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Programming embryonic stem cells to neuronal subtypes

Mirza Peljto and Hynek Wichterle

Dept. of Pathology and Cell Biology, Neurology, and Neuroscience, Center for Motor Neuron Biology and Disease, Columbia University Medical Center, New York, NY 10032, USA

Abstract

Richness of neural circuits and specificity of neuronal connectivity depends on the diversification of nerve cells into functionally and molecularly distinct subtypes. While efficient methods for directed differentiation of embryonic stem cells (ESCs) into multiple principal neuronal classes have been established, only a few studies systematically examined the subtype diversity of in vitro derived nerve cells. Here we review evidence based on molecular and in vivo transplantation studies that ESC-derived spinal motor neurons and cortical layer V pyramidal neurons acquire subtype specific functional properties. We discuss similarities and differences in the role of cell intrinsic transcriptional programs, extrinsic signals and cell-cell interactions during subtype diversification of the two classes of nerve cells. We conclude that the high degree of fidelity with which differentiating ESCs recapitulate normal embryonic development provides a unique opportunity to explore developmental processes underlying specification of mammalian neuronal diversity in a simplified and experimentally accessible system.

Keywords

neuronal subtypes; embryonic stem cells; neocortex; spinal cord; neural patterning; transplantation; motor pool; motor area; cell migration; axon guidance

Introduction

One of the tantalizing features of the central nervous system (CNS) is the precision with which nerve cells establish stereotypical neural circuits. Ongoing characterization of the developing CNS is revealing an unprecedented degree of neuronal diversity that corresponds with and likely underlies the richness and specificity of neuronal connectivity and function. Subtype diversification of spinal motor neurons, retinal amacrine cells, cortical layer V pyramidal neurons or olfactory sensory neurons underlies functionally relevant differences in axonal projections, dendritic arborization, and molecular and electrophysiological properties of these neurons [1-5]. How such neuronal diversity is established during mammalian development remains poorly understood in part due to technical challenges inherent to the analysis of complex and heterogeneous CNS tissue under poorly accessible experimental conditions. Recapitulation of normal neural development with pluripotent embryonic stem cells (ESCs) in vitro might therefore become a powerful and indispensable tool in the field of developmental neurobiology. It provides an unparalleled experimental

Correspondence: H. Wichterle, hw350@columbia.edu.

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access to the developing mammalian CNS previously enjoyed only by researchers studying oviparous vertebrates and lower organisms.

Major advances have been reported in directed differentiation of pluripotent cells into distinct classes of nerve cells (reviewed in [6-9]). These studies firmly established that differentiating pluripotent cells respond correctly to developmental signals that pattern the developing CNS along the rostro-caudal or dorso-ventral axes and give rise to nerve cells expressing congruent region specific markers and neurotransmitter phenotypes. In contrast, less attention has been paid to whether in vitro generated neurons also acquire refined subtype specific properties. This is largely due to the lack of understanding of mechanisms underlying subtype diversification of mammalian nerve cells in vivo. Modeling the acquisition of subtype specific nerve cell identities in a simplified in vitro system might therefore provide a powerful tool to dissect the roles of cell intrinsic genetic programs, extrinsic diffusible signals, and cell-cell interactions in the fine-grained diversification of developing nerve cells.

Motor neuron subtype diversity

Subtype diversification of neurons is particularly apparent in the motor system. Coherent transmission of motor signals relies on the existence of hundreds of spinal motor neuron subtypes, each communicating with a different muscle group in the periphery (Figure 1A-C). Developmentally encoded motor neuron subtype diversity prescribes not only the connectivity between motor neurons and their muscle targets, but also between motor neuron subtypes and efferent connections from cognate proprioceptive sensory neurons and from descending motor pathways transmitting commands originating from the motor cortex [4,10]. The presence of more than five hundred muscle groups in the mammalian body implies the existence of similar diversity of motor neuron subtypes.

Specification of somatic motor neuron subtypes can be deconstructed into several developmental steps (Figure 1A-C, Figure 2). First, a subset of spinal motor neurons is instructed by non-canonical Wnt signals to acquire the identity of axial muscle innervating median motor column (MMC) neurons [11,12]. Second, spinal motor neurons are diversified along the rostro-caudal axis by retinoic acid (RA), Wnt and Fgf signals that induce differential patterns of Hox gene expression along the spinal cord [13,14]. Non-MMC neurons at limb level spinal cord express Hox6 and Hox10 genes, respectively, and acquire the identity of limb innervating lateral motor column (LMC) neurons. Non-MMC neurons in the thoracic and cervical spinal cord acquire the identity of hypaxial motor column (HMC) neurons that innervate body wall muscles [4]. While subtype diversity of MMC and HMC neurons has not been systematically examined, diversification of LMC neurons into distinct subtypes is well-documented. In response to a local RA source, LMC neurons are subdivided into medial and lateral LMC divisions that innervate ventral and dorsal limb muscles, respectively [15,16]. Finally, LMC neurons within each division are organized into motor pools, groups of neurons dedicated to innervating a single muscle group in the limb [17]. Recent studies indicate that the establishment of motor pool diversity relies on the Hox-based transcriptional network that operates intrinsically in nascent motor neurons [4].

Specification of subtype identity in ESC-derived motor neurons

Detailed understanding of spinal motor neuron subtype diversity in vivo provides a platform to examine whether similar diversification of motor neurons can be achieved in vitro from differentiating ESCs. Initial methods for differentiation of ESCs to motor neurons relied on two principal patterning signals –RA that induces neuralization and caudalization of ESCs and sonic hedgehog (Hh) that directs ventralization of the spinal neural progenitor cells [18]. ESC-derived motor neurons express bona fide motor neuron markers such as Hb9, Isl1/2,

and Lhx3 transcription factors, biosynthetic enzyme choline acetyltransferase (ChAT), and vesicular acetylcholine transporter (VACHT) indicative of their cholinergic identity [18]. In addition, ESC-derived motor neurons develop electrophysiological properties typical of spinal motor neurons and form synaptic terminals that elicit clustering of acetylcholine receptors when co-cultured with myotubes [19,20].

Motor neurons derived under RA/Hh conditions acquire the identity of cervical level spinal motor neurons characterized by the expression of Hoxc4 and Hoxa5 genes [18,21,22] (Figure 1A). Motor neurons within the cervical spinal cord acquire MMC and HMC identities that can be distinguished by the persistent expression of Lhx3 in MMC neurons while this transient motor neuron marker is rapidly downregulated in HMC cells [12,23]. The majority of ESC-derived motor neurons expresses Lhx3 [18,24] indicating that in vitro derived motor neurons are of uniform MMC-like subtype identity. Interestingly, when transplanted into the developing chick neural tube, ESC-derived motor neurons innervated axial, hypaxial as well as limb muscles [18]. This result has been interpreted as a failure of MMC motor axons to navigate to their proper axial muscle target via a nerve branch overloaded by supernumerary transplanted axons [24,25]. Consistent with this view, transplantation of a limited number of motor neurons resulted in a more uniform projection of axons along the axial nerve branch [24]. As in vivo, the choice of ESC-derived motor neuron axons to innervate the axial nerve branch appears to depend on increased level of FGFR1 expression by MMC neurons [26,27].

Despite the uniform expression of Lhx3 in nascent ESC-derived motor neurons, we recently observed that a significant proportion of motor neurons downregulates Lhx3 expression and acquires expression profile characteristic of HMC neurons when cultured following dissociation (S. Nedelec and H. Wichterle, unpublished observation). Consistent with this observation, retrograde tracing of limb or hypaxially projecting engrafted ESC-derived motor neurons confirmed their HMC-like identity and lack of Lhx3 expression [21]. Therefore, we favor the conclusion that, similar to cervical spinal cord motor neurons, ESC-derived neurons acquire both MMC and HMC identity, but HMC neurons are initially masked by an extended duration of Lhx3 expression.

Examination of RA/Hh differentiated motor neurons failed to reveal whether in vitro generated cells are capable of acquiring highly specialized subtype identities that would enable them to engage in functional motor circuits. The general lack of molecular markers that would differentiate between cervical motor neurons innervating different muscle groups precludes this level of analysis. To overcome this limitation, retinoid independent differentiation conditions were recently developed that give rise to more caudally positioned motor neurons of brachial and thoracic identities [21]. In contrast to RA dependent generation of Hox4/5 expressing cervical motor neurons (likely by direct regulation of these Hox genes by RA), differentiation of caudal motor neurons relies on the patterning events driven solely by endogenous signaling centers formed in differentiating embryoid bodies. Embryoid bodies cultured in the absence of extrinsic factors express caudalizing signals of the Fgf and Wnt families as well as the ventralizing signal Hh providing a milieu conducive for the induction of Hox6, Hox8 and Hox9 genes and for the specification of brachio-thoracic motor neurons (Figure 1A, 2A).

Consistent with their Hox expression profile, a subset of ESC-derived motor neurons acquires LMC identity of limb innervating motor neurons marked by high-level expression of transcription factor FoxP1 [21,23]. Moreover, ESC-derived FoxP1 expressing motor neurons migrate into the lateral spinal territory occupied by the endogenous LMC neurons and project axons correctly into the limb when transplanted into the developing spinal cord. Finally, ESC-derived LMC neurons acquire subtype identity of Pea3 expressing cutaneous

maximus and Scip expressing flexor carpi ulnaris motor pools (Figure 1C) and, as in vivo, expression of Pea3 depends on a permissive GDNF signal [21,28]. Together these studies provide compelling evidence that ESCs can be differentiated into nerve cells that acquire not only generic but also some of the most refined subtype specific properties characteristic of neurons in vivo.

Differentiation of ESCs in vitro does not result in a single preferred or “default” neuronal subtype, but in a mixture of subtypes, not unlike the intrasegmental diversity of motor neuron subtypes observed in vivo. Such diversity could result from an intrinsic genetic program similar to the one controlling specification of diverse neural cells from a common progenitor in the developing *Drosophila* [29,30], or it could be an outcome of a diversification of a uniform population of “naïve” motor neurons in response to cell-cell interactions and paracrine signals. ESC differentiation in vitro provides a convenient system in which normal neighbor-neighbor relationships and extrinsic cues can be easily disrupted. Dissociation and mixing experiments demonstrate that specification of motor pools does not rely on extrinsic cues [21] and therefore is likely driven by an intrinsic genetic program [31]. In contrast, divisional identity of mouse ESC-derived motor neurons can be influenced by the extrinsic RA signal [21] analogous to the specification of divisional identity in the developing chick spinal cord [15].

Considering that motor pool diversification is controlled by a cell-intrinsic program, it is unlikely that differentiation protocols relying solely on extrinsic signals will ever produce a homogenous neuronal population. It remains to be determined whether combination of extrinsic programming with intrinsic modification of the transcriptional network might overcome this limitation and yield more uniform population of motor neurons. Such findings will have a positive impact on the utilization of ESC-derived neurons for biochemical and clinical applications in which defined and uniform populations of neurons are desirable. In future, it will be critical to determine whether human ESCs (hESCs) follow similar developmental principles and whether they can be coerced to differentiate into diverse and well-defined motor neuron subtypes. In contrast to mouse cells, many hESC-derived motor neurons express caudal brachial marker Hoxc8 under the RA/Hh differentiation protocol raising the possibility that some of them might be of LMC character [32,33].

Neocortical neuronal subtype diversity

While a strict developmental control of neuronal subtype diversity is a sensible way to ensure reproducible and reliable transmission of signals between the CNS and periphery, central connectivity might benefit from a greater degree of plasticity. Accordingly, specification of neuronal subtype diversity in the developing neocortex does not rely only on developmental programs and local paracrine signals, but also on patterns of innervation by efferent axons [34].

Neocortex develops in the rostral and dorsal aspect of the neural tube. Principal classes of neocortical neurons are generated in a stereotypic temporal sequence and settle according to their birth-date and molecular identity within distinct cortical layers [35]. The first born neurons are Cajal-Retzius cells occupying the marginal zone, corticothalamic neurons that settle in layer VI are generated next, followed by layer V pyramidal neurons, layer IV neurons, and Layer II/III pyramidal neurons. Neurons within each layer are further diversified. Based on their projections, layer V pyramidal neurons are subdivided into layer Va callosally projecting neurons (expressing Lmo4) and layer Vb corticofugal neurons (expressing Scip, Ctip2, Fez1) [1,36] (Figure 1E). Ongoing molecular studies indicate a further degree of intralaminar diversity among layer V neurons that yet remains to be correlated with defined functional attributes [37].

Furthermore, layer V pyramidal neurons are diversified into distinct cortical area subtypes according to rostro-caudal and medio-lateral position within the developing neocortex (Figure 1D). For example, corticofugal layer V pyramidal neurons in the visual (occipital) cortex express Coup-TF1 transcription factor and project axons to pontine nuclei and optic tectum (superior colliculus), while layer V pyramidal neurons in the motor cortex express Diap3, Igfbp4, and Crim1 and project axons to pontine nuclei, red nucleus, and the spinal cord [1]. The diversity of pyramidal neurons likely extends further, as distinct somatotopic areas of motor cortex contain corticospinal motor neurons targeting different spinal cord levels (Figure 1E), motor columns, and motor pools [38-43].

Programs controlling diversification of layer V neurons are not well understood (Figure 2B). Arealization of the developing neocortex is initiated by locally secreted patterning signals, including rostrally secreted Fgfs [44]. However, unlike in the developing spinal cord, genetic programs initiated by these patterning gradients are not the ultimate determinants of cortical neuron subtype identity. Postmitotic cortical neurons retain a great degree of plasticity and their ultimate specification critically depends on thalamocortical and other efferent inputs, as revealed by lesion and transplantation studies (reviewed in [45,46]). For example, grafts of late embryonic occipital cortex to postnatal somatosensory area acquire the architecture and molecular characteristics of barrel cortex and grafts into motor area result in the retention of corticospinal axonal projections that are normally eliminated in visual layer V neurons (reviewed in [34]). How intrinsic genetic programs are integrated with extrinsic functional inputs to generate the extraordinary level of subtype diversity of cortical neurons remains poorly understood.

Specification of principal classes of neocortical neurons from ESCs

Neural tissue induced in the absence of caudalizing signals in the developing embryo acquires rostral forebrain identity [47,48]. The same principle applies to the patterning of ESC-derived neurons - in the absence of exogenous factors, differentiating ESCs preferentially acquire rostral neural identity [49-57]. Neuralization of ESCs under these conditions critically depend on endogenously expressed Fgf5 signal [58] and can be improved by blocking residual BMP, Nodal, and Wnt signaling pathways [51,59] or by controlling the initial size of ESC aggregates [56].

Retinoid free differentiation protocols most closely conform to the principles of neural induction derived from studies of developing vertebrate embryos. Accordingly, ESCs differentiated as free floating embryoid bodies [51], adherent monolayers [50,60] or cocultures with stromal cells [61] under serum free conditions generate primarily forebrain cells expressing marker FoxG1 (also known as BF1). ESC-derived forebrain progenitors are patternable along the dorso-ventral axis by application of Wnt3a, Shh antagonist cyclopamine, or Shh (Figure 2A) and give rise to neocortical progenitors expressing Pax6 and Emx1 or basal forebrain progenitors expressing Nkx2.1, respectively [51,55,57,60,62]. The patterned progenitors differentiate into classes of forebrain neurons that display characteristics of neurons found in the rostral, caudal and dorsal regions of the cortex [51,56,57,60], ventral basal ganglia [51,57,62], hypothalamus [55], olfactory bulb [56], and optic vesicle [63,64].

Neocortical progenitors derived in vitro from ESCs not only express correct combination of molecular markers, but are also capable of arrangement into three-dimensional neural rosettes, whose cytoarchitecture is analogous to the developing neuroepithelium [56]. When differentiated into neurons, ESC-derived cortical progenitors generate all principal classes of cortical neurons found in vivo and their birth-date sequence conforms to the temporally progressive specification of cortical plate neurons observed in vivo [56,60,65]. ESC-derived

cortical progenitors differentiate first into Reelin and calretinin positive layer I Cajal Retzius neurons; followed by Tbr1 positive layer VI neurons; Ctip2 and Emx1 expressing layer V neurons; and finally to layers II and III neurons expressing Brn2 and Satb2 [56,60]. Although neurons of all cortical layers are generated in vitro, the proportion of early born deep layer neurons is significantly greater compared to the later born more superficial neurons. Considering how similar is the program of ESC differentiation to cortical development, it might help to define intrinsic genetic programs activated in maturing progenitors and to identify paracrine signals contributing to the temporal patterning of the developing neocortex [35,66].

Regional subtype diversity of in vitro derived cortical pyramidal neurons

Efficient derivation of deep layer cortical nerve cells, many of which exhibit properties of corticofugal layer V pyramidal neurons, raised a question whether in vitro generated pyramidal neurons are largely of one particular subtype identity, a mixture of different identities, or whether they retain plasticity and will acquire their final identities only in response to local cues and efferent innervation as has been demonstrated for primary embryonic cortical tissue.

To examine this question, Gaspard and colleagues implanted ESC -derived neurons into frontal (~ motor) cortex area in neonatal mice. One month later, engrafted ESC-derived neurons were observed to innervate targets that are typically innervated by visual and limbic cortices (visual and limbic thalamic nuclei and superior colliculus), whereas no innervation was detected in the pyramidal tract innervated by motor cortex [60]. These axonal pathfinding preferences indicate that the majority of in vitro generated corticofugal neurons acquire the identity of occipital and limbic cortical neurons and that their identity was not respecified in response to local cues and efferent inputs upon transplantation into the motor cortex. In light of the regional plasticity of late embryonic visual cortex grafted into the somatosensory area [67] this is a surprising result. Unfortunately, control grafts using primary cortical tissue were not performed by Gaspard et al. and therefore it remains possible that subtle technical differences might underlie the different outcome of the two studies. Alternatively, in vitro derived cortical neurons may fail to acquire the ability to respond properly to efferent inputs due to a defect in their programming. Or in vitro conditions provide signals that initiate the specification of visual and limbic cortical identities and terminate the critical period during which cortical identity can be respecified in heterotopic grafts. Resolving between these possibilities is of great interest as it may provide mechanistically important insights into the process of cortical neuron subtype specification.

Consistent with their predominantly visual cortical identity, in vitro generated cells express Coup-TF1, a marker of the occipital cortex [56,60]. Interestingly, this regional molecular identity can be altered by modulating FGF signaling, implicated in the anterior patterning of the developing neocortex [44]. Treatment of ES cells differentiating as embryoid bodies with Fgf8 results in nearly complete suppression of Coup-TF1 expression while blocking of endogenous Fgf signaling increases the number of Coup-TF1 positive cells [56]. Although these results demonstrate that pre-patterning of the neocortex into different regions can be recapitulated in vitro, neurons generated under the different conditions were not subjected to homotopic or heterotopic transplantations. Thus, it remains to be seen whether neurons derived under these conditions would innervate targets corresponding to their pre-patterned identity or whether they would exhibit subtype plasticity and innervate targets according to the site of transplantation.

Most recently, ES cells were efficiently converted into cortical neurons by co-culture with MS5 stromal cell line [61]. Majority of neurons generated under this condition express Ctip2 and Otx1, markers of deep layer V and VI corticofugal neurons. Interestingly, transplantation of these pyramidal neurons into motor, somatosensory and visual cortex resulted in striking differences in axonal pathfinding preference. Grafts in visual cortex did not extend axons to the pyramidal tract or spinal cord while a substantial number of axons from ES cell derived neurons grafted into the motor cortex were detected in both of these target areas of endogenous cortical motor neurons [61]. These observations are consistent with the idea that regional subtype identity of cortical pyramidal neurons is at least partially plastic and can be influenced by the site of transplantation. It remains to be determined whether differences in the culture conditions (stromal co-cultures vs. monolayer differentiation in the presence of cyclopamine) might underlie differential plasticity of in vitro derived cortical cells. In this regard it is of interest to note that developmental plasticity of neural progenitors can be maintained by activation of Hh signaling pathway [49], raising the possibility that inhibition of endogenous Hh signaling by cyclopamine in Gaspard's study might cause a loss of a residual pool of patternable progenitors.

The areal diversity of layer V pyramidal neurons and of their axonal projections can be compared to the diversity and axonal selectivity of motor neuron columnar subtypes. Analogously, the next important step will be to determine how pyramidal neurons acquire distinct subregional identities, akin to spinal motor pool subtypes, that correlate with refined patterns of cortical neuronal connectivity and that underlie the formation of precise topographic maps evident in cortical areas.

Conclusions and Future Directions

The qualitative leap forward in our ability to differentiate ESCs under conditions that recapitulate normal embryogenesis provides an opportunity to study mammalian neural development in an accessible and convenient system capable of producing virtually unlimited supply of neural cells. This review focused on spinal motor neurons and cortical pyramidal neurons, two classes of nerve cells that were recently demonstrated to acquire refined subtype specific identities in vitro. However, advances in programming numerous other classes of nerve cells exhibiting aspects of subtype diversity, including retinal cells [63,64,68], forebrain interneurons [51,57,62]; hypothalamic neurons [55], midbrain dopaminergic neurons [69-71], inner ear hair cells [72,73] and cerebellar neurons [74-77], indicate that this area of research is rapidly expanding. Characterization and experimental manipulation of class and subtype diversity of nerve cells generated under these protocols will advance our understanding of neuronal differentiation and will help to define molecular mechanisms controlling the complex and precise neuronal connectivity established during CNS development.

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References and recommended reading

Papers of interest are highlighted:

- special interest

•• outstanding interest

1. Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD. Neuronal subtype specification in the cerebral cortex. *Nat Rev Neurosci* 2007;8:427–437. [PubMed: 17514196]
2. MacNeil MA, Masland RH. Extreme diversity among amacrine cells: implications for function. *Neuron* 1998;20:971–982. [PubMed: 9620701]
3. Rockhill RL, Daly FJ, MacNeil MA, Brown SP, Masland RH. The diversity of ganglion cells in a mammalian retina. *J Neurosci* 2002;22:3831–3843. [PubMed: 11978858]
4. Dasen JS, Jessell TM. Chapter Six Hox Networks and the Origins of Motor Neuron Diversity. In: Academic Press, editor. *Current Topics in Developmental Biology*. Vol. 88. 2009. p. 169–200.
5. Buck L, Axel R. A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* 1991;65:175–187. [PubMed: 1840504]
6. Gaspard N, Vanderhaeghen P. Mechanisms of neural specification from embryonic stem cells. *Curr Opin Neurobiol* 2010;20:37–43. [PubMed: 20080043]
7. Zhang SC. Neural subtype specification from embryonic stem cells. *Brain Pathol* 2006;16:132–142. [PubMed: 16768754]
8. Elkabetz Y, Studer L. Human ESC-derived neural rosettes and neural stem cell progression. *Cold Spring Harb Symp Quant Biol* 2008;73:377–387. [PubMed: 19204067]
9. Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells. *J Cell Biochem* 2008;105:633–640. [PubMed: 18759328]
10. Dalla Torre di Sanguinetto SA, Dasen JS, Arber S. Transcriptional mechanisms controlling motor neuron diversity and connectivity. *Current Opinion in Neurobiology* 2008;18:36–43. [PubMed: 18524570]
11. Agalliu D, Takada S, Agalliu I, McMahon AP, Jessell TM. Motor neurons with axial muscle projections specified by Wnt4/5 signaling. *Neuron* 2009;61:708–720. [PubMed: 19285468]
12. Sharma K, Sheng HZ, Lettieri K, Li H, Karavanov A, Potter S, Westphal H, Pfaff SL. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell* 1998;95:817–828. [PubMed: 9865699]
13. Nordstrom U, Maier E, Jessell TM, Edlund T. An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. *PLoS Biol* 2006;4:e252. [PubMed: 16895440]
14. Liu JP, Laufer E, Jessell TM. Assigning the positional identity of spinal motor neurons. Rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* 2001;32:997–1012. [PubMed: 11754833]
15. Sockanathan S, Jessell TM. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* 1998;94:503–514. [PubMed: 9727493]
16. Kania A, Jessell TM. Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. *Neuron* 2003;38:581–596. [PubMed: 12765610]
17. Hollyday M, Jacobson RD. Location of motor pools innervating chick wing. *J Comp Neurol* 1990;302:575–588. [PubMed: 1702118]
18. Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 2002;110:385–397. [PubMed: 12176325]
19. Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. Functional properties of motoneurons derived from mouse embryonic stem cells. *J Neurosci* 2004;24:7848–7858. [PubMed: 15356197]
20. Mizuseki K, Sakamoto T, Watanabe K, Muguruma K, Ikeya M, Nishiyama A, Arakawa A, Suemori H, Nakatsuji N, Kawasaki H, et al. Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. *Proc Natl Acad Sci U S A* 2003;100:5828–5833. [PubMed: 12724518]
21. Peljto M, Dasen JS, Mazzoni EO, Jessell TM, Wichterle H. Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. *Cell Stem Cell* 2010;7:355–366. [PubMed: 20804971] • The authors provide evidence that ESCs give rise to brachial level LMC neurons that project their axons into the limb and migrate into the lateral portion of the developing spinal cord when engrafted into the chick spinal cord. Acquisition of motor pool

subtype identity is driven by an intrinsic transcriptional network established in motor neuron progenitors.

22. Okada Y, Shimazaki T, Sobue G, Okano H. Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev Biol* 2004;275:124–142. [PubMed: 15464577]
23. Dasen JS, De Camilli A, Wang B, Tucker PW, Jessell TM. Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* 2008;134:304–316. [PubMed: 18662545] • The authors demonstrate that the intrinsic Hox transcription network plays a critical role in the specification of motor pool identity. Inactivation of a Hox co-factor FoxP1 results in the loss of all motor pool identities and in the subsequent randomization of motor neuron axonal projections.
24. Soundararajan P, Miles GB, Rubin LL, Brownstone RM, Rafuse VF. Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. *J Neurosci* 2006;26:3256–3268. [PubMed: 16554476]
25. Sharma K, Leonard AE, Lettieri K, Pfaff SL. Genetic and epigenetic mechanisms contribute to motor neuron pathfinding. *Nature* 2000;406:515–519. [PubMed: 10952312]
26. Soundararajan P, Fawcett JP, Rafuse VF. Guidance of postural motoneurons requires MAPK/ERK signaling downstream of fibroblast growth factor receptor 1. *J Neurosci* 2010;30:6595–6606. [PubMed: 20463222]
27. Shirasaki R, Lewcock JW, Lettieri K, Pfaff SL. FGF as a target-derived chemoattractant for developing motor axons genetically programmed by the LIM Code. *Neuron* 2006;50:841–853. [PubMed: 16772167]
28. Haase G, Dessaud E, Garces A, de Bovis B, Birling M, Filippi P, Schmalbruch H, Arber S, deLapeyriere O. GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. *Neuron* 2002;35:893–905. [PubMed: 12372284]
29. Pearson BJ, Doe CQ. Specification of temporal identity in the developing nervous system. *Annu Rev Cell Dev Biol* 2004;20:619–647. [PubMed: 15473854]
30. Baumgardt M, Karlsson D, Terriente J, Díaz-Benjumea FJ, Thor S. Neuronal subtype specification within a lineage by opposing temporal feed-forward loops. 2009;139:969–982.
31. Dasen JS, Tice BC, Brenner-Morton S, Jessell TM. A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. *Cell* 2005;123:477–491. [PubMed: 16269338]
32. Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 2005;23:215–221. [PubMed: 15685164]
33. Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrision NL, Panagiotakos G, Socci ND, Tabar V, Studer L. Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* 2007;25:1931–1939. [PubMed: 17478583]
34. O'Leary DD, Chou SJ, Sahara S. Area patterning of the mammalian cortex. *Neuron* 2007;56:252–269. [PubMed: 17964244]
35. Okano H, Temple S. Cell types to order: temporal specification of CNS stem cells. *Curr Opin Neurobiol* 2009;19:112–119. [PubMed: 19427192]
36. Arlotta P, Molyneaux BJ, Chen J, Inoue J, Kominami R, Macklis JD. Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* 2005;45:207–221. [PubMed: 15664173]
37. Molnar Z, Cheung AF. Towards the classification of subpopulations of layer V pyramidal projection neurons. *Neurosci Res* 2006;55:105–115. [PubMed: 16542744]
38. Martin JH. Differential spinal projections from the forelimb areas of the rostral and caudal subregions of primary motor cortex in the cat. *Exp Brain Res* 1996;108:191–205. [PubMed: 8815029]
39. He SQ, Dum RP, Strick PL. Topographic organization of corticospinal projections from the frontal lobe: motor areas on the medial surface of the hemisphere. *J Neurosci* 1995;15:3284–3306. [PubMed: 7538558]

40. Steward O, Zheng B, Ho C, Anderson K, Tessier-Lavigne M. The dorsolateral corticospinal tract in mice: an alternative route for corticospinal input to caudal segments following dorsal column lesions. *J Comp Neurol* 2004;472:463–477. [PubMed: 15065120]
41. Thompson FJ, Fernandex JJ. Patterns of cortical projection to hindlimb muscle motoneurone pools. *Brain Res* 1975;97:33–46. [PubMed: 1175038]
42. Lacroix S, Havton LA, McKay H, Yang H, Brant A, Roberts J, Tuszynski MH. Bilateral corticospinal projections arise from each motor cortex in the macaque monkey: a quantitative study. *J Comp Neurol* 2004;473:147–161. [PubMed: 15101086]
43. Petersen NC, Butler JE, Taylor JL, Gandevia SC. Probing the corticospinal link between the motor cortex and motoneurons: some neglected aspects of human motor cortical function. *Acta Physiol (Oxf)* 2010;198:403–416. [PubMed: 20003100]
44. Grove EA, Fukuchi-Shimogori T. Generating the cerebral cortical area map. *Annu Rev Neurosci* 2003;26:355–380. [PubMed: 14527269]
45. O'Leary DD, Schlaggar BL, Tuttle R. Specification of neocortical areas and thalamocortical connections. *Annu Rev Neurosci* 1994;17:419–439. [PubMed: 8210182]
46. Frost DO, Metin C. Induction of functional retinal projections to the somatosensory system. *Nature* 1985;317:162–164. [PubMed: 4033796]
47. Stern CD. Neural induction: 10 years on since the 'default model'. *Curr Opin Cell Biol* 2006;18:692–697. [PubMed: 17045790]
48. Munoz-Sanjuan I, Brivanlou AH. Neural induction, the default model and embryonic stem cells. *Nat Rev Neurosci* 2002;3:271–280. [PubMed: 11967557]
49. Elkabetz Y, Panagiotakos G, Al Shamy G, Socci ND, Tabar V, Studer L. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes Dev* 2008;22:152–165. [PubMed: 18198334]
50. Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotech* 2003;21:183–186.
51. Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y. Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat Neurosci* 2005;8:288–296. [PubMed: 15696161]
52. Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 2001;30:65–78. [PubMed: 11343645]
53. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 2000;18:675–679. [PubMed: 10835609]
54. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 2001;19:1129–1133. [PubMed: 11731781]
55. Wataya T, Ando S, Muguruma K, Ikeda H, Watanabe K, Eiraku M, Kawada M, Takahashi J, Hashimoto N, Sasai Y. Minimization of exogenous signals in ES cell culture induces rostral hypothalamic differentiation. *Proceedings of the National Academy of Sciences* 2008;105:11796–11801.
56. Eiraku M, Watanabe K, Matsuo-Takasaka M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* 2008;3:519–532. [PubMed: 18983967] •The authors demonstrate successful generation of apico-basally polarized cortical tissue from ESCs. In addition, cortical neurons derived from ESCs are generated in a temporal sequence analogous to the generation of cortical neuron types in vivo. Cortical progenitors can be patterned by manipulating Fgf signaling into distinct cortical areas.
57. Li XJ, Zhang X, Johnson MA, Wang ZB, Lavaute T, Zhang SC. Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. *Development* 2009;136:4055–4063. [PubMed: 19906872]

58. Stavridis MP, Lunn JS, Collins BJ, Storey KG. A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. *Development* 2007;134:2889–2894. [PubMed: 17660197]
59. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 2009;27:275–280. [PubMed: 19252484]
60. Gaspard N, Bouschet T, Hourez R, Dimidschstein J, Naeije G, van den Aamee J, Espuny-Camacho I, Herpoel A, Passante L, Schiffmann SN, et al. An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* 2008;455:351–357. [PubMed: 18716623] •The authors demonstrate the generation of distinct layer specific types of cortical neurons from ESCs in a temporal manner that resembles in vivo cortical neuron development. Based on their axonal trajectories after engraftment in vivo, the authors concluded that the ESC-derived neurons appear to acquire the identities of visual and limbic area cortical neurons.
61. Ideguchi M, Palmer TD, Recht LD, Weimann JM. Murine embryonic stem cell-derived pyramidal neurons integrate into the cerebral cortex and appropriately project axons to subcortical targets. *J Neurosci* 2010;30:894–904. [PubMed: 20089898] •Authors of this article demonstrate the generation of cortical neurons using co-culture system with MS5 stromal cell line. ESC-derived neurons were capable of acquiring layer V pyramidal neuronal identities and depending on the site of implantation projected axons reminiscent of in vivo neurons found in the motor, somatosensory, and visual cortical areas.
62. Maroof AM, Brown K, Shi SH, Studer L, Anderson SA. Prospective isolation of cortical interneuron precursors from mouse embryonic stem cells. *J Neurosci* 2010;30:4667–4675. [PubMed: 20357117]
63. Lamba DA, Karl MO, Ware CB, Reh TA. Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2006;103:12769–12774. [PubMed: 16908856]
64. Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, Miyoshi H, Kamiya D, Honda Y, Sasai N, Yoshimura N, et al. Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. *Proc Natl Acad Sci U S A* 2005;102:11331–11336. [PubMed: 16076961]
65. Qian X, Shen Q, Goderie S, He W, Capela A, Davis A, Temple S. Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 2000;28:69–80. see comment. [PubMed: 11086984]
66. Guillemot F. Cell fate specification in the mammalian telencephalon. *Prog Neurobiol* 2007;83:37–52. [PubMed: 17517461]
67. Schlaggar BL, O'Leary DD. Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. *Science* 1991;252:1556–1560. [PubMed: 2047863]
68. Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, Yoshimura N, Akaike A, Sasai Y, Takahashi M. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat Biotechnol* 2008;26:215–224. [PubMed: 18246062]
69. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, Nishikawa SI, Sasai Y. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 2000;28:31–40. [PubMed: 11086981]
70. Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L. Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2004;101:12543–12548. [PubMed: 15310843]
71. Andersson E, Tryggvason U, Deng Q, Friling S, Alekseenko Z, Robert B, Perlmann T, Ericson J. Identification of Intrinsic Determinants of Midbrain Dopamine Neurons. *Cell* 2006;124:393–405. [PubMed: 16439212]
72. Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S. Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. *Cell* 2010;141:704–716. [PubMed: 20478259]
73. Li H, Roblin G, Liu H, Heller S. Generation of hair cells by stepwise differentiation of embryonic stem cells. *Proc Natl Acad Sci U S A* 2003;100:13495–13500. [PubMed: 14593207]

74. Su HL, Muguruma K, Matsuo-Takasaki M, Kengaku M, Watanabe K, Sasai Y. Generation of cerebellar neuron precursors from embryonic stem cells. *Dev Biol* 2006;290:287–296. [PubMed: 16406324]
75. Salero E, Hatten ME. Differentiation of ES cells into cerebellar neurons. *Proc Natl Acad Sci U S A* 2007;104:2997–3002. [PubMed: 17293457]
76. Tao O, Shimazaki T, Okada Y, Naka H, Kohda K, Yuzaki M, Mizusawa H, Okano H. Efficient generation of mature cerebellar Purkinje cells from mouse embryonic stem cells. *J Neurosci Res* 2010;88:234–247. [PubMed: 19705453]
77. Erceg S, Ronaghi M, Ivan Z, Lainez S, Garcia Rosello M, Moreno-Manzano V, Xiong C, Rodriguez-Jimenez FJ, Planells R, Alvarez Dolado M, et al. Efficient differentiation of human embryonic stem cells into functional cerebellar-like cells. *Stem Cells Dev*. 2010

Figure 1. Functional diversity of cortical projection neurons and spinal motor neurons

A) Spinal cord motor neurons acquire distinct columnar identities depending on their rostro-caudal position and *Hox* gene expression profile. Motor neuron columnar identity determines the cell body position and general muscle target specificity of motor neurons: medial motor column (MMC) neurons innervate axial muscles, hypaxial motor column (HMC) cells innervate hypaxial muscles, lateral motor column (LMC) neurons innervate limb muscles and preganglionic motor column (PGC) neurons innervate sympathetic ganglia.

B) LMC and MMC neurons can be distinguished not only by the differential cell body position (lateral vs medial) and axonal projections (limb vs. axial muscles), but also by differential expression of FoxP1 and Lhx3 transcription factors.

C) LMC neurons are further subdivided into the Lhx1⁺ lateral (l) and Isl1⁺ medial (m) divisions, harboring motor neurons innervating dorsal and ventral limb muscles, respectively. Each division is further parceled into motor pools that innervate distinct limb muscles. For example, LMCm contains a Scip expressing motor pool innervating flexor carpi ulnaris (FCU) muscle and Pea3 expressing pool innervating cutaneous maximus muscle. LMCl also contains a Pea3 expressing motor pool, but this one is dedicated to innervate latissimus dorsi (LD) muscle.

D) Cortical projection neurons found within layer V of the neocortex target distinct regions of the CNS. Cortical motor neurons (blue) project axons to pons and spinal cord. Neurons from the auditory cortex (grey) project axons to inferior colliculus while neurons from the visual cortex (orange) project to pons and superior colliculus. Schematic adapted from [6].

E) Layer V cortical neurons are subdivided into Scip, Ctip2, and Fez1 expressing corticofugal and Lmo4 expressing callosal projection neurons. Corticofugal pyramidal neurons acquire diverse areal subtype identities that define and stabilize their general axonal trajectories (D). Finally, neurons in individual cortical areas are further functionally diversified. For example, corticospinal layer Vb neurons in the motor cortex innervate distinct spinal cord segments (e.g. cervical vs. thoracic), forming a refined somatotopic map within the motor cortex.

Figure 2. Generation of neuronal subtype diversity from ESCs

A) Specification of diverse classes of nerve cells from ESCs depends on the spatial and temporal patterning of differentiating cells. Spatial patterning of the neuroepithelium along the rostro-caudal (R-C) and dorso-ventral (D-V) axes in response to diffusible patterning signals (RA, Wnt, FGF, Shh, BMPs) results in the formation of molecularly distinct neural progenitor domains, including neocortical progenitors (rostral, dorsal identity) and motor neurons progenitors (caudal, ventral identity). Temporal patterning further increases the diversity of nerve cells generated from each progenitor domain (e.g. early born layer V cortical neurons and spinal motor neurons vs. late born oligodendrocytes and layer II/III pyramidal neurons).

B) Principal classes of nerve cells are diversified into numerous neuronal subtypes endowed with specialized functional properties. Spinal motor neurons acquire distinct columnar identities controlling their general axonal projections (in part controlled by Wnt4/5 signals), limb innervating LMC neurons acquire divisional identities (LMCm vs. LMCi, in response to paracrine RA signaling) and motor pool identities (controlled by intrinsic Hox regulatory network). Analogously, cortical layer V projection neurons can be subdivided into Va callosally and Vb corticofugally projecting neurons. Layer Vb neurons are further diversified according to their rostro-caudal and medio-lateral position within the neocortex (in response to FGF gradient and afferent inputs). Finally, layer Vb neurons within each cortical area are further diversified to achieve the fine topographic functional organization characteristic of the mature neocortex.