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**Humanized Chimeric mouse models to study human neural development
and pathogenesis of brain diseases**

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Abstract

The development of humanized neural chimeric mouse models consists in grafting human neuronal progenitor cells (NPC) or differentiated neurons derived from induced pluripotent stem cells (iPSC) into the mouse brain at different timepoints. As the main features of cortical networks and their alterations during brain development cannot be reproduced *in vitro*, these *in vivo* models have been used to investigate the mechanisms by which reprogrammed human neurons and non-neuronal cells integrate and migrate into the mouse brain at early stages of brain development and display functional activities several months after their grafting. Here, we describe the neonatal grafting technique of human NPC which we use in our laboratory. We also present a new method based on the grafting of human NPC into the brain of mouse embryos, *in utero*. A third method consists in the stereotaxic grafting of human NPC into selected brain regions of adult mice. The iPSC technology combined with the use of chimeric mouse models offers numerous possibilities to study human neural development within well-controlled and defined temporal windows and to model neuropathological disorders.

Key words

Xenografting, *in utero* transplant, human stem cells, induced pluripotent stem cells, chimeric mouse models, neurodevelopment, neurodevelopmental disorders.

Running Head: Humanized mouse models and neural development

1. Introduction

The reprogramming of human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) into neuronal progenitor cells (NPC) and mature neurons is of major interest to study brain development during the critical periods of establishing diversity, connectivity, and functional maturation of neurons within the central nervous system (CNS). These properties are particularly important within the cortical regions and the targeted areas of their innervation. A specificity of the cerebral cortex resides in the existence of multiple cortical sections that are structurally and functionally organized into connected units to exert different functions through a large variety of neuronal subtypes [1]. At later stages of development, post-mitotic neocortical neurons conserve a high degree of plasticity which allows the postnatal reorganization of specific neural microcircuits *in vivo* [2].

To date, different ESC and iPSC reprogramming protocols have been developed which can be used to produce various neuronal subtypes [3]. In particular, the reprogramming of stem cells into human cortical neurons has made it possible to study various developmental features after their grafting within the prefrontal cortex [4]. Interestingly enough, it has been shown that grafted ESC-derived human cortical neurons can integrate functionally into the lesioned mouse brain, when their cellular identity corresponds to that of the brain area under grafting [5]. In humans, the cortex is organized into six layers [6] which contain neurons with selective spatial organization and distinct biochemical, morphological, and electrophysiological characteristics [7-10]. The axonal projections of these cells are also specific not only to their cortical location, but also to the neuronal phenotype. Thus, the excitatory pyramidal cortical neurons project into various but specific regions of the brain while the interneurons' projections remain local within the cortical layers.

Xenografting of human neuronal progenitor cells into a rodent host allows their study *in vivo* on an extended time scale (up to 10-12 months) [11-12] as compared to *in vitro* studies (70-80 days in culture) [13]. In addition to such an important temporal aspect, the growth of human xenografted neurons takes place in a vascularized environment, which is essential for the survival of the transplanted cells. It has also been shown that the oligodendrocytes of host mice have the ability to myelinate the axons of grafted human neurons [4], which is an essential process to their long-term functional activity. The cortical identity of the grafted neurons, which depends on the layer in which they are positioned, as well as the temporal characteristics of their development are also maintained after transplantation [4, 11, 14]. Thus, the maturation of human neurons can take place over several months despite their development in a more mature murine environment [11]. A recent study has shown that when NPC are transplanted together with deep-layer cortical neurons into the brain of adult mice, the first synapses are visible after only 20 days of development and their number increases gradually overtime [14].

A summary of the xenografting studies carried out to date with human cells is presented in Table 1. It should be noted that most of these studies have been performed in newborn or adult rodents with ES- or iPS-derived human neurons. The main phenotypes studied are also indicated, whether they are morphological and / or functional.

Here, we first describe the step-by-step protocol used for neonatal transplants. This protocol has provided the means to analyze some of the processes which take place during the early stages of neuronal maturation, and on the role of SHANK3, a postsynaptic protein which is altered in autism spectrum disorders (ASD) as we reported previously [15]. In the course of this study, we also found that the maturation of the same reprogrammed neurons was slower *in vivo* as compared to *in vitro* conditions, using the same cells in both studies [15, 16].

We will then describe a step-by-step protocol which consists in grafting human-derived NPC in mice *in utero*. This new method, which has not been used in mice to date, provides the means to study the early stages of human development and neural precursors events before they start differentiating. Another important aspect resides in the fact that *in utero* grafting allows the use of immunocompetent mice. Finally, we describe a transplantation method in adult mice which can be particularly adapted to the grafting of inhibitory neurons which have limited projections to cortical areas. We will highlight the diverse applications of each of these methods for studying different pathologies at their earliest stages during brain development.

2. Materials

2.1 Media

-NPC culture medium: The culture medium consisted of 500 ml of DMEM/F-12 mixed with 500 ml of Neurobasal medium and supplemented with 2 vials (5 ml each) of N2 supplement (Gibco™, ref. 17502048), 2 vials (10 ml each) of B27 supplement (Gibco™, ref. 17504044), 10 ml of Penicillin-Streptomycin (Penicillin = 10,000 units/ml and Streptomycin = 10,000 units/ml) and 0.1mM of 2-βmercaptoethanol. Growth factors were selectively added as described in Figure 1.

-Flask coating: Culture Flasks were initially coated with Geltrex (Gibco™ ref. A1413302) diluted to 1/100 in DMEM/F-12 + GlutamaX and incubated for at least one hour at 37°C.

-The following is the list of primary antibodies and their corresponding secondary antibodies:

Primary Antibody			Secondary Antibody		
Name	Ref	Dilution	Name	Ref	Dilution
anti-GFP Rabbit serum	A6455, Invitrogen	1/750	Donkey anti-Rabbit Alexa 488	DZP030, Jackson INTERCHIM	1/200
anti-GFP Rabbit serum	A6455, Invitrogen	1/750	Donkey anti-Rabbit Alexa 594	Jackson INTERCHIM	1/200
Rabbit anti-VGluT1	135303, Synaptic Systems	1/1000	Donkey anti-Rabbit Alexa 488	DZP030, Jackson INTERCHIM	1/200
DAPI	D9542, Merck	1/200			

-Equipment: Stretcher apparatus (Narishige PB-7 needle puller), Glass pipettes: dimensions:1.0 OD x 0.78 ID x 100L mm, Sterile Towel Drape 18"x26" - 46cmx66cm (4410-IMC) Imcoinc; Harvard Syringe Pump (Harvard apparatus PHD 2000); Vibratome (Leica Biosystems, V1000S); Microtome (Leica SM 2010R); Confocal laser-scanning microscope with a 488nm laser for GFP excitation, and a 555 nm laser for mCherry excitation.

3. Methods

3.1 Neuronal progenitor cells (NPC) from human iPSC lines

The purpose of this section is to specifically describe the protocols which allow amplification and labeling of neuronal progenitor cells (NPC) before their grafting into the mouse brain.

For this study, we used two control cell lines from Coriell Biorepository (Coriell Institute for Medical Research), the GM04603 (<https://www.coriell.org/Search?q=GM04603>) and GM01869 (<https://www.coriell.org/Search?q=GM01869>). Their derivation into NPC and their

characterization have been described elsewhere [17]. For protocol optimization we used the WTSli002-A line (<https://cells.ebisc.org/WTSli002-A/> Wellcome Trust, UK) as in a previous study [18]. Protocol modifications result from the constant development of new commercial products to simplify and optimize iPSC-derivation protocols, without altering the cell differentiation. For the illustrations presented here, we only selected the original data obtained with the GM04603 iPSC line. Fig. 1A shows the experimental conditions used, according to Boissart et al (2013) [17], to derive human iPSC with slight modifications. Figure 1B shows NPC amplification and labeling, with the selected growth and transcription factors used in this study. Figure 1C describes the map of a lentiviral vector for GFP labeling [15].

3.2 NPC amplification and labeling

All the following steps were performed in sterile conditions under the flow hood. For their labeling, NPC were transduced with a CMV-GFP-lentivirus (green fluorescence) or with a CMV-mCherry-lentivirus (red fluorescence) as described previously [13]. Lentiviral vectors were prepared according to a published protocol [19]. The map of the CMV vector is detailed in Figure 1C. NPC were transduced with 99.5 ng of lentivirus in a 6-well culture plate filled with 500 µl of fresh culture medium for 3 hours. The volume was completed to 2 ml and incubated for the next 48 hours, and then rinsed with culture medium. For co-transplantation with two distinct populations [15], transduced NPC should be initially purified by FACS to inject equivalent levels of green or red fluorescence intensities within cells. In the case of co-transplantation, the two populations of labeled NPC should be amplified using the same culture medium.

For amplification, NPC were thawed in T75 flasks at a density of 50,000 cells/cm² in 25ml of NPC medium supplemented with Rock Inhibitor Y27632 (10µM). The next day, medium was

changed in order to remove the Rock inhibitor. Once confluency was reached, cells were passaged into a T175 flask. Medium was changed every 2 to 4 days during NPC amplification.

3.3 Grafting into mouse neonates

Cultured NPC were dissociated and resuspended in DPBS (10^5 cells/ μ l) before their transplantation into the neonatal brain. GFP-labeled cells were transplanted into the motor cortex of immunodeficient NOD/SCID mice (Charles River Laboratories), at postnatal days 1–2. Pups were first locally anesthetized using a lidocaine gel. The head of newborn pups was gently held horizontally between the operator's fingers. Bregma was used as a visible landmark to localize the injection site. We used the published coordinates Dorso-Lateral = + 1 mm, Antero-Posterior = – 1 mm, depth = – 1 mm to target the cortex, or depth = – 2 mm to target the ventricles. Two μ l of resuspended cells were slowly injected unilaterally using a Hamilton syringe (Hamilton, ref. 65460). The syringe needle was set for injecting at 1- or 2-mm depth and kept in the pups' brain for 30 s following the injection before its removal. The injection lasted over 1 minute. This protocol was approved by the Institut Pasteur Ethics Committee and the French "Ministère de l'Éducation Nationale, de la Recherche et de l'Innovation" under reference APAFiS #20686. The accuracy of injection was monitored by immunofluorescence labeling of GFP-labeled transplants, after brain slicing at different periods post-injection. All pups per litter were injected and their survival was evaluated at ~60%. For analysis, we recommend keeping only the mice that present well-defined GFP-labeled transplants within the same cortical areas. We excluded the brains with small number of labeled transplanted cells from analysis even though they presented similar phenotypes.

3.4 *In utero* grafting of mouse embryos

This protocol was approved by the Institut Pasteur Ethics Committee and the French “Ministère de l’Education Nationale, de la Recherche et de l’Innovation” under reference APAFiS #25221. Swiss OF1 mice (Charles River Laboratories), were habituated to the animal facility at least 3 days and surgeries were performed at the embryonic stage of E17.5. We observed that this developmental stage allows high rate of pup’s survival (~65%).

3.4.1 Material and equipment settings

A Hamilton syringe was connected to the pump by a plastic capillary, at the end of which a sterile glass micropipette was fixed (~ 50 µm diameter). Parafilm can be used to tightly connect plastic and glass micropipette. Before each surgery, the Hamilton syringe was filled with sterile distilled water and placed in a Harvard pump. A set of glass injection micropipettes were prepared by hand, using the needle puller, without any further autoclaving. These are the only type of needles that can be used for *in utero* injection. Once embryos were ready to be injected, the glass micropipette was carefully filled with cell suspension (~10⁵ cells / µl), to avoid the formation of air bubbles. Injection settings were as follows: Infusion rate: 30 000 µl / min, Target volume: 2 µl, Diameter: 0.4600 mm. Depending on experimenter, the injection can be performed using a magnification loupe or under a dissection microscope, or directly without any magnification device. At the stage E17.5, the ventricles can be easily distinguished in the swiss OF1 mouse strain. Cold light and sterile surgical instruments are necessary and should be ready to use.

3.4.2 *In utero* Surgery Procedures

Before their grafting, cell suspensions were prepared according to the protocol described in section 2.2 and kept in a water bath at 37°C. For the *in utero* approach, the injection solution also contains 20 mM EGTA [11, 20] in sterile DPBS to dissociate apical junctions in the neuroepithelium and avoid cell aggregation upon transplantation. For transplantation, we injected 2 µl containing $2 \cdot 10^5$ cells within one lateral ventricle of E17.5 embryos. Thirty minutes before surgery, buprenorphine (0.05 to 0.1 mg/kg) was injected subcutaneously at the back of the neck of pregnant mouse, for initial analgesia. The pregnant mouse was then anesthetized in a box with isoflurane and oxygen delivered via a continuous flow anesthesia unit and then placed on a heating pad which was also connected to the anesthesia unit. A drop of ocryl gel was applied on each eye to avoid eye dryness. The abdomen was shaved locally for ventral laparotomy at the white midline level. A betadine solution was applied on the skin, then rinsed with 70% ethanol using cotton swabs. A lidocaine gel was applied locally, before and after the laparotomy. Before starting the surgery, the sterile towel drape was positioned on the mouse abdomen. An incision of 1 to 1.5 cm was made in the lower abdomen in a manner to cut both the skin and the thin fascia layer. Abdominal organs should not be injured during this critical step. The whole uterus was carefully extracted from the abdomen, right and left ovaries were identified, and the total number of fetuses was counted. During surgery, drops of sterile PBS kept at 37°C were consistently applied on the exposed fetuses. Then, each fetus was held to clearly identify the lateral ventricle. The fetus was stabilized between thumb and forefinger within or close to the amniotic cavity. Any fetus rotation or damage should be avoided as it can affect embryo survival. The sharp glass pipette was carefully inserted through the fetal skull, and cells were injected into each fetus, except the first one to be delivered, on each uterine horn. After the injection, the pipette was carefully

withdrawn with well-controlled movements. Note that leakage of amniotic fluid should not be observed. Injections should not take more than 15-20 minutes per mouse. If the duration of injection is longer, some of the injected fetuses can be put back into the abdomen to keep them at the body temperature. Then, the uterus was carefully reentered into the abdominal cavity in a way to avoid any twist between the embryos and with a controlled holding pressure. The peritoneal cavity was rehydrated with 0.5 to 1 mL of sterile PBS to replace any fluid loss during the surgery. Then, the incision was closed with two types of sutures: for fascia and skin, we used sterile resorbable suture (Prolene™, EH7471, Ethicon, LLC) and non-resorbable suture (Coated Vicryl, V302, Ethicon, LLC), respectively. After the surgery, the pregnant mouse was replaced in its own cage and positioned on a warming pad until the mouse was fully awake. Analgesia treatment (acetaminophen 4mg/20ml) was administered in drinking water for 3 to 5 days after the surgery, and stress (noise, excessive manipulation of cages...) was minimized to avoid preterm delivery. Hydrogel was also given to mice for one day after surgery. We observed that grafted cells' distributions and density may slightly vary between each embryo, due to a possible aggregation of cells in the pipette and during injection. We could observe that the presence of EGTA significantly reduces cell aggregation, and increase the possibility of identifying single cells within the mouse brain, as described previously [11].

For a first *in utero* test or training, it is recommended to inject 1-2 µl of fast green or bromophenol blue solutions, without cells. If correctly injected into lateral ventricle, the solution will immediately fill the whole embryonic ventricular systems. In that case, the pregnant mouse and embryos should be sacrificed immediately by profound anesthesia followed by cervical dislocation.

3.5 Grafting into the dentate gyrus of adult mouse brain

The protocol for cell preparation is identical to that for *in utero* grafting. Experiments were performed with adult NOD-SCID mice (Charles River Laboratories).

As for *in utero* grafting, the mice received an injection of buprenorphine (0.1 mg/kg) for initial analgesia 30 min before anesthesia by isoflurane. The mouse was placed on a stereotaxic frame connected to an isoflurane unit and to a heating pad. A drop of ocryl gel was applied on each eye to avoid dryness. The skin was shaved and cleaned with betadine followed by 70% ethanol. For a local anesthesia, 150µl of Lidocaine (7 mg/kg) were injected subcutaneously at the skull level at least 10 min before the skin incision. Using a drill, two holes were done at the following coordinates A/P: -1.8; M/L: +/-1.25 for targeting the DG according to the Paxinos and Franklin Atlas [21]. Twenty nanoliters of cell suspensions were injected bilaterally at a rate of 3nl/s in three steps along the D/V axis: 15 injections at -1.82 depth, 20 at -1.76 and 15 at -1.72 to achieve a final volume of 1 µl/ hemisphere. To ensure reproducible injection volumes, we recommend to wait 10 seconds between each injection and 5 min before removing the glass capillary from the brain tissue.

For mouse recovery, metacam (0.05mg/kg) and 200 µl of sterile saline was injected separately subcutaneously before the end of anesthesia (~50 µl/ mouse). As post-operative care, metacam (1mg /Kg /day) diluted in diet gel was given for at least 3 days. Mice were monitored for 5 days.

3.6 Immunofluorescence labeling

For immunofluorescence labeling, mice received a lethal dose of a mixture of ketamine (Imalgen, 100 mg/kg, Merial) and Xylazine (Rompun, 10 mg/Kg, Bayer), and were transcardially perfused with 15 ml of DPBS, followed by 50 ml of ice-cold 4%

paraformaldehyde (PFA). Brains were removed and post-fixed overnight. Forty μm -coronal sections were obtained using a vibratome or a microtome, collected in 12-well or 24-well culture plates containing DPBS supplemented with 0.02% azide, and stored at 4°C until use. Free-floating sections were rinsed twice in DPBS (pH 7.4) and then incubated in DPBS solution containing 0.5% Triton X-100 and 10% serum at room temperature for 30 min to block nonspecific binding. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. In order to improve the labeling of neuron morphology, immunofluorescence was performed using an anti-GFP antibody in permeabilized conditions. An additional DAPI staining diluted in DPBS was performed for 10 min at room temperature.

3.7 Representative data

In this chapter, we describe three different approaches for grafting the human NPC which result in the integration of human cells into the brain of adult mouse. Fig. 1 illustrates the different steps of the protocol which we used for amplification, banking, and labeling of NPC before their grafting. We have previously shown that the neonatal engraftment of human NPC can lead to neurons that are able to differentiate into glutamatergic neurons [12, 15]. Fig. 2 illustrates the precise site of injection and the cortical graft containing a large density of neuronal cell bodies together with numerous axonal projections within distinct brain regions. The full cartography of axonal projections in the brain has been published elsewhere [15]. Using the same human NPC, we set up the grafting into the lateral ventricle of mouse embryo (E17.5). Our results show that two weeks after their grafting, the cells are able to integrate within the brain parenchyma and start to migrate along known migratory pathways including cingulum bundle, corpus callosum, and fimbria (Fig. 3A). At this early stage of post-grafting, a large number of cells remain close to the injection site. By contrast, the cells are found to be

more dispersed after one month and in the process of integrating the distinct brain regions including the striatum (Fig. 3A, B). Two months post-grafting, the cells start to differentiate into excitatory cells (Fig. 3C). At this stage, the co-localization of VGlut labeling with the human cells is observed both in neuronal cell bodies and projections (Fig. 3C). Fig. 4 illustrates the grafting of the human NPC into the dentate gyrus (DG) of adult immunodeficient mice, three weeks post-grafting. Our data show that cells can survive and integrate within the hippocampal structures following a stereotaxic injection in adult mice.

4. Notes

4.1 The three humanized mouse models

The use of humanized chimeric mouse models allows the study of neuronal development and associated pathogenesis and neurodegenerative diseases using human cells in an *in vivo* condition. Under the experimental approaches described here, the neonatal grafting allows the grafted progenitors to predominantly differentiate into excitatory neurons which can mature, migrate, and project their axons in different brain regions. The iPSC technology has offered the possibility to study some specific phenotypes, including cell differentiation and maturation, neuronal morphology and functional activities by electrophysiological recordings of cultured cells under *in vitro* conditions [4]. However, *in vitro* models using human iPSC-derived neurons are limited as they do not offer the possibility to study disease pathogenesis at different stages of brain maturation within a such complex environment, and in the presence of external cues that are necessary for neuronal patterning. As shown in Table 1, the neonatal grafting of iPSC-derived neuronal progenitors has been successfully used by several authors. By comparing neonatal and *in utero* grafting, we observed that in both cases, the engrafted cells form initial clusters during the first month post-grafting which

tend to remain over time, at least up to 7 months [12]. For neonatal grafting, it has been shown that grafted NPC appear to respond to region specific signals and are also responsive to neural patterning cues *in vitro* [4; 12; 41]. These human cells progressively expand and migrate in the murine forebrain. It has been difficult to understand to what extent the undifferentiated NPC or the immature neurons can integrate and recapitulate physiological neurogenesis *in vivo*. Moreover, most studies have been limited due to the use of immunodeficient NOD-SCID mice, which have the tendency to form tumors at 7-8 months of age [49]. So far, these issues have not been considered for studying neurodevelopmental disorders. Interest has been focused on early stages of neuronal maturation, which has allowed the discovery of a panel of morphogenetic abnormalities that are linked to early development of neurons with ASD-associated mutations [15, 50]. It should also be noted that transplanted human PSC-derived NPC can be tumorigenic, if contaminated with residual pluripotent stem cells with sustained proliferation of residual stem cells [51]. As shown in Fig. 1, we used the SMAD inhibitor SB431542, based on previously reported studies [17], in order to avoid abnormal cell proliferation. For *in utero* grafting, we observed large densities of graft-derived fibers projecting into migratory pathways and in the host tissue as early as 15 days post-grafting (Fig. 3). The *in utero* grafting method presents some advantages as it can be applied to immunocompetent mice with no graft rejection and no inflammation at injection sites (data not shown), while mouse neurogenesis continues which may favor further development of human cells.

Our results indicate that the cells can survive at least for 3 months after grafting and give rise to glutamatergic neurons (Fig. 3). We also observed that they develop dendritic spines after neonatal grafting (data not shown), using the same GFP-labeled human iPSC line [15]. To our knowledge, there is no report on the *in utero* grafting of human iPSC-derived neuronal cells in

rodent brain. The available data from using mouse embryonic cells grafted in the brain of rat embryos, were analyzed within 7 weeks post-grafting [22]. However, human cells are expected to be found uniformly throughout the brain regions, including hippocampus, cerebral cortex and striatum at later stages, up to 13 months, as observed in neonatally grafted mice [41]. Finally, we describe the grafting of NPC within hippocampus of the adult mice. This grafting method may help targeting a specific brain region, and to analyze more specifically how grafted cells can integrate within specific circuits. We illustrate this approach with NPC giving rise to excitatory neurons after grafting into the dentate gyrus of hippocampus. The same approach could be applied to interneurons that can be derived from human iPSC as well [40]. Interestingly, interneurons represent a large and diverse population of hippocampal neurons that are critical for information and memory processing. By grafting well-characterized mature interneurons, it is possible to identify more specifically how a specific human interneuron subtype may morphologically and functionally interact within the surrounding host cells.

4.2 Applications for neurodevelopmental disorders

Neurodevelopmental disorders are either due to various inherited mutations or acquired causal factors, and lead to dysfunction of brain development and cognitive deficits.

The pathogenesis of neurodevelopmental disorders has not been well elucidated and no effective treatment has so far been found for these disorders. The establishment of accurate models which allow to investigate neurological disorders represents an important step towards better understanding and appropriate treatment of neurodevelopmental pathologies. To study the brain-associated diseases *in vitro* has been not possible, and to obtain the tissues from human brain represents a difficult alternative. The use of patient-

derived iPSC however, offers a unique opportunity to investigate pathological changes that may occur in the brain at early stages of neuronal development. In addition, human iPSC provide an auto-renewal source of cells which are identical to the donor [52]. Under the action of specific growth and transcription factors, they can differentiate into neurons of different phenotypes that are found within the six cortical layers, including excitatory and inhibitory neurons [53]. The grafting of iPSC-derived neurons into mouse brain offers the possibility to decipher cellular mechanisms in different temporal windows, from the neurogenic stages with undifferentiated cells to later stages of well-differentiated mature neurons, under control and pathological conditions. For genetic diseases, the iPSC from patients carry the genetic pattern of mutations from these donors and should display similar pathological and phenotypical changes. For autism spectrum disorders (ASD), recent studies have shown that the events underlying such disorders, can occur during neonatal development, together with strong synaptic defects that are known to occur at later stages of development. In a previous *in vitro* study, we investigated the iPSC-derived pyramidal cortical neurons from patients with ASD carrying *de novo* heterozygous mutations in the postsynaptic SHANK3 protein. SHANK3 is a synaptic scaffolding protein located at the postsynaptic density of excitatory synapses [54]. The haploinsufficiency of *SHANK3* gene provokes a developmental disorder known as the 22q13.3 deletion syndrome (or Phelan-McDermid syndrome). *SHANK3* gene is also associated with pathogenesis and neuropathology of ASD, which involves synaptic dysfunctions and an imbalance in synaptic excitation/inhibition ratio. Using iPSC-derived neurons from ASD patients, we observed altered spinogenesis in all the patients who were included in the study [16]. In a subsequent study, we used the same cells from a control individual at precursor stages (NPC), and transplanted them into the cortex of newborn mouse brain [15]. We analyzed the integration of NPC, the maturation of the derived excitatory

neurons and their axonal projections into the adult brain. We then co-transplanted the NPC from a control individual and from a patient carrying one *de novo* heterozygous mutation in *SHANK3*. In human neurons with the *SHANK3* mutation, we observed a reduction in cell soma size of selective neuronal populations and in axonal projections, as compared to control neurons, at 30 days post-transplantation. However, we did not observe any alteration in spinogenesis at this early stage, which are expected to occur at later stages of development. This indicates that *in vivo* models can recapitulate the early stages of the disease, which may help understand the initial mechanisms before the appearance of the well-described phenotypes observed in mouse models or in human cells kept in culture.

4.3 Conclusion

One of the strong aspects of our different grafting approaches is that humanized mouse models can be used for both neurodevelopmental as well as neurodegenerative diseases, depending on the stage at which the phenotypes of grafted cells are evaluated. At the early stages of development, regulation of cortical neurogenesis is very important as it is involved in neuronal fate [55]. For example, recent discoveries point out the permissive role of the amyloid precursor protein (APP) in the regulation of human cortical neurogenesis [56]. APP is also the precursor of β -amyloid peptide which can lead to progressive accumulation of β -amyloid oligomers, followed by their aggregation in amyloid plaques, one of the major hallmarks of the neurodegenerative Alzheimer's disease (AD). It would be possible to test whether the physiological functions of APP are related to the onset of AD, using *in vivo* approaches such as mouse chimeric models, xenografted *in utero* and appropriate genetic tools, to follow and control the production of endogenous APP within the whole brain or locally in specific brain regions. Humanized mouse models not only offer the possibilities to

decipher the early events which underlie neuronal maturation and plasticity but also to investigate the pathological events that occur in the brain of patients with neurological disorders, including those with AD. Such models should also facilitate the preclinical evaluation of therapeutic measures and treatments as well as their consequences within the human neurons, *in vivo*. Finally, these models can help to set up appropriate transplantation therapies using neural stem cells. The xenografting strategies offer the possibility to compensate neural loss and neural tissue injury and should allow the discovery of therapeutic strategies for the future, in the context of both developmental disorders and degenerative diseases. One main challenge will be to face the complexity of human brain and consider its unique spatial and temporal development.

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Legend to Figure 1: *In vitro* cell differentiation, amplification and labeling before engraftment.

A. Human iPSC are first amplified and then differentiated in early cortical progenitors, then to NPC with further amplification before banking. B. Description of NPC amplification and labeling before grafting into mouse brain. GFP labeling can be started directly from the stock of frozen NPC. C. Schematic representation of the lentiviral construct for transduction of NPC with GFP (green fluorescence) using a human cytomegalovirus promoter (CMVp).

Legend to Figure 2:

Engrafting of human iPSC-derived NPC into the cortex of newborn mice. A. Intracranial injection of human iPSC-derived NPC into the cortex of neonatal mice. The Lambda and Bregma landmarks are used to target the motor cortex as indicated in red. The injection site is located approximately 1 mm posterior to the bregma and 1 mm lateral to the sagittal suture, with 1 mm depth, as indicated by the red symbol. B: Representative engraftments into the cortex of 50-day old mice after neonatal injection of NPC. Graft location within the somato-sensory cortex. Most of GFP-labeled neurons remain within the graft site, and extend their dendrites. C. Axonal projections within the somato-sensory cortex (Bregma \approx -0.955 mm). D. Axonal projections in the somato-sensory cortex (Bregma \approx 1.545 mm). E. Long-range axonal projections in the retrohippocampal region (Bregma \approx -2.78 mm) F. Long-range axonal projections in the somato-sensory cortex (Bregma \approx -2.78 mm).

Scale bars = 200 μ m.

Legend to Figure 3:

Engrafting of human iPSC-derived NPC *in utero*, at different time points after transplantation

A. Representative engraftments at 2 weeks post-transplantation (2WPT) (Bregma \approx -0.1mm) (Upper panel) and at 1-month post-transplantation (1MPT) (Bregma \approx -0.94mm) (Lower panel). Graft core remains close to the lateral ventricle (LV) area. All pictures were taken using a 10X objective. Scale bar of mosaic (left panel) = 500 μ m and scale bar of pictures (right panel) = 200 μ m. B. Projections of human NPC along different brain regions (cing = cingulum bundle, ILA = Infralimbic area, cc = Corpus callosum, ST = Striatum area) at 3 MPT at 10X magnification (scale bar = 500 μ m). C. Excitatory phenotype of grafted human NPC shown by VGluT expression at 63X magnification (Scale bar = 30 μ m), and at 2MPT.

Legend to Figure 4:

Engrafting of human iPSC-derived NPC into the dentate gyrus (DG) of adult mice, at 3 weeks post-injection. Representative engraftments into the hippocampus area of NOD-SCID mice as shown by the graft core in the DG (Bregma = -1.80mm) (A) and projections (B) at low (left panel) and higher (right panel) magnifications. Low magnification mosaics are taken with a 10X objective (scale bar = 1000 μ m) and higher magnification pictures with a 20X objective (scale bar = 100 μ m). All pictures were acquired as a Z-stack with a confocal microscope to maximize the graft detection. In green, GFP-labeled human NPC and in blue cell nuclei stained with DAPI.

Table 1: Reported studies using xenotransplantation of human stem cell-derived neurons

Human cell origin	Host	Brain region	Graft Integration	Functionality	Ref.
Fetal brain cells	Rat embryo	Brain ventricles	Widespread incorporation of neurons	n.d.	[22]
Embryonic NSC	Adult rat	SVZ & Hp	Migration along the routes taken by endogenous precursors. Substantial migration within the striatum	n.d.	[23]
Fetal brain cells	Mouse neonate	Brain ventricles	Proliferation, migration, and neural differentiation (7MPT)	n.d.	[24]
NSC	Monkey fetus at 12-13 week-gestation	Brain ventricles	At 16-17 weeks of gestation: Two subpopulations: -Differentiated neurons & glia; -Undifferentiated cells (SVZ)	n.d.	[25]
ESC-derived NPC	Mouse neonate	Brain ventricles	At 1-4 WPT: Incorporation of cells in cortex, Hp, OB, septum, thalamus, hypothalamus, striatum midbrain and some white-matter regions - Differentiation into neurons and astrocytes	n.d.	[26]
ESC	Chick embryo	<i>In ovo</i> grafting into somites; 1.5-2 days of development	6-7 days post-surgery Differentiation and migration into DRG, neural tube	n.d.	[27]
Immortalized NS-like cells	Rat with focal cerebral ischemia	i.v. injection	Differentiation into neurons & astrocytes in lesioned areas; survival up to 56 days after grafting (cortex & striatum)	Generation of AP & synaptic integration-Miniature EPSC	[28]
Differentiated ESC	Mouse embryo (E14)	Brain ventricles	2 MPT: Widespread incorporation including within cortex and hippocampus	8 MPT: Action Potentials	[29]
ESC-derived NPC	Young / adult rat brain	Brain ventricles	Integration into SVZ; migration along the rostral migratory stream to olfactory bulb; contribution to neurogenesis	n.d.	[30]
ESC-derived DA neurons (+ astrocytes)	6-OHDA-lesioned adult rat	Striatum	Substantial neuronal recovery	Behavioral testing	[31]
ESC-derived striatal progenitors	Adult rat	Striatum	Xenograft overgrowth & persistent proliferation of NPC	n.d.	[32]
ESC-derived DA neurons	Parkinsonian models in mouse, rat & monkey	Striatum	4-5 MPT. No neural overgrowth. <i>In vivo</i> survival. Restoration of behavioral symptoms	Membrane potential oscillations	[33]

ESC-derived retinal progenitors	Mouse neonate	Subretinal space & intravitreal space	Migration in mouse retina. Differentiation into functional photoreceptors	Restoration of visual functions in <i>Crx</i> -deficient mice	[34]
ESC-derived neural rosettes	Adult rat	Striatum	Survival and integration 4 weeks after transplantation	n.d.	[35]
iPSC-derived DA neurons from patients with Parkinson	Adult rat	Striatum	4-16 WPT: High level of cell survival, arborization with few projections into host striatum	Reduced motor asymmetry of 6-OHDA-lesioned rats	[36]
ESC-derived cortical neurons	Mouse neonate	Cortex	Integration, establishment of specific axonal projections & dendritic patterns of cortical neurons	9 MPT: Firing patterns, synaptic potentials	[4]
ESC-derived MGE-like cells	Mouse	Hippocampus	Synaptic connections with endogenous neurons	Improvements in learning and memory deficits	[37]
PSC-derived MGE progenitors	Mouse neonate (P2)	Cortex	3 to 7 MPT: Cells migration in the cortex; Maturation into GABAergic interneurons subtypes	7 MPT: Firing patterns, synaptic potentials	[38]
Fetal NSC	Monkey	Midbrain	Generation of neurons that project long fibers in adult brain (+GDNF)	n.d.	[39]
ESC- & iPSC-derived interneurons	Mice	Hippocampus	Differentiation method to MGE or CGE cells & subclasses of GABAergic interneurons	n.d.	[40]
ESC-derived cortical neurons	Adult mouse	Lesioned Visual cortex	Restoration of damaged cortical pathways	Mature electrical neuronal profile	[5]
iPSC-derived NPC	Mouse neonate (P0)	Brain ventricles	- Migration and wide dispersion of transplanted cells into the mouse brain (detection in the cerebellum at 6 MPT) - Differentiation into excitatory neurons, astrocytes & oligodendrocytes	Functional mature neurons at 13 MPT	[41]
MGE-derived progenitors	Mouse neonate (P1-4)	Dorsal hippocampus	Differentiation of transplanted cells in PV & SOM interneurons	Functional inhibitory synapses with the host	[42]
ESC-derived interneurons	Adult Rat	Prefrontal Cortex	<i>In situ</i> differentiation of PV interneurons after grafting	Firing analysis of the host pyramidal cells	[43]

iPSC-derived progenitors and cortical excitatory neurons	Adult mouse	Somato-sensory cortex	Graft Vascularization 5 MPT: long range selective projections	Inhibitory and excitatory inputs, Firing patterns	[14]
PSC-derived cortical interneurons	Mouse	Cortex	Protocol optimization for cell banking before grafting. Graft integration & cell migration	n.d.	[44]
iPSC-derived interneuron precursors	Rat model of epilepsy	Hippocampus	Synapses formation between graft-derived axons and host hippocampal excitatory neurons in DG & CA1 field	Involvement of graft-derived interneurons in seizure control	[45]
Embryonic PSC-derived cortical neurons	Mouse neonate (P0-1)	Brain ventricles	Integration in the mouse cortex as single cells	10 MPT: mature firing patterns	[11]
H9 stem cells-derived NPC	Adult mouse and rat	Frontal cortex	Transduction with human channel rhodopsin. At 3MPT: differentiation into GABAergic neurons. Dense terminal fields and innervation of dendrites in host cortical neurons	Light stimulation of hChR2-hNP transplants: variability in grafted neuron response	[46]
MGE-derived interneurons	ASD model and WT mouse neonate (P1-3)	Prefrontal cortex	-At 8 WPT: Integration into PFC, anterior cingulate, dorsal insular, primary motor and somatosensory areas. - In WT mouse: higher differentiation into SOM interneurons - In ASD mouse model: higher differentiation into PV interneurons	Rescue of social interaction of ASD model mouse	[47]
iPSC-derived NPC	Mouse neonate	Frontal cortex / brain ventricles	-At 2 MPT: Axonal projections in the mouse cortex - Differentiation into pyramidal neurons, SOM neurons, astrocytes and oligodendrocytes - 4MPT: Graft vascularization	n.d.	[12]
iPSC-derived NPC	Mouse neonate	Frontal cortex or brain ventricles	At 50 DPT into: Brain ventricles: Migration to olfactory bulbs following the rostral migratory stream. Cortex: axonal projections into different brain areas following white matter fibers (fimbria and stria terminalis)	n.d.	[15]

ESC-derived midbrain DA or cortical glutamate neurons	Adult Parkinson Mouse model	Substantia Nigra or Striatum	Projection of axons in different brain areas depending on neuron subtypes	-Functional (/ anatomical) synaptic inputs dependent of neuronal subtypes (/ graft site) - Rescue of motor deficits by dopaminergic grafted neurons	[48]
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Abbreviations. AP: Action potentials; ASD: autism spectrum disorders; CGE: Caudal ganglionic eminence; DPT: days post transplantation; DRG: Dorsal root ganglion, EPSC: Excitatory postsynaptic current; Hp: Hippocampus; MGE: Medial ganglionic eminence; MPT: Month post transplantation; OB: Olfactory bulb; PSC: pluripotent stem cells; PFC: Prefrontal cortex; PSC: Pluripotent stem cells; PV: Parvalbumin; SOM: Somatostatin. SVZ: Subventricular zone.

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Figure 1

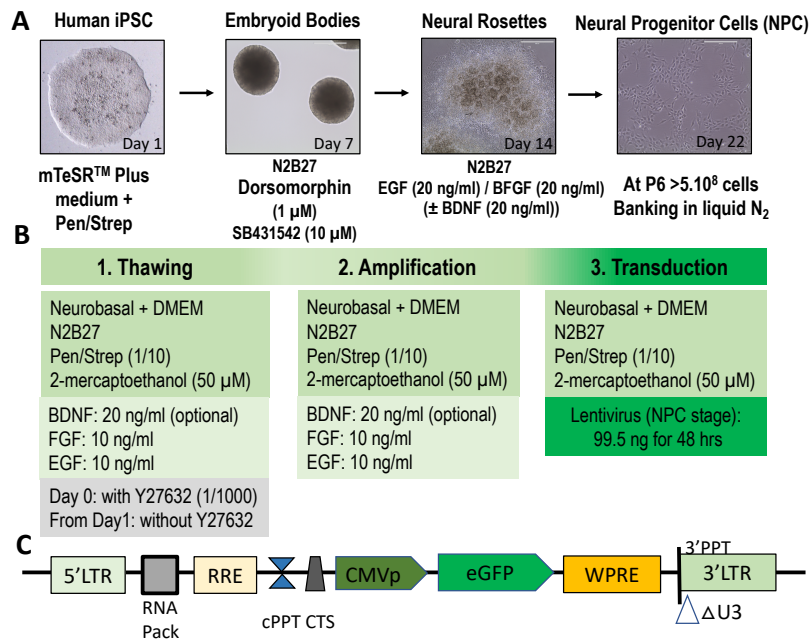


Figure 2

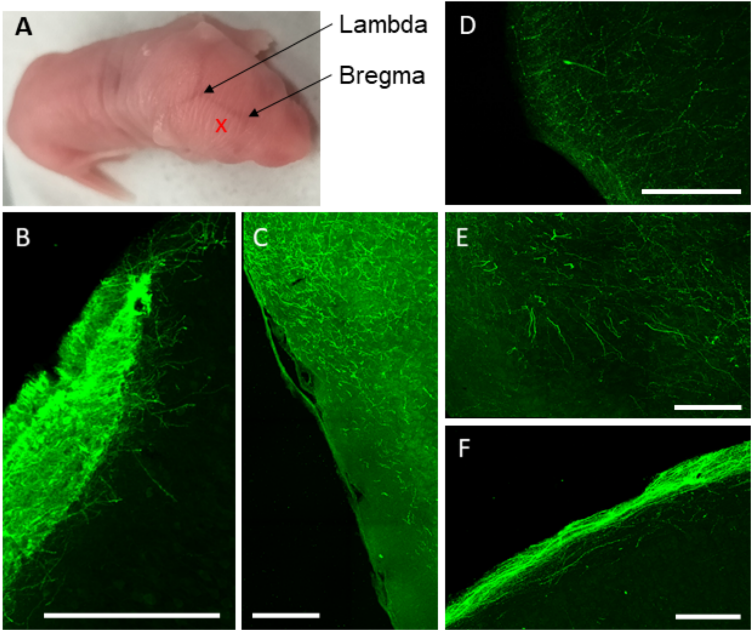


Figure 3

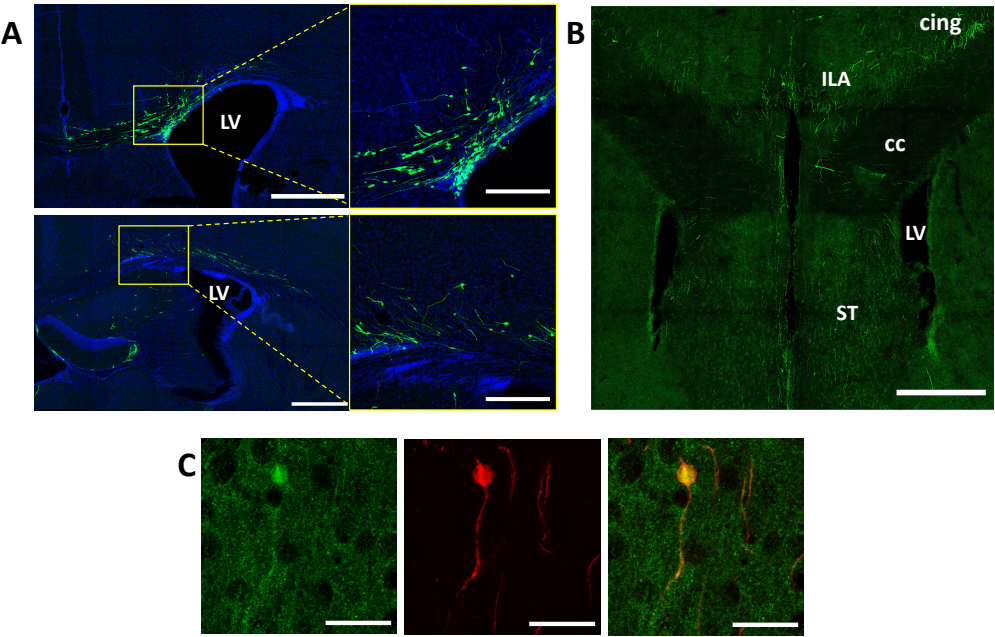


Figure 4

