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Anatomic and Molecular Development of Corticostriatal Projection Neurons in Mice

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ABSTRACT

Corticostriatal projection neurons (CStrPN) project from the neocortex to the ipsilateral and contralateral striata to control and coordinate motor programs and movement. They are clinically important in multiple disorders: they are thought to be the predominant cortical population that degenerates in Huntington's disease; they are implicated in other neurodegenerative conditions such as Parkinson's disease; and their injury contributes to multiple forms of cerebral palsy. Together with their well-studied functions in motor control, these clinical connections make them functionally, behaviorally, and clinically an important population of neocortical neurons. Little is known about their development. "Intratelencephalic" corticostriatal projection neurons (CStrPN_i), projecting to the contralateral striatum, with their axons fully within the telencephalon ("intratelencephalic"), are a major population of CStrPN. CStrPN_i are of particular interest because they share hodological characteristics, and thus developmental axon guidance characteristics, of both callosal projection neurons (CPN) and corticofugal projection neurons (CFuPN); CStrPN_i send axons contralaterally before descending into the contralateral striatum. The relationship of CStrPN_i development to that of broader CPN and CFuPN populations remains unclear; evidence suggests that they might be evolutionary "hybrids" between CFuPN and deep layer CPN – in a sense "chimeric" with both callosal and corticofugal features. In this report, we investigated the development of CStrPN_i in mice – their birth, maturation, projections, and expression of important molecular developmental controls over projection neuron subtype identity.

INTRODUCTION

Corticostriatal projection neurons (CStrPN) are the cortical efferent neurons of cortico-basal ganglia circuitry; their degeneration is a predominant feature to Huntington's disease (HD), and they are implicated in the pathophysiology of several neurological conditions including Parkinson's disease and multiple forms of cerebral palsy (Albright 1996; Martin et al. 1997; Reading et al. 2004; Rosas et al. 2002; Sieradzan and Mann 2001; Sotrel et al. 1991; Stephens et al. 2005; Vonsattel et al. 1985). A major population of CStrPN, termed intratelencephalic CStrPN (CStrPN_i), projects to targets within the telencephalon (Reiner et al. 2010; Reiner et al. 2003). CStrPN_i have the unique attribute of being both corticofugal because they project to the striata bilaterally, and callosal because their axons cross the midline (Suppl. Fig. 1A). Adult CStrPN_i have been well-studied, and are considered to be the major corticostriatal population (Cowan and Wilson 1994; Donoghue and Kitai 1981; Jones et al. 1977; Lei et al. 2004; Reiner et al. 2010; Reiner et al. 2003; Royce 1982; Wilson 1986, 1987; Wise and Jones 1977; Wright et al. 1999; Wright et al. 2001). In addition to CStrPN_i, there is a small population of subcerebral projection neurons (corticospinal and related cortico-brainstem neurons) (Arlotta et al. 2005; Lai et al. 2008) that have axon collaterals to the ipsilateral striatum (pyramidal tract-type corticostriatal projection neurons, CStrPN_p) (Lei et al. 2004; Reiner et al. 2003; Sheth et al. 1998; Wilson 1987) (Suppl. Fig. 1B). Despite their significance, very little is known about CStrPN_i development.

We applied a range of developmental analyses to investigate the generation, axon outgrowth and projections, target innervation, molecular development, and pruning of CStrPN_i in mice. We first investigated the temporal range of birth of CStrPN_i; we report that CStrPN_i in mice are born predominantly during the initial infragranular phase of neocortical development (embryonic day (E)12.5 – E14.5). We applied both anterograde

and retrograde axonal projection analyses; we find that the earliest that CStrPN_i can be distinguished from pure callosal projection neurons (CPN; neurons having only contralateral cortical targets) is when they first invade the contralateral striatum at around postnatal day (P)3 – P4. At this stage, but not before, at least some CStrPN_i also simultaneously project to the ipsilateral striatum. We investigated the medio-lateral and rostro-caudal distribution of CStrPN_i through postnatal development until their stabilization; by two weeks after birth, there is substantial loss of CStrPN_i from caudal neocortex, and the distribution in mice reaches a pattern similar to that of adult rat CStrPN_i (McGeorge and Faull 1987, 1989). We applied analysis of recently identified molecular controls over CPN and corticofugal projection neurons (CFuPN; neurons that send their axons away from the neocortex) as indicators of alternative differentiation pathways; our data indicate that CStrPN_i have molecular features of both CPN and CFuPN. For example, at P4 we find that, while CStrPN_i express *Satb2*, a critical molecular regulator of CPN development, many also express *Sox5*, a molecular control over sequential generation of CFuPN subtypes, in striking contrast to pure CPN. These results indicate that CStrPN_i possess dual callosal and corticofugal anatomic and molecular characteristics, motivating further work identifying molecular controls over the development of this unique, functionally and clinically important population of projection neurons.

MATERIALS AND METHODS

All mice used in these experiments were handled according to guidelines of the National Institutes of Health (NIH), and all procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital.

CStrPN_i Birthdating Analysis

The timing of CStrPN_i birth was assessed through bromodeoxyuridine (BrdU) pulse-labeling. Briefly, timed-pregnant females (day of vaginal plug is taken to be embryonic day (E) 0.5) at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, and E16.5 were pulse-labeled with BrdU (100 mg/kg body weight) by a single intraperitoneal injection. Retrograde labeling protocols were subsequently performed on the pups from these litters at P12 in order to label CStrPN_i, as described below, and brains processed for analysis at P14. Results are from at least 3 different mice from at least 2 independent litters, and bars representing standard error of the mean are shown (Fig. 2A).

Retrograde Labeling

Retrograde labeling was performed as previously described (Arlotta et al. 2005; Fricker-Gates et al. 2002). Postnatal day (P)3 pups or younger were anesthetized with 4 to 5 minutes of hypothermia, while pups of age P6 and older were deeply anesthetized via an intraperitoneal injection of 0.015 cc/g body weight of Avertin (1.25% of 2-2-2 tribromoethanol in a solvent containing 0.63% isoamyl alcohol by weight in ddH₂O). For specific, distinct experimental goals, a number of retrograde labels, 20-40 nl each, were used: 1) red and green fluorescent microspheres (Lumafluor); 2) FluoroGold (FG; Fluorochrome), and 3) Alexa 555- or Alexa 647-conjugated beta subunit of the cholera toxin (CTB555 and CTB647 respectively; Invitrogen). Microspheres/CTB/FluoroGold

injections were performed using an ultrasound-guided microinjection system (Vevo 770, Visual Sonics, Toronto, Canada) for pups younger than P3, and stereotaxically (Stoelting, IL) for older mice 24-48 hours before perfusion and brain collection. *Fezf2-lacZ* mice were generated by Hirata and colleagues (Hirata et al. 2004).

Immunocytochemistry:

Mice were deeply anesthetized with a lethal dose of anesthetic (Avertin) or hypothermia, and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde, rinsed with PBS, and sectioned coronally at 50 μ m on a vibrating microtome (Leica). Sections were blocked in 8% donkey or goat serum, 0.3% BSA (Sigma), and 0.3% Triton x-100 (Sigma) for one hour at room temperature before incubation with primary antibodies overnight at 4°C. Primary antibodies and dilutions used were: 1) rat anti-BrdU (1:500; Accurate Chemical and Scientific, NY); 2) rabbit anti-Cux1 (1:200; Santa Cruz Biotechnology, CA); 3) goat anti-Lmo4 (1:200; Santa Cruz Biotechnology, CA); 4) rat anti-Ctip2 (1:500; Abcam, MA); 5) goat anti-Sox5 (1:200; Santa Cruz Biotechnology, CA (discontinued)) or rabbit anti-Sox5 (1:500; Abcam, MA); 6) goat anti-cholera toxin, β -subunit (1:4000; List Biological Labs, CA); and 7) rabbit anti-beta galactosidase (1:3000; MP Biomedical). Before applying the blocking solution in BrdU immunocytochemistry protocols, sections were immersed in 2 N HCl for 2 hours at room temperature, and rinsed three times in PBS for five minutes each. We used anti-beta galactosidase immunocytochemistry on retrogradely labeled *Fezf2-lacZ* heterozygous brain tissue to investigate whether CStrPN_i express *Fezf2*. Alexa fluorophore conjugated secondary antibodies from Invitrogen were used at a dilution of 1:500.

Anterograde Labeling with Dil Crystals

Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI_{C-18}(3)) crystals (Invitrogen) were used to anterogradely label CStrPN_i projections from the cortex. Crystals were placed into post-fixed brains of P0, P2, P3, and P4 mice. Brains were incubated at 37°C for 1-2 weeks in PBS to allow for anterograde transport of dye along axons innervating the contralateral striatum.

Visualization and Analysis

Image acquisition was performed using either: 1) a Nikon Eclipse E1000 epifluorescence microscope with a QImaging Retiga EX cooled CCD digital camera (QImaging Corporation, Surrey, Canada); 2) a Nikon 90i epifluorescence microscope with a Clara DR-328G cooled CCD digital camera (Andor Technology, Belfast, Northern Ireland); or 3) a BioRad Radiance 2100 Rainbow laser-scanning confocal microscope based on a Nikon E800 microscope. Images were assembled in Adobe Photoshop and Illustrator (CS3), with adjustments for contrast, brightness, and color balance to obtain optimal visual reproduction of data.

RESULTS

Retrograde labeling of intratelencephalic corticostriatal projection neurons (CStrPN_i)

We developed a reproducible approach to retrogradely label CStrPN_i by injecting tracers in the dorsolateral sector of the striatum (Fig. 1A). Using DAPI staining to better visualize the laminar architecture (Fig. 1B), we identified the location of CStrPN_i (Fig. 1C-D). As observed in rats (McGeorge and Faull 1987, 1989) and primates (Jones et al. 1977), CStrPN_i are mostly located in layer V and lower layer II/III, though we also occasionally found CStrPN_i in the deeper infragranular layers (Fig. 1C).

CStrPN_i are predominantly born between E12.5 and E14.5

In the mouse, deep layer neurons are predominantly born between E10.5 and E13.5, while superficial layer neurons are born between E14.5 and E16.5. To assess the time-course of CStrPN_i birth, timed-pregnant females were pulse-labeled with BrdU at each embryonic day from E10.5 to E16.5; the resulting mice were retrogradely labeled for CStrPN_i at P12, and brains were collected at P14. The percentage of BrdU-positive CStrPN_i was quantified in the cortex contralateral to the injection site (Fig. 2A-C). A small number of CStrPN_i are born at E10.5 and E11.5 ($5 \pm 2.5\%$ and $6 \pm 2.2\%$, respectively). The greatest percentage of CStrPN_i birth occurs at E12.5 and E13.5 ($32 \pm 0.9\%$ and $42 \pm 4.0\%$, respectively), while this percentage decreases progressively at E14.5, E15.5, and E16.5 ($26 \pm 3.8\%$, $13 \pm 2.0\%$ and $5 \pm 0.7\%$, respectively) (Fig. 2D; statistics reported \pm standard error of the mean). These data indicate that most CStrPN_i are born between E12.5 and E14.5, comparable to other populations of early-born, evolutionarily older, corticofugal and deep layer callosal projection neurons (Molyneaux et al. 2007).

CStrPN_i collaterals reach their targets between P3 and P4

In order to establish distinct and potentially critical developmental stages during postmitotic CStrPN_i maturation (e.g. midline crossing, axonal entry into the striatum, axonal branching, completion of innervation, axonal pruning), it is important to understand the timing and course of their axonal projections into the contralateral striatum. To visualize the fine axonal projections of CStrPN_i during development, crystals of the carbocyanine dye Dil were placed in the cortex of fixed brains at ages P0, P2, P3, and P4 to anterogradely label fibers in the contralateral hemisphere (Fig. 3A). Sufficient time was allowed for full Dil diffusion into the most distant axonal terminals and growth cones. At P0 and P2, no corticostriatal axons contralateral to the Dil deposit were observed entering the striatum yet. The corpus callosum overlying the striatum contained pioneering axons that had already crossed the midline but did not exhibit entry or collateralization into the striatum (Fig. 3B-C). By P3-P4, collateralization of callosal axons was observed to occur into the dorsolateral striatum. These axons display multiple secondary collateral branches (Fig. 3D-F). These data indicate that CStrPN_i initially reach the contralateral striatum around P3-P4, defining ~P3-P4 as the earliest developmental age at which the CStrPN_i population can be identified by connectivity. In the absence of subtype-specific markers or other genetic reagents, axonal connectivity enables future investigation of subtype-specific molecular controls, using established approaches originally applied to corticospinal motor neurons and callosal projection neurons (e.g. Arlotta et al. 2005; Molyneaux et al., 2009).

We next asked when CStrPN_i collaterals invade the ipsilateral striatum. We specifically investigated whether ipsilateral collaterals enter the striatum before, at the same time as, or after collaterals reach the contralateral striatum. At P0, P1, and P2, we

retrogradely labeled from the entire contralateral callosum with CTB555 and simultaneously injected the volume of the ipsilateral striatum with CTB647, and collected brains 24 hours later (at P1, P2, and P3, respectively). We did not observe any double-labeled neurons at any of these stages, indicating that ipsilateral striatal collaterals do not enter in advance of contralateral striatal innervation. However, bilateral striatal labeling at P3 (Fig. 3G) revealed double-labeled neurons (Fig. 3H-J). These data indicate that CStrPN_i simultaneously project to both striata beginning at P3-4 in mouse.

Previous work (Mizuno et al. 2007; Wang et al., 2007) indicates that contralateral cortex is innervated by CPN at around P5, coincident with or just after we find that CStrPN_i axons are entering the contralateral striatum. Taken together, these data indicate that CStrPN_i send their ipsilateral and contralateral axonal projections simultaneously to their bilateral targets at ~ P3-P4 in the mouse.

Distribution of CStrPN_i during postnatal development

To directly examine the rostro-caudal and medio-lateral distribution of CStrPN_i, we injected fluorescent latex microspheres into the dorsolateral striatum at P12-13, and the contralateral cortex was examined for retrogradely labeled neurons at P15. These experiments identified that CStrPN_i are predominantly located in the M1/M2 regions of the cortex (Fig. 4Ca-d) consistent with the published distribution in adult rats (McGeorge and Faull 1987, 1989) and primates (Jones et al. 1977). Thus, at the frontal pole, CStrPN_i populate the entire dorsoventral and mediolateral expanse of the cortex. At the level of the anterior commissure, the distribution is substantially confined to deep layer II/III and layer V (Fig. 4Cc). A smaller population of CStrPN_i is located in layer VI. Further caudal, at the level of the hippocampus (Fig. 4Cd), CStrPN_i become sparse. A population of CStrPN_i located latero-ventrally (Fig. 4Cb-d) might represent a subpopulation of CStrPN_i crossing the midline through the anterior commissure, rather

than through the corpus callosum (Lent and Guimaraes 1991). We found that the rostro-caudal location of CStrPN_i in >3 month-old adult mice is the same as that at P14 (data not shown). Taken together, these results confirm that the distribution of mouse CStrPN_i is similar to the distributions in rats and primates.

We next investigated the distribution of CStrPN_i through early postnatal development, to investigate whether pruning, neuronal elimination, and/or refinement of other types occurs. We retrogradely labeled the dorsolateral sector of contralateral striatum at P3, when CStrPN_i first project axons into the contralateral striatum (Fig. 3). At this early developmental stage, there is a substantially more diffuse distribution of CStrPN_i in the neocortex along the rostro-caudal axis, mainly in the deeper layers (Fig. 4Ca-d). Comparison of the distribution of CStrPN_i at P15 with the P3 distribution (Fig. 4Ba-d vs. 4Ca-d) shows that, while the distribution remains the same rostrally, the caudal projections to the contralateral striatum are largely absent at P15. This could be due to either retraction of initial collaterals to the contralateral striatum, or death of an early developmental population of caudal CStrPN_i (Innocenti and Price 2005; Spreafico et al. 1995).

Some CStrPN_i project to both striata and the contralateral cortex

To establish whether subsets of CStrPN_i might project to multiple contralateral and ipsilateral targets, red and green fluorescent latex microspheres were simultaneously injected into both striata of 2 week old mice (Fig. 5A). These experiments identified a subset of double-labeled neurons (Fig. 5B-D; ~15% of CStrPN_i), indicating that at least a subset of CStrPN_i project bilaterally to both striata, consistent with what is known in rats and primates.

We next investigated whether mouse CStrPN_i have collaterals in the contralateral cortex, as is the case for rats and non-human primates (Jones et al. 1977; Parent and

Parent 2006; Takada et al. 1998; Tokuno et al. 1999; Wise and Jones 1977; Yeterian and Pandya 1998). We employed a broad grid of diffusible FG injections spanning rostro-caudally and medio-laterally to broadly infiltrate unilateral cortex with FG as a retrograde label (Mitchell and Macklis 2005), and we simultaneously stereotaxically targeted the contralateral striatum with fluorescent latex microspheres as a second, distinct retrograde label (Fig. 5F-H). Consistent with data regarding rat and primate CStrPN_i, these experiments demonstrate the presence of mouse CStrPN_i with projections to both the contralateral striatum and the contralateral cortex.

In a further experiment investigating whether at least some CStrPN_i might project to all three targets at once, P11-P12 mice were bilaterally retrogradely labeled from both striata with distinct red vs. green fluorescent latex microspheres, and with broad injection of FG in the contralateral cortex, as described above (Fig. 5I; ~8% of CStrPN_i). These experiments reveal that a subset of CStrPN_i are triple labeled, indicating that a subset of CStrPN_i send collaterals to both striata as well as the contralateral cortex (Fig. 5J-M), consistent with what has been reported in primates and rats (Jones et al. 1977; Parent and Parent 2006; Takada et al. 1998; Tokuno et al. 1999; Wise and Jones 1977; Yeterian and Pandya 1998). The actual number of CStrPN_i that have axons extending into both striata and/or contralateral cortex is likely to be higher than the estimates we provide here, since retrograde labeling studies typically do not capture all neurons projecting to a region, especially when axon terminals are distributed over a wide area like the dorso-lateral sector of the striatum. As a broad population, CStrPN_i appear to broadly distribute information both contralaterally to striatum and homotopic cortex, and ipsilaterally within the striatum.

CStrPN_i express a hybrid “signature” of known molecular controls of neocortical projection neuron subtype identity

Projection neurons in the neocortex can be broadly classified based on their projection patterns into neurons that send their axons either to targets within the opposite hemisphere (callosal projection neurons, CPN) or to targets outside the neocortex (corticofugal projection neurons, CFuPN) (Molyneaux et al. 2007). CStrPN_i are a unique population of anatomically “hybrid” neurons that send their axons both across the midline via the corpus callosum, and also away from the cortex to the subcortical striatum. We investigated whether this hybrid connectivity / hodology might be reflected in simultaneous, hybrid molecular character.

In order to establish whether critical molecular controls for CPN and CFuPN might be expressed distinctly or in combination by developing CStrPN_i, we performed immunocytochemistry (ICC) for select, recently identified transcriptional regulator controls over development of these distinct populations of cortical projection neurons. *Satb2* is a chromatin binding protein necessary for CPN differentiation, and is not expressed by mature CFuPN (Alcamo et al. 2008; Britanova et al. 2005; Britanova et al. 2008). *Fezf2* (Arlotta et al., 2005; Chen et al. 2005a; Chen et al. 2005b; Molyneaux et al. 2005) and *Ctip2* (Arlotta et al. 2005;) are two recently identified, functionally important transcription factors controlling specification (*Fezf2*) and connectivity (*Ctip2*), expressed specifically by CFuPN (and not by cortico-cortical CPN) (Chen et al. 2008; Han et al. 2011; Ip et al. 2011; McKenna et al. 2011; Molyneaux et al., 2007; Shim et al., 2012). We found that CStrPN_i express *Satb2* at P4 (Fig. 6A-D), but do not express either *Fezf2* (Fig. 6E-H) or *Ctip2* (Fig. 6I-L). Whether CStrPN_i might express CFuPN controls earlier in their development cannot be addressed until specific controls over, and, therefore, markers of early CStrPN_i development are identified, enabling analysis prior to their innervation of the contralateral striatum (now required for their identification).

The transcription factor *Sox5* is of particular interest in this regard, since it is specifically expressed by the major classes of CFuPN (subplate, corticothalamic, and all

subcerebral subtypes), but not by cortico-cortical CPN, and it controls sequential generation of these subtypes by its own progressive down-regulation (Lai et al. 2008). Quite interestingly, our experiments reveal that approximately 50% of CStrPN_i express Sox5 at P4, and that by P14, it is expressed by all CStrPN_i (Fig. 6M-P; U-W, Y). These results suggest that CStrPN_i originate as CFuPN rather than as pure cortico-cortical CPN, and acquire their hybrid ability to cross contralaterally via the corpus callosum via unique mechanisms, with increasing Sox5 repression of subcerebral characteristics.

Additional results reinforce this interpretation. Lmo4, a LIM domain-containing protein, is expressed by cortico-cortical CPN in layers II/III and V, but is excluded from corticospinal motor neurons postnatally (Arlotta et al. 2005; Azim et al. 2009; Bulchand et al. 2003). During earlier development, Lmo4 is expressed by both CPN and CFuPN, but its expression is refined and is subsequently restricted to CPN (Azim et al. 2009). The current experiments reveal that Lmo4 expression by CStrPN_i increases with time; at P4, only some express Lmo4 (Fig. 6Q-T), but by P14 most CStrPN_i express Lmo4 (Fig. 6U-V, X-Y). Reinforcing the earlier BrdU birthdating results and these transcriptional regulator expression results that together reveal that CStrPN_i partially resemble deep layer, CFuPN early, then acquire hybrid deep layer CPN / CFuPN character, the current experiments reveal that CStrPN_i do not express Cux1 protein (Fig. 6Z, a-c). Cux1 is expressed by neurons in layers II/III and IV, and data indicate that it might act as a determinant of superficial layer neuron fate (Nieto et al. 2004). The absence of Cux1 from CStrPN_i reinforces that CStrPN_i neurons are an infragranular, evolutionarily older population, distinct from superficial layer CPN.

Taken together, these results reveal that CStrPN_i exhibit hybrid molecular characteristics of both early-born, deep layer CPN with some shared features of typical,

non-callosally projecting CFuPN. This might indicate hybrid and / or novel developmental controls enabling this combination of otherwise typically distinct characteristics.

DISCUSSION

By integrating information from regions of the neocortex and the striatum, corticostriatal projection neurons (CStrPN) participate in the modulation of neuronal activity associated with cognitive, affective, and performance aspects of motor function (Dure et al. 1992). Here, we investigated in mice, the birth, axon extension, and maturation of intratelencephalic CStrPN (CStrPN_i), a predominant population of corticostriatal projection neurons in mice (Reiner et al. 2010; Reiner et al. 2003).

CStrPN_i distribute information via bilateral connectivity in mice, rats, and primates

We find that at least a subset CStrPN_i in mice project to both striata, and a subset also projects contralaterally to the homotopic cortex (Fig. 5), similar to what is known in rats and primates (Cowan and Wilson 1994; Donoghue and Kitai 1981; Jones et al. 1977; McGeorge and Faull 1987; Reiner et al. 2003; Wise and Jones 1977). There is evidence from rat and monkey investigations that there are likely multiple subsets of CStrPN_i, some that project only bilaterally to both contralateral and ipsilateral striata, and others that project to both striata as well as contralateral cortex (Cowan and Wilson 1994; Kincaid and Wilson 1996; Parent and Parent 2006; Wilson 1987; Wright et al. 2001). It will be of interest in the future to investigate whether molecularly identifiable subsets exist, and whether such information enables anatomical identification of homologous subsets of CStrPN_i in mice, toward further investigation into molecular controls over the specificity of their axonal, synaptic, and functional connections.

The experiments reported here establish that mouse CStrPN_i are largely confined to motor (M1) and pre-motor (M2) cortices; CStrPN_i most densely populate broad rostral

regions of the cortex, and are more confined to medial areas more caudally. CStrPN_i reside mostly in layer V, but smaller subsets are also located in deep layer II/III and in layer VI (Fig. 1), consistent with results previously described in rats (McGeorge and Faull 1987). Together, M1 and M2 cortex coordinate planning and execution of movement (Reiner et al. 2010). CStrPN_i in M1 and M2 project to the dorso-lateral striatum, and thus into the motor circuitry of the basal ganglia, consistent with their function in motor cognition and planning across species.

CStrPN_i are born during the early phase of cortical neurogenesis

Consistent with their predominantly layer V location in infragranular cortex, the current experiments find that most CStrPN_i are born between E12.5 and E14.5 (Fig. 2), during the early phase of cortical development when corticofugal and deep layer callosal projection neurons are born (Angevine and Sidman 1961; Fame et al. 2011; Molyneaux et al. 2007). Neurons have been identified in turtle dorsal cortex that resemble deep layer output neurons of the mammalian neocortex (Reiner, 1991; Reiner, 1993). These and other data suggest that deep layer output neurons existed prior to mammalian neocortical evolution. The predominant location of CStrPN_i in layer V, along with their early generation and projection to subcortical targets in the striata, suggest that CStrPN_i are developmentally related to evolutionarily older corticofugal neurons. CStrPN_i appear to be largely distinct from superficial layer CPN (~ 80% of CPN) that constitute the most extensive evolutionary addition underlying the striking and relatively rapid expansion of cortical thickness from sauropsids to rodents to primates (Fame *et al.* 2011).

CStrPN_i have hybrid callosal and corticofugal features, and undergo postnatal refinement of projections

One important distinguishing feature in neocortical projection neuron subtype specificity and diversity is whether or not axons cross the midline within the cerebrum (Koester and O'Leary 1993, 1994; O'Leary and Koester 1993; Richards et al. 1997). We find that CStrPN_i extend their axons across the midline along with other deep layer CPN, then send collaterals subcortically to the striata by P3-P4 (Fig. 3), developing hybrid callosal and corticofugal connectivity. Interestingly, axons of pure cortico-cortical CPN enter the contralateral cortex at the same time (Mizuno et al. 2007) that CStrPN_i axons enter the striata. Also, we found that at least some CStrPN_i project to both the contralateral striatum and the contralateral cortex. Identification of molecular controls regulating midline crossing by CStrPN_i axons without disabling later subcortical axonal growth and collateralization might likely elucidate multi-step axonal guidance mechanisms relevant to other forebrain neuronal populations.

These experiments also establish that CStrPN_i express a hybrid set of both callosal and corticofugal molecular developmental controls. At P4, all CStrPN_i express *Satb2* (Fig. 6A-D), a chromatin binding protein that regulates callosal identity of projection neurons (Alcamo et al. 2008; Britanova et al. 2008) while they do not express *Fezf2-lacZ* or *Ctip2* (Fig. 6E-L), transcription factors required for specification and axon outgrowth and fasciculation of subcerebral projection neurons (Arlotta et al. 2005). Consistent with the interpretation that CStrPN_i are related to deep layer CPN, but not to superficial layer, evolutionarily newer CPN, these experiments reveal that CStrPN_i do not express *Cux1*.

Additional molecular expression analyses reinforce that CStrPN_i initially resemble deep layer CFuPN, then transition to suppress subcerebral transcriptional control and adopt more CPN signature. At P4, when CStrPN_i axons and collaterals are just beginning to enter the striata, about 50% of CStrPN_i express *Sox5*, a transcription factor

expressed by all CFuPN with lower expression levels as sequential populations are generated, whose decreasing transcriptional repression of coordinately regulated sets of genes regulates the sequential generation of CFuPN subtypes (Lai et al. 2008). At this same stage, only a subset of CStrPN_i express *Lmo4*, a LIM-domain containing transcriptional co-regulator excluded from corticospinal motor neurons and all subcerebral projection neurons (Arlotta et al., 2005). Interestingly, at P14, all CStrPN_i co-express *Sox5*⁺ and *Lmo4*⁺ (Fig. 6Q-Y), indicating progressively increased repression of subcerebral molecular character, and progressively increased expression of CPN character. This emerging hybrid expression of these otherwise exclusionary transcriptional regulators in the CStrPN_i population suggests the progressive maturation of CStrPN_i to possess hybrid characteristics of callosal contralateral projection with ultimate subcortical CFuPN connectivity.

Our data establish substantial refinement of the CStrPN_i population and / or their projections in caudal regions of the neocortex. There is substantial loss of projections to the contralateral striatum from the caudal neocortex during early postnatal development (Fig. 5). There are at least two possible explanations for this refinement. First, there might be retraction of collaterals to the striatum by caudally located CPN, with these neurons maintaining their projections to the contralateral cortex itself. Such exuberant projections to inappropriate targets, with subsequent retraction during the early postnatal period by both callosal projection neurons (of which CStrPN_i are a subset) and subcerebral projection neurons have been reported in cats and rodents (Arlotta et al., 2005; Innocenti, 1981; Innocenti and Price, 2005; Low et al., 2008; O'Leary et al., 1981; Polleux et al., 2001; Stanfield et al., 1982; Stanfield and O'Leary, 1985; Weimann et al., 1999). A second possibility is that there is well characterized developmental apoptosis ongoing in the neocortex during this same time period in rodents (Spreafico *et al.* 1995;

Verney et al. 2000). Therefore, it is possible that immature CStrPN_i in caudal cortex might undergo programmed cell death. The progressive stabilization of more rostral CStrPN_i axon collaterals might support the final distribution of CStrPN_i in adult cortex.

Taken together, these results indicate that CStrPN_i are quite a unique and hybrid population of cortical projection neurons, with a mixed set of cardinal features and molecular expression of both CFuPN and CPN. Data from the current experiments indicate a developmental origin common to CFuPN, with molecular-genetic modifications to enable telencephalic midline crossing, then later progressive acquisition of increased mature CPN character. These results suggest possible evolutionary origin from CFuPN, with acquisition of molecular mechanisms enabling bilateral collateral distribution of motor planning and control information via distributed bilateral axonal connectivity. Future identification and functional analysis of specific molecular controls over the development of this clinically-important neuronal population might potentially both elucidate the circuitry underlying complex mammalian motor control, and provide insight into the pathophysiology of important neurological disease, including Huntington's disease.

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FIGURE LEGENDS

Figure 1. Intratelencephalic corticostriatal projection neurons (CStrPN_i) are located predominantly in Layer V of the cerebral cortex. (A) Example of a unilateral injection site in the dorsal striatum to label contralateral CStrPN_i; postnatal day (P) 8 mouse injected with red fluorescent latex microspheres. (B) DAPI stain (pseudo-colored green) shows cortical lamina (dashed lines) in this P14 neocortical coronal section at a level immediately caudal to the anterior commissure. (C) CStrPN_i retrogradely labeled in the same P14 mouse with Alexa 555-conjugated cholera toxin beta subunit (CTB555; enhanced with antibody to CTB secondarily labeled with Alexa 546). (D) Merge of B and C, showing distribution of CStrPN_i predominantly in layer V and superficial layer VI. Scale bars, 200 μm.

Figure 2. Intratelencephalic corticostriatal projection neurons (CStrPN_i) are born predominantly between embryonic day (E)12.5 and E14.5 in the mouse. (A-C) CStrPN_i (A) are identified by retrograde labeling from the contralateral striatum in postnatal mice, as described in the text. BrdU immunocytochemistry (B) identifies cells born on the day of injection. Retrogradely labeled neurons co-localizing BrdU (C; arrowheads), are identified as CStrPN_i born shortly after the time of BrdU pulse administration at specific embryonic ages. (D) Quantification of the percentage of the total CStrPN_i population birth from E10.5 to E16.5, during mouse cortical development. Each time shown represents 3-4 independent biological replicates. Error bars represent standard error of the mean. Scale bars, (A-C) 100 μm.

Figure 3. Corticostriatal projection neuron (CStrPN_i) axons enter the contralateral striatum around P3-P4; a subset also project to the ipsilateral striatum during the same period. (A) Schematic of a brain coronal section demonstrating placement of Dil crystals (red circles; anterograde label), and area of analysis in (B-F) (red rectangle). (B-D) Development of axonal projections through the corpus callosum to the contralateral hemisphere and striatum at indicated postnatal stages. (B) At P0, rare pioneering axons are seen (arrow), prior to entry into the contralateral striatum. (C) By P2, there is a substantial increase in the number of axons in the contralateral corpus callosum (arrows). (D) The number of axons in the contralateral corpus callosum continues to increase at P3-4. In addition, some callosal axons enter the contralateral striatum (Str; boundary represented by yellow dashed line). (E-F) Representative confocal stacks of boxed areas in (D) showing axons innervating the contralateral striatum at P3-4. (E) The primary axon of a CStrPN_i in the contralateral corpus callosum (arrow), and main striatal axon collateral in the contralateral striatum (arrowheads). (F) Secondary collateral branches (arrowheads) deep in the contralateral striatum. (G) Schematic of brain coronal section demonstrating bilateral retrograde labeling from both striata, with two distinct colors of fluorophor-conjugated β subunit of cholera toxin at P3: Alexa 555 (contralateral; red) and Alexa 647 (ipsilateral; green). (H-J) Neurons projecting to both ipsilateral and contralateral striata are identified at P4 (arrowheads). Scale bars, (B, L) 50 μm , (C) 100 μm , (E) 30 μm , (F) 20 μm .

Figure 4. CStrPN_i (intratelencephalic corticostriatal projection neurons) become progressively refined in distribution during early postnatal development. (A) Schematic showing unilateral striatal retrograde label injection (represented by green micropipette), with retrogradely labeled CStrPN_i cell bodies in the boxed hemisphere contralateral to the injection site, magnified in (B) at P4 and (C) at P15. (Ba-d) Composite image showing distribution of CStrPN_i (retrogradely labeled from contralateral striatum) in coronal brain sections from 3 different P4 brains, from rostral (Ba) to caudal (Bd). CStrPN_i are broadly distributed rostrally across layers and medio-laterally, and are distributed across the medio-lateral extent of layer V and superficial layer VI in mid- and more caudal cortex. (Ca-d) Representative coronal brain sections from P15 mouse showing distribution of CStrPN_i (retrogradely labeled from contralateral striatum), from rostral (Ca) to caudal (Cd). CStrPN_i become much more restricted in their caudal distribution from P4 to P14, predominantly located in more medial motor cortex at middle rostro-caudal levels (Cb, Cc), and essentially absent caudally (Cd). The small subpopulation located far laterally in Cb-d might represent CStrPN_i projecting their axons contralaterally through the anterior commissure (see text). Scale bars, 200 μm.

Figure 5. Some CStrPN_i (intratelencephalic corticostriatal projection neurons) innervate both striata and the contralateral cortex. (A, E, I) Retrograde labeling approaches from multiple simultaneous targets *in vivo* in mice to identify potential multiple projections by individual CStrPN_i. The boxed areas in A, E, I represent the approximate areas shown in the images to the right of each: the box in (A) corresponds to images in (B-D); the box in (E) corresponds to (F-H), and the box in (I) corresponds to (J-M). (A) Red and green microspheres were simultaneously injected into both striata *in vivo* to investigate whether bilateral striatal projections exist from the same individual neurons. Most labeled neurons in the cortex are double-labeled CStrPN_i (white arrowheads in B, C, and merged image D), indicating that most CStrPN_i project to both striata, as is the case in rats and primates. (E) To investigate whether some or most CStrPN_i have a contralateral cortical collateral target, mice were simultaneously injected in the contralateral striatum with latex microspheres (red) and FluoroGold (green). Examination of the cortex reveals many double-labeled neurons (white arrowheads in F, G, and merged image H; ~15% of CStrPN_i), indicating that many CStrPN_i have a contralateral cortical collateral. (I) To investigate whether individual CStrPN_i can project to both striata and the contralateral cortex simultaneously, mice were injected in each striatum with green or red fluorescent latex microspheres, and injected with FluoroGold in the contralateral cortex. J, K, L, and the merged image M show triple-labeled neurons (~8% of CStrPN_i), indicating that at least a subset of CStrPN_i in mice send collaterals to all three targets, as is known to be the case in rats and primates. Brains were collected 2

days after retrograde labeling, as described in the text, either at 2 (*A-D; I-M*) or 4 (*E-H*) weeks of age. Scale bars, (*B-D, F-H*) 100 μm , (*J-M*) 20 μm .

Figure 6. CStrPN_i (intratelencephalic corticostriatal projection neurons) express transcriptional regulators that are characteristic of both callosal and corticofugal projection neurons. (A-L) At postnatal day (P)4, CStrPN_i express Satb2 (A-D; (zoomed panels; filled white arrowheads)), a protein that regulates acquisition of callosal identity of projection neurons, but do not express Fezf2 (as determined by surrogate expression of beta galactosidase from a Fezf2-lacZ reporter) (E-H) or Ctip2 (I-L) (zoomed panels; empty white arrowheads), transcription factors required for specification and axon outgrowth and fasciculation of subcerebral projection neurons. (M-T) At P4, some CStrPN_i express Sox5 (~50%; M-P; (zoomed panels; empty and filled white arrowheads)), a transcription factor that regulates sequential generation of corticofugal projection neuron subtypes, and Lmo4 (Q-T; (zoomed panels; empty and filled white arrowheads)), a transcription factor excluded from corticospinal motor neurons (CSMN) and all subcerebral projection neurons. (U-Y) By P14, most CStrPN_i express Sox5 and Lmo4 (V-Y; (zoomed panels; filled white arrowheads)) indicating progressively increased repression of subcerebral molecular character, and progressively increased expression of CPN character. (Z, a-c) As expected, CStrPN_i do not express Cux1 (a-c; (zoomed panels; empty arrowheads)), a protein implicated in superficial layer fate. Scale bars, 50 μm.

Supplemental Figure 1. Intratelencephalic corticostriatal projection neurons (CStrPN_i) are both callosal and corticofugal. (A) Major types of projection neurons in the mammalian neocortex. In the classification of cortical projection neurons based on hodology, two broad classes exist: 1) commissural neurons (of which the major class is callosal projection neurons, CPN), which cross the midline (blue circle; depicted by pink and orange arrows on a representative coronal view of the rodent brain); and 2) corticofugal projection neurons (CFuPN), which project away from the cortex, and typically respect the midline (yellow circle; depicted by pink arrows on a representative coronal view of the rodent brain). CStrPN_i (green intersection of CFuPN and CPN) are an anatomically “hybrid” population that both cross the midline and send projections corticofugally. (B) The two main types of corticostriatal projection neurons shown in a coronal representation of the rodent brain, compared to “pure” CPN (green neuron). Rodent CStrPN_i largely project to both striata, as well as the contralateral cortex (red neuron); identified to be the case in mice in this report. Pyramidal-type corticostriatal projection neurons (CStrPN_p) are subcerebral projection neurons (corticospinal motor neurons and related brainstem projecting neurons), that send an axon collateral into the striatum (blue neuron). CStrPN_i are thought to be the major population of corticostriatal projection neurons. See text for details.

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