How to make striatal projection neurons

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Medium spiny neurons (MSNs) are the main projection neurons of the striatum and are preferentially lost in Huntington's disease (HD). With no current cure for this neurodegenerative disorder, the specificity of neuronal loss in the striatum makes cell transplantation therapy an attractive avenue for its treatment. Also, given that MSNs are particularly vulnerable in HD, it is necessary to understand why these neurons degenerate in order to develop new therapeutic options. Both approaches require access to human MSN progenitors and their mature neuronal derivatives. Human embryonic stem cells and HD patient induced pluripotent stem cells (together referred to as hPSCs) may serve as an unlimited source of such tissue if they can be directed toward authentic striatal neuronal lineage. Understanding the MSN differentiation pathway in the brain is therefore of paramount importance for the generation of accurate protocols to obtain striatal cells *in vitro*. The focus of this mini review will be on striatal development and current methods to generate MSNs from hPSCs.

Introduction

Medium spiny neurons (MSNs) comprise 90% of the neuronal population in the striatum and are the main output projection cells of this brain region. Understanding how and when MSNs are born during normal human development is of particular interest to the Huntington's disease (HD) research community as this cell population is the main target of neurodegeneration in HD.[1,2] Although tremendous effort has been made toward uncovering molecular mechanisms causing preferential MSN degeneration, modeling HD using patient-derived induced pluripotent stem cells (iPSCs) has been hampered by a low yield of these neurons in HD iPSC cultures.[3] Fate committed neuronal progenitors have been demonstrated to survive and integrate better in the host brain than non-dividing neurons following transplantation.[4] Thus, there is great demand for a source of MSN progenitor cells in order to develop cell transplantation therapies aimed at replenishing the lost neuronal population in patients' brains.

Human pluripotent stem cells (hPSCs) are emerging as an attractive donor cell source that can be standardized for clinical applications. MSNs derived from hPSCs present us with unlimited access to live human neurons. Their value extends beyond their use in the field of cell replacement therapy to the potential to study human development, as well as compare healthy and diseased neurons derived from patient iPSCs. This latter application provides a useful platform for high throughput screening of potential drugs before taking them into animal models. However, in order to exploit these possibilities, reliable and efficient differentiation paradigms are necessary to produce cell types of interest. This mini review will focus on current knowledge of striatal development and will provide an overview of existing protocols to generate MSNs from hPSCs.

Striatal Development

The striatum is developed from the lateral ganglionic eminence (LGE) in the ventral telencephalon, also known as the subpallium (**Fig. 1**). The LGE is the birthplace of MSNs and a small population of olfactory bulb interneurons.[5] The two other neurogenic domains of the subpallium, the medial and caudal ganglionic eminences (MGE and CGE, respectively), give rise to cortical and striatal interneurons and globus pallidus projection neurons.[6-8] The LGE is patterned under the influence of antagonistic morphogen gradients between sonic hedgehog (SHH) from the floorplate and the bone morphogenic proteins (BMPs) derived from the dorsal roofplate.[9-11] Working downstream and in concert with the dorsal-ventral patterning morphogens are domain-specifically expressed transcription factors (TFs), such as PAX6 and GLI3 dorsally in the developing cortex and NKX2.1 in the MGE and CGE. A coordinated action between these
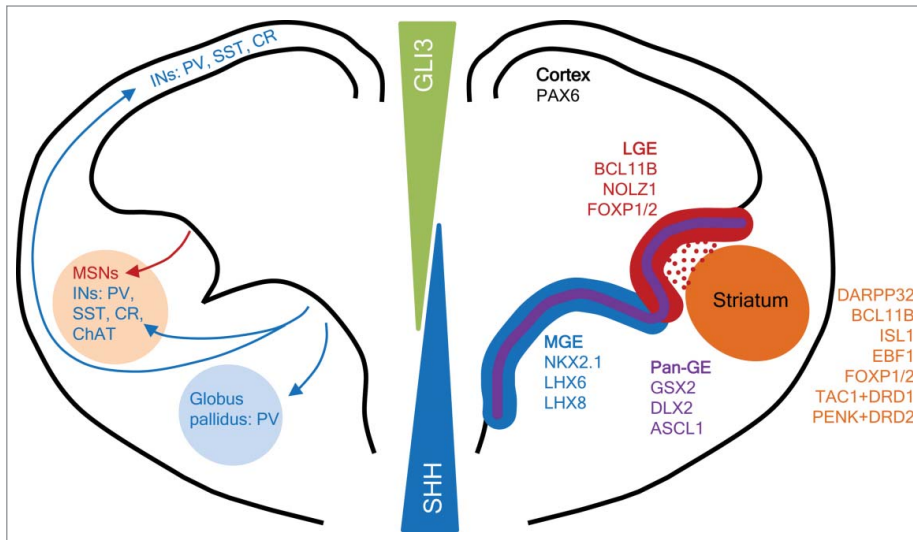
Figure 1. Schematic representation of a coronal hemisection of the developing telencephalon showing the cell migration (left) and gene expression (right) profile of morphologically defined progenitor domains of the cortex and medial (MGE) and lateral ganglionic eminences (LGE). Dorsal-ventral patterning of the telencephalon is achieved through gradients of morphogens SHH and GLI3. MSN progenitors develop in the LGE and populate the striatum, along with interneurons (INs) that migrate from the MGE. Cortical INs bypass the striatum and migrate from the MGE to the cortex. Parvalbumin$^+$ (PV$^+$) globus pallidus projection neurons also originate in the MGE. Abbreviations: GE: ganglionic eminence; SST: somatostatin; CR: calretinin; ChAT: choline acetyltransferase. Figure adapted from.[56]

extrinsic and intrinsic regulators is critical for the proper establishment of the LGE and striatum.

GSX2 is a crucial homeodomain protein in early subpallial development involved in the maintenance of molecular identity of LGE progenitors.[12] It is first detected in the forebrain from embryonic day 9 (E9) in mice and is expressed in the later developing ganglionic eminences, ventral thalamus and hypothalamus. In the absence of *Gsx2*, the mutant LGE is reduced in size and the subpallial markers DLX2 and ASCL1 (also named MASH1) are lost from all but the most ventral aspect of the LGE and replaced by the dorsal regulators PAX6 and NGN1/2 at E11.5.[13] Furthermore, expression levels of *Ebf1*, important in the transition of progenitors from the subventricular zone (SVZ) to the striatal mantle, and *Gad67*, a biosynthetic enzyme that catalyzes the formation of the neurotransmitter GABA, are also greatly reduced in the *Gsx2*$^{-/-}$ LGE at E12.5.[14] However, these molecular abnormalities are less prominent at later stages when aforementioned markers are expressed throughout the LGE up to the cortical-striatal boundary, suggesting that other genes are also at play.[13,14] When expressed ectopically in the dorsal telencephalon, *Gsx2* is able to up-regulate DLX1/2 and ASCL1 at the expense of cortical markers, further highlighting its participation in the specification of the LGE identity.[5]

The mis-specification of early LGE progenitors caused by the loss of *Gsx2* affects the development of subsets of early born striatal neurons of the patch compartment. At E18.5, *enkephalin (Enk)* transcript levels are significantly decreased and *dopamine receptor 2 (Drd2)* expression is missing in the nucleus accumbens and perirhinal aspect of the ventral telencephalon, regions that are believed to be populated by early born striatal cells.[14,15] This is further supported by a partial loss of cells positive for a mature MSN marker DARPP32 (the dopamine- and cAMP-regulated phosphoprotein) and fewer and smaller patches in the striatal SVZ at this age.[14,16, 17] Although all MSNs express this protein in the adult striatum (i.e. in both the patch and matrix compartments), it is largely restricted to earlier born patch neurons at prenatal stages.[18] The striatal defects in *Gsx2*$^{-/-}$ mutants are exacerbated by the loss of *Gsx1*, a gene closely related to *Gsx2*, resulting in no DARPP32$^+$ neurons in the patch compartment of the double mutant striatum.[16] This demonstrates a requirement for at least one of these genes for the differentiation of striatal patch MSNs. In contrast, later born striatal matrix neurons expressing calbindin and *Ebf1* appear to be unaffected in *Gsx2*$^{-/-}$ and *Gsx2/1*$^{-/-}$ mice, indicating that *Gsx*-independent mechanisms might be important at later stages of striatal neurogenesis.[14,16]

Once the LGE progenitor domain is established, these cells give rise to the striatal complex through the activity of several regulatory genes, including *DLX1/2*, *ASCL1* and *EBF1*. ASCL1 is a TF that is autonomously involved in neurogenesis and nonautonomously represses differentiation of adjacent progenitors through Notch signaling.[19] *Ascl1* mutants exhibit only moderate alterations in striatal development where *Dlx1* and *Gad67* are still expressed in the subpallium, while *Gsx2/Ascl1* double mutants show severely affected LGE development similar to that seen in *Gsx2/1*$^{-/-}$ mice.[20] After *Ascl1* activity in the early stages of neurogenesis, *Dlx1/2* repress its expression and Notch signaling, promoting later steps in LGE differentiation.[21,22] Single knock outs of either *Dlx1* or *2* do not show any evidence of forebrain defects, while *Dlx1/2*$^{-/-}$ mice have disrupted molecular identity of the SVZ in the LGE and reduced migration of later born striatal matrix neurons.[23] EBF1 controls later striatal differentiation and loss of *Ebf1* expression leads to defects in SVZ to mantle transition and increased progenitor death.[24] A dramatic atrophy of the postnatal striatum is observed, characterized by the reduction of the matrix compartment, whereas the patch compartment remains relatively preserved.

GSX2 and DLX2 function upstream of other TFs such as HELIOS and NOLZ1, which promote cell cycle exit and terminal striatal differentiation.[25,26] Although *Helios* is expressed downstream of *Gsx2* and *Dlx1/2*, it is not directly regulated by these 2 genes. It is preferentially expressed in the developing matrix compartment co-localizing with BCL11B (also known as CTIP2) and FOXP1, well established markers of MSNs, however *Helios's* exact role in striatal development remains unknown.[25]

Nolz1 overexpression in neural progenitors promotes cell cycle exit and the acquisition of a neuronal phenotype.[26] It relies on *Gsx2* for normal expression since it is lost in *Gsx2*^{-/-} mutants (thus, it may have a role in the development of MSNs of the patch compartment) and regulates retinoic acid (RA)-induced neurogenesis during LGE development. ASCL1, DLX1/2 and NOLZ1 in cooperation with RA signaling activate GAD67, initiating GABAergic fate specification in subpallial progenitors.[26,27] While loss of RA signaling in *Raldh3*^{-/-} embryos does not affect MAP2^{+} and DARPP32^{+} neurogenesis in the basal ganglia, it prevents striatal neurons from acquiring a GABAergic fate, suggesting that RA signaling is required to stimulate GABA synthesis in LGE-derived progenitors.[27,28]

After the active phase of neurogenesis (E14 in the rat and 50 d post fertilization in the human), juvenile post-mitotic neurons in the mantle zone of the LGE and striatal anlage cease to express GSX2 and DLX2 and are identified instead by the expression of BCL11B, FOXP1, FOXP2, ISL1 and EBF1 (**Fig. 1**).[25,29] Mature MSNs can be identified by co-expression of BCL11B and DARPP32, while MSNs in patch and matrix compartments of the striatum can be distinguished by their expression of FOXP2 and FOXP1, respectively.[30,31] Co-localization of these proteins is necessary to reliably identify cells as MSNs, as each of them is individually expressed in cortical neurons.[29] Two circuits of the basal ganglia originate from distinct populations of MSNs expressing D1 dopamine receptors (DRD1) and substance P (direct) and DRD2 and ENK (indirect).[32] Despite numerous studies of subpallial development described above our understanding of the induction of MSN fate in the developing LGE is still limited.

Derivation of MSNs from ESCs

Given the right cues embryonic stem cells (ESCs) have the capacity to differentiate into any cell types in the body. After ESCs exit the pluripotent state and become neuralized, different patterning factors are generally applied to direct newly derived naïve neuroectoderm cells toward a regionally-specific progenitor fate, which subsequently differentiate into a defined neuronal cell type. The first group to report a method for MSN differentiation *in vitro* used mouse stromal cell co-culture for neural induction of hESCs.[33] Neural precursor cells were treated with BDNF, SHH and DKK1 to induce anterior-ventral identity, then neural rosettes were mechanically isolated and expanded in the same differentiation medium before maturation in the absence of SHH and DKK1. This method proved to be fairly inefficient at inducing a neuronal fate in hESCs (22% MAP2^{+} cells) but produced a >50% yield of DARPP32^{+} cells within the neuronal population after 60 d in culture. This suggested that SHH and DKK1 were somewhat effective in patterning hESCs-derivatives toward an MSN fate. Although differentiated progenitors gave rise to DARPP32^{+} neurons *in vivo* when transplanted into a quinolinic acid-lesioned rat striatum, there was overgrowth of neural progenitors 3–5 months after the graft. The ventralizing effect of SHH was further investigated by Ma et al.,[34] who used an embryoid body method to generate neuroepithelial cells that were manually isolated and treated with a dose of SHH that effectively down-regulated PAX6 and up-regulated ASCL1, MEIS2 and *GSX2*, while keeping NKX2.1 expression to a minimum of 30%. This approach was far more efficient at producing neurons (93% βIII-tubulin1^{+} cells) and generation of MSNs with 80% of neurons co-labeling with DARPP32 and GABA. Four months after transplantation, no graft overgrowth was observed and DARPP32^{+} and GABA^{+} neurons represented more than half of grafted cells. Transplanted animals showed functional recovery on rotarod test and improvement in parameters of fine gait movements. Although this differentiation paradigm appears highly efficient in generating MSNs, the manual isolation of neural colonies makes this method unsuitable for large scale and automated MSN production.

Both Carri et al.[35] and Nicoleau et al.[36] took advantage of the highly efficient neural conversion of hESCs based on the dual inhibition of Smad signaling discovered by Chambers et al.,[37] and confirmed the efficacy of the monolayer differentiation method (51% and 58% MAP2^{+} cells, respectively). Ventral telencephalic specification was also induced by SHH treatment, resulting in BCL11B^{+} and GABA^{+} neurons and an overall 20% DARPP32^{+} neurons *in vitro*.[35] Transplanted MSN progenitors survived *in vivo* but showed a tendency to overgrow and no behavioral recovery. Nicoleau et al.[36] optimized Wnt signaling inhibition by DKK1 or a chemical Wnt antagonist, XAV-939, and demonstrated an enhancement of FOXG1^{+} forebrain cell production in a dose-dependent manner. They also showed that a lower concentration of SHH was more efficient at inducing the LGE fate, while a higher concentration directed cells toward the MGE phenotype with 80% NKX2.1^{+} cells. Together, XAV-939 and SHH-treated cultures generated 28% DARPP32^{+} neurons with evidence of the 2 MSN subpopulations expressing DRD1 or DRD2 receptors *in vitro*. In addition, transplanted LGE progenitors matured into DARPP32^{+} neurons co-expressing BCL11B and FOXP1 5 months post-grafting, although behavior was not assessed on these animals.

In contrast to the above conceptually similar MSN differentiation methods, we developed a new protocol for generating MSNs from hPSC by using Transforming Growth Factor β (TGFβ) family protein Activin A (henceforth activin).[38] The TGFβ superfamily members are secreted molecules that include TGFβs, Nodal, Activins, growth differentiation factors (GDFs) and BMPs.[39] They regulate a variety of developmental processes through specific Activin-like kinase receptors (ALKs), which induce the phosphorylation and stimulation of regulatory Smads. TGFβs, Nodal and Activins act through ALKs 4/5 to recruit Smads 2/3, while GDFs and BMPs activate Smads 1/5/8 through ALKs 2/3/6. Activated Smad complexes translocate to the nucleus where they regulate gene expression through recruitment of co-Smad4 and other homeodomain proteins, including co-activators and co-repressors. Previous studies demonstrated the presence of Activin subunits, receptors and phosphorylated Smad2 (pSmad2) in the developing subpallium and co-localization of pSmad2 with DLX2 throughout.[40,41] The two proteins were also confirmed to interact *in vivo*, and pSmad2 was found

to bind to the *DLX2* gene enhancer.[40] In line with these findings, we treated hPSC-derived forebrain neural progenitors with activin to induce an LGE-derived MSN fate.[38] We showed that activin induced markers specific to the LGE, including *BCL11B*, *NOLZ1* and *FOXP2* at progenitor stage. While it also upregulated pan-GE markers *GSX2* and *DLX2*, it had no effect on the MGE markers *NKX2.1* and *LHX8*. We demonstrated that induction of these LGE transcripts by activin can be completely blocked by a specific activin/Smad inhibitor, SB431542, suggesting that the specification of striatal characteristics in this system requires activation of the Smad2/3 pathway.

Furthermore, activin stimulated a large increase in *BCL11B* expression after just 12 hours of treatment, hinting that it may have a direct regulatory effect on its expression. Increase in the levels of LGE-specific TFs upon addition of activin remained higher for *BCL11B* than others, suggesting that it might be central to MSN fate specification. *In vitro*, activin-patterned neural precursors efficiently generated mature MSNs immunoreactive for BCL11B, GABA, GAD65/67 and PSD95 with 20–50% neurons co-expressing DARPP32 depending on the hPSC line. Upon transplantation in a rat model of HD, progenitors survived and differentiated into DARPP32^{+} neurons of both the direct and indirect subtypes of MSNs. Despite indications of glutamatergic and dopaminergic innervation from the host brain, the recipient animals did not show a behavioral improvement. It is worth noting that the behavioral recovery observed with SHH-treated cell-derived grafts might be attributed, at least in part, to the presence of interneurons generated by SHH.[34,35] Also, LGE grafts derived with the activin protocol were generally lower in mass than those reported in SHH-based studies, which might affect the behavioral analysis. In fact, we demonstrated that activin acts as a potent pro-differentiation factor by promoting cell cycle exit via SHH signaling inhibition and by enhancing RA signaling.[42] This 'pro-differentiation' effect of activin treatment might explain why we do not see overgrowth of neural progenitors after transplantation.

All of the studies using SHH-mediated generation of MSNs have faced the challenging trade-off between SHH and DKK1/XAV-939 dosage and the increase in levels of NKX2.1. While the treatment with patterning factors lead to up-regulated expression of pan-GE markers FOXG1, OTX2 and GSX2, LGE-specific TF BCL11B was unaffected and other LGE specific TFs not analyzed in MSN progenitors. It is possible that the observed results in MSN differentiation of hESCs are due to a generic ventral patterning of neural ectoderm by SHH rather than a specific promotion of striatal progenitor phenotype. This notion is supported by mouse genetics studies, which demonstrated that the primary role of SHH is to repress GLI3 function and vice versa to achieve normal dorsal-ventral patterning in the telencephalon.[9] In the absence of either of them, the cortical-striatal border is shifted either ventrally (*Shh* mutants) or dorsally (*Gli3* mutants) while expression of pan-ventral genes *Gsx2* and *Dlx2* remains persistent in homozygous mutants. We showed that cyclopamine-induced inhibition of SHH signaling in activin-treated cultures does not affect up-regulation of the LGE-enriched genes.[38] This is in line with our previous findings that

activin is initiating differentiation in forebrain progenitors via GLI3-mediated suppression of SHH pathway.[42] Therefore, it seems that activin is able to induce the LGE characteristics in telencephalic neural precursors while simultaneously inhibiting the MGE fate.

Despite the robust effect of activin in LGE fate induction, very little is known about the TGFβ/Activin signaling pathway in subpallial development. Members of the TGFβ superfamily have been implicated in a variety of neurodevelopmental processes, including axon specification and synapse formation, as well as adult neurogenesis, and have been attributed broad neuroprotective functions in the adult brain.[43-48] Moreover, TGFβ ligands, receptors and Smad signaling molecules show strong expression in the adult murine striatum, particularly in neurons of the striatal matrix compartment, and TGFβ signaling was found to be reduced in HD striatal tissues.[49,50] Thus, this signaling pathway might play a role not only in LGE development but also in adult striatal homeostasis and its disruption might contribute to MSN vulnerability in HD.

Direct Conversion of Somatic Cells into MSNs

A non-hPSC route to generate neurons, direct reprogramming, or induced neuron (iN) technology, has been rapidly developing over recent years. The iN method converts post mitotic somatic cells (often fibroblasts) directly into functional neurons by combinatorial expression of lineage specific TFs.[51] In a recent study, Victor et al.[52] implemented this method to generate human MSNs via ectopic expression of brain-enriched microRNAs, miR-9/9* and miR-124, with striatal TFs. Interestingly, out of 16 TFs tested, only BCL11B induced DARPP32 expression in the neurons produced using the microRNAs. This further highlights the central role of BCL11B in MSN fate specification. DLX1 and DLX2 were added to promote GABAergic fate, while MYT1L increased the number of MAP2^{+} cells. Transplanted fibroblasts reprogrammed with the 2 microRNAs and all 4 of these factors survived in the murine striatum for 6 months and matured into MSNs expressing DARPP32, BCL11B, GABA and FOXP1.

The iN technology serves as a valuable model to study gene regulation in cell fate decisions but it has disadvantages over the current hPSC differentiation methods with regard to biomedical applications. Firstly, the iN method converts post mitotic somatic cells directly into neurons, hence it does not offer the opportunity for harvesting fate committed neuronal progenitors. These have been demonstrated to survive and integrate better in the host brain than non-dividing neurons following transplantation, and thus are better suited for applications in cell therapy.[4] Secondly, iNs bypass developmental stages that precede final maturation and are therefore unsuitable for applications focusing on neuronal development. Finally, iN MSNs are derived from primary fibroblasts established from skin biopsies, which are generally limited in scale and proliferation capacity before undergoing senescence, unlike hPSCs that can be cultured indefinitely *in vitro*. There is an upper limit to how many iNs could be

generated from a given tissue preparation. Thus, for applications in regenerative medicine and drug screening, which are currently the most sought-after applications of *in vitro* derived human neurons, the present iN approach cannot replace hPSC differentiation.

Ongoing Challenges

Stem cells hold great promise to become the source of striatal progenitors for clinical applications and a platform for *in vitro* disease modeling and high throughput drug screening. However, current MSN generation protocols are 40-50% efficient at best in generating striatal neurons *in vitro*, suggesting that there is still room to improve methods for MSN differentiation. Although mouse genetics studies have identified several TFs as important for MSN development, absence of these TFs does not prevent formation of the striatum. Thus, one of the main ongoing challenges remains to uncover the exact mechanism by which striatal neurons acquire their fate *in vivo*. It is possible that MSNs generated in SHH-based protocols might be a 'by-product' of general ventralization of forebrain progenitors induced by SHH. On the other hand, induction of the LGE fate by activin might be a more targeted event of striatal lineage specification. Further investigation is required to dissect the different components of this pathway that could be driving LGE characteristics in hPSC-derived neural progenitors. We found that BCL11B is the first striatal marker to be robustly upregulated within just 12 hours of activin treatment and it continues to increase with prolonged exposure to activin.[38] This suggests that activin might have a direct regulatory effect on the expression of this TF. There is an increasing amount of evidence that supports BCL11B as a central factor for MSN fate specification and maturation. Loss of BCL11B in mice leads to dramatically reduced striatal DARPP32 expression and aberrant cellular organization into patches.[53] Moreover, it has been implicated in BDNF (brain derived neurotrophic factor) signaling, which is also important for DARPP32 expression in mouse MSNs.[54,55] Consensus binding sites for this factor include regions upstream of striatum-enriched genes such as DARPP32, FOXP1 and ARPP19. We consider BCL11B the likely mediator down-stream of Activin-Smad signaling in MSN fate specification and it would be interesting to demonstrate direct interaction and binding of this TF to striatal genes.

Conclusion

In conclusion, *in vitro* derivation of mature MSNs from hPSCs and iNs holds great promise for disease modeling and drug discovery. The development of cell therapies to slow down the progression of HD will benefit greatly from efficient generation of MSN progenitors, which survive better than their mature derivatives post-grafting. Moreover, the value of hPSCs also extends to the potential to study human MSN development *in vitro*. However, there is still room to improve MSN differentiation methods and our understanding of the induction of the LGE fate *in vivo*.

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