Cholinergic systems are essential for late-stage maturation and refinement of motor cortical circuits

Dhakshin S. Ramanathan,1,3,4 James M. Conner,1 Arjun A. Anilkumar,1 and Mark H. Tuszynski1,2
1Department of Neurosciences, University of California, San Diego, La Jolla, California; 2Veterans Affairs Medical Center, San Diego, California; 3Department of Psychiatry, University of California, San Francisco, California; and 4Veterans Affairs Medical Center, San Francisco, California

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Ramanathan DS, Conner JM, Anilkumar AA, Tuszynski MH. Cholinergic systems are essential for late-stage maturation and refinement of motor cortical circuits. J Neurophysiol 113: 1585–1597, 2015. First published December 10, 2014; doi:10.1152/jn.00408.2014—Previous studies reported that early postnatal cholinergic lesions severely perturb early cortical development, impairing neuronal cortical migration and the formation of cortical dendrites and synapses. These severe effects of early postnatal cholinergic lesions preclude our ability to understand the contribution of cholinergic systems to the later-stage maturation of topographic cortical representations. To study cholinergic mechanisms contributing to the later maturation of motor cortical circuits, we first characterized the temporal course of cortical motor map development and maturation in rats. In this study, we focused our attention on the maturation of cortical motor representations after postnatal day 25 (PND 25), a time after neuronal migration has been accomplished and cortical volume has reached adult size. We found significant maturation of cortical motor representations after this time, including both an expansion of forelimb representations in motor cortex and a shift from proximal to distal forelimb representations to an extent unexplainable by simple volume enlargement of the neocortex. Specific cholinergic lesions placed at PND 24 impaired enlargement of distal forelimb representations in particular and markedly reduced the ability to learn skilled motor tasks as adults. These results identify a novel and essential role for cholinergic systems in the late refinement and maturation of cortical circuits. Dysfunctions in this system may constitute a mechanism of late-onset neurodevelopmental disorders such as Rett syndrome and schizophrenia.

THE DEVELOPMENT OF NEURAL CIRCUITS has been characterized across several distinct stages (Katz and Crowley 2002): a very early (i.e., prenatal/early postnatal) period of axonal path finding associated predominantly with intrinsic molecular and genetically specified cues (Kolodkin and Tessier-Lavigne 2011), followed by a subsequent period of synaptic refinement driven by both intrinsic (Feller 1999; Feller and Scanziani 2005; McLaughlin et al. 2003) and experience-dependent neural activity (Fox 1992; Friel et. al. 2005, 2007; Glazewski and Fox 1996; Glazewski et al. 1998; Hensch 2005). Most studies of this latter period of experience-dependent developmental plasticity in rodents have focused on the early, preadolescent developmental period that occurs before the end of critical periods (Feldman 2001; Fox 1992; Friel et al. 2005, 2007; Glazewski and Fox 1996; Glazewski et al. 1998; Hensch 2005). Although several studies have demonstrated that sensory cortical circuits continue to mature during adolescence (Daw et al. 1992; Fox and Wong 2005; Thomases et al. 2013; Zuo et al. 2005), there are few studies investigating later-stage, adolescent maturation of motor cortical systems. To gain further insight into adolescent development of motor systems, and particularly the development of fine distal motor control, we first characterized whether late-stage adolescent development is associated with a shift from proximal to distal forelimb representations, as would be predicted by maturation of skilled/fine motor control of the distal forelimb. Next, we examined whether the basal forebrain cholinergic system is required for later-stage refinement of motor cortical circuits. Prior studies demonstrate the importance of acetylcholine (Hohmann et al. 1998; Koh et al. 2005; Ricceri et al. 2002), phenotypic specification (Hohmann and Berger-Sweeney 1998; Koh et al. 2005; Ricceri et al. 2002; Zhu and Waite 1998), dendrite formation (Robertson et al. 1998; Sherren and Pappas 2005), and synaptic (Janesich et al. 2011) and neuronal function (Aramakis et al. 2000), ultimately impairing the development of cortical sensory representations (Baskerville et al. 1997; Bear and Singer 1986; Gu and Singer 1993; Kuczewski et al. 2005; Nishimura et al. 2002). However, the role of acetylcholine in subsequent refinement and maturation of cortical circuits (the later, adolescent period) is largely unknown. Indirect evidence suggests that adolescent cortical circuits are partially sensitive to cholinergic modulation. For example, exogenous delivery of nicotine results in lasting changes in metabotropic glutamate receptor (mGluR) expression and synaptic plasticity in the cortex specifically during adolescence (Counotte et al. 2011; Doura et al. 2008; Goriounova et al. 2012; Schochet et al. 2005; Slotkin et al. 2004). Despite this, no studies have directly assessed whether endogenous cholinergic systems are required for normal refinement of cortical circuits during late-stage development. Given the vast body of work implicating cholinergic systems in experience-dependent plasticity in adults (Bakin and Weinberger 1996; Conner et al. 2010; Dimyan and Weinberger 1999; Froemke et al. 2007; Kilgard and Merzenich 1998; Ramanathan et al. 2009; Rasmusson 2000; Rasmusson and Dykes 1988; Webster et al. 1991a, 1991b; Weinberger 2003, 2004, 2007), we hypothesized that cholinergic inputs exert an...
essential role in the maturation of adolescent cortical circuits associated particularly with establishment of distal forelimb motor skilled movements.

To probe these hypotheses, we divided our study into two parts. First, we characterized the temporal course of cortical motor map development from postnatal day 7 (PND 7) through maturity. We find that motor cortex first becomes organized into detectable functional domains at PND 15. By the time the isocortex reaches its mature size (by PND 25, it is roughly 80% of mature cortical volume; Calabrese et. al. 2013), distinct proximal and distal forelimb representations have emerged; these are subsequently modified throughout adolescence (PND 30–60). Second, we assessed the role of cholinergic systems in adolescent development of the motor cortex: we find that motor cortex first becomes organized during development in rodents has demonstrated that the isocortex

enlarges significantly until about PND 25 (Calabrese et. al. 2013), by which time it is almost at adult size. We focused our investigation on the maturation of motor cortical circuits after this time point to minimize the contribution that simple enlargement of cortex has on motor map changes. A total of 32 animals underwent electrophysiological mapping between PND 25 and 90 to characterize developmental changes in motor map topography.

Part 2. We next examined the role of cholinergic inputs to the cortex in supporting circuit maturation and refinement in later, adolescent stages of cortical motor development (Fig. 1 and Table 1). For this purpose, corticopetal cholinergic innervation was selectively removed at PND 24 (n = 11 male rats), a time period before onset of adolescence but after most cortical migration/volume expansion has occurred, as previously described (Calabrese et al. 2013). Cholinergic inputs were ablated using the selective immunotoxin 192-IgG-saporin (Advanced Targeting Systems, San Diego, CA). This immunotoxin consists of a ribosomal inactivator coupled to an antibody targeting the p75 neurotrophin receptor, which is only expressed in the forebrain by cholinergic neurons. 192-IgG-saporin lesions result in selective removal of cholinergic systems (Conner et al. 2003, 2005; Holmberg and Berger-Sweeney 1998; Kilgard and Merzenich 1998; Ramanathan et al. 2009; Robertson et al. 1998; Weinberger 2007) while preserving adjacent noncholinergic projection systems in both juvenile and adult rats (Conner et al. 2003, 2010; Leanza et al. 1996; Ramanathan et al. 2009). Controls were injected with artificial cerebrospinal fluid (ACSF; n = 11). After 192-IgG-saporin lesions were introduced at PND 24, rats survived to maturity. To examine the effects of juvenile cholinergic lesions on motor map topographies, we then performed terminal cortical mapping on 12 animals between PND 120 and 125 (6 192-IgG-saporin and 6 ACSF controls). In addition, to examine the effects of juvenile cholinergic lesions on behavior, 10 animals (5 192-IgG-saporin and 5 ACSF) underwent skilled forelimb reach training; again followed by terminal cortical mapping (between PND 120 and 125). At completion of the studies, brains were examined to verify completeness of cholinergic lesions. Three of these animals showed incomplete cholinergic lesions on post mortem histopathology and were excluded from further analysis (2 animals from the no-training group and 1 animal from the training group).

Animal Subjects

Fisher 344 rats were used in this study. In addition, further analysis was performed on motor maps derived from 12 rats used in a prior study by Calabrese et al. 2013. These 12 rats were injected with either 192-IgG-saporin or ACSF control as adults (PND 90), followed by terminal cortical mapping after 6 wk. To assess whether cholinergic lesions alone are enough to cause significant motor map changes (Ramanathan et al. 2009).

MATERIALS AND METHODS

Experimental Design

Part 1. We first characterized the normal postnatal development of cortical motor representations in Fisher 344 rats (Fig. 1 and Table 1). Forty-three male and female rats between the ages of PND 7 and 90 were mapped using standard intracortical microstimulation techniques (Conner et al. 2003; Kleim et al. 1998). Time points beginning at PND 7 were tested, but peripheral movements could not be evoked by intracortical microstimulation in rats younger than PND 15, indicating probable immaturity of the motor cortex and corticospinal circuitry at these early time points. Recent work quantifying volume changes during development in rodents has demonstrated that the isocortex...
study (Ramanathan et al. 2009). All procedures and animal care adhered strictly to AAALAC, Society for Neuroscience, and institutional guidelines for experimental animal health, safety, and comfort.

Cholinergic Lesions

192-IgG-saporin was diluted to a concentration of 0.375 mg/ml in ACSF. PND 24 rats were anesthetized using a mixture of ketamine, xylazine, and acepromazine and placed in a standard rat stereotactic apparatus (David Kopf Instruments, Tujunga, CA). A total of 0.5 μl of the 192-IgG-saporin immunotoxin [or vehicle (ACSF) in control animals] was pressure-injected intraparenchymally into the nucleus basalis magnocellularis at stereotaxic coordinates anterioposterior, −1.0 mm from bregma; mediolateral, ±3.0 mm from midline; and dorsoventral, 5.5 mm from cortex using a 27-gauge Hamilton syringe. Injections were carried out at a rate of 0.1 μl/min, and the injection needle was left in place for 3 min following the injection to permit diffusion of the injected liquid. After the final injection, rats were placed back into a standard housing environment (3 rats per standard Plexiglas cage) and matured beyond adulthood, after which animals underwent either electrophysiological mapping (at PND 120) or 4 wk of handling/behavioral training (starting at PND 75) followed by electrophysiological mapping starting at PND 115. ACSF- and 192-IgG-saporin-injected animals were housed randomly together in the same cages, allowing for identical handling and other conditions during development.

The effectiveness of 192-IgG-saporin lesions in depleting cholinergic cortical innervation was assessed using acetylcholinesterase (AChE) histochemistry, as previously described (Conner et al. 2003, 2005; Ramanathan et al. 2009). Briefly, rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and coronal brain sections were obtained on a cryostat at 40-μm intervals. A series of sections spaced 240 μm apart were processed for AChE histochemistry using the modified Tago method (Di Patre et al. 1993). Unbiased sampling techniques were used to estimate the degree of loss in cholinergic innervation to the sensorimotor cortex (Conner et al. 2001; Geula and Mesulam 1996). The density of cholinergic innervation to the sensorimotor cortex was estimated by counting the number of AChE-positive fibers crossing the two inclusion boundaries of a 15-μm counting frame. A stereology computer program (StereoInvestigator) controlled placement of the counting frame within a pre-scribed sampling area that included cortical layers II and III of sensorimotor cortex (Zilles et al. 1985). The distance between sampling sites was 150 μm. Previous studies have indicated that 192-IgG-saporin lesions result in >98% loss in cholinergic innervation to the sensorimotor cortex (Conner et al. 2003). Lesions producing a loss in cortical cholinergic innervation of <90% were considered incomplete, and these animals were excluded from additional analysis.

Functional Intracortical Microstimulation

Intracortical microelectrode stimulation (ICMS) techniques were used to map the motor cortex (Conner et al. 2003; Klein et al. 1997; Ramanathan et al. 2009). The experimenter was blinded to prior experimental treatment in all cases. Animals were anesthetized with ketamine hydrochloride (70 mg/kg ip) and xylazine (5 mg/kg ip) and received supplementary doses of the anesthesia mixture as needed to maintain animals in an adequately anesthetized state appropriate for motor mapping (stable respirations with intact foot pinch reflex). Recording procedures were completed more rapidly in the smaller brains of juvenile animals (<PND 60), and they did not require supplementary anesthesia. Pulled-glass stimulating electrodes (input impedance ~0.5 MΩ at 300 Hz) filled with 3 M NaCl were used. Microelectrode penetrations were made at 0.5-μm intervals at a depth of ~1,800 μm (corresponding to cortical layers V and VI). Stimulation consisted of a 30-ms train of 200-μs duration monophasic cathodal pulses delivered at 200 Hz from an electrically isolated, constant-current stimulator (ISO-Flex stimulus isolator; AMPI, Jerusalem, Israel) under the control of a programmable pulse generator (Master-8; AMPI). Pulse trains were delivered 1.2 s apart, and evoked movements were examined with the animal maintained in a prone position and the limbs supported in a consistent manner (see example of elbow movement shown in Fig. S1). (Supplemental material for this article is available online at the Journal of Neurophysiology website.) At each site, the current was gradually increased until a movement was detected (threshold current). If no movement was detected at a maximum stimulus intensity of 200 μA, the site was defined as “nonresponsive.” If multiple body parts were evoked at the minimal stimulation intensity, that region of cortex was interpreted to represent both body parts. The area of any particular motor representation was determined by multiplying the number of responsive sites evoking a movement of the forelimb by 0.25 mm² (the product of 0.5 × 0.5-mm sampling intervals); regions wherein stimulation evoked movement of two body parts were counted as one-half of the normal area. The outcome of this procedure is a somatotopic map of body part representations across motor cortex in animals, which was then analyzed for changes in either areal representation or the minimal stimulation required to evoke movements of certain body parts.

Motor map changes during development can be attributed to one of two basic phenomena: a simple enlargement of cortical volume and/or plasticity of neural systems that reflects maturation of cortical systems that underlie these motor representations. In this study, we were primarily interested in how cholinergic systems interfere with this latter aspect of development (i.e., later-stage changes in neural systems involved in motor control) and thus restricted analysis to time periods when there was minimal increase in volume. A recent quantitative study of changes in rodent brain size during development showed that the volume of the isocortex is almost complete (~80% of adult size) by PND 25 (Calabrese et al. 2013), with the remaining growth linear until adulthood. Thus we focused on motor map development that occurs after PND 25. For statistical analysis, we divided the developmental time period from PND 25 to PND 90 into four developmental epochs, PND 25–30 (preadolescence), PND 31–44 (early adolescence), PND 45–60 (late adolescence), and PND 90 (adulthood), based on prior estimates of rodent adolescence (Spear 2000). By dividing developmental epochs in this way, we were able to isolate changes in motor maps that occur during adolescence while minimizing potentially confounding effects of volume changes on these changes. For each stage of development, overall motor map size and the size of individual motor representations were determined for the caudal forelimb area (Conner et al. 2003, 2005; Ramanathan et al. 2006; Rouiller et al. 1993), an area associated with skilled forelimb behavior in rats (Conner et al. 2003; Klein et al. 1998, 2004; Rouiller et al. 1993).

Skilled Motor Training

Motor training was carried out using single-pellet retrieval boxes as described previously (Conner et al. 2003; Ramanathan et al. 2009; Whishaw et al. 1990). Animals were deprived of food for 2 days until they achieved 90–95% of their initial body weight. Food was then restricted to 1.5 large food pellets per day to maintain this weight, and supplemental food was given between training sessions if daily weight fell below 90% of baseline. Behavioral training was performed over 4 wk, consisting of 1 h of handling, food deprivation, and habituation to the testing apparatus, followed by 2) 11 days of skilled forelimb motor training. The reach task requires animals to extend their forepaw to reach through a small slit in a Plexiglas chamber and to grasp and retrieve a 45-mg food pellet positioned on a platform 2 mm away from the internal wall of the chamber. Animals performed 50–60 reaches per day. Total reaches, accuracy, and identity of preferred limb were recorded.

A “reach” was counted when the rat extended its forelimb through the slot. Percentage success consisted of the proportion of reaches that
resulted in successful pellet retrieval. The animal order of testing was randomized each day.

Statistics
Differences between two treatment groups were tested using analysis of variance (ANOVA) or Student’s t-test with a significance threshold of $P < 0.05$. Mapping studies, behavioral training, and histological quantification were performed with the examiner blinded to group identity. Analysis of covariance (ANCOVA) was performed to assess for developmental changes in motor map topographies while controlling for differences in stimulation intensities observed during development.

RESULTS
Development of Cortical Motor Representations
The first part of this study characterized the normal course of postnatal maturation of cortical motor representations in rats. We initially attempted to elicit movements with intracortical microstimulation between PND 7 and 14 and were unable to evoke any limb movements during this early time period. The first time point at which motor movements could be elicited was PND 15 (Fig. 2), indicating that electrophysiologically identifiable maps first form within this time frame. Motor maps then underwent a significant and continuing expansion in both size and topographic organization over time (Fig. 2). Before the onset of adolescence (between PND 25 and 30), cortical motor maps evoked through ICMS were small relative to their final adult size.

To characterize development, refinement, and maturation of the motor map, animals were divided into three previously identified developmental time periods: preadolescent (PND 25–30), early adolescent (PND 31–44), late adolescent (PND 45–60), and adult (PND 90) (Fig. 3) (Spear 2000). As stated

Fig. 2. Motor map development in Fisher 344 rats. Representative motor maps obtained from animals at 4 different time points. Motor maps are first identified at PND 15 and expand during the preadolescent period of PND 15–29 (PND 15 and 28 shown); before PND 15, intracortical microstimulation fails to elicit forelimb movements. Adolescence begins at PND 30, and representative maps are shown during early adolescence between PND 30 and 45 (PND 38 shown) and during late adolescence between PND 45 and 60 (PND 48 shown). During early adolescence, distinct movements from different body parts can be elicited and motor maps continue to expand in overall size and undergo significant topographic reorganization. Between PND 45–60 (PND 48 shown), motor maps have undergone refinement to more mature patterns with specific proportionate expansion of distal forelimb representations, including the wrist. Relative sizes of maps are shown. The cortex size/total volume panel (top right) is based on data from the Duke Center for In Vivo Microscopy (2014) and Calabrese et al. 2013.
previously, we focused our analysis on maturation of maps after PND 25 because of data suggesting that the cortex has reached almost adult volume at this time (Calabrese et al. 2013). Three aspects of development were quantified: enlargement of forelimb motor representations over time, reorganization of forelimb motor representations over time, and changes in the minimal stimulation intensities required to evoke movements over time (Young et al. 2012). A detailed analysis of individual movement representations within the caudal forelimb area (CFA; a caudal part of motor cortex encoding shoulder, elbow, wrist, and digit movements) revealed significant evolution through adolescence (Fig. 3A). The overall size of the caudal forelimb area (including all forelimb representations; shoulder, elbow, and wrist) increased throughout adolescent development [overall ANOVA: F(3,28) = 31.2, P < 0.0001]. This region enlarged significantly by 55% in early adolescence compared with the preadolescent group from 1.5 ± 0.25 to 2.3 ± 0.22 mm² (P < 0.05). It enlarged again in late adolescence, from 2.3 ± 0.2 to 4.0 ± 0.24 mm², an increase of 78% compared with the preadolescent group (P < 0.0001). There was no further change in the late-adolescent group compared with adult animals (P = 0.9). We subsequently broke this down for proximal and distal body parts. The shoulder representation showed quite different changes during development [overall ANOVA: F(3,28) = 5.7, P < 0.01]. After initially showing a trend for enlargement in early adolescence by 67%, from 0.8 ± 0.2 to 1.3 ± 0.18 mm² (P = 0.06), this region subsequently diminished in size by 73% in late adolescence, from 1.4 ± 0.2 to 0.37 ± 0.19 mm² (P < 0.001). Again, there was no further change in shoulder size in late-adolescent compared with adult animals (P = 0.5). By contrast, the distal forelimb representation (wrist and digits), associated with more complex forelimb movements such as grasping, enlarged most during later stages of adolescence [overall ANOVA: F(3,28) = 25.5, P < 0.0001]. There was only a small and nonsignificant increase in wrist area in early adolescence compared with preadolescence (0.6 ± 0.2 vs. 0.9 ± 0.2 mm²; P = 0.3). However, in late adolescence there was a large and significant enlargement in wrist size (from 0.9 ± 0.2 mm² in early adolescence to 2.1 ± 0.2 mm² by late adolescence, an increase of 140%; P < 0.0001). Again, there was only minimal (and nonsignificant) change between late-adolescent and adult animals (P = 0.4). The region representing elbow demonstrated a more consistent enlargement across both early and late adolescent time points [overall ANOVA F(3,28) = 6, P = 0.003]. The elbow representation expanded significantly by 54% during early adolescence, from 0.89 ± 0.17 mm² to 1.4 ± 0.15 mm² (P < 0.05). This was followed by a
smaller but still significant increase during late adolescence (from 0.89 ± 0.17 to 1.4 ± 0.15 mm², a 37% increase; \( P < 0.05 \)). Thus we find a general pattern in which the most proximal forelimb regions (i.e., shoulder regions) expand earlier during development, whereas the most distal forelimb representations (i.e., wrist) expand later. These map changes cannot be attributed simply to changes in volume during these time windows (at most, an additional 20–25% after PND 25 until adulthood, and generally linear over that time) (Calabrese et al. 2013). However, to ensure this is the case, we performed a follow-up ANCOVA in which we used isocortex volume (data extracted from Calabrese et al. 2013) as a nuisance covariate. All of the above findings remain significant.

To assess reorganization of forelimb motor cortical representations through adolescent brain development, we also quantified the percentage of the total forelimb region devoted to proximal vs. distal forelimb representations across developmental epochs. We found that significant changes occurred in the proportion of caudal forelimb devoted to both wrist [ANOVA: \( F(3,28) = 12.4, P < 0.001 \)] and shoulder [ANOVA: \( F(3,28) = 15.2, P < 0.001 \)], whereas no significant changes occurred in the proportion of forelimb cortex devoted to elbow over time [\( F(3,28) = 1.3, P = 0.3 \)]. Further breakdown reveals that the proportion of the caudal forelimb area allocated to the shoulder representation remained relatively constant during the early phase of adolescent development (36% and 37% of the total forelimb area during preadolescence and early adolescence, respectively) before undergoing a significant reduction in proportional representation during late adolescence, at which time it comprised only 8.5% of the total caudal forelimb area (Fig. 3A; \( P < 0.0001 \) for both preadolescent and early-adolescent animals compared with late-adolescent and adult animals). The distal forelimb representation (wrist and digits), which is most associated with the ability to perform fine motor skills (Conner et al. 2003), underwent an opposite pattern of development. Before the onset of adolescence, the distal forelimb representation comprised only 23 ± 5% of the caudal forelimb area, and this proportion remained relatively similar in early adolescence (25 ± 5%; \( P = 0.7 \)). By late adolescence, when more complex motor skills emerge, there was a significant increase in the size of the caudal forelimb area devoted to these distal forelimb regions (49 ± 6%; \( P < 0.01 \) for late adolescence compared with both earlier time points measured), a proportion nearly equal to that of adults (53 ± 5%; \( P = 0.3 \) compared with late adolescence; Fig. 3A). Thus, during adolescence, the cortical motor representation of the forelimb increases in overall size and is accompanied by a late-adolescent reorganization of the individual components comprising the forelimb representation, resulting in a significant shift in allocation within the caudal forelimb area from more proximal (shoulder) to more distal (wrist) representations. In addition to cortical map size, the minimum stimulation intensity required to evoke movements during ICMS paradigms is thought to be a useful measure of cortical excitation/inhibition and synaptic efficacy (Young et al. 2011, 2012). We measured mean stimulation threshold over developmental epochs and found a significant reduction only in the late adolescent period (Fig. 3B; ANOVA: \( P < 0.01 \); post hoc Fisher’s: \( P < 0.001 \) for late adolescent and adult periods compared with earlier periods).

It is important to address a potential confound that could influence these findings: differences in motor map size over developmental ages could simply reflect differences in anesthetic depth if very young animals were more deeply anesthetized, requiring greater stimulation currents to evoke movements. This possibility is not likely to have affected our findings for three reasons. 1) Differences in depth of anesthesia would most likely confound interpretation of the earliest developmental animals; thus we limited analyses to animals PND 25 and older. 2) Among the 12 animals mapped between PND 25 and 35, maximum stimulation thresholds were increased to 300 \( \mu A \), a stimulus intensity that exceeds the usual maximum threshold of 200 \( \mu A \) used in older animals; this high stimulation threshold had no effect on the evocation of caudal forelimb movements. Caudal forelimb cortex size after 300-\( \mu \)A stimulation changed by only 5% (from 1.95 to 2.07 mm²; \( P > 0.5 \)). 3) Furthermore, in these same 12 animals, we reassessed motor maps multiple times over the course of a 1- to 2-h period while monitoring anesthetic state visually (respiratory rate, motor tone) and using tail and foot-pinch reflex monitoring. In each subject, we terminated motor mapping when animals reached a state that required additional anesthesia (based on visual observations of respiratory rate and heightened response to tail pinch or foot pinch). Reported maps were constructed on the basis of the largest map that was evoked over the recording session. Thus, in these younger animals, significant care was taken to ensure that the largest possible motor maps were being evoked. Finally, our results are consistent with prior developmental motor mapping studies addressing other topics that have been conducted in both rodents (Young 2012) and cats (Chakrabarty and Martin 2000), demonstrating strong consistency across multiple labs and species. Collectively, it appears highly unlikely that our findings are a simple artifact of differing age sensitivity to anesthetic depth.

Expansion of the distal forelimb region during late stage development occurs in parallel with a reduction in threshold stimulation intensities. Whereas declining stimulation intensities are thought to be associated with maturation of cortical circuits during development (Chakrabarty and Martin 2000; Young 2012), differences in stimulation thresholds in adult animals are postulated to reflect variations in anesthetic state. To address the possibility that reduced stimulation intensities in development are a result of fluctuations in anesthetic state, we performed a standard analysis for assessing confounding variables (Maxwell et al. 1984), ANCOVA, wherein stimulation intensities are treated as a “nuisance” covariate while the ANOVA is performed across developmental periods (preadolescence, early adolescence, late adolescence, and adulthood). If the distal forelimb expansion during late adolescence is explained completely by changes in stimulation thresholds over time, the ANCOVA model would no longer detect a significant effect of age, nor would it detect significant differences in early vs. late adolescent distal forelimb areas on post hoc analysis. The findings of this analysis suggest no effect of anesthetic state: that is, even when controlling for stimulation intensities, an overall significant effect of development on map size is found for the entire CFA (\( P < 0.001 \)), with post hoc testing showing significant enlargement between both preadolescence and early adolescence (\( P < 0.05 \)) and between early and late adolescence (\( P = 0.001 \)), but not between late adolescence and adulthood (\( P = 0.7 \)). For the distal forelimb regions (wrist area) in particular, there was likewise an overall significant effect of development (\( P < 0.001 \)), with post hoc
testing revealing no significant differences between preadolescence and early adolescence ($p = 0.3$), significant differences between early and late adolescence ($p < 0.01$), and no changes between late adolescence and adulthood ($p = 0.2$). Collectively, statistical analysis continues to indicate a significant enlargement in distal forelimb regions even when for changes in stimulation thresholds with age are controlled for with the use of an ANCOVA model, demonstrating that the enlargement of distal forelimb regions during late adolescence cannot be explained simply by differences in anesthetic sensitivities.

### Basal Forebrain Cholinergic Mechanisms in Motor Map Formation During Development

The preceding characterization of motor development indicates that during late adolescence, there is a marked reorganization from a predominance of proximal to distal forelimb representations. Interestingly, such a shift is also known to result from skilled forelimb motor training, and this learning-induced reorganization is blocked by lesions of the cholinergic system in adult animals (Conner et al. 2003; Kleim et al. 1998). To characterize the role of cholinergic systems in this developmental shift of motor maps from a predominance of proximal forelimb representations to the mature pattern of predominant distal forelimb representations, we used the selective immunotoxin 192-IgG-saporin to specifically eliminate cholinergic inputs at PND 24. This developmental time point was chosen for several reasons: 1) as stated above, this time point allows for the evaluation of the role of cholinergic systems on late maturation of cortical circuits, as opposed to cholinergic involvement in changes in volume, axonal innervation, and other aspects of basic cortical patterning that occur much earlier in development (Bear and Singer 1986; Pappas et al. 1996; Robertson et al. 1998; Sherren and Pappas 2005). Related to this, cholinergic axons are growing into their innervation targets before PND 25: basal forebrain cholinergic axonal innervation of the cortex reaches mature levels at PND 16 (Calarco and Robertson 1995; Janiesch et al. 2011; Mecwar and Descarries 2001), and cholinergic activity measured by choline acetyltransferase and AChE assays reach adult levels between PND 25 and 30 (Thal et al. 1992). Thus a lesion at PND 24 allows for normal innervation of cholinergic projections to the cortex before lesion, likely minimizing potential compensatory changes that might occur with earlier cholinergic lesions. Additionally, prior evidence suggests that the 192-IgG-saporin toxin requires several days to completely eliminate cholinergic inputs (Book et al. 1994; Pappas et al. 1996); thus PND 24 lesions ensure complete destruction of cholinergic inputs before the onset of adolescence at PND 30. 2) 192-IgG-saporin injections resulted in complete lesions of the cholinergic system at this time point, but not before. Pilot studies in our laboratory were unsuccessful in generating consistently complete cholinergic lesions (defined as >90% loss of cholinergic fibers measured in adults) when 192-IgG-saporin was injected at earlier ages. This result is consistent with prior studies in which this immunotoxin was injected at earlier postnatal ages (PND 4–10), also demonstrating incomplete cholinergic lesions when assessed in adulthood, with reductions of cortical cholinergic innervation varying from 52% to 75% (Hohmann and Berger-Sweeney 1998; Koh et al. 2005; Leanza et al. 1996; Pappas et al. 1996; Ricceri et al. 1999, 2002; Zhu and Waite 1998). By contrast, 192-IgG-saporin delivered at PND 24 resulted in complete and long-term reductions of cholinergic neurons and their afferent target innervation >95%.

In all experiments, the effectiveness of developmentally induced cholinergic lesions was assessed histologically by quantifying the extent of depletion of cortical AChE terminal fiber labeling (Conner et al. 2003) (Fig. 4). Three animals with <90% depletion of cortical cholinergic innervation density (compared with vehicle-injected controls) were excluded from the study on the basis of this analysis. All remaining animals exhibited >90% reductions in cortical cholinergic axon density (average: 96 ± 2% decrease in sensorimotor cortex innervation compared with intact animals); only these latter subjects are reflected in the group numbers reported in this study. This degree of depletion is comparable to reports of cholinergic lesions by 192-IgG-saporin injections in adult animals (Conner et al. 2003, 2005; Ramanathan et al. 2009). Consistent with previous studies (Conner et al. 2003, 2005; Leanza et al. 1996), additional sections immunostained for parvalbumin, a marker for GABAergic interneurons within the basal forebrain, demonstrated that juvenile 192-IgG immunotoxin injections did not impact noncholinergic cell populations adjacent to the injection...
site. Following immunotoxic cholinergic lesions at PND 24, all animals were returned to their home cages and underwent intracortical microstimulation mapping at PND 120 to characterize motor map topography (a time point chosen to ensure complete development and full maturation of the motor cortex). Twelve animals were initially randomized to receive either ACSF or 192-IgG-saporin; subsequent analysis of cholinergic axons demonstrated that two of these animals had incomplete cholinergic lesions (<90%), and they were excluded from the analysis presented below, resulting in a total of 10 animals used in the final analysis (4 in the cholinergic lesion group and 6 in the ACSF group; Table 1).

Preadolescent cholinergic lesions significantly altered subsequent adolescent development of mature cortical motor maps compared with ACSF-infused controls (Fig. 5, A and B). The overall size of the caudal forelimb area (including both proximal and distal regions, i.e., elbow, wrist, and shoulder), when measured at PND 120 (adulthood), was significantly reduced by 33% in animals that underwent cholinergic depletion at PND 24 compared with vehicle-treated controls, from 5.3 ± 0.23 to 3.4 ± 0.28 mm² \( F(1,8) = 5.2, P < 0.01; \) Fig. 4C. A multivariate analysis across all of the regions noted above (elbow, wrist, and shoulder) showed an overall significant effect across all regions \( F(4,5) = 10.6, P < 0.05. \) However, this effect was driven entirely by a reduction in distal (i.e., wrist) forelimb representations \( F(1,8) = 31.5, P = 0.001, \) from 3.1 ± 0.1 to 2.0 ± 0.1 mm². By contrast, the elbow \( (P = 0.4) \) and shoulder \( (P = 0.3) \) representations were not significantly different between these two groups. Further analysis of other motor representations demonstrates that cholinergic lesions did not significantly affect maturation of other regions of the motor cortex, including representations for rostral forelimb area \( (0.58 ± 0.1 \text{ mm}^2 \) in ACSF animals vs. \( 0.55 ± 0.1 \text{ mm}^2 \) in 192-IgG-saporin animals, \( P > 0.8, \) neck \( (1 ± 0.14 \text{ mm}^2 \) in ACSF animals vs. \( 0.75 ± 0.18 \text{ mm}^2 \) in 192-IgG-saporin animals, \( P > 0.3, \) or vibrissa \( (1.6 ± 0.24 \text{ mm}^2 \) in ACSF animals vs. \( 2 ± 0.3 \text{ mm}^2 \) in 192-IgG-saporin animals, \( P > 0.3. \)) Thus cholinergic lesions performed after the emergence of cortical motor maps at PND 24 specifically abolished further maturation/enlargement of only distal caudal forelimb motor cortical representations that are required for the generation of more skilled and complex motor behaviors. These findings are consistent with adult lesions of cholinergic systems, showing that cholinergic systems are primarily important for mediating cortical motor map plasticity associated with skilled movements of the distal forelimb (Ramanathan et al. 2009).

Our primary hypothesis in this study is that cholinergic inputs have a direct effect on the developmental emergence of normal cortical motor representations. However, it is also possible that cholinergic mechanisms are simply required to maintain normal motor representations and that their elimination would result in alterations in evoked motor maps independent of development. To address this distinction, we reanalyzed data obtained from rats in a previous study (Ramanathan et al. 2009). In that study, 12 naive adult animals (male, 225–250 g, PND 90–120) received injections of either 192-IgG-saporin \( (n = 6) \) or vehicle \( (ACSF, n = 6) \) into the nucleus basalis magnocellularis and were returned to their cages for an additional 6 wk before undergoing intracortical microstimulation mapping. Analysis of these maps confirms that cholinergic lesions alone in mature animals had no effect on ICMS-evoked motor maps. Both overall size of the distal caudal forelimb area \( (4.23 ± 0.23 \text{ mm}^2 \) in 192-IgG-saporin animals compared with \( 4.14 ± 0.23 \text{ mm}^2 \) ACSF-injected animals, \( P > 0.8) \) and the size and organization of individual movement representations (unpaired \( t \)-test: \( P > 0.6 \) for elbow and wrist, 192-IgG-saporin compared with vehicle-injected groups) were comparable in vehicle and cholinergic-lesioned animals. These results are consistent with reports from prior studies that lesions of the cholinergic system do not affect established motor maps (Conner 2003; Ramanathan 2009).

**Behavioral Consequences of Adolescent Cholinergic Lesions**

In this study, we have shown that juvenile cholinergic lesions result in a specific reduction of caudal forelimb representations (particularly distal representations). Prior studies have demonstrated that plasticity within the caudal forelimb area of the motor cortex, and in particular distal forelimb representations, is essential for learning skilled motor behaviors in rats (Conner et al. 2003; Kleim et al. 1998; Ramanathan et al. 2009). Skilled motor learning is accompanied by significant plasticity within the caudal forelimb area (Conner et al. 2003, 2005, 2010; Kleim et al. 1998; Nudo et al. 1996;

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**Fig. 5.** Cortical motor map maturation requires cholinergic mechanisms. Sample motor maps demonstrate effects of juvenile cholinergic lesions on subsequent development of motor maps. A: cholinergically depleted animals (Chol lesion) had a smaller distal forelimb representation (yellow) compared with control (ACSF injected) animals. B: total distal caudal forelimb area (wrist, elbow, and shoulder area) was significantly reduced following SAP (**\( P < 0.01, \) \( t \)-test), driven primarily by a significant reduction in the size of the wrist representation (**\( P < 0.001 \).)**
Ramanathan et al. 2009) and focal cortical lesions within this region after learning abolishes skilled motor performance (Conner et al. 2005; Ramanathan 2006). However, the behavioral consequences of developmental cholinergic lesions on motor learning are unknown. Thus we performed a final experiment to assess whether these juvenile cholinergic lesions and the resulting reduction in distal forelimb representations result in impairments in skilled motor learning after animals are fully mature.

For this purpose, animals received 192-IgG-saporin injections (n = 5) or comparable injections of ACSF (n = 5) on PND 24. At PND 75 animals underwent 4 wk of skilled forelimb reach training. As in the prior analysis, all 192-IgG-saporin-lesioned animals included in the analysis demonstrated >95% reductions in cortical cholinergic innervation analyzed as adults (1 animal was excluded due to an incomplete cholinergic lesion, for a total of 9 animals analyzed, including 4 in the saporotoxin group and 5 in the control group; Table 1).

Consistent with the prior analysis, animals with developmental cholinergic lesions exhibited a significant, 35% reduction in distal caudal forelimb map size compared with ACSF controls (P < 0.01; Fig. 6A) and a 41% reduction in the size of the wrist representation (P < 0.001; mapping/analysis of cholinergic lesions were performed after skilled motor learning so as not to interfere with behavior). ACSF-injected animals exhibited the expected progressive increase in success rate of pellet retrieval over time (Fig. 6B; repeated-measures ANOVA; P < 0.05), whereas animals that received cholinergic lesions at PND 24 showed significantly impaired motor learning (Fig. 6B; repeated-measures ANOVA: main effect group, P < 0.05; group X time, P < 0.001; post hoc comparisons show significant differences starting on day 4). As previously demonstrated following cholinergic lesions in adult animals (Conner et al. 2003), learning was not completely abolishe in animals with juvenile cholinergic lesions (repeated-measures ANOVA: P < 0.001 over time for 192-IgG-saporin animals).

To compare anesthetic levels between the cholinergic lesioned and control animals, we performed an analysis of the minimal stimulation thresholds required to evoke caudal forelimb movements. Across the 19 animals included in this part of the study (8 animals with cholinergic lesions and 11 control animals), we found no significant group differences in threshold stimulation intensities (89.7 ± 6.7 μA in control animals vs. 99.1 ± 9.22 μA in cholinergic lesion animals, P = 0.4). Thus impairments in caudal forelimb development caused by juvenile cholinergic lesions are not likely attributable simply to differences in anesthetic state during motor mapping.

**DISCUSSION**

The present study reveals an essential role for basal forebrain cholinergic systems in the maturation and refinement of cortical motor circuitry during later stages of adolescent development, a period when corticospinal projections are established but fine motor control and dexterity are still evolving (Dayanidhi et al. 2013; Martin et al. 2004, 2005). We find that motor maps undergo a significant expansion in size and a reorganization of topography during this time period, with a shift from more proximal forelimb representations in early adolescence to a predominance of distal forelimb representations at maturity. Selective damage to the cholinergic system just before the onset of adolescence disrupts the expansion of distal forelimb representations and results in long-lasting impairments in skilled motor learning in adulthood. Importantly,
we do not find that adolescent cholinergic lesions entirely abolish motor maps: the effects of cholinergic lesions are specific only to reorganization/expansion of distal forelimb representations that mediate more skilled distal forelimb movements, a finding that is consistent with the specific importance in adulthood of cholinergic inputs to the cortex in shaping complex, but not simple, behavioral adaptations (Conner et al. 2010; Ramanathan et al. 2009).

During development there is a significant increase in the overall size of the forelimb motor representation. In addition, there is a significant reorganization of the individual components of forelimb function, ultimately resulting in a shift in allocation from more proximal (shoulder) to more distal (wrist) representations over time. Because this increase in size of the distal representation is disproportionate to the rest of the motor map, it does not reflect simple enlargement of this cortical region over time, but rather a refinement and reallocation of neural circuitry toward more skilled motor control of regions requiring greater dexterity (Kleim et al. 1998). This refinement of motor cortex development in rats parallels an earlier stage of cortical map development previously observed in cats (Chakraborty and Martin 2000), wherein proximal forelimb representations develop several weeks earlier than distal forelimb representations (Chakraborty and Martin 2000); the present study extends this observation to later-stage adolescent development. Early stages of motor map development seem to depend on maturation of the corticospinal tract and synapse formation within the spinal cord (Harrison et al. 2012; Martin et al. 2004, 2005). However, the ultimate expression of cortical maps is also heavily influenced by intracortical changes in excitation and inhibition (Friel et al. 2007; Jacobs and Donoghue 1991) and changes in synaptic and dendritic organization (Chakraborty and Martin 2000; Feldman et al. 1999; Florence and Kaas 1995; Huntley 1997; Wang et al. 2011). Given the importance of cortical cholinergic systems in late-stage enlargement of distal forelimb regions in particular, we suggest that this maturation is likely a result of cholinergic modulation of intracortical excitability or synapse maturation.

It is interesting that we observe motor map enlargement from preadolescent to early adolescent time periods despite a lack of expansion in cortical volume during this same time period. We speculate that two factors may be contributing to motor map enlargement during this time period of development: 1) enhanced innervation of the spinal cord (which in cats occurs during preadolescent time periods; Martin 2005), and 2) maturation of intracortical microcircuits. One model of such development may thus be that early enlargement of motor map topographies is associated with path finding/targeting of corticospinal neurons (Martin et al. 2004, 2005), whereas late adolescent changes are related to experience-dependent modifications of intracortical motor circuits, a process that is dependent on acetylcholine.

Cholinergic systems appear to specifically affect the maturation and expansion of distal forelimb motor representations, but not more proximal motor representations. As noted above, this finding is consistent with data from studies investigating the impact of cholinergic lesions on motor learning in adult animals. These prior studies indicate a specific requirement for cholinergic systems in modulating plasticity of distal forelimb motor circuits during highly demanding, skilled motor behaviors (Bakin and Weinberger 1996; Conner et al. 2003, 2010; Kilgard and Merzenich 1998; Ramanathan et al. 2009; Sarter et al. 2003; Stephan et al. 2009; Weinberger 2003). On the other hand, adaptations of the motor cortex that occur while acquiring simple motor tasks, or in expressing simple and rapid responses to injury, do not require cholinergic systems (Conner et al. 2003, 2005; Ramanathan et al. 2009). The establishment of more complex behaviors and adaptations appears to require greater reorganization of cortical circuitry, a need that may be met through the coordinated activity of multiple inputs converging on the key effector circuitry (Conner et al. 2010). In the case of skilled grasp motor learning, this key circuitry is located in the distal forelimb representation of the motor cortex, and the present findings suggest that acquisition of distal forelimb skills during development also requires complex rearrangements of neural circuitry, reflected by dependence on cholinergic inputs. We used 192-IgG-saporin lesions to remove basal forebrain cholinergic inputs to the cortex, a model that is well established (Pappas et al. 1996; Roberston et al. 1998); we presume the resulting perturbations in motor maps and motor behaviors are a specific consequence of reduced cholinergic activity in cortex, since there is no evidence that cholinergic neurons release other factors into cortex.

The finding that the cholinergic system is involved in the refinement of cortical circuits during adolescence may have clinical implications for neurodevelopmental disorders that develop or worsen in adolescence, such as schizophrenia (Lewis and Levitt 2002) and Rett syndrome (Cho and Zoghbi 2007). Indeed, the potential importance of aberrant cholinergic signaling has already been suggested as a possible mechanism in the development of schizophrenia: schizophrenics exhibit increased use of nicotine (Lohr and Flynn 1992), altered interactions between cholinergic and glutamatergic synapses (Timofeeva and Levin 2011), and abnormal regulation of cholinergic activity that may be related to hallucinations (Sarter and Bruno 1998). In addition, several animal models of schizophrenia, including adolescent delivery of kynurenic acid (an α7-nicotinic receptor antagonist and glycine-site NMDA antagonist) (Akagbosu et al. 2010; Trecartin and Bucci 2011) and neonatal ventral hippocampal lesions (Alexander et al. 2009; Brooks et al. 2011), implicate disrupted cholinergic signaling as a key factor in abnormal neurodevelopment. In the case of Rett syndrome, both early developmental changes and later-stage adolescent motor deterioration occur (Chahrour and Zoghbi 2007). Several rodent models of Rett syndrome (Guy et al. 2001; Shahbazian et al. 2002) demonstrate normal developmental into adolescence, followed by progressive neurologic and motor abnormalities (Guy et al. 2001; Shahbazian et al. 2002). More importantly, Rett syndrome has been directly linked to dysfunctional cholinergic transmission in number of both animal and post-mortem human studies of Rett syndrome (Brašić et al. 2012; Hohmann and Berger-Sweeney 1998; Kaufmann et al. 1997; Nag and Berger-Sweeney 2007; Ricceri et al. 2011, 2013; Wenk and Mobley 1996; Wenk et al. 1993). Our findings suggest that dysfunction of cholinergic systems may impair late developmental plasticity of frontal cortical circuits and thus may be one locus of dysfunction in delayed neurodevelopmental disorders involving frontal cortical circuits such as schizophrenia and Rett syndrome.
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AUTHOR CONTRIBUTIONS
D.S.R., J.M.C., and M.H.T. conception and design of research; D.S.R. performed experiments; D.S.R., J.M.C., and A.A.A. analyzed data; D.S.R., J.M.C., A.A.A., and M.H.T. interpreted results of experiments; D.S.R. and M.H.T. prepared figures; D.S.R. drafted manuscript; D.S.R., J.M.C., and M.H.T. edited and revised manuscript; D.S.R., J.M.C., A.A.A., and M.H.T. approved final version of manuscript.

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Several studies have investigated the role of cholinergic agents in the development of motor cortex. For example, Hohmann et al. (2011) found that neonatal lesions of the basal forebrain cholinergic system result in abnormal cortical development. Similarly, Delaney et al. (1998) reported that cholinergic neurons play a crucial role in the refinement of cortical maps.

Furthermore, the study by Nag and Berger-Sweeney (2002) suggested that cholinergic agents can influence synaptic plasticity. Their research showed that postnatal dietary choline supplementation alters the development of cortical circuits, particularly synaptic plasticity. These findings are consistent with a previous study by Slotkin et al. (2002), which demonstrated that neonatal lesions of the basal forebrain cholinergic system result in abnormal cortical development.

Additionally, Nishimura et al. (2006) reported that cholinergic agents could modulate the development of motor cortex. Their study demonstrated that increased levels of cholinergic activity could influence the development of motor cortex.

In conclusion, the role of cholinergic agents in the development of motor cortex is critical. Future research should continue to explore the mechanisms by which cholinergic agents influence motor cortex development and how these findings can be applied to clinical settings.


