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Motor Cortex Maturation Is Associated with Reductions in Recurrent Connectivity among Functional Subpopulations and Increases in Intrinsic Excitability

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Behavior is derived from the configuration of synaptic connectivity among functionally diverse neurons. Fine motor behavior is absent at birth in most mammals but gradually emerges during subsequent postnatal corticospinal system maturation; the nature of circuit development and reorganization during this period has been largely unexplored. We investigated connectivity and synaptic signaling among functionally distinct corticospinal populations in Fischer 344 rats from postnatal day 18 through 75 using retrograde tracer injections into specific spinal cord segments associated with distinct aspects of forelimb function. Primary motor cortex slices were prepared enabling simultaneous patch-clamp recordings of up to four labeled corticospinal neurons and testing of 3489 potential synaptic connections. We find that, in immature animals, local connectivity is biased toward corticospinal neurons projecting to the same spinal cord segment; this within-population connectivity significantly decreases through maturation until connection frequency is similar between neurons projecting to the same (within-population) or different (across-population) spinal segments. Concomitantly, postnatal maturation is associated with a significant reduction in synaptic efficacy over time and an increase in intrinsic neuronal excitability, altering how excitation is effectively transmitted across recurrent corticospinal networks. Collectively, the postnatal emergence of fine motor control is associated with a relative broadening of connectivity between functionally diverse cortical motor neurons and changes in synaptic properties that could enable the emergence of smaller independent networks, enabling fine motor movement. These changes in synaptic patterning and physiological function provide a basis for the increased capabilities of the mature versus developing brain.

Key words: **corticospinal; development; excitability; motor system; neural circuits; synaptic plasticity**

Introduction

At birth, synaptic circuitry of the cortex is diffuse, nonspecific, and overlapping (Goldman-Rakic, 1987; Katz and Callaway, 1992; Innocenti and Price, 2005), with axonal/dendritic patterning established by such factors as gene expression, clonal lineage, and environmental guidance cues (Katz and Shatz, 1996; Yu et al., 2009; Yu et al., 2012). This initial configuration can differ greatly from the mature pattern of synaptic connectivity (Huberman et al., 2008; Ohtsuki et al., 2012; Ko et al., 2013). Consequently, most animals are born with limited abilities that advance as synaptic circuitry is refined over time. In the rat motor system, for example, fine motor movements are absent at birth and do not emerge for several weeks, closely corresponding to the development of the corticospinal system of the primary motor cortex (M1) (Lawrence and Hopkins, 1976; Martin, 2005).

Although the development of corticospinal projections within the spinal cord is well documented (Gribnau et al., 1986; Stanfield, 1992; Meng et al., 2004), little is known with regard to how local network properties within M1 evolve during maturation of the motor system. In adulthood, layer 5 corticospinal neurons of the motor cortex are essential for fine motor control (Whishaw et al., 1993; Piecharka et al., 2005; Anderson et al., 2007; Lemon, 2008; Rosenzweig et al., 2010; Wahl et al., 2014), and the pattern of corticospinal interconnectivity is selectively altered following changes to the motor repertoire (Biane et al., 2012). Moreover, corticospinal neurons exhibit changes in dendritic morphology, spine dynamics, and representational motor maps within M1 during the acquisition of new motor skills (Nudo et al., 1996; Kleim et al., 1998; Conner et al., 2005; Xu et al., 2009; Yang et al., 2009; Wang et al., 2011; Fu et al., 2012).

Modification of corticospinal circuitry within M1 is likely critical for the emergence of fine motor behavior during postnatal maturation. Indeed, progressive changes in representational motor maps during early life (Chakrabarty and Martin, 2000; Young et al., 2012) suggest that network and cell-intrinsic properties of

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corticospinal neurons undergo considerable change during development. Broad changes in dendritic morphology and spine dynamics among layer 5 neurons during development further indicate that the reorganization of corticospinal circuitry may enable fine motor performance (Eayrs and Goodhead, 1959; Markus and Petit, 1987; Zuo et al., 2005).

We hypothesized that the emergence of fine motor behavior will be accompanied by increases in connectivity between functionally diverse cortical motor neurons, enabling coordination of movement across joints. We examined changes in intrinsic circuitry among corticospinal neurons during the time frame of postnatal fine motor development to elucidate neural modifications associated with the emergence of fine motor behavior. We used two subpopulations of corticospinal neurons that project to different levels of the spinal cord and appear to be associated with distinct forelimb behaviors (Brichta et al., 1987; Callister et al., 1987; McKenna et al., 2000; Wang et al., 2011; Tosolini and Morris, 2012). This use of distinct corticospinal subpopulations allowed us to probe the evolving nature of recurrent corticospinal interactions within and across subpopulations during development.

Materials and Methods

Injection of distinct retrograde tracers into the C4 and C8 spinal cord segments predominantly labels discrete populations of layer 5 corticospinal neurons. In rats, colocalization is observed in a minority of L5 corticospinal neurons when distinct retrograde tracers are injected at the C3 and C7 spinal segments (Akintunde and Buxton, 1992; Wang et al., 2011); such double-labeled neurons were excluded from the current study. This segregation of corticospinal projection patterns is also observed in primates, whereby colocalization of retrograde tracers from upper (C2–C4) and lower (C7–T1) spinal segments is <4% in corticospinal projections arising from primary motor cortex (He et al., 1993). Because these corticospinal innervation patterns are established soon after birth (Stanfield, 1992; O'Leary and Koester, 1993; Curfs et al., 1994), these dissociable populations can be stably examined over the course of motor system development.

As weight and age are directly related over the time points currently examined, age was reliably estimated via body weight growth curves provided by the animal supplier (Harlan Laboratories). At the earliest time point currently investigated [at 20 g or postnatal day 18 (P18)], the efficacy of M1 to activate muscles is low (Meng et al., 2004; Young et al., 2012), and, as such the cortical contribution to movement is likely minimal. Correspondingly, fine motor behaviors have yet to emerge, although basic motor behaviors associated with the brainstem motor system (e.g., righting, climbing, and rearing with support) are present (Altman and Sudarshan, 1975; Martin, 2005). Accordingly, we recorded from animals across a continuum of ages beginning at 20 g (P18) and continuing through 250 g (~P75). To address whether there was a developmental change in the synaptic organization of recurrent corticospinal subnetworks, we categorized animals as "immature" or "mature," with 125 g (~P45) as a cutoff. P45 was considered mature because cortical dendrites and spinal innervation patterns have attained their adult form at this time point (O'Leary and Koester, 1993; Curfs et al., 1994; Kamiyama et al., 2006), and the dynamics of dendritic spine growth and elimination have plateaued near adult levels (Markus and Petit, 1987; Kamiyama et al., 2006). Further, representational motor maps have largely stabilized (Young et al., 2012), and rats are capable of performing and learning fine motor behaviors (Markus and Petit, 1987; Young et al., 2012).

Neuronal labeling. A total of 175 male Fischer 344 rats were the subjects of this study, with physiological recordings occurring between 20 and 250 g (P18 and P75). Twenty-three animals were recorded in the time period of 18–30 d (21–60 g); 41 animals in the time period of 31–45 d (61–125 g); 74 animals in the time period of 46–60 d (126–185 g); and 26 animals in the time period of 61–75 d (186–250 g). Eleven animals were used exclusively for assessing retrograde labeling.

All procedures and animal care adhered to the American Association for the Accreditation of Laboratory Animal Care; the Society for Neuroscience; and institutional guidelines for experimental animal health, safety, and comfort. Animals were anesthetized with a cocktail (2 ml/kg) containing ketamine (25 mg/ml), xylazine (1.3 mg/ml), and acepromazine (0.25 mg/ml). To label corticospinal neurons projecting to the C8 cervical spinal cord, the overlying dura between C7 and T1 was resected and a glass micropipette (tip <40 μm) containing red or green fluorescent latex microspheres (Lumafluor) was inserted into the dorsal horn of spinal cord (Wang et al., 2011). Using a Picospritzer II (General Valve), ~350 nl of fluorescent latex microspheres was injected into each side of the spinal cord (Fig. 1). To label corticospinal neurons projecting to the upper cervical spinal cord, the same procedure was repeated between C3 and C4 spinal vertebra, using a different colored dye (green or red) than that used for C8 injections. In all cases, tracer diffusion was assessed postmortem in 50 μm coronal slices of the spinal cord (Fig. 1*C*). Animals with tracer diffusion into the dorsal columns were excluded from further study.

Histology. To assess retrograde labeling of corticospinal neurons, a total of 11 animals received injections of retrograde tracer, as described above. Injections were performed at P20 ($n = 4$), P45 ($n = 5$), or P80 ($n = 2$). Two weeks following injections, animals were deeply anesthetized with a 25 mg/ml ketamine, 1.3 mg/ml xylazine, and 0.25 mg/ml acepromazine cocktail, and transcardially perfused with 250 ml of cold PBS, pH 7.4, followed by 250 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted, postfixed for 2 h in the same fixative, and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose for 96 h at 4°C. Coronal sections (50 μm) were cut on a freezing sliding microtome and stored in tissue cryoprotectant solution (TCS) at 4°C. Confocal images were captured using 5 μm *z*-stacks using an Olympus Fluoview FV1000 camera to ensure neuronal localization of the labeled objects. Various criteria were used to identify labeled cells, including morphology, size, and signal versus background. Stereological methods were not used due to our interest in whether the proportion, and not the absolute number, of double-labeled cells changed with animal age. Cells were manually counted, and three or more sections were analyzed per hemisphere for each subject.

Electrophysiological slice preparation. Three to 12 days following tracer injection, rats were anesthetized and perfused for 3 min with ice-cold, oxygenated, modified sucrose artificial CSF (ACSF) containing the following (in mM): 75 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 0.5 $CaCl_2$, 1NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 52.6 sucrose, 10 HEPES, 10 choline chloride, 1 pyruvate, and 1 L-ascorbic acid, at ~300 mOsm/L and pH 7.4. The brain was rapidly dissected and 330-μm-thick slices spanning the motor cortex were cut at 15° anterior to the mid-coronal plane to match the projection pattern of layer 5 corticospinal neurons (Wang et al., 2011). Cortical slices were cut and collected in ice-cold, oxygenated, modified sucrose ACSF. Slices were transferred to an interface chamber containing the same modified sucrose ACSF solution and incubated at 34°C for 30 min. Slices were then held at room temperature (23°C) in the interface chamber for at least 45 min before initiating recordings. Recordings were made in a submersion-type recording chamber and perfused with oxygenated ACSF containing the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 26.0 $NaHCO_3$, and 20 glucose (~300 mOsm/L) at 23°C at a rate of 2–3 ml/min.

Electrophysiology. All recordings were performed within the caudal forelimb area of the primary motor cortex. Neurons were selected based on emission spectra (red or green), reflecting tracers injected at either the C8 or C4 spinal segment; individual cells expressing both red and green retrograde tracers were not used, so that analyses could be focused on cortical neurons predominantly terminating in C8 or C4 spinal cord segments. These were then visualized under infrared differential interference contrast videomicroscopy (BX-51 scope and Rolera XR digital camera, Olympus). Whole-cell voltage and current-clamp recordings were made at room temperature using pulled patch pipettes (4–7 MΩ) filled with internal solution containing the following (in mM): 150 K-gluconate, 1.5 $MgCl_2$, 5.0 HEPES, 1 EGTA, 10 phosphocreatine, 2.0 ATP, and 0.3 GTP. Postsynaptic data were analyzed exclusively from cells with a resting membrane potential of less than or equal to −50 mV, with a drift of <6 mV over the entire recording period, with access resistance of ≤35 MΩ, with the ability to evoke multiple spikes with a >60 mV peak amplitude from threshold. Series resistance was not compensated but

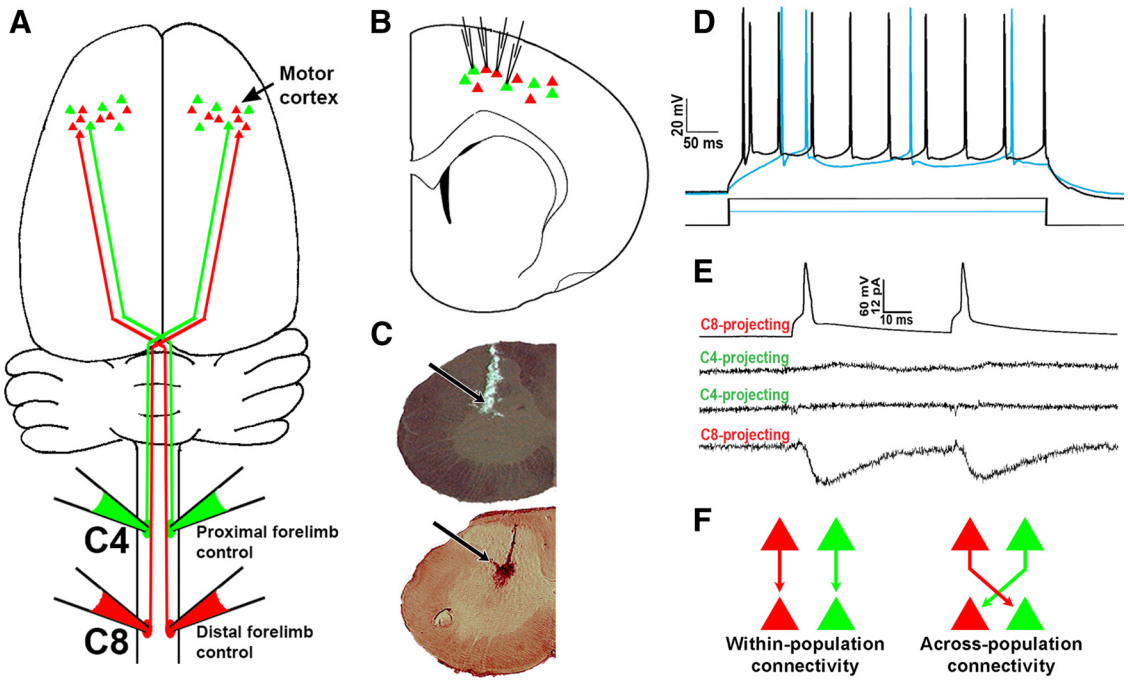

Figure 1. Experimental overview. ***A***, Retrograde tracer injections at levels C4 and C8 of the spinal cord enabled the identification of distinct corticospinal projection populations originating in layer 5 motor cortex. ***B***, Labeled cells were targeted for *in vitro* whole-cell patch clamp of up to four neurons simultaneously in animals aged P18 through P75. ***C***, Tracer injections targeted the dorsal horn and intermediate zone (indicated by arrow) and did not inadvertently spread into the corticospinal tract. ***D***, All recorded cells displayed a regular spiking pattern to suprathreshold current injections (blue trace, 200 pA current injection; black trace, 400 pA). ***E***, Quadruple recordings exhibiting an excitatory response in a single postsynaptic neuron. ***F***, Sample configuration of within- and across-population connections.

was continuously monitored via negative voltage steps. In a minority of cell pairs (~15%), the "presynaptic" neuron was fired in cell-attached mode. Numerous properties reflective of cell health remained stable across the ages examined, including resting membrane potential, input resistance, and spiking properties, suggesting slice health was comparable across recording sessions.

Data acquisition and analysis. Whole-cell patch-clamp recordings were obtained using Multiclamp 700B patch amplifiers (Molecular Devices), and data were analyzed using pClamp 10 software (Molecular Devices). To characterize basic membrane properties, a series of hyperpolarizing and depolarizing current steps were applied for 500 ms in 10–45 pA increments at 5 s intervals. The action potential threshold was determined for the first spike at the lowest level of depolarizing current required to evoke at least one spike. Action potential spike measurements were taken from the first action potential on the first sweep to reach the threshold. Spike height was measured as the peak membrane voltage relative to threshold, and half-width was measured at the half amplitude of the action potential. Input resistance was determined from the slope of the linear regression taken through the voltage–current relationship in the hyperpolarizing range.

To determine connectivity among C4- and C8-projecting cell populations, simultaneous whole-cell recordings were made in up to four retrogradely labeled cells. In many cases, a recorded cell in one pipette was replaced with a new cell while keeping the other patched cells intact. Repeating this procedure allowed us to test many (up to 11) synaptic partners for a particular cell, although the mean (±SD) number of tested synaptic partners per cell was 3.7 ± 2.1. The distribution of C4- and C8-projecting cells, which are almost entirely intermingled across the primary motor cortex (Wang et al., 2011), enabled all types of paired recordings to be obtained within a single field (C4 → C4, C8 → C8, C4 → C8, and C8 → C4). "Within-population" cell pairs are defined as cell pairs where both neurons terminated in the same spinal cord segment (i.e., C4 → C4 and C8 → C8). "Across-population" cell pairs consist of neurons each projecting to a different segment (C4 → C8 and C8 → C4; Fig. 1*F*).

Data were collected from cells >25 μm below the cutting plane of the slice surface (mean ± SD cell depth from slice surface = 66 ± 25 μm). Connectivity was determined by evoking paired action potentials spaced 50 ms apart in the presynaptic cell while monitoring responses in postsynaptic cells held at −65 mV in voltage clamp. Presynaptic action potentials were evoked by a 7 ms depolarizing current injection of 2 nA (up to 10 nA in cell-attached recordings). Individual sweeps were separated by 5 s. A cell pair was considered connected if the postsynaptic cell displayed a monotonic rise and decay characteristic of EPSCs that was of appropriate onset latency (~3 ms) and was repeatable across presynaptic stimulation trials.

The responses to 30 to ≥100 evoked action potentials were measured for each paired recording. In connected cell pairs, the failure rate was calculated as the percentage of single trials in which the postsynaptic peak current was <2 SDs below baseline current noise. All traces were manually inspected for signal consistency, including monotonic rise and decay, and reliable onset latency. Postsynaptic response amplitude was calculated as the averaged current over a 1.5 ms time window of peak response current compared with baseline, which was defined as the average current in a 17 ms window before presynaptic firing. Response potency, latency, rise time, decay time, and half-width were calculated using only traces where a postsynaptic response was detected (failures omitted). Response latency was measured from the peak of the presynaptic spike to the onset of the EPSC. Rise time was calculated as the time between 20% and 80% of peak EPSC amplitude. Decay time was calculated as the time between 80% and 20% of peak EPSC amplitude. For paired-pulse analysis, the peak response to each pulse was averaged over all trials (i.e., failures were not omitted), and the average response of the second response was divided by that of the first pulse. To preserve the relative differences in magnitude for ratios above and below a value of 1, the logarithm of each ratio was used for statistical comparisons.

Current–spike relationship and medium afterhyperpolarization (AHP) were measured in cells requiring less than −150 pA to hold at a membrane potential of −65 mV. AHP was calculated in cells for which a 300 pA depolarizing current delivered for 500 ms evoked at least four spikes. Reported *p* values reflect the main effect of training for a two-way

ANOVA (independent variable 1 = training; independent variable 2 = number of spikes elicited by current injection).

Statistical comparisons were performed using JMP software, version 10.0 (SAS Institute Inc.). Comparisons of connectivity were made using Fisher's exact test. Pairwise comparisons used Student's *t* test unless otherwise noted (e.g., Wilcoxon test for synaptic potency comparisons). Linear regression analysis was used for all comparisons where both variables were continuous (e.g., synaptic failure rate by body weight). The significance level was set at 0.05. In text, data values are presented as the mean ± SD.

Results

Corticospinal excitatory interconnectivity diminishes during maturation

Corticospinal neurons exhibited a significant reduction over postnatal development in the total number of excitatory interconnections (<125 g: 61 connections detected/1256 connections tested; >125 g: 69 connections detected/2213 connections tested; $p = 0.02$, Fisher's exact test). Notably, all of the reduction in excitatory connectivity occurred among cell pairs projecting to identical spinal segments (i.e., within-population cell pairs). Indeed, interconnectivity within the C4-projecting and the C8-projecting populations exhibited significant reductions in excitatory interconnectivity upon reaching maturity (C4 → C4: $p = 0.02$; C8 → C8: $p = 0.02$; Fisher's exact test; Fig. 2*A*). Thus, the reduction in within-population connectivity during maturation was not due to changes within a specific projecting population but appears to be a general property of motor cortex development. In contrast, connection probability across neuronal populations (i.e., neurons projecting to different spinal cord segments; interconnections between C8-projecting and C4-projecting layer 5 cortical neurons) did not change (C4 → C8: $p = 1.0$; C8 → C4: $p = 0.64$; Fig. 2*A*). Logistical regression analysis confirmed that within-population connectivity was reduced as animals aged ($p < 0.01$), whereas across-population connectivity was stable ($p = 0.7$; Fig. 2*B*). These findings suggest that early cortical development biases the formation of excitatory connections to pools of neurons projecting to similar spinal cord segments, and this within-population connectivity is refined as maturation occurs.

The preceding changes could not be attributed to differences in intersomatic distance or depth of recorded cells below the slice surface. The average distance between recorded cell pairs increased with age, as would be expected as the brain increases in size, from 126 to 137 μm, and this increase was similar for both within- and across-population cell pairs (Fig. 3*A*). The average depth of recorded cells below the slice surface changed by only 3 μm when comparing immature (<125 g) and adult (>125 g) animals (68 vs 65 μm, respectively; Fig. 3*B*).

We also assessed whether corticospinal axons expanded or reduced the number of spinal segments to which they projected as a function of developmental age, since the extent of their terminations could influence the interpretation of intracortical connectivity data. To accomplish this, we compared retrogradely labeled neurons in the motor cortex of animals that received retrograde tracer injections into C4 and C8 spinal segments at developmental days P20, P45, and P80. Across these time points, there was no difference in the proportion of layer 5 corticospinal neurons that were double labeled for both retrograde tracers ($p = 0.9$, ANOVA; Fig. 4), indicating that axon terminals did not proportionately expand or contract their projections across spinal segments over these postnatal time periods.

Synaptic efficacy diminishes during maturation

In connected cell pairs, we analyzed unitary EPSCs (uEPSCs) to examine whether neurons alter their synaptic properties upon maturation. We found no significant differences between within- and across-population groups in immature animals (<125 g) for any of the uEPSC responses measured, including failure rate ($p = 0.15$, *t* test), synaptic potency ($p = 0.18$, Wilcoxon test), paired-pulse ratio ($p = 0.15$, *t* test), onset latency ($p = 0.98$, *t* test), rise time ($p = 0.34$, *t* test), and decay time ($p = 0.15$, *t* test). Similarly, uEPSC response properties did not differ when comparing within- and across-population cell pairs in mature animals weighing >125 g ($p > 0.05$). Together, these results indicate that synaptic signaling is comparable between cells that project to the same or differing spinal segments. Thus, we pooled within- and across-population responses to determine whether there was an overall change in synaptic transmission as a function of age (Fig. 5). Notably, failure rate, paired-pulse ratio, and uEPSC coefficient of variation all significantly increased with age ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively; linear regression). These findings indicate a reduction in presynaptic release probability as animals mature. Notably, the variance in all cases was relatively large, leading to weak coefficient of determination (R^2) values (Fig. 5). Such a large variance suggests that corticospinal synapses are highly variable, and remain capable of substantial modification throughout development and into adulthood. Synaptic potency trended toward decreasing with age ($p = 0.08$); however, this was driven by a minority of responses with exceptionally high amplitudes in young animals (sixfold greater than the mean; Fig. 5*D*). Other measured response properties did not vary with age (Fig. 5), including onset latency ($p = 0.83$), rise time ($p = 0.17$), and decay time ($p = 0.22$).

Intrinsic excitability increases during maturation

Although changes to intrinsic neuronal properties have been well documented during early development (P0–P20; McCormick and Prince, 1987; Connors, 1994; Kasper et al., 1994; Zhang, 2004; Etherington and Williams, 2011), less is known about how properties are altered through postnatal development into maturity. With respect to the corticospinal system, changes from P20 and beyond may be of particular importance, as skilled motor behavior does not emerge until maturity (Martin, 2005). Therefore, alterations beyond the juvenile period are likely important for the evolution of fine motor control. We find that several properties related to neuronal excitability are altered during late development, beyond P20 (Fig. 6). Action potential threshold ($p < 0.01$; Fig. 6*A*) and afterhyperpolarization amplitude ($p < 0.001$; Fig. 6*B*) both decrease with age. Additionally, spike frequency in response to depolarizing current injections increases with age ($p < 0.01$; Fig. 6*C*). Thus, the intrinsic excitability of corticospinal neurons increases as the motor system matures.

We did not find progressive differences with age for resting membrane potential ($p = 0.37$), input resistance ($p = 0.37$), or for the spiking parameters of action potential half-width ($p = 0.21$) and spike height ($p = 0.22$; data not shown). Although such measures are known to change during development, these modifications occur mostly before the age ranges examined in the current study (McCormick and Prince, 1987; Kasper et al., 1994; Zhang, 2004).

Discussion

Connection probability between cortical neurons is associated with numerous factors, including intersomatic distance, laminar position, intrinsic electrophysiological properties, efferent

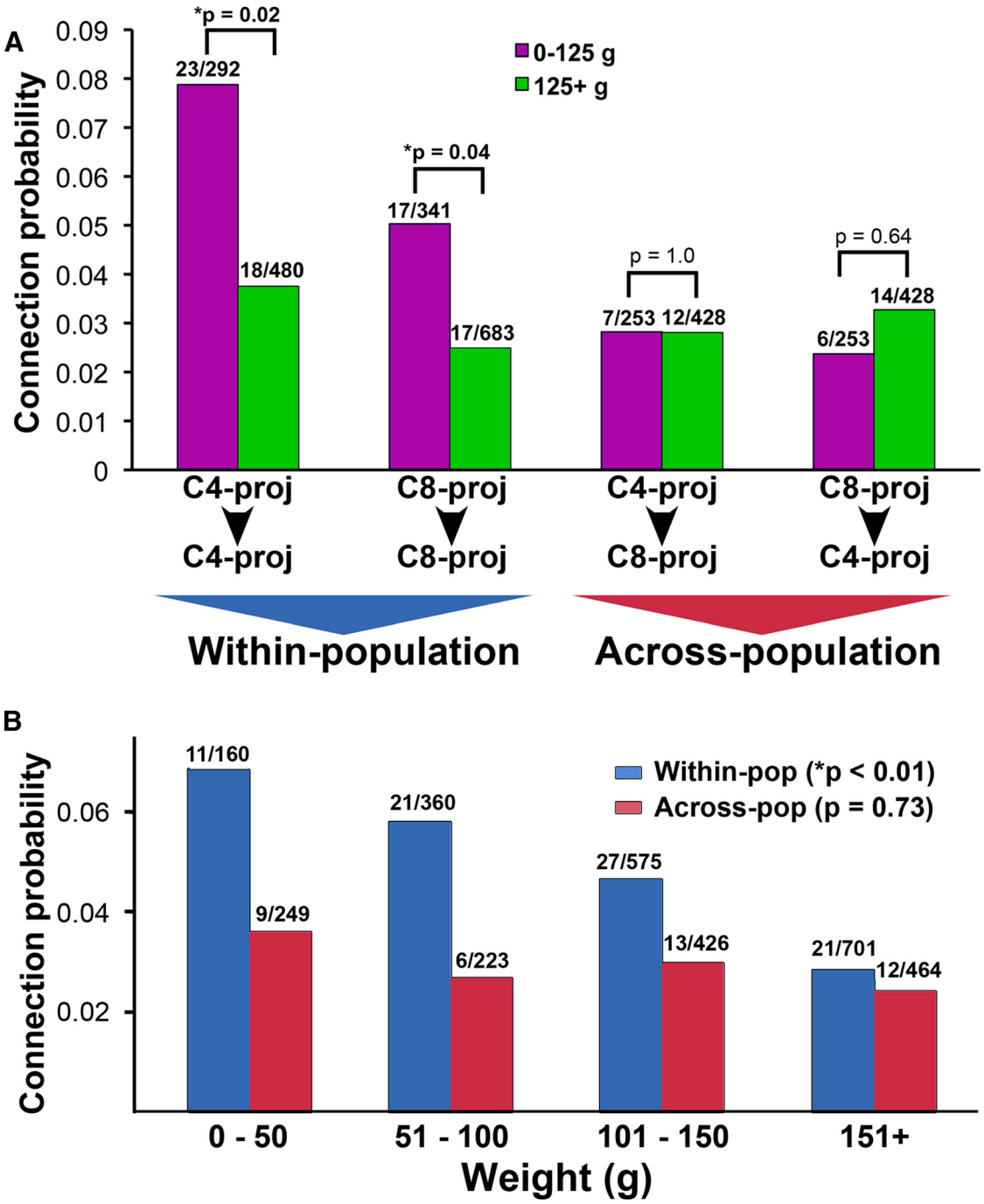

Figure 2. Age-related reductions of within-population connectivity occur among both C4–C4 and C8–C8 cell pairs. ***A***, Both C4–C4 and C8–C8 cell pairs exhibit a significant reduction in connection probability in maturity. Thus, elevated within-population connectivity appears to be a general property of the postnatal corticospinal system. Across-population connectivity did not change with age for either cell pair group (C4–C8 or C8–C4). The difference in connection probability between C4–C4 and C8–C8 cell pairs in developing (0–125 g) animals was not significant ($p = 0.14$, Fisher's exact test). Purple bars, 0–125 g; green bars, ≥125 g. ***B***, Connection probability, binned in 50 g animal weight increments, shows a progressive decline of connection probability specifically for within-population cell pairs (C4–C4 and C8–C8). Reported p values are based on logistic regression of connection probability over the entire (unbinned) weight range. proj, Projecting; pop, population.

target structures, animal age, and the functional relationship of neurons (Thomson et al., 2002; Markram et al., 2004; Bannister, 2005; Yoshimura et al., 2005; Morishima and Kawaguchi, 2006; Brown and Hestrin, 2009; Lefort et al., 2009; Anderson et al., 2010; Ko et al., 2011, 2013; Kiritani et al., 2012; Kaneko, 2013; Ueta et al., 2013). How developmental changes in synaptic patterning and transmission support the expression of mature behavior, however, is still unresolved. We now examine changes in corticospinal circuitry during the developmental time frame coinciding with the emergence of fine motor behavior. We find that, before maturation at P45, neuronal connectivity is more frequent between cells targeting the same spinal cord segment (within-population connectivity) versus different spinal segments (across-population connectivity); within-population connectivity is gradually reduced as the circuitry matures, until within- and across-

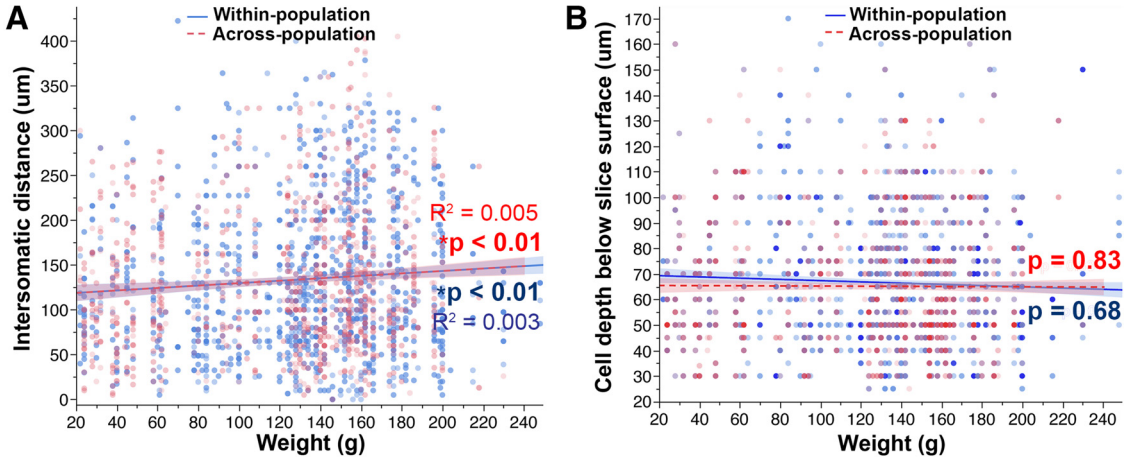

Figure 3. Intersomatic distance and depth of recorded neurons. ***A***, ***B***, Dots symbolize the intersomatic distance between corticospinal neurons in which synaptic connectivity was assessed (***A***) and corticospinal depth below the cutting surface for individual neurons belonging to within-population (blue) or across-population (red) cell pairs (***B***). Note: an individual cell can belong to both cell pair groups, as in a C8-projecting cell that was tested for connectivity with another C8-projecting cell (within population) as well as with a C4-projecting cell (across population). The distance between cell pairs increased with weight similarly for within- and across-population groups. The depth of recorded cells showed a marginal, though significant, tendency to decrease with weight for within-population neurons. Overlapping data are signified by increasing color intensity. Lines and shaded area indicate linear line of fit and 95% confidence of fit, respectively.

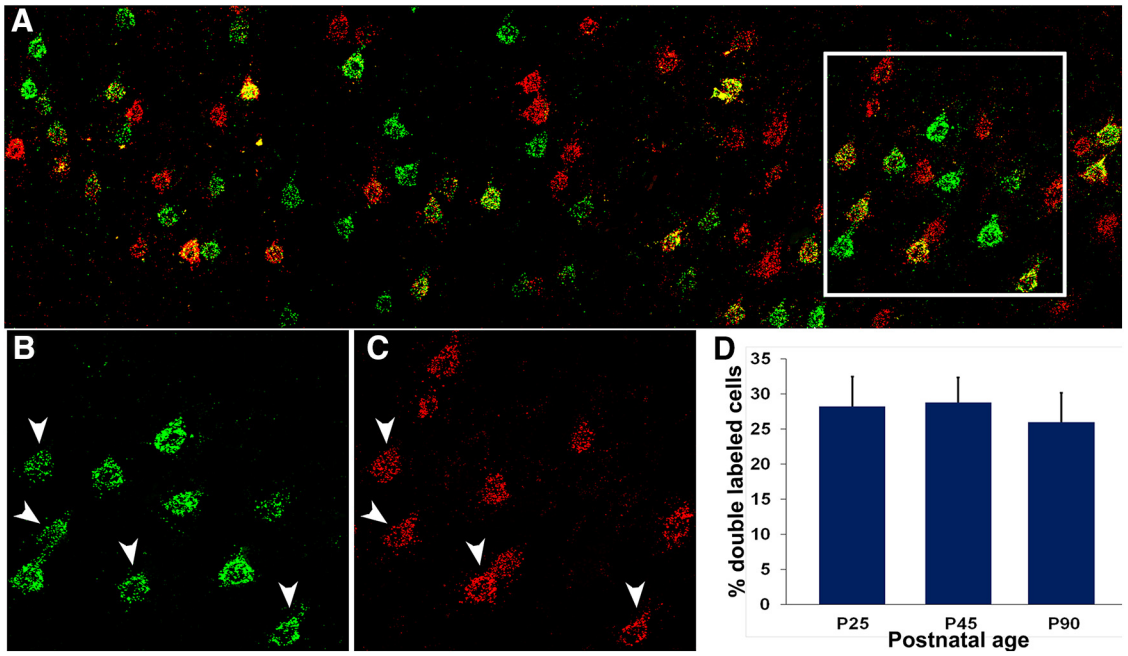

Figure 4. The proportion of layer 5 corticospinal neurons with axon collaterals in both C4 and C8 segments does not change with age. ***A***, Layer 5 of M1 caudal forelimb area shows intermingling of both C4-projecting (green) and C8-projecting (red) corticospinal neurons. A considerable minority of cells simultaneously expressed both retrograde tracers (yellow), presumably reflecting cells with collateral branches in both C4 and C8 segments of the spinal cord. Scale bar, 50 μm. ***B***, ***C***, Single-channel images of corticospinal neurons within the white box in ***A***. Arrowheads denote double-labeled cells. ***D***, The proportion of double-labeled cells was unaltered with age ($p = 0.9$). Only single-labeled neurons were targeted for electrophysiological recordings.

population connectivity are of uniform probability in adulthood. Accompanying this rearrangement of synaptic circuitry is a general reduction in synaptic efficacy and an increase in intrinsic excitability.

Developmental alterations in recurrent corticospinal circuitry

The overrepresentation of within-population synaptic coupling in immature animals (<P45) demonstrates that connectivity is initially favored between corticospinal neurons terminating within the same spinal segment. Such innate connectivity among anatomically, and presumably functionally, related neurons would almost certainly confer an advantage for the initial development of motor behavior. For example, during the time frame of our earliest recordings (~P20), corticospinal input to the spinal cord is relatively weak, as spinal terminals are not fully developed (Meng et al., 2004; Martin, 2005). Thus, increased interconnectivity during development (<P45) may serve to amplify descending signals by stimulating concerted activity among large corticospinal ensembles to generate cortically mediated movement. Nevertheless, many of these initial connections, such as coupling between corticospinal neurons controlling separate muscle pools that may be irrelevant for, or even interfere with, functional behaviors, may prove excessive or even counterpro-

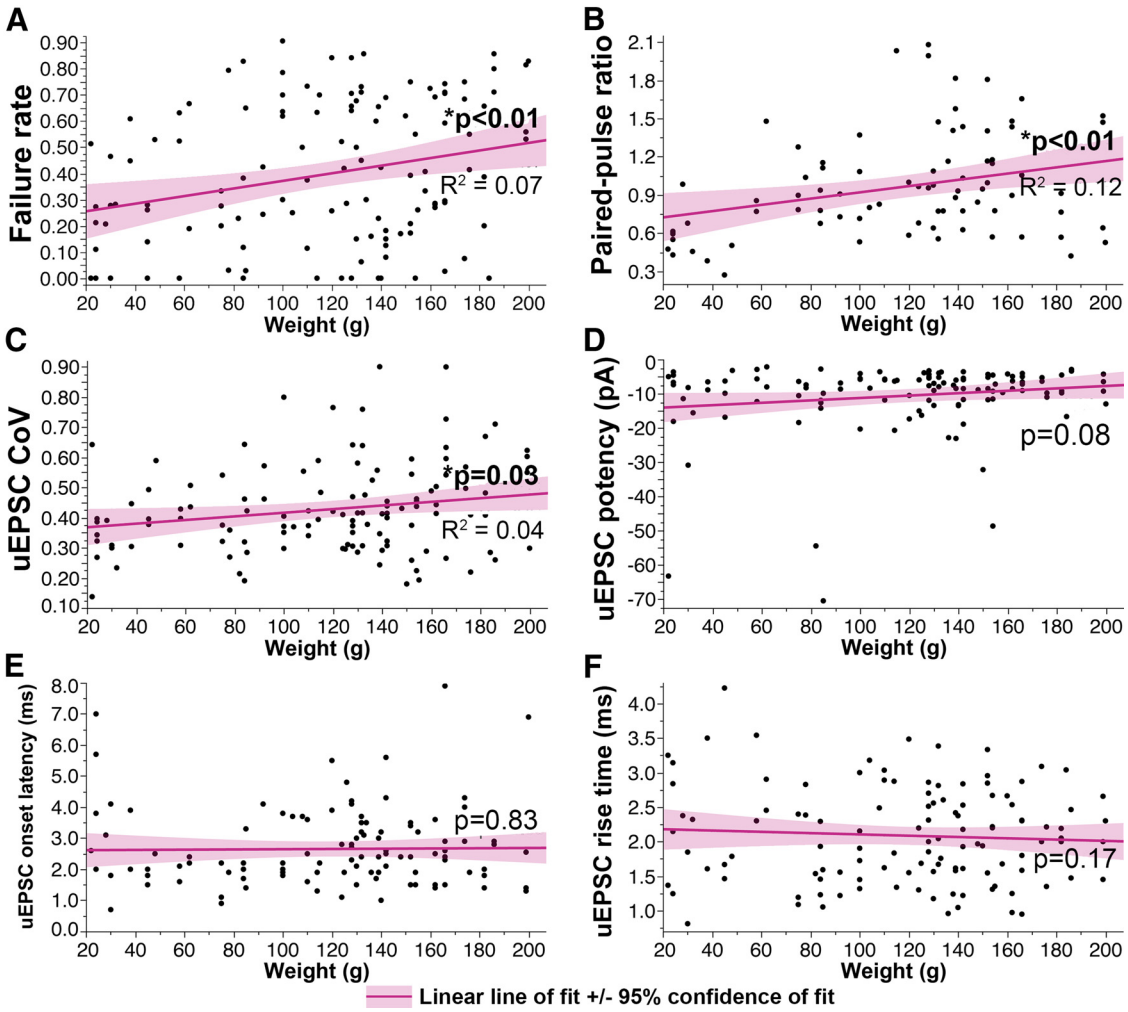

Figure 5. Presynaptic release probability decreases among recurrent corticospinal connections as the motor system matures. ***A–C***, Failure rate (***A***), paired-pulse ratio (***B***), and uEPSC coefficient of variation (CoV; ***C***) all showed a tendency to increase with age, suggesting a change in presynaptic function. However, note the large variability of these features, indicating that corticospinal inputs show wide functional variance and remain highly modifiable in adulthood. ***E***, ***F***, Other features, including response onset latency (***E***) and 20 – 80% of rise time (***F***), showed no change with maturation.

ductive. Only after these excessive connections are eliminated can fine motor control emerge, as evidenced by the progressive reduction in connectivity as animals mature. Reduced interconnectivity may also generate a greater number of independent networks, supporting enhanced fractionation and resolution of movement. In addition, the comparatively expanding role of across-population connections may be significant for the emergence of complex, multijoint behaviors during maturation (see below).

Whether the initial levels of across-population connectivity reflect random synaptic sampling or organized assembly remains to be determined. Moreover, some fraction of across-population cell pairs may in fact be within-population cell pairs due to axon collaterals that terminate in overlapping spinal segments (e.g., C4- and C8-projecting neurons with mutual collaterals in segment C6). Indeed, a minority of retrogradely labeled layer 5 corticospinal neurons contained both retrograde tracers that were injected into C4 and C8 across all ages examined, demonstrating the presence of axonal collaterals traversing both C4 and C8 spinal segments; we recorded only from single-labeled neurons to focus on functionally distinct neuronal populations.

Functional segregation of corticospinal subpopulations projecting to distinct spinal segments

In rats, the C8 spinal cord segment primarily contains lower motor neurons that activate muscles controlling distal forelimb movements of the wrist and digits (McKenna et al., 2000; Tosolini and Morris, 2012). Lower motor neuron pools located in the C4 spinal segment are principally associated with the control of proximal forelimb, shoulder, and neck musculature (Brichta et al., 1987; Callister et al., 1987). The extent to which these largely segregated pools of lower motor neurons, associated with unique aspects of motor function, are controlled by distinct populations of corticospinal cells is unknown. While retrograde tracing studies in rats and primates have consistently indicated that only a minority of corticospinal projections collateralize across upper and lower cervical spinal segments (Akintunde and Buxton, 1992; He et al., 1993; Wang et al., 2011), electrophysiological studies in cats have suggested that individual corticospinal neurons may collateralize more widely across cervical spinal segments (Shinoda et al., 1986). In addition, corticospinal projections in rats do not terminate directly on lower motor neurons but communicate with lower motor neuron pools through polysynaptic connections, including propriospinal, reticulospinal, and intersegmen-

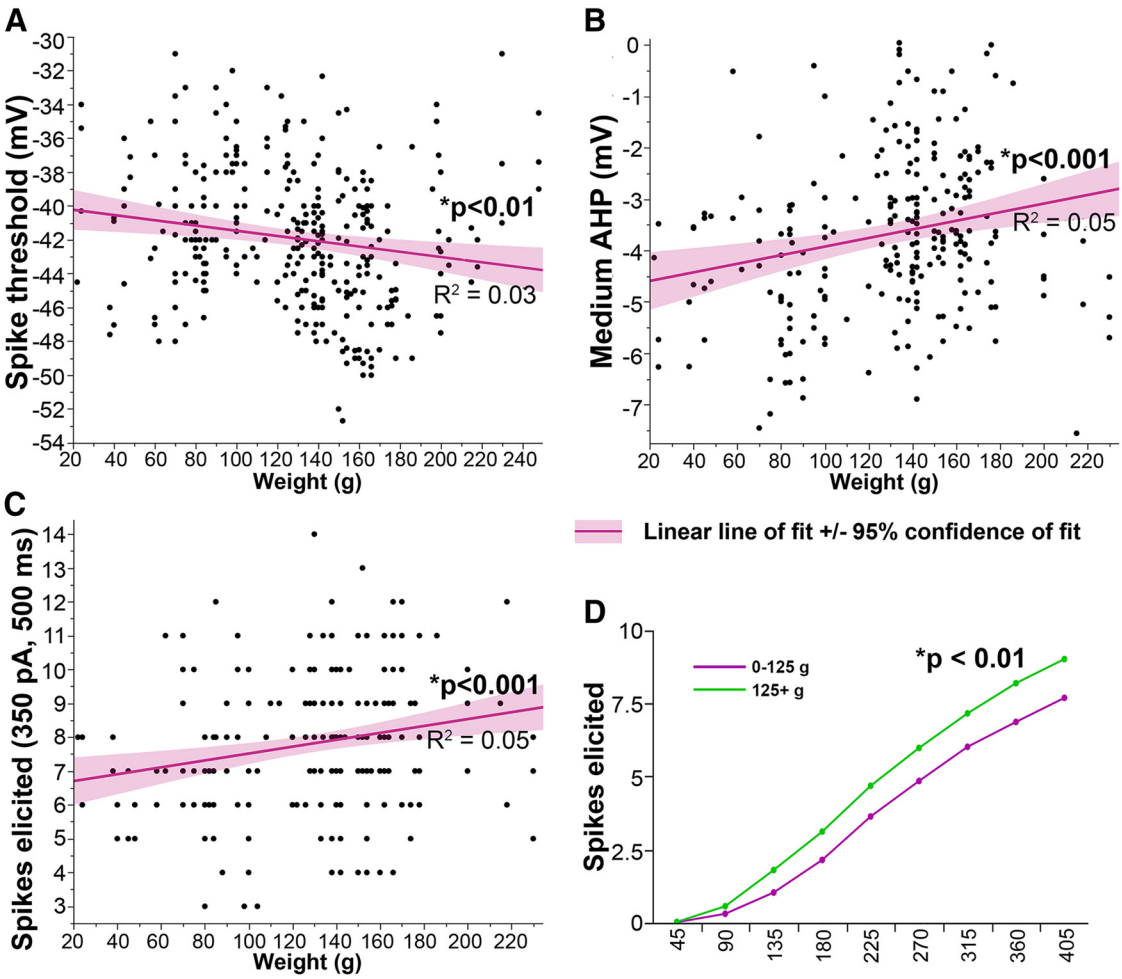

Figure 6. Intrinsic excitability increases over the course of development. ***A***, ***B***, Spike threshold (***A***) and peak medium AHP (***B***) decreased with age (note negative scale for medium AHP). ***C***, Spiking activity increased with age, as shown by the age-related increase in action potentials elicited in response to a 500 ms depolarizing current injection of 350 pA. ***D***, Furthermore, spiking activity was increased over multiple current injection levels for adult (125+ g: green) vs immature (0–125 g: purple) animals (repeated-measures ANOVA).

tal interneurons (Alstermark et al., 2004; Isa et al., 2007). Recent studies have supported a clear functional distinction between corticospinal neurons retrogradely labeled from the C8 spinal segment relative to those labeled from the C4 spinal segment: in response to skilled grasp training, a task that is associated specifically with the refinement of outputs controlling distal but not proximal forelimb movements (Kleim et al., 1998; Wang et al., 2011), corticospinal neurons specifically back-labeled from the C8 spinal segment elaborate numerous structural changes, including significant increases in spine density and dendritic complexity. In contrast, corticospinal neurons retrogradely labeled from the C4 spinal segment exhibit no structural change with skilled grasp training (Wang et al., 2011). These latter findings suggest a clear functional distinction between corticospinal populations projecting to the C8 or C4 spinal segments, indicating that C8-projecting cells may predominantly be involved with distal forelimb control.

Developmental changes in synaptic efficacy and intrinsic excitability

Unitary EPSC properties did not differ for within- or across-population cell pairs, suggesting that synaptic function is similar regardless of projection target similarity between individual neurons. We did, however, find a global trend for decreased presynaptic release probability as the motor system developed, consistent with findings for other neural populations and cortical regions (Reyes and Sakmann, 1999; Frick et al., 2007; Etherington and Williams, 2011; Ko et al., 2013). Furthermore, intrinsic excitability increased with age. These results are indicative of a developmental switch in how information is transferred between neurons: whereas single action potentials can more effectively transmit excitation in immature animals, mature circuits are more likely to rely on sustained activation for effective transmission. These different modes of signaling may promote different functions. For example, enhanced synaptic efficacy early on may be important for establishing and stabilizing nascent synapses (Feldmeyer and Radnikow, 2009). Over time, however, this relatively large synaptic strength could hinder fine motor control by allowing excessive synaptic connections to influence postsynaptic activity. For example, globally reducing synaptic strength would decrease the ability of functionally unrelated neurons to induce undesirable activity in recurrent targets, thereby decreasing noise within the motor system and destabilizing synapses between functionally disparate cell pairs via Hebbian or spike timing-dependent plasticity.

Complex, multijoint behavior and the progressive loss of within-population connectivity

That the age-related reduction in corticospinal neuron connectivity was restricted to cell pairs projecting to the same spinal cord

segment is intriguing and suggests that the role of within-population networks decreases over the time frame examined. Indeed, many connections among neighboring cortical neurons present during development might not be functionally beneficial in adulthood and would be lost with increasing experience-dependent plasticity (Katz and Callaway, 1992; Ko et al., 2013). Similarly, motor experience may drive reorganization of preliminary corticospinal networks when animals begin to engage in complex motor behaviors that require coordination not just among a particular muscle group, but across multiple body parts/spinal segments. This view is supported by intracortical microstimulation (ICMS) studies of M1: as the motor system develops and animals engage in complex movements, cortical stimulation increasingly evokes movement about multiple joints, instead of a single joint (Chakrabarty and Martin, 2000; Graziano et al., 2002; Martin et al., 2005; Ramanathan et al., 2006; Graziano, 2008). Because ICMS output is highly dependent on local signaling within M1 (Jacobs and Donoghue, 1991; Hess and Donoghue, 1994), this increase in multijoint representations may reflect an expanding role of across-population connections during maturation.

Conclusions

The results of this study show that the emergence of fine motor behavior coincides with several alterations within the network of corticospinal neurons in primary motor cortex. A progressive reduction in neuronal interconnectivity as maturity is reached may increase the number of independent corticospinal networks, thereby enabling greater fractionation of motor behavior, and allowing neurons with similar output to segregate into separate functional networks encoding distinct motor behaviors. This reorganization is likely experience dependent, and may be augmented by decreased synaptic efficacy ensuring that only functionally related cell pairs remain connected. Additionally, increased excitability promotes sustained activation of corticospinal neurons, amplifying descending signals and facilitating cortical control of movement.

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