, considering P0 the day of birth.

1. Prepare for the transplantation experiment following the steps below.
 1. Autoclave all the surgical material or, if not possible, sterilize with another approved method.
 2. Mount the injecting syringe in the holder without any glass capillary. Rinse the needle with water.

NOTE: The needle must be 33 G.
 3. Curve 90° the tip of a 30 G insulin needle.
 4. Create a homemade Play-Doh-like stage for positioning the pup with the head flat (**Figure 2A**).
 5. Fetch the mice cage with the mother and pups before preparing anything for the surgery to allow them to acclimatize to the new environment.
 6. Prepare a tray with wet ice and an empty cage with some paper for the isoflurane.

2. Anesthetize the mouse pup.
 1. Soak a piece of paper with isoflurane (see **Table of Materials**) and place it inside the empty cage. Carefully take one pup and place it into the cage with the paper soaked in isoflurane.

NOTE: Never leave less than three pups with the mum. Do not exceed 20-30 s of isoflurane anesthesia, or the pup may die.
 2. Verify the effect of the anesthesia by movement cessation.
 3. Immediately after anesthesia, place the pup onto a wet tissue on the surface of wet ice. Keep the animal on ice for 3 min until the upper limbs become whiteish (**Figure 2B**, blue arrow). This usually takes 2-3 min.
 4. Use this time for loading the syringe with the cell suspension. Resuspend the cells carefully with a pipette before.
 5. Place the pup on the stereotaxic frame. Use the ear bars in the opposite direction (**Figure 2A**, magenta arrows).

NOTE: The back side of the ear bars is less pointy, and as P2 pups do not have ears, use the flatter side to avoid hurting the animal.
 6. From the side, check if the head is straight. The head must be flat; use the Play-Doh-like stage to adjust for that.

NOTE: Pups at this age have not yet opened their eyes, so no additional step in this regard needs to be done. Pups do not have fur at this age, so no shaving of the area is required. No specific pain treatment is given as it is not an open incision to the skin, and no suturing is involved.

7. Keep the pup covered on ice (on top of tissue paper) for the entire duration of the surgery.

NOTE: Do not place the ice in direct contact with the pup's skin.
3. Perform the injection.
 1. Clean the surface of the skin using a soft tissue soaked in ethanol.
 2. Identify lambda and set the coordinates to zero on the digital display console of the stereotaxic instrument (**Figure 2C**, yellow dashed line). Sagittal and lambdoid sutures are easily visible by the eye, since they are vascularized, as red lines.

NOTE: If experiencing difficulties visualizing lambda, place a small piece of ice on the skin and wait a couple of minutes for it to cool down. Then, remove the piece of ice, and the subtle whitening of the skin will allow you to visualize.
 3. Relocate the injection needle to the desired coordinates. In the described protocol, the targeted region was the hippocampus, and the coordinates were as follows: anterior-posterior (AP) +0.85 and medio-lateral (ML) +1.35.
 4. Use a 90° bent insulin needle to penetrate the skull and create a tiny hole.
 5. Bring down the injection needle until it has crossed the skull, and zero the dorso-ventral (DV) coordinates. Lower the needle until the desired DV coordinates. In the described protocol, the coordinates were DV -1.1.
 6. Inject according to the predetermined time intervals.

NOTE: In the described protocol, the exact timings were the following: (i) after going down to the DV coordinate, wait 3 min, (ii) inject 1 µL of volume for 5 min, and (iii) after all the volume has been injected, wait 3 min.
 7. Retract the needle slowly.
4. End the procedure.

NOTE: The surgery cannot last more than 15 min to ensure the pup's survival and avoid any damage derived from the anesthesia by hypothermia.

 1. Warm up the pup with the hands until it starts moving before giving it back to the mother.
 2. Give DOX at a concentration of 1 mg/mL in 0.5% sucrose solution as drinking water for at least 2 days before and 3 weeks post transplantation (PT) to continue the cell differentiation *in vivo*.

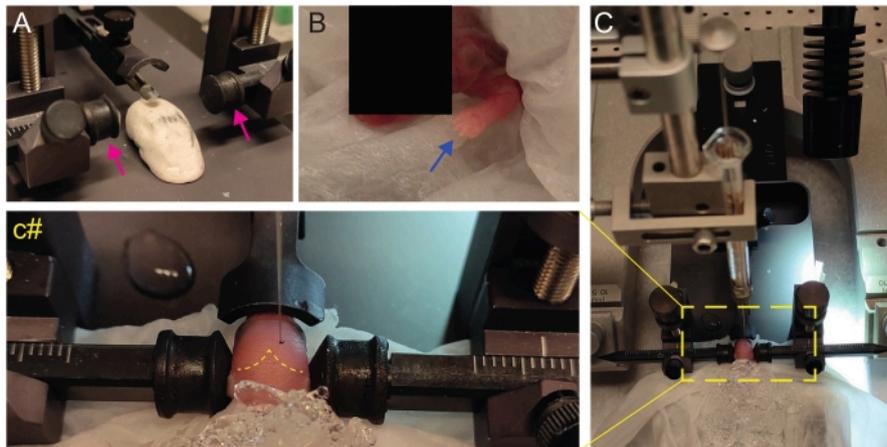


Figure 2: Stereotaxic transplantation in newborn mice pups at P2. (A) A Play-Doh-like stage for holding the pup's body in position and inverted ear bars (magenta arrows). (B) White front paw (blue arrow) indicative of the reduced blood flow in that area so that the pup is experiencing anesthesia by hypothermia. (C) Overview of the setup with the pup already covered by ice over the soft tissue paper. (c#) Closed-zoom of the head of the pup, with the injection needle already inserted into the brain (yellow dashed line indicating lambda and lambdoid sutures). This figure is adapted from Gonzalez Ramos et al.²⁷.

[Please click here to view a larger version of this figure.](#)

Representative Results

Following the protocol presented here and illustrated in **Figure 1A**, hdIN precursors were not proliferative yet at 7 DIV as defined by (i) negative immunoreactivity for the cell cycle marker Ki67 and (ii) expressing neuronal markers such as microtubule-associated protein 2 (MAP2) (**Figure 1B-E**). This characterization was performed on leftover cells replated for 24 h after having undergone all the procedure steps. In addition, the gene expression analysis published previously indicated that a rapid transition from the pluripotent state to a neuronal phenotype occurs around 4 DIV and 7 DIV⁸. Overall, these results confirmed the presence of postmitotic cells and the absence of risk for teratoma formation.

Next, the hdIN precursors' survival after early postnatal transplantation into the hippocampus of wild-type (WT) mice was tested by immunohistochemistry against the human cytoplasmatic marker STEM121. The hdIN precursors were transplanted into the right dorsal hippocampus of naïve immunocompetent mice at P2, which were then sacrificed at P14 and 2 months PT. Grafted cells were found across the whole dorsal hippocampus, as well as dispersed through the corpus callosum and the contralateral hippocampus, at both time points. Moreover, at both time points, grafted hdINs expressed *Ascl1*, one of the induction transcription factors (**Supplementary Figure 1**), and were not proliferative, as indicated by the absence of Ki67 expression (**Supplementary Figure 2**).

Importantly, no immune reaction or local inflammation against the transplanted cells was found either at P14 or 2 months PT, as assessed by the absence of reactive microglia identified using Iba1, CD68, and galectin-3 (Gal3) (**Figure 3**), the extent

of astrogliosis determined by the glial fibrillary acidic protein (GFAP) and inflammatory cytokines such as interleukine-1 (IL-1), and the absence of cytotoxic T lymphocytes (CD8) (**Figure 4**).

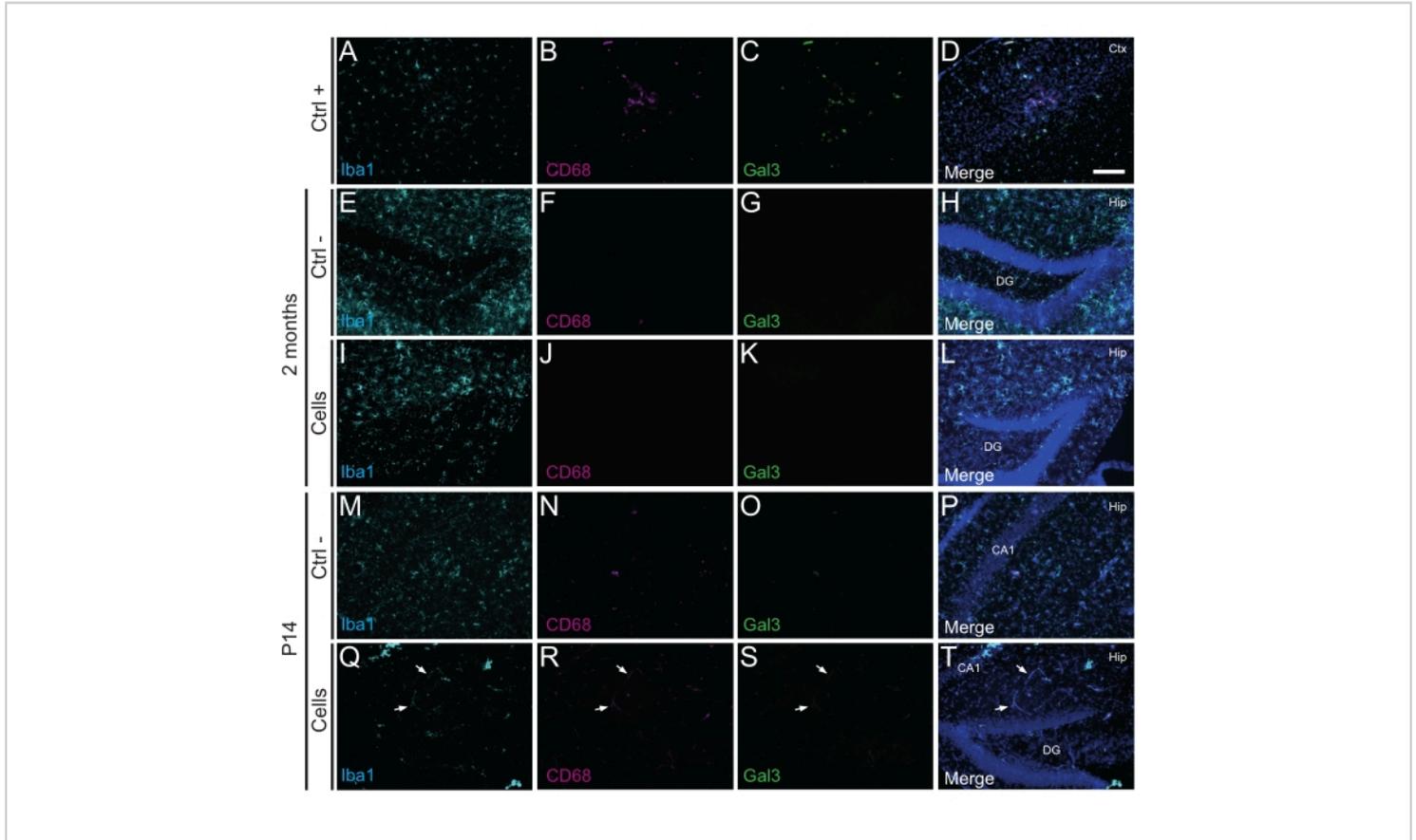


Figure 3: hdINs at P14 and 2 months PT into the hippocampus of newborn WT mice without triggering immune rejection from the host tissue. Immunofluorescence for Iba1, CD68, and Gal3 markers in brain tissue from (**A-D**) the proximity of an ischemic core area in an electrocoagulation stroke mouse model (positive control, Ctrl +), (**E-H**) negative control animals (Ctrl-) at 2 months and (**M-P**) P14, and (**I-L**) animals that have undergone cell transplantation at 2 months and (**Q-T**) P14. The white arrows indicate some examples of blood vessels visible at all channels due to autofluorescence. Abbreviations: Ctx = cortex; Hip = hippocampus; DG = dentate gyrus; CA1 = cornu ammonis 1. Scale bar: 50 μ m. [Please click here to view a larger version of this figure.](#)

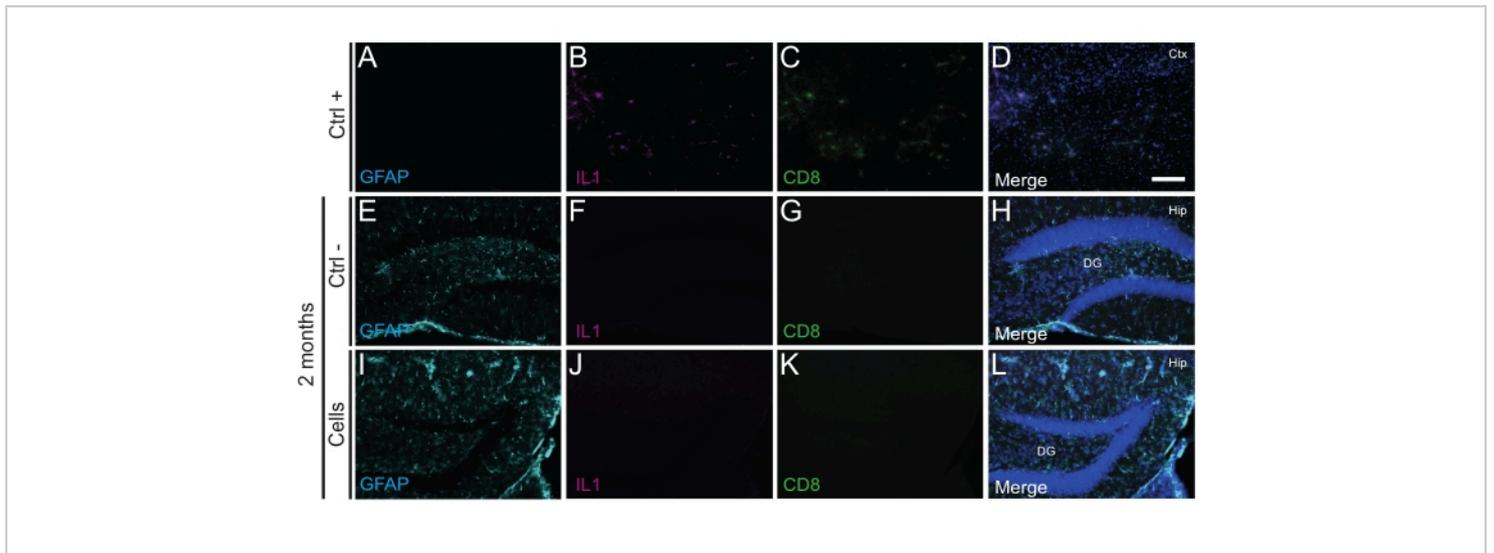


Figure 4: hdINs at 2 months PT into the hippocampus of newborn WT mice without triggering immune rejection from the host tissue. Immunofluorescence for IL1, GFAP, and CD8 markers in brain tissue from (A-D) the proximity of an ischemic core area in an electrocoagulation stroke mouse model (positive control, Ctrl +), (E-H) negative control animals (Ctrl-) at 2 months, and (I-L) animals that have undergone cell transplantation at 2 months. Abbreviations: Ctx = cortex; Hip = hippocampus; DG = dentate gyrus. Scale bar: 50 μ m. [Please click here to view a larger version of this figure.](#)

Similarly, hdIN precursors were also transplanted into the hippocampus of *Cntnap2* KO mice, a model for autism spectrum disorder and cortical dysplasia-focal epilepsy syndrome. In the *Cntnap2* KO mice, indeed, the hdINs survived up to 9 months PT and were localized at the injection site, although they were also dispersed across the ipsilateral

and even the contralateral hippocampus as observed in the WT mice (**Figure 5**). Moreover, most of the grafted hdINs were immunoreactive for interneuron markers, as expected from previous results *in vitro*^{8,26} and in adult rodents *in vivo*²⁵.

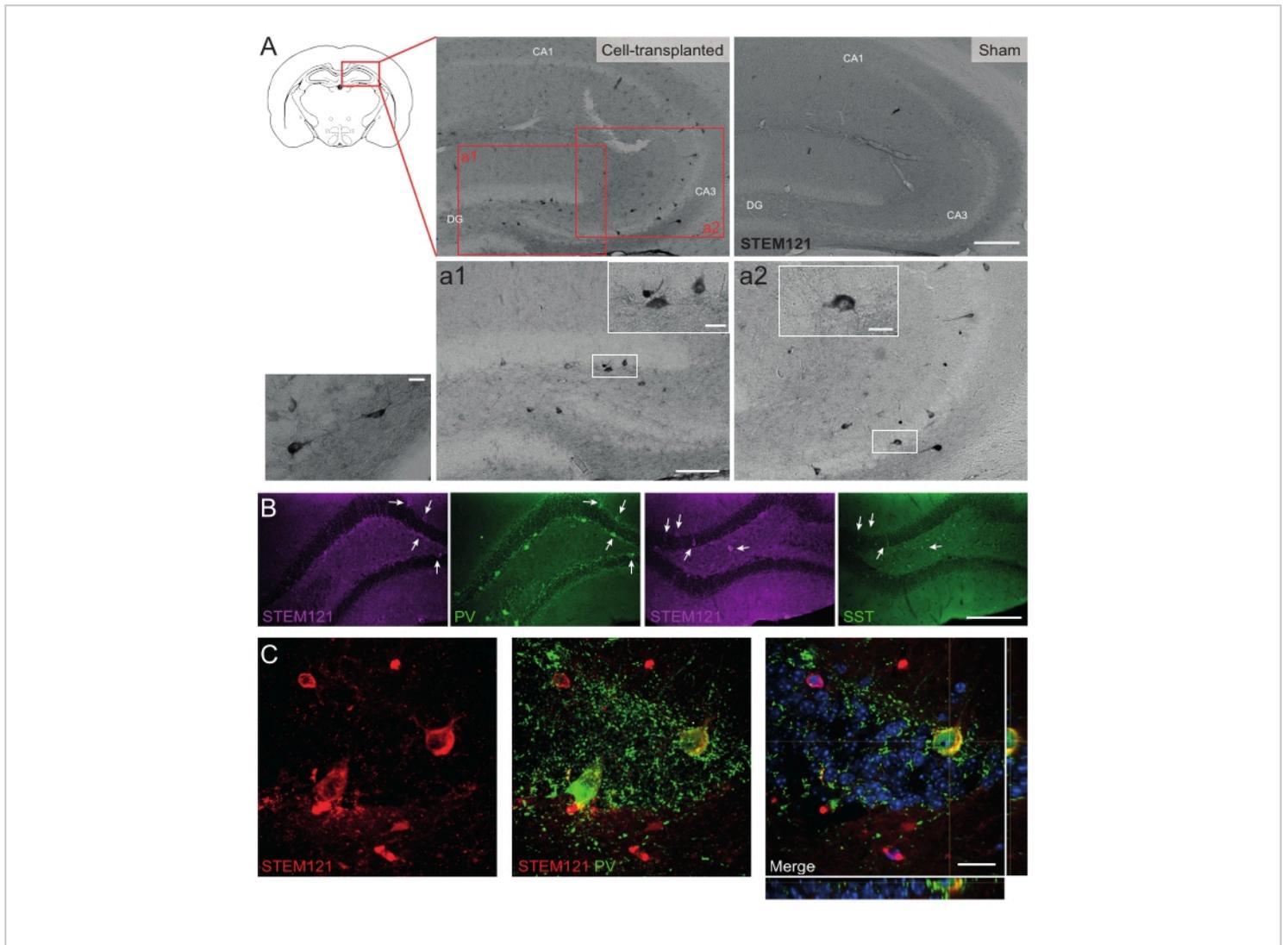


Figure 5: Grafted hdINs in the hippocampus of *Cntnap2* KO mice at 9 months PT. (A) Immunohistochemistry against the cytoplasmatic human marker STEM121 in cell-transplanted (left) and sham (right) mice. (a1 and a2) Magnified images of STEM121 positive cells. (B) Immunofluorescence for STEM121 (magenta) and the interneuron markers parvalbumin (PV) and somatostatin (SST). White arrows indicate double positive cells for STEM121 and the respective interneuron marker. (C) Orthogonal view of a grafted hdIN immunoreactive for STEM121 and PV. Scale bar: 200 μm (A and B), 100 μm (a1 and a2), 20 μm (small square magnification in a1 and a2, and C). [Please click here to view a larger version of this figure.](#)

Supplementary Figure 1: Grafted hdINs at 2 months PT into the hippocampus of newborn WT mice expressing *Ascl1*. Immunofluorescence against *Ascl1* and the cytoplasmatic human marker STEM121 at the (A) CA3 and (B) DG in cell-transplanted WT mice. (a) Magnified image

of a STEM121 positive cell. The white arrows indicate double-positive cells for STEM121 and *Ascl1*. Scale bar: 100 μm . [Please click here to download this File.](#)

Supplementary Figure 2: Post-mitotic grafted hdINs at 2 months PT into the hippocampus of newborn WT mice.

Immunofluorescence against the proliferative marker Ki67 and the cytoplasmatic human marker STEM121 in (A) naïve and (B) cell-transplanted WT mice. (b) Magnified image of a STEM121 positive cell. The yellow arrow indicates a cell positive for Ki67 and negative for STEM121. The white arrow indicates cells positive for STEM121 and negative for Ki67. The white asterisk points out a lateral ventricle. Scale bar: 100 μ m. [Please click here to download this File.](#)

Discussion

The present protocol describes a robust, fast, simple, and widely accessible methodology to generate hdIN precursors *in vitro* and its use as early interventional cell therapy in preclinical models of neurodevelopmental disorders.

Even though some of the characteristic phenotypes of neurodevelopmental disorders arise during adolescence or adulthood, pathophysiological alterations are already present during early development. For this reason, early intervention would be highly warranted for achieving beneficial effects by acting in critical brain developmental periods before symptomatology or clinical manifestation. In the future, genetic screening and the development of biomarkers will afford prophylactic or pre-symptomatic treatment, representing a game changer for those patients. Therefore, hdIN precursors were transplanted early after birth in the *Cntnap2* KO mouse model when epileptogenic changes in the neuronal network might be ongoing²⁸ and at a timepoint at which cellular alterations have been described in this animal model²⁹. It is important, however, to consider the potential pitfalls of age extrapolation and the timing of certain processes in the mouse versus the human brain.

Focusing on the procedure itself, the differentiation protocol presented here is based on the use of transcription factors, which allow for fast and highly efficient programming of stem

cells compared with other protocols elsewhere based on small molecules^{10,30}. A potential drawback of this approach could be the requirement for lentiviral vectors, which carries a risk of insertional mutagenesis. Two critical steps in the protocol are the addition of the antibiotics and the anti-mitotic agent to the medium to select for cells expressing the transcription factors and eliminate proliferative cells, avoiding the risk of teratoma formation, respectively. Although only hdIN precursors were tested in this study, the procedure is expected to be feasible with other cell sources and programming/differentiation protocols. Nevertheless, other neuronal subtypes and/or models should be validated.

The hdIN precursor's age for transplantation, 7 DIV, was decided based on (i) the absence of proliferative cells, assessed by immunoreactivity against Ki67, (ii) together with the previously reported observation of decreases in the expression of pluripotency genes such as *POU5F1* and the appearance of the neuronal marker *MAP2* and the interneuron marker *GAD1* at that timepoint⁸. However, the original work describing this protocol performed transplantations at 14 DIV after DOX withdrawal⁹. This raises questions about whether DOX in the mother's drinking water can reach cells grafted into the brains of nursing pups *via* the milk, or if 7 DIV of DOX induction is enough to establish the GABAergic fate. Although Yang et al. identified 14 days of DOX as sufficient to generate stable neuronal cells *in vitro*⁹, Gonzalez-Ramos et al.⁸ detected *GAD1* gene expression already at 7 DIV, indicating that the downstream activation of *GAD67* by *Ascl1* and *Dlx2* has already occurred at this time point. Hence, patterning has begun at 7 DIV and might be less dependent on the DOX treatment. Moreover, evidence in rodents and humans indicates the presence of DOX in breast milk^{31,32}, and the results presented here show that grafted hdINs were immunoreactive for *Ascl1* at 2 weeks and 2

months PT and interneuron markers later on at 9 months PT. Within the grafted population, besides PV and SST positive neurons, other markers for subpopulations of interneurons were also found in lower amounts, such as calretinin (CR) and calbindin (CB).

A challenging aspect of this procedure is the coordination of the timings for both differentiation and the age of the pups. Usually, mouse gestation takes 21 days after setting up the mating cage, albeit this can vary sometimes. This scenario does not occur when performing cell transplantations in adult rodents when everything can be carefully planned and arranged. Nevertheless, this can be easily mitigated by setting up two to three mating cages with a 2 day interval or two to three differentiation batches with a 2 day time-lapse from each other.

Although the mice used in this study were neither immunodeficient nor immunosuppressed, the transplanted cells survived up to 9 months *in vivo*, and markers of immune reaction against xenogeneic cells or local inflammation were not observed at either P14 or 2 months PT. Immune rejection of grafted xenogeneic cells is triggered against MHC/peptides, and the key cellular mediators of graft rejection are T lymphocytes and microglial cells^{33,34}. Therefore, immunoreactivity to markers of T cells, as well as reactive microglia, was explored. No signs of immune rejection of the grafted cells in the host tissue were detected either by levels of reactive microglia or by the presence of T lymphocytes in WT mice at P14 or 2 months. Moreover, no local inflammation was observed based on the assessed levels of astrogliosis and inflammatory cytokines. This outcome could partly be dependent on neonatal immune tolerance^{35,36,37}, observed by other cell identities, locations, and animal models^{35,38}. Englund et al. identified regional differences in the outcome

of the grafted cells in terms of migration and maturation, including the observation of grafted cells in the adjacent white matter³⁵.

Finally, a greater dispersion of the grafted cells within the hippocampus was observed compared to other studies transplanting into adult rodents, where hdINs remained as a grafted core²⁵. This dispersion also differed from results observed previously by Yang et al.⁹, which could be explained in this case by the age of the cells at the time of transplantation.

Disclosures

The authors declare no competing interests.

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