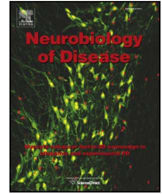
Neurobiology of Disease 79 (2015) 28–40

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Review

Reconstruction of brain circuitry by neural transplants generated from pluripotent stem cells

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A R T I C L E   I N F O

Article history:
Received 11 March 2015
Revised 9 April 2015
Accepted 15 April 2015
Available online 22 April 2015

Keywords:
Regeneration
Transplantation
Induced pluripotent
Embryonic stem
Cell replacement
Integration

A B S T R A C T

Pluripotent stem cells (embryonic stem cells, ESCs, and induced pluripotent stem cells, iPSCs) have the capacity to generate neural progenitors that are intrinsically patterned to undergo differentiation into specific neuronal subtypes and express *in vivo* properties that match the ones formed during normal embryonic development. Remarkable progress has been made in this field during recent years thanks to the development of more refined protocols for the generation of transplantable neuronal progenitors from pluripotent stem cells, and the access to new tools for tracing of neuronal connectivity and assessment of integration and function of grafted neurons. Recent studies in brains of neonatal mice or rats, as well as in rodent models of brain or spinal cord damage, have shown that ESC- or iPSC-derived neural progenitors can be made to survive and differentiate after transplantation, and that they possess a remarkable capacity to extend axons over long distances and become functionally integrated into host neural circuitry. Here, we summarize these recent developments in the perspective of earlier studies using intracerebral and intraspinal transplants of primary neurons derived from fetal brain, with special focus on the ability of human ESC- and iPSC-derived progenitors to reconstruct damaged neural circuitry in cortex, hippocampus, the nigrostriatal system and the spinal cord, and we discuss the intrinsic and extrinsic factors that determine the growth properties of the grafted neurons and their capacity to establish target-specific long-distance axonal connections in the damaged host brain.

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Introduction

The use of cell transplants for brain repair is based on the idea that grafted neurons can become integrated into damaged brain circuitry, replace lost neurons, and reconstruct some critical aspects of damaged neuronal connectivity. The idea that immature neurons or neural precursors can be used to re-establish lost or damaged axonal

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.04.003
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connectivity and boost the regenerative capacity of the central nervous system (CNS) has been pursued experimentally in rodents since the 1970s, but it is only recently that serious attempts have been made to use cells derived from embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs) with this goal in mind. Significant improvements in protocols for directed differentiation of human pluripotent stem cells and innovative approaches to study connectivity of transplanted neurons have pushed the field forward considerably. In particular, recent studies have provided new interesting insights into the capacity of ESC- and iPSC-derived neurons to establish new functional connections in brain and spinal cord in various lesion and disease models.

In this review we summarize these recent developments in the perspective of earlier studies using intracerebral and intraspinal transplants of primary neurons derived from fetal brain, and discuss the intrinsic and extrinsic factors that determine the growth properties of the grafted neurons and their capacity to establish target-specific long-distance axonal connections in the damaged host brain. We will limit ourselves to studies using cells derived from pluripotent stem cells – ESCs and iPSCs – and focus on four transplantation targets where some of the most interesting studies have been performed, *i.e.*, cortex, hippocampus, nigro-striatal system, and spinal cord.

Studies of graft–host connectivity derived from grafted primary neurons using classical anatomical and immunohistochemical methods

The early studies on anatomical integration of neural transplants relied on histochemical or immunohistochemical methods for selective visualization of specific neuronal systems, defined either by their transmitter content, or on the use of species-specific antibodies that allowed visualization of, *e.g.*, mouse, pig or human neurons and their projections in the rat brain. These tools were combined with classic anterograde and retrograde tracers injected into the graft tissue or into selected targets in the host brain. The transmitter-specific methods allowed visualization of grafted dopaminergic, noradrenergic and serotonergic neurons in their entirety, including the totality of their axonal projections in the host brain (see, *e.g.*, Bjorklund et al., 1976, 1979; Daszuta et al., 1988) or spinal cord (see, *e.g.*, Nygren et al., 1977; Nornes et al., 1983; Foster et al., 1985). The acetylcholine-esterase (AChE) staining method was similarly effective in tracing axonal projections from basal forebrain cholinergic neurons grafted to the hippocampus (see, *e.g.*, Bjorklund and Stenevi, 1977; Dunnett et al., 1982; Gage et al., 1984). The M2 and M6 antibodies, specific for mouse neurons and glia, were introduced by Ray Lund and colleagues as a tool to trace connections from mouse cells grafted to the rat brain (Lund et al., 1985; Hankin and Lund, 1987) and have since become a standard method in studies of mouse-to-rat xenografts in the brain. Other species-specific antibodies recognizing either human or pig neuronal epitopes, such as neurofilament, NCAM or Thy-1, have been effectively used for the same purpose, again in a xenograft setting (see, *e.g.*, Wictorin et al., 1990a; Stromberg et al., 1992; Isacson and Deacon, 1996).

The early studies using transmitter specific histochemical methods or immunohistochemistry with species specific antibodies revealed an impressive capacity of grafted immature neurons or neuroblasts, obtained from fetal brain, to grow axons over long distances and in a target-directed manner, extending not only within gray matter, but also along white matter tracts, such as observed from pontine noradrenergic, serotonergic or cortical neurons grafted to the lesioned spinal cord (Nornes et al., 1983; Foster et al., 1985; Li and Raisman, 1993), from striatal projection neurons grafted to the lesioned striatum (Wictorin et al., 1990b, 1991; Isacson and Deacon, 1996; Armstrong et al., 2002) and from septal cholinergic neurons grafted to the lesioned septum (Leanza et al., 1996).

The ability of grafted neuroblasts to re-establish target-specific innervation patterns in previously denervated brain regions is particularly intriguing. Some of the most interesting experiments along these lines were performed with fetal cells transplanted to the subcortically or cortically denervated hippocampus (reviewed in Bjorklund et al., 1990; Dunnett, 1991). In the adult rat hippocampus, denervated of its subcortical afferents by a lesion of the septum or the fimbria–fornix pathway, grafted fetal cholinergic, noradrenergic and serotonergic neurons were seen to reproduce innervation patterns in dentate gyrus and hippocampal subfields that mimicked closely those of the intrinsic afferents that had been removed by the fimbria–fornix lesion (Fig. 1G,H) (Bjorklund and Stenevi, 1977; Gibbs et al., 1986; Nilsson et al., 1988; Leanza et al., 1996). The graft-derived axons were shown to form functional synaptic contacts with host target neurons (Segal et al., 1985; Clarke et al., 1986), restore hippocampal acetylcholine levels and release (Nilsson et al., 1990), and ameliorate some aspects of lesion-induced deficits in hippocampus-dependent learning and memory, as observed both in rats (Dunnett et al., 1982, 1993; Gage et al., 1984; Gage and Bjorklund, 1986; Segal et al., 1987; Hodges et al., 1991) and monkeys (Ridley et al., 1992). The ingrowth of axons was blocked by the presence of an intact cholinergic innervation, and stimulated by removal of the intrinsic afferents, suggesting that axonal outgrowth from the grafted neurons was very precisely regulated by the re-innervated target (Bjorklund et al., 1979; (Lewis and Cotman, 1982). In an interesting study using microdialysis to monitor changes in release of acetylcholine in the graft-reinnervated hippocampus, Nilsson et al. (1990) and Nilsson and Bjorklund (1992) showed that the activity of the grafted septal cholinergic neurons was markedly increased during behavioral activation by sensory stimulation, or by activation of host brain afferents, suggesting that they were under regulatory control from the host brain (Fig. 1A–F).

Interestingly, the graft-derived innervation patterns differed not only between neurons of different transmitter phenotypes, but also between different subtypes of cholinergic neurons, suggesting that the growth and terminal arborization of the ingrowing axons were precisely regulated by the denervated target: each neuron type was seen to produce distinctly different innervation patterns, the ingrowth was inhibited by an intact innervation of the same type, and it was stimulated by additional denervating lesions (Gibbs et al., 1986; Nilsson et al., 1988). This is further supported by work from Geoffrey Riasman's lab, showing that the axonal connections established by transplants of fetal entorhinal cortex in the adult mouse hippocampus and dentate gyrus are restricted to the appropriate terminal zones, and that these connections are formed only after removal of the intrinsic host entorhinal afferents (Zhou et al., 1989).

Studies of graft–host connectivity using transgenic GFP-expressing reporter mice

The introduction of uorescent reporters, such as green- uorescent protein, GFP, has provided a new set of powerful and versatile tools to visualize and trace axonal projections derived from grafted neurons with a sensitivity and specificity that goes beyond what has been possible with classic tract-tracing techniques. The access to transgenic GFP-expressing reporter mice and stem cell lines, in particular, has made it possible to study the connectivity of intracerebral neural grafts in a new and more refined way and opened up for more detailed studies on the ability of grafted neurons to serve as tools for reconstruction of damaged brain and spinal cord circuitry. To be useful for tracing of axonal projections, however, the uorescent reporter has to be expressed at a sufficiently high level to fill out all axonal and dendritic projections, and the expression of the reporter has to be maintained also in fully mature neurons after transplantation. Down-regulation of the reporter in the mature cells can indeed be a problem, as observed in transplants from GFP transgenic mice where the expression of GFP was driven by the chicken β-actin or the prion promoters (Eriksson et al., 2003; Kelly et al., 2007).

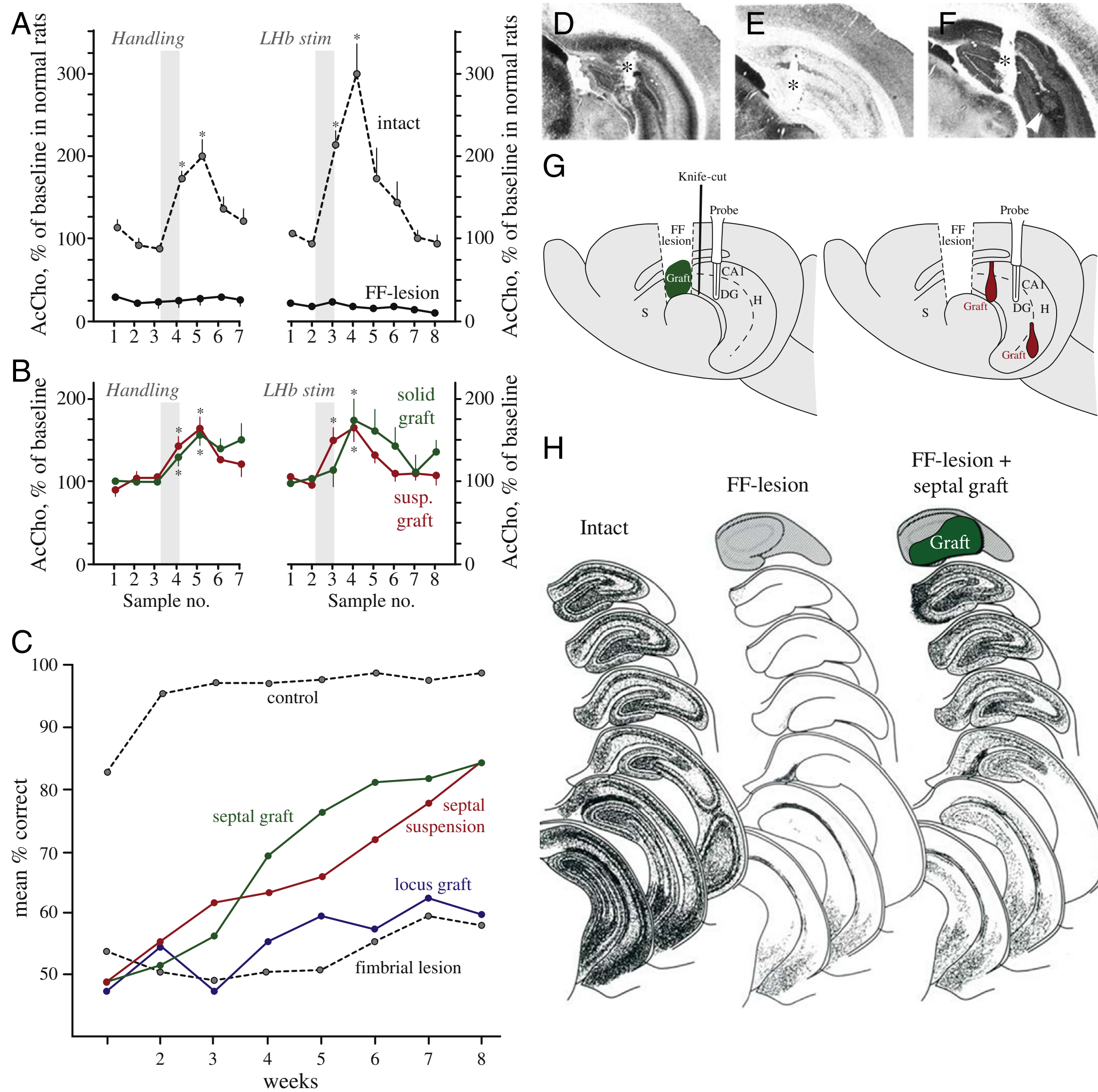

Fig. 1. Summary of results obtained in adult rats with transplants of fetal septal cholinergic neurons grafted to the de-afferented hippocampus. G: The cells were implanted either as a solid tissue graft into a pre-formed cavity transecting the fornix-fimbria (FF) pathway (left panel), or as a cell suspension deposited into the dorsal and ventral hippocampus (right panel). H: Extent and patterning of the graft-derived cholinergic innervation, as visualized using AChE histochemistry, 3 months survival. The FF lesion, which transects the septo-hippocampal connections, denervates the hippocampus of its cholinergic input (middle panel), and the septal graft is efficient in providing most of the denervated hippocampus with a new cholinergic innervation. The laminar pattern formed by the graft-derived fibers in hippocampus and dentate gyrus (as seen in the right hand panel) matches closely the normal laminar innervation patters (as seen in the left-hand panel). C: Performance of intact, FF-lesioned and FF-lesioned + grafted rats in a rewarded T-maze alternation task. The lesion-only rats perform close to chance (50%) and do not learn the task over time. Rats with septal graft, rich in cholinergic neurons, but not the noradrenergic-rich locus coeruleus grafts, reversed this impairment. Behavioral recovery correlated significantly with AChE-positive fiber ingrowth into the denervated hippocampus. A and B: ACh release, as monitored by microdialysis, was recovered to normal levels in the graft-reinnervated hippocampus (*cf.* A and B), and showed a similar, albeit smaller, increase in response to either sensory stimulation (handling) or stimulation of host afferents (by electrical stimulation of the lateral habenula, LHb). D–F show placement of the dialysis probe in the dorsal hippocampus (AChE-stained sections). Data compiled from Dunnett et al. (1982) and Nilsson et al. (1990).

The usefulness of this approach has been demonstrated in studies from Afsaneh Gaillard's and Mohamed Jaber's lab using fetal tissue derived from mice expressing GFP under a synthetic promoter, the chicken β-actin-CMV enhancer, transplanted to the damaged motor cortex or to the lesioned nigrostriatal pathway in adult mice (Gaillard et al., 2007, 2009), and also in our own work where we have studied the connectivity of grafted dopamine neurons, transplanted to either the substantia nigra or the striatum, labeled by GFP driven by either the tyrosine hydroxylase (TH) promoter (Sawamoto et al., 2001; Thompson et al., 2005, 2009) or by GFP inserted into the Pitx3 locus (Grealish et al., 2010) (Fig. 2). More recently, Tuszynski and collaborators have used cells from a transgenic rat expressing GFP under the ubiquitin promoter to trace connectivity established by spinal cord precursors transplanted to the lesioned rat spinal cord (Lu et al., 2012). In all these cases the expression of the GFP reporter is maintained long-term in the fully mature neurons after transplantation, and in line with earlier observations these studies provide striking demonstrations of the ability of transplanted neural precursors and neuroblasts to re-establish long-distance axonal connections with the appropriate denervated targets: the intra-cortical grafts with striatum, thalamus and cervical spinal cord; the intra-nigral dopamine neuron grafts with the striatum; and the intra-spinal grafts extending axons in rostral and caudal directions over a distance of about 25 mm from the graft site.

Generation of transplantable neurons from pluripotent stem cells

The “transplantability” of neurons, *i.e.*, their ability to survive, differentiate, integrate and function after transplantation to the lesioned

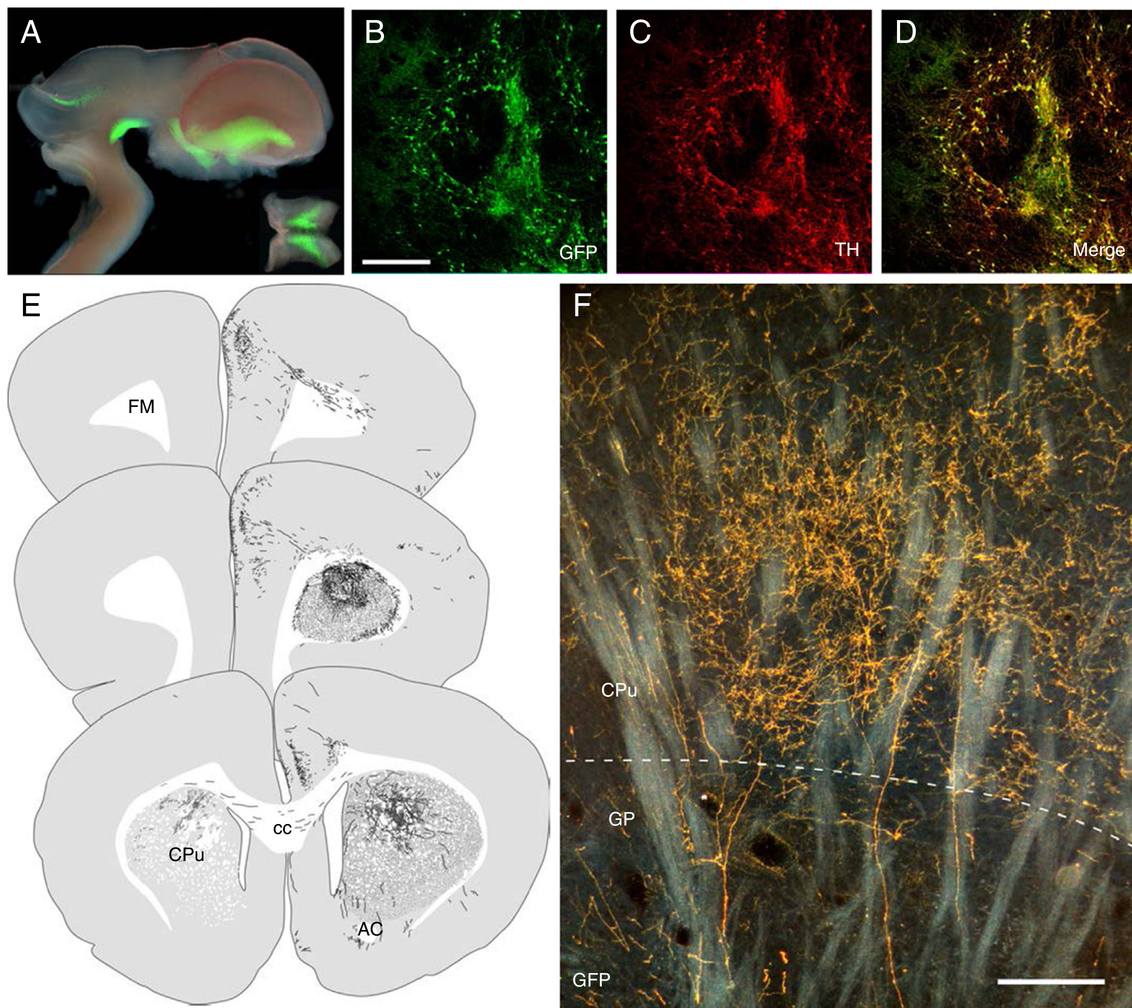

Fig. 2. The embryonic (E12) brain of the TH-GFP reporter shows GFP expression (green) in developing dopamine neurons in the ventral mesencephalon (A, inset shows typical example of VM a piece used for transplantation). After intra-striatal transplantation of the VM, the grafted dopamine neurons express both GFP and TH (B–D). Reconstructions of immunohistochemical detection of GFP+ fiber growth in coronal sections illustrate the graft-specific patterns of dopamine neuron integration in the host brain after intra-striatal VM grafting (E). A darkfield image of GFP+ fibers labeled with the DAB chromophore by immunohistochemistry in an animal that received VM cells grafted into the midbrain shows that the grafted dopamine neurons can extend axons over long distances in order to innervate the striatum (F). The dashed line indicates the boundary between the globus pallidus and the striatum. Abbreviations: AC, anterior commissure; cc, corpus callosum; CPu, caudate-putamen unit; FM, forceps minor; GP, globus pallidus. Scale bars: B, 50 m; F, 200 m.
Modified from Thompson et al. (2005) and Thompson et al. (2009).

adult brain or spinal cord, is critically depending on their stage of differentiation. Studies using transplants of cells derived from fetal tissue show that they need to be harvested just before, or at the time of cell cycle exit, before they have started to extend more elaborate processes but after they have become genetically committed to their mature phenotype. This is well illustrated by studies performed on the survival of grafted midbrain dopamine neurons (Brundin et al., 1985; Torres et al., 2007; Bye et al., 2012) showing that survival is optimal when the grafted cells are harvested around the time of cell cycle exit (embryonic day 10–11 (E10–11) in the mouse, as determined by BrdU-labeling), and that survival declines sharply at later stages of differentiation. In a study using FACS sorting to isolate dopamine neuron precursors from the developing mouse midbrain, we have shown that the dopamine neurons that survive and mature after transplantation into the dopamine-denervated striatum are primarily derived from Nurr1-positive early postmitotic neuroblasts that have not yet started to express the characteristic dopamine neuron marker, TH (Thompson et al., 2006; Jonsson et al., 2009). At this stage of development midbrain tissue contains a mixture of post-mitotic and still dividing neuroblasts. Using TH-GFP reporter mice as donors, Bye et al. (2012) have found that the inclusion of still dividing precursors is advantageous and likely to contribute to a high yield of surviving dopamine neurons. Studies on cortical projection neurons transplanted to the lesioned adult cortex have given similar results, showing that good survival, integration and targeted axonal outgrowth are obtained when the cells are taken from E14–19 mouse donors, *i.e.*, at the early postmitotic stage (Hernit-Grant and Macklis, 1996; Fricker-Gates et al., 2002; Gaillard et al., 2007).

Early attempts to generate transplantable neurons from mouse or human ESCs were hampered by either poor overall survival or by inclusion of proliferative cells that generated expanding tumors at the graft site (REFs?). Cell preparations rich in immature progenitors that could survive the transplantation procedure likely included many unspecified cells capable of uncontrolled growth *in vivo*. On the other hand, long differentiation procedures intended to deplete cultures of these primitive cell types are likely to result in a substantial proportion of terminally differentiated cells that do not survive transplantation. The development of more refined cell derivation protocols has provided ways to solve these problems. These more refined protocols have been inspired by insights into the role of cell intrinsic and extrinsic mechanisms, as well as timing, derived from studies of regional patterning and phenotypic specification of neuronal subtypes during embryonic development. Good examples of such protocols, applied to either mouse or human pluripotent cells, are the ones developed by Vanderhaeghen and collaborators for generation of transplantable cortical neurons based on

manipulation of sonic hedgehog (SHH) and/or bone morphogenic protein (BMP) signaling (Gaspard et al., 2008, 2009; Espuny-Camacho et al., 2013); the ones developed in Wichterle's and Studer's labs for generation of transplantable spinal motoneurons, induced either by addition of two patterning morphogens, retinoic acid (RA) and SHH (Wichterle et al., 2002; Lee et al., 2007a) or in the absence of extrinsic morphogenic factors (Peljto et al., 2010); the ones from the Studer and Chung labs for the specification of inhibitory interneurons (Maroof et al., 2013; Kim et al., 2014); and the ones developed in Studer's and Parmar's labs for generation of transplantable midbrain dopamine neurons based on the generation of cells with midbrain floorplate characteristics, using a combination of SHH and WNT activators, as well as inhibitors of SMAD signaling (Kriks et al., 2011; Kirkeby et al., 2012).

An alternative approach has been to convert ESCs or iPSCs into expandable neural progenitor cells or cell lines. An advantage of this approach is that it avoids the inclusion of contaminating pluripotent cells, thus reducing the risk of transplant overgrowth or tumor formation. In several recent studies such cells have been shown to survive transplantation to the brain or spinal cord in neonatal or adult hosts, and develop into neurons with mature phenotypes, without any signs of overgrowth (Steinbeck et al., 2012; Lu et al., 2014).

The problem of cell fusion

The ability of transplanted cells to fuse with cells in the host CNS has been considered to be a very rare phenomenon and mostly limited to cells derived from the bone marrow. It is well known that systemically injected bone marrow-derived cells can fuse with Purkinje cells in the cerebellum, and that this phenomenon is greatly enhanced by inflammation or tissue damage (Johansson et al., 2008; Nygren et al., 2008; Espejel et al., 2009). More recently, however, two carefully executed studies (Cusulin et al., 2012; Brilli et al., 2013) have provided compelling evidence that transplanted neural stem cells, derived from fetal brain or ESCs, can undergo fusion with mature, postmitotic neurons in the adult brain. In the experiments of Brilli et al. (2013) cells passaged long-term in attached neural stem cell cultures were transplanted into hippocampus, cortex or striatum in adult mice. Cell fusion, documented by different complementary methods, was notably frequent in granule cells of the dentate gyrus, but was observed also in cortex and striatum. In hippocampus about 0.2% of the injected GFP-positive cells, grafted into wild-type or ROSA26 transgenic mice, displayed a mature neuronal phenotype, and they appeared as soon as 7 days after transplantation. Notably, the GFP-expressing granule cells and pyramidal-like neurons, appearing after injection into the cortex, exhibited the authentic polarity, position and orientation typical for these neuron types.

In Cusulin et al.'s (2012) study, GFP-expressing mouse ES cell derived neural stem cells were transplanted into cerebral cortex in neonatal rat or mouse pups. GFP-expressing cells with a mature cortical pyramidal morphology appeared within 3 weeks. Evidence of cell fusion was obtained by use of species-specific antibodies, and by pre-labeling of host pyramidal neurons with retrogradely transported Lumafluor beads. Moreover, around 17% of the GFP-positive neurons were seen to contain two distinct nuclei. In parallel *in vitro* experiments Cusulin et al. (2012) obtained evidence that microglia play a critical role in this fusion event, indicating that the immature neural stem cells fuse first with microglia, and that the fused NS/microglia cells in a second step fuse with neurons.

These observations show that cell fusion is a phenomenon that has to be taken into consideration in studies using transplants of expanded neural stem cells, particularly in connection with traumatic injury or damage-induced inflammation were microglial activation may come into play. Some types of host neurons, such as dentate granule cells and cortical pyramidal neurons, may be more prone to undergo fusion, and the ability of the grafted cells to fuse seems to vary markedly depending on the type of cell used. Thus, Brilli et al. (2013) observed that progenitors derived from neurosphere cultures were less prone to fusion than neural stem cells grown long-term in attached cultures. In retrospect, it seems clear that some of the more striking results reported in previous literature, such as in our own experiments using transplants of the immortalized RN33B cell line (Englund et al., 2002), may have to be interpreted with caution. In these studies graft-derived, fully mature and perfectly oriented pyramidal-like neurons were observed, but the possible role of cell fusion was not investigated. Nevertheless, cell fusion is unlikely to represent a major problem in the interpretation of results obtained with ESC- or iPSC-derived neural progenitors and this is reflected in a number of studies where cell fusion has specifically been examined but not observed (MacLaren et al., 2006; Gaillard et al., 2007; Magavi and Lois, 2008; Michelsen et al., 2015).

Establishment of axonal connections from ESC- or iPSC-derived neurons grafted to the brain or spinal cord

The new generation of cell differentiation protocols has opened up for interesting studies of ESC- and iPSC-derived cells implanted in diverse brain regions, such as the cortex, hippocampus and substantia nigra, as well as in the damaged spinal cord. A common feature of these cell differentiation procedures is that they take advantage of insights into how morphogens, such as RA, SHH, BMP, fibroblast growth factors (FGFs) and WNTs, act to generate neuronal diversity in the CNS during embryonic development (Gaspard and Vanderhaeghen, 2010; Peljto and Wichterle, 2011). The first protocol devised along these lines was that of Wichterle et al. (2002) using a combination of RA (as a neuralizing and caudalizing agent) and SHH (as a ventralizing agent) to generate spinal cord motor neurons from mouse ESCs. These cells were shown to express phenotypic markers characteristic for one of the motor neuron subtypes (the cervical median motor column neurons), capable of innervating multiple muscle targets following transplantation to the developing chick spinal cord. In a later modification of this protocol, designed to mimic more closely the patterning events in the developing spinal cord, Peljto et al. (2010) have been able to generate also brachial and thoracic, limb innervating motor neuron subtypes with the ability to correctly target their axons in the developing chick limb, suggesting that ESC-derived motor neurons can be programmed to develop into specific and diverse motor neuron subtypes.

Work from Studer's lab (Lee et al., 2007a) has shown that this approach is adaptable also to human ESCs, using a modified protocol whereby the cells are neuralized on MS5 stromal feeders and specified using RA and SHH. Neurons generated under these conditions expressed features of both rostral and caudal motor neuron identities. Following transplantation to the developing chick spinal cord processes positive for human NCAM and GFP were seen to exit the cord and extend over several millimeters toward peripheral muscular targets. In this study the cells were transplanted also to the adult rat spinal cord, but the survival time (up to 6 weeks) did not allow assessment of connectivity of the grafted neurons. Attempts to use mESC- or hESC-derived motor neurons for transplantation in injured rat spinal cord have reported survival and functional maturation of the grafted cells, but so far no or very limited growth of axons toward peripheral targets (Harper et al., 2004; Erceg et al., 2010).

In a recent study, Lu et al. (2014) have shown that neurons generated from human pluripotent stem cells are capable of extensive axonal growth throughout the injured spinal cord. Neural progenitor cells, derived from human iPSCs, were transduced to express GFP, enclosed in a fibrin matrix containing a cocktail of growth factors, and transplanted into a C5 spinal cord hemisection lesion in adult immunodeficient rats. In these grafts around 70% of the cells expressed NeuN at 3 months post-grafting and 18% the astrocyte marker GFAP. Around 7% of the cells remained proliferative at 3 months. Similar approaches using human ES or iPS-derived neural progenitors have been explored by other investigators (Nori et al., 2011; Fujimoto et al., 2012) but the extent of fiber outgrowth reported by Lu et al. (2014) far exceeds that

obtained previously: GFP-expressing axons were seen to extend in very large numbers, primarily along white matter tracts, throughout the rostro-caudal extent of the cord, and also rostrally as far as the olfactory bulb, a distance of more than 9 cm. The outgrowth pattern appeared to be largely non-selective, suggesting that the relatively undifferentiated cells within the graft take their guidance cues from the local host environment, resulting in long-distance growth associated with myelinated fiber tracts, in place of any intrinsically specified programs for the establishment of target-directed connectivity. Axons leaving the myelinated fiber bundles were seen to ramify in the gray matter of the spinal cord to form synaptophysin-positive, bouton-like terminals in close apposition to host neurons and dendrites. These terminal arborizations, however, were quite sparse, suggesting that the grafted progenitors are poorly specified with a limited capacity to establish target-specific axonal connectivity in the host CNS.

In terms of the capacity of implanted cells to establish target-directed patterns of connectivity, more interesting results have been obtained in studies using ESC-derived cortical neurons. A number of recent studies have generated transplantable cortical progenitors from mouse (Gaspard et al., 2008; Ideguchi et al., 2010; Michelsen et al., 2015) or human (Denham et al., 2012; Steinbeck et al., 2012; Espuny-Camacho et al., 2013) pluripotent stem cells by taking advantage of the default tendency for these cells to acquire a dorsal forebrain identity after neural induction. Inhibition of BMP signaling using Noggin (or the small molecule inhibitor LDN193189) facilitates rapid and efficient neural specification and acquisition of a molecular phenotype consistent with early cortical progenitor identity. Interestingly, in these culture systems neurons of distinct cortical layer identities were generated sequentially, as in normal cortical development.

Following transplantation to the cortex in neonatal mice or rats the ESC-derived, GFP-labeled cells develop into mature pyramidal-like neurons with the capacity to extend axons along major corticofugal pathways, including the corpus callosum, the external and internal capsules and the cerebral peduncles (Fig. 3). The outgrowth patterns suggest that the axons grow in a highly specific and target-directed manner. The GFP-positive graft derived innervations are consistent

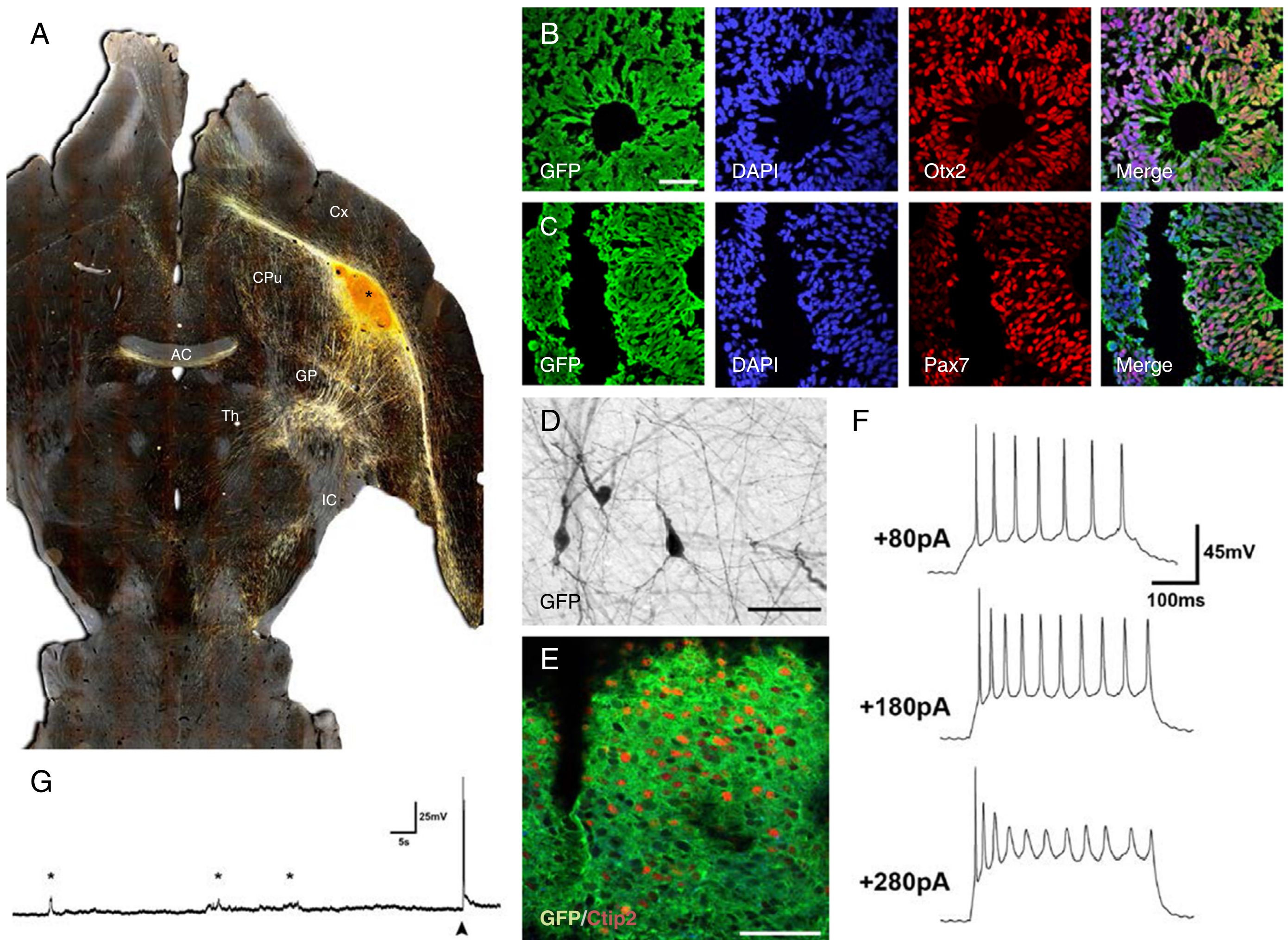

Fig. 3. A darkfield image of immunohistochemistry for GFP in a horizontal section of rat brain 12 weeks after transplantation of GFP+ neural progenitors generated from human ESCs (A). The GFP+ axons emanating from the graft (*) grow over long-distances in the host brain, predominately in caudal a direction *via* myelinated host fiber bundles. Prior to transplantation the neural progenitors displayed phenotypic properties consistent with cortical progenitor identity, including expression of the forebrain specific transcription factor Otx2 (B) and the dorsal marker Pax7 (C). In the resulting grafts, many of the GFP+ neurons have morphological features of differentiated neurons, including pyramidal subtypes (D). At the molecular level expression of the transcription factor Ctip2 (E) suggests some of the grafted cortical neurons may have features of a deep-layer cortical phenotype. Patch-clamp recordings show that the grafted human ES-derived neurons were capable of firing action potentials in response to current injection, but at the 12 week time-point, many cells failed to sustain a firing pattern in response to increasing current, suggesting an immature neuronal phenotype (F). Excitatory post-synaptic potentials are clearly evident on the voltage trace recorded from an individual neuron (*) and in some cases they depolarized the neuron sufficiently to fire action potentials (filled arrow, G). Abbreviations: AC, anterior commissure; Cx, cortex; CPu, caudate-putamen unit; GP, globus pallidus; IC, internal capsule; Th, thalamus. Scale bars: A, 3 mm; B, 50 m; D, 50 m; E, 200 m.
Modified from Denham et al. (2012).

with pyramidal neurons of diverse layer identities: layer IV-type neurons innervating thalamus, layer V-type neurons innervating striatum and brainstem, and layer II–V-type neurons innervating ipsi- and contralateral cortex. In Espuny-Camacho et al.'s (2013) study the sublayer specificity of the projection patterns was supported by retrograde tracing, performed at 9 months after transplantation in immunodeficient NOD-SCID mice, showing that many of the human pyramidal-like neurons labeled from thalamus expressed the layer IV-marker TBR1, and that the neurons labeled from the superior colliculus expressed the layer V-marker CTIP2. Observations from studies by Gaspard et al. (2008) indicate that the laminar commitment of the neurons depends on the length of culture, such that the ones generated first (at 12 days of culture) project primarily to thalamus (consistent with deep-layer neurons) and those generated later, at 14–17 days of culture, project more prominently to cortex (consistent with neurons of more superficial layers). More recently, similar experiments using mouse ESCs showed that, in addition to laminar identity, *regional* cortical phenotype is also important for establishing specific patterns of connectivity (Michelsen et al., 2015). For example, after lesioning of the visual cortex in adult mice, re-establishment of the occipital cortical pathways could be achieved when transplanting ESC-derived progenitors with molecular features of occipital cortex but not after transplantation of fetal motor cortex. At the functional level, electrophysiological recordings showed that grafted occipital cortical neurons, but those from motor cortex, had a similar capacity to respond to visual stimulus through increased firing activity as nearby cortical neurons in the mouse visual cortex. These findings suggest that the cells generated in these protocols possess intrinsically specified programs for the establishment of target-directed connectivity, and functional integration characteristic for each pyramidal neuron subtype. Overall, the capacity for the establishment of long-distance, targeted connectivity of ESC-derived cortical neurons appears quite similar to that obtained with fetal cortical tissue, transplanted to the cortex in neonates or lesioned adult hosts (Gaillard and Roger, 2000; Gaillard et al., 2007).

A notable feature of grafts generated from human pluripotent cells is the protracted nature of the growth properties *in vivo*. This can be observed both at the level of the cellular composition of the grafts and also the development of graft-derived innervation patterns in the host brain over time. The studies by Denham et al. (2012) and Steinbeck et al. (2012) show the persistence of immature neural progenitors up to 3 months after grafting, including active migration of neuroblasts from the graft core into the surrounding host parenchyma. In Steinbeck et al.'s study there was a progressive increase in the percentage of mature, NeuN + neurons between 12 and 48 weeks and a corresponding decline in the presence of Ki67 + diving cells and nestin + neural precursors. Although the growth of GFP + axons occurred over remarkably long distances after implantation into the neonatal or adult brain, patterns of terminal arborization in specific host nuclei were notably sparse still by 12 weeks post-grafting. Similarly, in the study by Espuny-Camacho et al. (2013), the level of graft-derived innervation of the host brain is seen to increase substantially between 2 and 6 months, reflecting the slow and protracted maturation of the grafted human cells.

Further support for the ability of pluripotent cells to generate committed precursors of highly specific neuronal subtypes comes from studies aimed at the generation of transplantable midbrain dopamine neurons. Early experiments along these lines were successful in generation TH-positive neurons in high frequency and large numbers from both mouse and human ES and iPSCs (Yurek and Fletcher-Turner, 2004; Roy et al., 2006; Wernig et al., 2008; Hargus et al., 2010; Rhee et al., 2011). The limited capacity of these cells to re-innervate appropriate denervated targets in the adult rat or mouse brain, however, suggested that most of the cells were either incompletely specified and/or not fully matured. During embryonic development midbrain dopamine neurons are derived from floorplate cells, determined by an interplay between SHH, FGF and WNT signaling (Ono et al., 2007; Bonilla et al., 2008). Based on these insights, protocols have been developed to derive midbrain floorplate-like cells from human ES and iPSCs, characterized by their expression of two key transcription factors, FoxA2 and Lmx1A (Kriks et al., 2011; Kirkeby et al., 2012; Sundberg et al., 2013). These cells survive transplantation and develop into fully mature TH-positive neurons that express phenotypic markers characteristic for the two major dopamine neuron subtypes, A9 and A10, without any signs of overgrowth or tumor formation. The neurons generated by these floorplate protocols are highly efficient in re-innervating both A9 and A10 targets after transplantation either to the striatum (Kriks et al., 2011; Kirkeby et al., 2012; Sundberg et al., 2013) or into the substantia nigra (Grealish et al., 2014) in adult rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway.

In Grealish et al.'s (2014) study the hESC-derived dopamine neuron precursors were transplanted unilaterally into the lesioned substantia nigra and axonal outgrowth was studied using antibodies directed against human NCAM and TH. Grafts of authentic fetal human ventral midbrain (VM) dopamine neurons, obtained from 5.5–8 week old fetuses, were studied in parallel. Six months post-grafting the appearance of the TH + neurons were closely similar in both types of grafts with respect to both morphology and phenotypic expression. From both types of graft hNCAM + axons could be traced in large numbers along the medial forebrain bundle and the nigrostriatal pathway to provide innervations in appropriate A9 and A10 targets, including striatum, nucleus accumbens, amygdala, olfactory tubercle and infralimbic and cingulate cortex, reaching about 10 mm from the graft deposit (Fig. 4). Quantification showed that the number of hNCAM + axons extending rostrally was similar for fetal VM neurons and midbrain-patterned hESCs. Fiber density measurements showed that fetal and hESC-derived neurons had a similar propensity to innervate A9 and A10 targets, although the A9-specific innervation in the dorsolateral striatum was overall of higher density in rats with fetal VM grafts. These data support the view that the hESC-derived grafts generated in the floorplate protocol contain the two principal midbrain dopamine neuron types, similar to fetal VM tissue grafts, but that the A10 subtype is relatively more abundant in the hESC-derived grafts.

Although not characterized further, the midbrain patterned hESCs contained not only TH-positive neurons but also other neuron types, not expressing the TH marker. Consistent with this, a significant fraction of the outgrowing hNCAM + axons did not stain for TH, suggesting that the non-dopaminergic neurons in the graft contributed to the overall axonal outgrowth pattern. This is not unique for hESC-derived transplants. It is well known that fetal VM transplants have a mixed composition. The TH + neurons comprise only some 5–10% of all neurons contained in these grafts, and we have previously shown that non-TH + neurons contribute a widespread innervation not only to areas normally innervated by TH + neurons, but also to areas not receiving such innervation (Thompson et al., 2008). Similar non-TH + innervations were observed also in Grealish et al.'s 2014 study, from fetal VM as well as from the hESC-derived grafts, and most prominently in areas of the cortex and in hypothalamus. It is unclear whether these non-TH + innervations represent aberrant axonal projections, or contributions from other midbrain neuron types.

Functional integration of transplanted neurons generated from pluripotent stem cells

Important insights into the functional properties of neural grafts generated from pluripotent stem cells have been gained from studies both at the cellular level of the grafted neurons and also by assessing their functional impact in animal models of disease. Patch-clamping experiments have shown that neurons derived from ESCs or iPSCs develop appropriate electrophysiological properties, including evoked and spontaneous firing of action potentials and evidence for functional afferent input from host neurons. The use of GFP-reporter lines has been particularly useful in these studies in that the grafted neurons can be

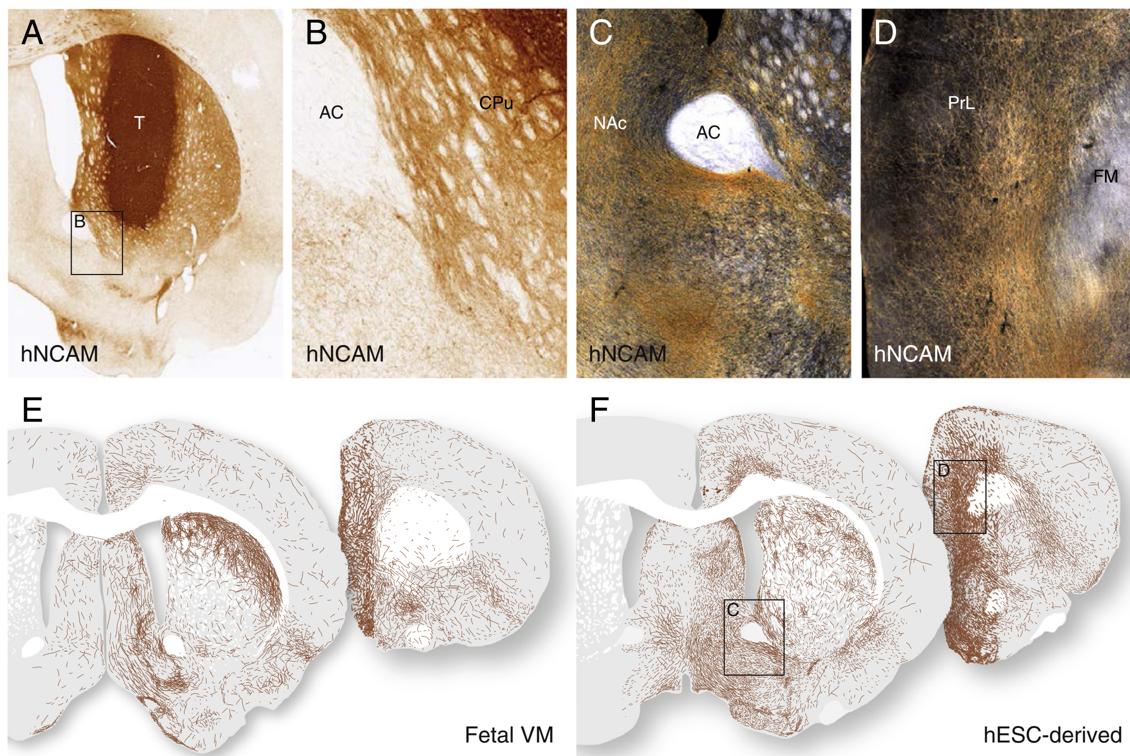

Fig. 4. Extent of axonal outgrowth obtained from hESC-derived neurons transplanted either to the striatum (A and B) or into the substantia nigra (C,D and F) in immunosuppressed, 6-OHDA lesioned rats, 6 months survival. E illustrates for comparison the innervation patterns generated from an intra-nigral graft of human fetal midbrain neurons. From both types of graft axons, stained with an antibody specific for human NCAM, were seen to extend along the medial forebrain bundle and the nigro-striatal pathway to reinnervate the appropriate forebrain target areas, including the caudate-putamen (CPu), nucleus accumbens (NAc) and the prelimbic frontal cortex (PrL). AC, anterior commissure; FM, forceps minor; T, transplant. Modified from Grealish et al. (2014).

quickly identified in acute brain slices prepared from grafted animals. Numerous studies (Wernig et al., 2008; Koch et al., 2009; Denham et al., 2012; Steinbeck et al., 2012; Espuny-Camacho et al., 2013; Tornero et al., 2013; Avaliani et al., 2014) have given us a good idea of the intrinsic electrophysiological properties of grafted neurons derived from human ESCs or iPSCs. Some of the initial patch-clamping studies from the Brustle lab showed that GFP+ neural progenitors generated from ESCs displayed voltage-dependent inward and outward currents and repetitive action potentials in response to depolarization after grafting into neonatal mice (Koch et al., 2009). The recorded neurons had immature electrophysiological properties at 6–9 weeks but developed more complex firing patterns and acquired spontaneous postsynaptic currents indicative of afferent inputs at 18–24 weeks. Similarly, Denham et al. (2012) reported firing patterns of consistent with an immature neuronal phenotype at 12 weeks post-grafting, while in the study by Espuny-Camacho et al. (2013), grafted human ES-derived cortical neurons patch-clamped at the much later (9 month) time-point display membrane and firing properties typical for mature pyramidal neurons. Similar observations have been reported by Nicholas et al. (2013)) in *in vitro* studies, showing that inhibitory telencephalic interneurons generated from human ESCs continue to mature between 15 and 30 weeks.

Collectively, these results support the view that the development and maturation of implanted neurons generated from human ESCs is notably protracted. In our on-going work we have found this to hold true also for grafts generated from human iPSCs. After transplantation into athymic rats, the capacity of patch-clamped neurons to sustain a firing pattern in response to increasing current continues to develop between 12, 26 and 50 weeks *in vivo* (Fig. 5A). At these later time-points the grafts have established extensive patterns of axonal growth in the host brain (Fig. 5B).

The utilization of optogenetic approaches has provided evidence that neurons generated from pluripotent stem cells are not only capable of developing intrinsic electrophysiological properties *in vivo*, but importantly, that they can establish functional patterns of afferent and efferent connectivity in the host brain. In a study by Avaliani et al. (2014) long-term passaged neural progenitors produced from human iPSCs were transplanted into the hippocampus of adult athymic rats in which the nearby host neurons were transduced to express the light-sensitive channelrhodopsin-2 (ChR2) cation channel. Six months after grafting, selective light-activated stimulation of host neurons in acute slice preparations induced post-synaptic currents in ~70% of the grafted neurons, thus showing that neurons generated from pluripotent stem cells are capable of receiving functional synaptic input from host circuitry.

Similar approaches have been used to show that implanted neurons can establish functional *efferent* connections with host neurons. Cunningham et al. (2014) investigated the capacity of inhibitory interneurons generated from human ESCs to functionally integrate into hippocampal circuitry in a mouse model of temporal lobe epilepsy. In this study the grafted cells were engineered to stably express GFP and ChR2 prior to transplantation. Photo-stimulation in acute slice preparations evoked currents in the grafted GFP+ cells, and importantly, also induced inhibitory post-synaptic responses in nearby host neurons. Steinbeck et al. (2015) have recently reported similar findings after transplantation of hESC-derived dopamine neuron progenitors in a mouse model of Parkinson's disease (PD). In these experiments the ES derived donor cells were engineered to express the light-sensitive *anion* channel, halorhodopsin, so that the differentiated neurons could effectively be 'silenced' upon exposure to light. The innervation of the striatum by the host midbrain dopamine system was removed unilaterally prior to transplantation and by 4–6 months, the grafted dopamine

neurons were able to partially re-innervate the host striatum. Patch-clamp recordings in acute slice preparations showed that the grafted neurons displayed a similar electrophysiological profile to that shown previously for fetal mouse dopamine neurons (Sorensen et al., 2005), and stimulation of the grafted neurons produced dopamine receptor (D1) dependent excitatory post-synaptic potentials in host medium spiny projection neurons. This effect was significantly diminished in response to optogenetic silencing of the grafted cells.

This optogenetic strategy has also been used in gain-of-function experiments to study the capacity of motor neurons generated from mouse ESCs to functionally innervate host muscle fibers. Bryson et al. (2014) showed that 30 days after implantation into the partially denervated sciatic nerve of adult mice, ChR2-expressing motor neurons were able to send long-distance, cholinergic axonal projections to hind-limb muscles and, upon light-stimulation, induced twitch contractions in the innervated muscle fibers. These findings strongly suggest that neurons generated from pluripotent stem cells are capable of establishing patterns of functional efferent connectivity with appropriate target cells after transplantation.

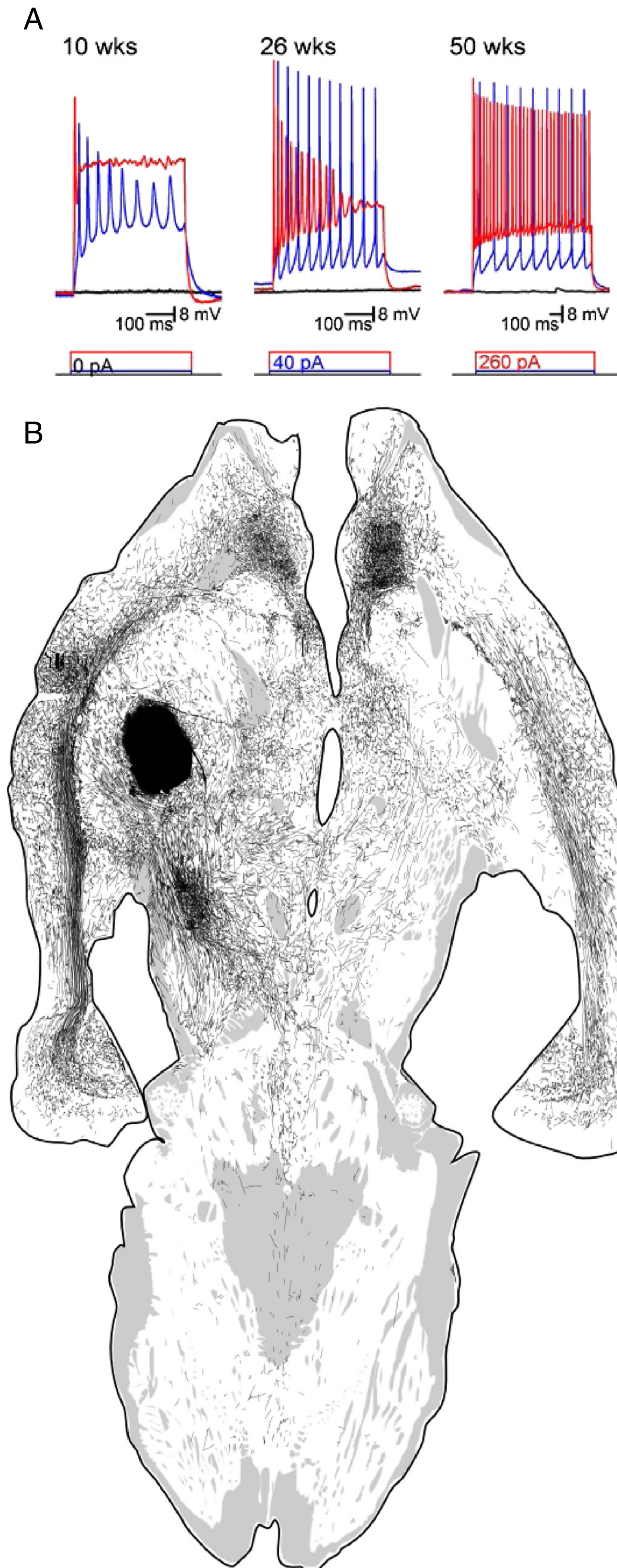

Fig. 5. The progressive maturation of neurons generated from human iPSCs is illustrated by action potential firing patterns in response to 400–1000 ms depolarizing current steps at 10, 26 and 50 weeks after transplantation (A). Representative traces of patch-clamped neurons in acute slice preparations show that action potentials in younger neurons 'collapse' with increasing current steps while the more mature neurons at 50 weeks maintain an increased firing rate for longer duration. Schematic re-construction of GFP+ fibers in these grafts shows that grafted neurons generated from human iPSCs are capable of establishing extensive patterns of axonal growth over long distances in the adult brain (B). Abbreviations: ms, milliseconds; mV, millivolts; pA, picoamps.

Use of neural transplants for reconstruction of damaged brain circuitry

Studies using transplants of neuroblasts derived from embryonic or fetal brain provide compelling evidence that such cells can be used to restore, at least in part, previously damaged brain circuitry. In animal models of PD there is considerable evidence that the functional impact of grafted dopamine neurons is mediated by reinnervation in the previously denervated striatum accompanied by reinstatement of dopamine neurotransmission, and that this mechanism is likely to underlie the therapeutic benefits seen in some of the PD patients that have received grafts of fetal dopamine neuroblasts (for review, see Bjorklund and Lindvall, 2000; Lindvall and Bjorklund, 2011). Similarly, fetal striatal progenitors have been shown to integrate into basal ganglia circuitry, restore functional connections with downstream striatal targets and link up with host cortical afferents, after transplantation into rats with excitotoxic lesions of the striatum (see Bjorklund and Lindvall, 2000; Dunnett et al., 2000 for review).

The studies performed using transplants of fetal cholinergic neurons in rats with lesions of the septo-hippocampal pathway, discussed above, provide further examples of circuitry reconstruction. In these experiments restoration of cholinergic afferents and synaptic cholinergic neurotransmission in the previously denervated hippocampus and dentate gyrus, was accompanied by reversal of some of the lesion-induced deficits in hippocampus-dependent learning and memory (Bjorklund et al., 1990; Dunnett, 1991). More recently, the studies of transplants of GABAergic interneurons, derived from the fetal medial ganglionic eminence (MGE) or from pluripotent stem cells, show that these cells can become functionally integrated into dysfunctioning host circuitry, and that they can be used to modify inhibitory signaling and assist to restore normal functions in the host brain (see Southwell et al., 2014, for a recent review).

Transplantation studies using neural progenitors generated from pluripotent stem cells point to the possibility to use ESC-or iPSC-derived neurons as therapeutic agents for brain repair. The results obtained in disease models highlight the possibility to generate neural progenitors from pluripotent stem cells, and that these progenitors can be patterned to give rise to specialized neuronal subtypes with *in vivo* properties that match the ones generated during normal embryonic development. Importantly, this includes the capacity for functional

synaptic integration in order to replace or modify neural circuitry in the damaged CNS as a therapeutic approach for reconstruction of damaged circuitry and functional repair. In the study by Steinbeck et al. (2015), sensorimotor deficit induced by lesion of the host midbrain dopamine system was significantly ameliorated in animals that received grafts of iPSC-derived dopamine neurons, and optogenetic silencing of the grafted cells, performed in freely moving animals, was shown to reverse the functional impact of the graft. These findings support a general principle – well established in earlier studies using primary fetal tissue – that therapeutic impact following transplantation is underpinned by functional integration of the grafted neurons within the host circuitry (for review see Thompson and Bjorklund, 2012), and extend on similar work in models of PD showing improvement of motor function using donor cells generated from either ESCs (Chung et al., 2011; Kriks et al., 2011; Doi et al., 2012; Ganat et al., 2012; Grealish et al., 2014) or iPSCs (Wernig et al., 2008; Hargus et al., 2010; Rhee et al., 2011). Collectively, this body of work suggests that dopamine neurons generated from pluripotent stem cells share the capacity of their fetal counterparts to restore motor function in the dopamine depleted brain through functional integration into the host circuitry.

Other studies that convincingly link functional integration of grafted stem cell-derived neurons to therapeutic impact in disease models include those that involve transplantation of inhibitory interneurons in models of epilepsy. When grafted into rodent models of temporal lobe epilepsy (TLE), interneurons generated from mouse ESCs (Maisano et al., 2012) or human iPSCs (Cunningham et al., 2014) have been shown to significantly reduce the frequency of spontaneous seizure activity. In both studies the uorescent-reported labeled interneurons were seen to migrate widely throughout the hippocampus before differentiating as GABA or GAD expressing interneuron subtypes with various morphological and molecular profiles reminiscent of endogenous hippocampal interneurons. In the study by Maisano et al. (2012) patch-clamp recordings in slice preparations at 2–4 months showed that the grafted interneurons had similar membrane and firing properties to endogenous hippocampal interneurons in the host brain, although overall less mature compared to the host neurons. Nonetheless, recordings under voltage-clamp showed that the stem cell-derived interneurons displayed patterns of spontaneous excitatory postsynaptic currents consistent with synaptic integration into host circuitry. As discussed above, further support for the capacity of stem cell-derived interneurons to functionally integrate into existing circuitry in the epileptic brain comes from transplantation work using ChR2-expressing cells, where selective stimulation of human iPSC-derived grafted interneurons was seen to elicit inhibitory postsynaptic responses in host neurons (Cunningham et al., 2014). These postsynaptic responses were completely abolished in the presence of the GABAA receptor antagonist bicuculline, indicating functional inhibitory neurotransmission. These animals also displayed a significantly reduced frequency of spontaneous seizures (~0.1 seizures/day) compared to un-grafted control animals with TLE (~1.9 seizures/day).

The results from these studies are similar to those obtained in experiments using primary fetal tissue prepared from the MGE. In these studies the grafted interneurons were seen to migrate over large distances (Wichterle et al., 1999), form functional synaptic connections with host neurons, and reduce seizure frequency in rodent models of TLE (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Hunt et al., 2013; Henderson et al., 2014).

Alternative mechanisms of stem cell-induced repair

In other models of CNS damage the relationship between functional integration and therapeutic impact is not always as clear. This is particularly the case in models of cerebral ischemia and spinal cord damage, both of which have received significant attention in the context of transplantation using neural progenitors derived from pluripotent stem cells (for reviews, see Lukovic et al., 2012; Hao et al., 2014). These types of injury are particularly challenging as targets for cell replacement therapy in that they typically involve damage or loss of multiple neuronal pathways involving a range of neuronal subtypes and with patterns of pathology that vary widely between patients. In these injury models, there is good evidence to suggest that mechanisms not related to neuronal replacement, such as neuroprotection and stimulation of host plasticity, play the major role in reducing functional deficits resulting from the injury.

This so-called 'by-stander' effect is best exemplified by transplantation studies where the donor cells are not expected to differentiate into neural cell types. For example, grafting of mesenchymal stem cells has been shown in a number of studies to at least partially mitigate against the functional consequences of stroke (Kang et al., 2003; Andrews et al., 2008; Lin et al., 2011) or spinal cord injury (Lee et al., 2007b). Studies using glia-restricted progenitors have also shown promising results despite the lack of neuronal contribution to the resulting grafts (Lepore et al., 2008; Kondo et al., 2014). Depending on the injury and donor cell type, key mechanisms are likely to include the release of neurotrophic factors that can protect vulnerable tissue following injury, stimulation of angiogenesis and attenuation of in ammatory or excitotoxic signaling pathways in the damaged area and surrounding tissue (for reviews, see Carletti et al., 2011; De Feo et al., 2012).

These observations highlight that, despite the capacity for pluripotent stem cells to differentiate into therapeutically relevant neuronal cell types, one cannot assume functional circuit reconstruction as the default mechanism following transplantation in the damaged CNS. We (Denham et al., 2012), and others (Steinbeck et al., 2012) have shown that the neural progenitor cell types, such as those previously described to exert neuroprotective effects on surrounding host tissue, persist for long periods in neural grafts generated from human pluripotent stem cells. Thus the potential contribution of neuroprotection, as opposed to neuronal replacement, is important to recognize particularly in experimental paradigms where the grafting occurs acutely after injury. This point is well made in a study by Tornero and colleagues (2013), where cortical progenitors generated from human iPSCs were implanted into immune-compromised rats 48 h after stroke induced through medial cerebral artery occlusion. Tests of motor function showed that deficits caused by the ischemic injury were partially but significantly attenuated within 8 weeks of receiving human cell grafts. This timeframe for recovery is inconsistent with functional maturation and integration of human neurons and is most likely explained by a graft-mediated reduction in the progressive secondary neurodegeneration that is known to occur following stroke (Polentes et al., 2012).

The same interpretation likely applies to other grafting studies in stroke models where a similarly short time-coarse of recovery has been reported following transplantation of human cells in the early phase after injury (Daadi et al., 2008; Chen et al., 2010; Gomi et al., 2012; Oki et al., 2012; Mohamad et al., 2013). In the study by Chen et al. (2010), implantation of neural progenitors generated from human iPSCs was found to significantly reduce infarct volume in rats that received medial cerebral artery occlusion. Similarly, in models of spinal cord injury, rapid recovery of motor function following transplantation of human neural progenitors derived from fetal tissue (Cummings et al., 2005; Iwanami et al., 2005) or pluripotent stem cells (Keirstead et al., 2005; Sharp et al., 2010; Nori et al., 2011; Fujimoto et al., 2012) suggests mechanisms other than functional replacement of neurons. These studies variously present evidence for a range of other possible mechanisms, including sparing of host tissue, host axonal regrowth and angiogenesis, as well as re-myelination by grafted oligodendrocytes.

Concluding remarks

The use of ESC- or iPSC-derived neuronal progenitors for repair of damaged brain or spinal cord circuitry is an intriguing possibility, not

only experimentally, but in a longer perspective also in clinical therapy. Clearly, neural grafts generated from stem cells have the capacity to provide early therapeutic benefit through alternative mechanisms, such as neuroprotection or stimulation of host plasticity. However, this does not preclude that both mechanisms may operate in parallel and that additional benefit might be achieved through restoration of neuronal circuitry at later time-points. Studies to investigate this possibility need to be conducted over timeframes consistent with reports on the electrophysiological maturation and integration of grafted neurons, at least 6–9 months when using human cells (Koch et al., 2009; Espuny-Camacho et al., 2013; Avaliani et al., 2014). Notably, this timeframe is in line with clinical observations on the delayed onset for improvement of motor functions in PD patients following transplantation of human fetal tissue (Lindvall and Hagell, 2000) and the slow maturation of grafted human ESC-derived dopamine neurons observed in rat models of PD (Grealish et al., 2014). Grafting of cells in the chronic phase after injury, at a time when the secondary phase of neurodegeneration is largely complete, may also provide a useful approach for more directly assessing the ontribution of cell replacement to recovery of function. Additionally, the use of cell lines expressing reporter genes, such as GFP, and the application of new tools, such as optogenetics and trans-synaptic tracing using pseudo-rabies virus, will provide valuable means to better understand the relationship between properties of the grafted cells and their capacity for restoration of graft–host connectivity, and the functional impact in specific models of CNS damage and disease.

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