Intracortical Pathway Involving Dysgranular Cortex Conveys Hindlimb Inputs to S-I Forelimb-Stump Representation of Neonatally Amputated Rats

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Stojic, Andrey S., Richard D. Lane, and Robert W. Rhoades. Intracortical pathway involving dysgranular cortex conveys hindlimb inputs to S-I forelimb-stump representation of neonatally amputated rats. J Neurophysiol 85: 407–413, 2001. Reorganization of the primary somatosensory cortex (S-I) forelimb-stump representation of rats that sustained neonatal forelimb removal is characterized by the expression of hindlimb inputs that are revealed when cortical GABA receptors are pharmacologically blocked. Recent work has shown that the majority of these inputs are transmitted from the S-I hindlimb representation to the forelimb-stump field via an, as yet, unidentified pathway between these regions. In this study, we tested the possibility that hindlimb inputs to the S-I forelimb-stump representation of neonatally amputated rats are conveyed through an intracortical pathway between the S-I hindlimb and forelimb-stump representations that involves the intervening dysgranular cortex by transiently inactivating this area and evaluating the effect on hindlimb expression in the S-I forelimb-stump representation during GABA receptor blockade. Of 332 S-I forelimb-stump recording sites from six neonatally amputated rats, 68.3% expressed hindlimb inputs during GABA receptor blockade. Inactivation of dysgranular cortex with cobalt chloride (CoCl₂) resulted in a significant decrease in the number of hindlimb responsive sites (9.5%, P < 0.001 vs. cortex during GABA receptor blockade before CoCl₂ treatment). Results were also compiled from S-I forelimb recording sites from three normal rats: 14.1% of 136 sites were responsive to the hindlimb during GABA receptor blockade, and all of these responses were abolished during inactivation of dysgranular cortex with CoCl₂ (P < 0.05). These results indicate that the S-I hindlimb representation transmits inputs to the forelimb-stump field of neonatally amputated rats through a polysynaptic intracortical pathway involving dysgranular cortex. Furthermore, the findings from normal rats suggest that this pathway might reflect the amplification of a neuronal circuit normally present between the two representations.

INTRODUCTION

Reorganization of the forelimb-stump representation in the primary somatosensory cortex (S-I) of rats with neonatal forelimb removal is characterized by the expression of unexpected hindlimb inputs, the majority of which are only revealed when the effects of cortical receptors for γ-amino butyric acid (GABA) are pharmacologically blocked (Lane et al. 1997). These findings led to the following question, what is/are the source(s) of the hindlimb inputs revealed during GABA receptor blockade? Earlier work suggested that hindlimb inputs expressed in the S-I forelimb-stump field of neonatally amputated rats reflected changes occurring at subcortical levels of the dorsal column-medial lemniscal pathway. Lane et al. (1995) demonstrated that neonatal forelimb removal in rats results in the invasion of cuneate nucleus by sciatric nerve afferents and the development of cuneate neurons (40%) with receptive fields that included both the forelimb-stump and hindlimb. Furthermore, Stojic et al. (1998) reported that 19% of neurons in the forelimb-stump representation of the ventral posterolateral (VPL) nucleus had receptive fields that included both a forelimb-stump and hindlimb receptive field, and that of these cells, 33% projected to the S-I forelimb-stump representation. However, recent work by Lane et al. (1999) suggests that these subcortical changes may contribute very little to the reorganization of the S-I forelimb-stump representation. Electrolytic lesions of the S-I hindlimb representation [or transient inactivation with cobalt chloride (CoCl₂)] of neonatally amputated rats reduces the number of hindlimb responsive sites in the S-I forelimb-stump representation by 80% (Lane et al. 1999).

The neural circuit by which these inputs from the S-I hindlimb representation reach the forelimb-stump field is not known. Latency data from Lane et al. (1999) suggest that these inputs might be conveyed via a polysynaptic pathway. In the current study, we test the possibility that hindlimb inputs to the S-I forelimb-stump representation of neonatally amputated rats are conveyed through a polysynaptic intracortical pathway between the S-I hindlimb and forelimb-stump representations which includes a synapse in the dysgranular cortex interposed between these two regions by transiently inactivating this dysgranular cortex during GABA receptor blockade.

METHODS

All protocols described here were developed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Medical College of Ohio.

Neonatal forelimb removal

Neonatal forelimb removals were carried out as previously described (Lane et al. 1995). Briefly, postnatal day 0 (>12 h old) rats

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were anesthetized by hypothermia, and the left forelimb amputated by making a circular incision through the skin and muscle distal to the shoulder. The shaft of the humerus was exposed and severed between its proximal and middle thirds. The skin was closed with cyanoacrylate adhesive following cauterization of the brachial artery and application of 0.7% bupivicaine. The pups were then rewarmed and returned to their mothers.

**Recordings from S-I**

A total of six rats that sustained neonatal forelimb removal and three normal rats were used in this study. Rats were initially anesthetized with ketamine (60 mg/kg) and xylazine (15 mg/kg) administered intraperitoneally and prepared for recordings as has been previously described (Lane et al. 1999). The trachea was cannulated, and the left brachial plexus and sciatic nerves were exposed. The animal was then placed in a stereotaxic head holder and ventilated mechanically. A bipolar stimulating electrode was placed on the brachial plexus, just proximal to the origins of the median, ulnar, and radial nerves, and another on the sciatic nerve ~15 mm distal to the sciatic notch. Rats were then paralyzed with gallamine triethiodide (30 mg, delivered intraperitoneally) and kept under light anesthesia during the recording session by adminstering urethan (1.1 g/kg) as needed. A midline incision was then made in the scalp. The cisterna magna was opened to drain cerebral spinal fluid, and a large craniotomy was made over the right cerebral cortex, contralateral to the amputated forelimb. Recordings from normal animals were also made from the right cerebral cortex, contralateral to the amputated forelimb.

Temporary inactivation of dysgranular cortex with CoCl₂

This procedure was modified from a protocol used to inactivate the S-I hindlimb representation by Lane et al. (1999). A 30 mM solution of CoCl₂ dissolved in saline was injected into 6–10 sites (0.2 μl/site) in dysgranular cortex around the anterior, posterior, and lateral borders of the S-I hindlimb representation (as determined by physiologic mappings). The injections were made at a depth of 600–800 μm below the pial surface. Inactivation of dysgranular cortex was determined by recording spontaneous activity and stimulus-driven neuronal activity with a saline recording electrode affixed along side the CoCl₂ injection pipette. Experimental observations indicate that a series of four mappings were made in each animal as outlined in Fig. 1. First, the forelimb-stump and hindlimb representations were mapped under normal anesthetized conditions (Fig. 1A). After this, the GABA receptor blockers bicuculline methiodide (BMI, for GABA_A) and saclofen (SAC, for GABA_B) were applied to the surface of the cortex (methods described in the following text), and the forelimb-stump representation was remapped to identify recording sites in the S-I forelimb-stump representation that expressed dual forelimb-stump and hindlimb receptive fields (Fig. 1B). Following identification of these dual receptive field sites in the S-I forelimb-stump field, the dysgranular cortex interposed between the hindlimb and forelimb-stump fields was temporarily inactivated with a series of CoCl₂ injections as described in the following section and the forelimb-stump representation was remapped (Fig. 1C). Finally, the S-I forelimb-stump recordings sites were reevaluated after the effects of the CoCl₂ injections into dysgranular cortex had dissipated (Fig. 1D).

**Application of BMI and SAC to the cortical surface**

Blockade of GABA receptors was accomplished using methods previously described (Lane et al. 1997). In brief, a 30 μl solution containing equal parts of 50 μM BMI and 50 μM SAC was applied to the cortical surface. Experimental observations have previously demonstrated that the antagonists are effective by 10 min following application and continue to be effective at blocking GABA receptor activity for ~30 min (Stojic et al. 2000), a time sufficient to complete mapping of the S-I forelimb-stump representation. If more than 30 min was required to complete a particular mapping sequence, additional 30 μl aliquots of the GABA blockers were applied as needed.

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single injection was effective in silencing an area of cortex with a radius of ~250 μm for ~30 min. The spread of CoCl₂ from the injection sites as just described opened the possibility their direct effect could have extended into parts of the S-I hindlimb and forelimb-stump representations. This issue was resolved by monitoring all recording sites within the S-I hindlimb and forelimb-stump regions during CoCl₂ inactivation. If ≥10% of recording sites in either representation became unresponsive to stimulation of their principle cutaneous region of innervation, the mapping experiment was suspended to allow the effects of CoCl₂ to dissipate, then fresh CoCl₂ injections were made into dysgranular cortex and the mapping experiment resumed.

Identification of recording sites

Specific recording sites in the S-I forelimb-stump and hindlimb representations, as well as the sites of CoCl₂ injection into dysgranular cortex, were marked by making small electrolytic lesions at each site (1–2 s duration, 3–4 V). After this, animals were killed with a lethal dose of CO₂ and perfused transcardially, first with a 0.9% heparanized phosphate buffer saline, followed by a 4% paraformaldehyde solution. The brain was then allowed to postfix overnight. Following this, the brain was extracted, and the right cerebral cortex was removed, flattened and sectioned into 50 μm sections on a freezing microtome. The sections were then processed for cytochrome oxidase (CO) using the methods of Wong-Riley (1979).

Analysis of physiologic data

The results from the six neonatally amputated rats and three normal rats were used to compare the percentage of dual forelimb-stump and hindlimb responsive recording sites within the S-I forelimb-stump representation under four conditions: prior to GABA receptor blockade; during GABA receptor blockade, prior to silencing of dysgranular cortex; during GABA receptor blockade and silencing of dysgranular cortex; and after recovery from CoCl₂ treatment. These data were evaluated statistically using a two-way repeated measures ANOVA. Mean differences in percentages of recording sites with dual forelimb-stump and hindlimb receptive fields under pairs of treatment conditions described just above were evaluated using Scheffe post hoc analysis. The accepted level of significance was $P < 0.05$.

RESULTS

Effect of CoCl₂ inactivation of dysgranular cortex on the expression of hindlimb receptive fields in the S-I forelimb-stump representation of neonatally amputated rats

Inactivation of dysgranular cortex resulted in a significant reduction in the expression of hindlimb receptive fields in the S-I forelimb-stump representation revealed during GABA receptor blockade. An example of this is illustrated in the case from a neonatally amputated rat shown in Fig. 2. Prior to GABA receptor blockade, 1 of 57 (1.8%) recording sites in the S-I forelimb-stump field was responsive to hindlimb stimulation (not shown). When the effects of cortical GABA receptors were blocked with BMI and SAC, 35 sites (61.4%) demonstrated responsivity to the hindlimb (Fig. 2A) in addition to the forelimb-stump. This is consistent with previous results (Lane et al. 1997, 1999). CoCl₂ inactivation of dysgranular cortex resulted in the selective loss of hindlimb expression at most of these sites (31 of 35 sites, 88.5%, Fig. 2B). In this case as well as all others, CoCl₂ inactivation of the dysgranular cortex caused <10% of the recording sites in either representation to become unresponsive to stimulation of their principle cutaneous region of innervation.

Figure 3 shows physiologic recordings made from electrodes placed in the S-I forelimb-stump field, dysgranular cortex, and hindlimb representation during CoCl₂ inactivation of dysgranular cortex (see Fig. 2C for positions of recording electrodes and sites of CoCl₂ injections). Note that the loss of hindlimb receptive field expression in the S-I forelimb-stump representation corresponded with CoCl₂-mediated inactivation of neuronal activity in dysgranular cortex while expression of forelimb persisted. Recording sites within the hindlimb representation remained responsive to cutaneous hindlimb stimulation and sciatic nerve activation throughout the experiment.
After the effects of CoCl$_2$ were allowed to dissipate, hindlimb responses returned to all of the recording sites in the S-I forelimb-stump representation that expressed dual receptive fields prior to CoCl$_2$ treatment.

Of 332 S-I forelimb-stump recording sites from six neonatally amputated rats that were responsive to forelimb-stump stimulation, 3.1% were also responsive to hindlimb stimulation prior to GABA receptor blockade (Fig. 4, $n$ before GRB). During cortical GABA receptor blockade, 68.3% of the forelimb-stump recording sites became dually responsive to hindlimb as well as forelimb-stump stimulation ($P < 0.001$ vs. unblocked cortices, Fig. 4, $n$, during GRB). During inactivation of dysgranular cortex with CoCl$_2$, only 9.5% of forelimb-stump sites remained responsive to stimulation of the hindlimb ($P < 0.001$ vs. cortex during GABA receptor blockade and CoCl$_2$ inactivation, Fig. 4, $n$, GRB + CoCl$_2$). Following recovery from CoCl$_2$ treatment, most of the forelimb-stump recording sites (66.6 ± 26.9% of all forelimb-stump recording sites) that expressed dual (forelimb-stump/hindlimb) receptive fields prior to CoCl$_2$ treatment again showed hindlimb responsivity ($P > 0.05$ versus GABA receptor blocked cortex prior to CoCl$_2$ treatment, Fig. 4, $n$, recovery + GRB).

A total of 136 S-I forelimb recording sites from three normal adult rats were tested. Prior to GABA receptor blockade, 1.9% of 136 recording sites responded to hindlimb stimulation in addition to the forelimb (Fig. 4, $n$, before GRB). During GABA receptor blockade, the number of hindlimb responsive
sites increased to 14.1 \pm 6.0\% (Fig. 4, \textit{during GRB}). This sevenfold increase was not statistically significant primarily due to the small sample size. In a previous study (Lane et al. 1997) in which six normal rats were tested, a significant difference was observed between the number of hindlimb responsive sites present prior to versus during GABA receptor blockade. Inactivation of dysgranular cortex between the S-I hindlimb and forelimb-stump representations of normal rats totally abolished all hindlimb responses within the S-I forelimb field (0\%, \(P < 0.05\) vs. GABA receptor blocked cortex prior to CoCl$_2$ treatment, Fig. 4, \textit{GRB + CoCl$_2$}). Following recovery from CoCl$_2$ treatment (while maintaining GABA receptor blockade), hindlimb responses returned to all S-I forelimb recording sites that prior to CoCl$_2$ treatment were responsive to the hindlimb (Fig. 4, \textit{recovery + GRB}).

Role of intracortical circuit in the expression of hindlimb inputs in the S-I forelimb-stump representation of neonatally amputated rats

The results of this study indicate that the majority of hindlimb inputs expressed in the reorganized S-I forelimb-stump representation of neonatally amputated rats during GABA receptor blockade are transmitted through a polysynaptic intracortical pathway between the S-I hindlimb and forelimb-stump representations. These findings are consistent with other studies that have indicated that intracortical connections play an important role in the functional reorganization of adult cortical representations that follow nerve injury (Darian-Smith and Gilbert 1994; Das and Gilbert 1995; Doetsch et al. 1988; Florence et al. 1998; Jacobs and Donoghue 1991; Pons et al. 1991). However, these results, and those of Lane et al. (1999) differ from previous reports in that the intracortical pathway described here does not involve direct connections between the S-I hindlimb and forelimb-stump fields (see Lane et al. 1999; Stojic et al. 1998). Rather, the intracortical circuit described in this study involves neuronal elements that are not only within the S-I hindlimb and forelimb-stump representations, but between the two fields in the region of dysgranular cortex (Chapin et al. 1987; Fabri and Burton 1991). The presence of hindlimb-forelimb-stump circuit synapses within this region is supported by the following evidence: the ability of CoCl$_2$ treatment of dysgranular cortex to inactivate this circuit; and the fact that CoCl$_2$ blocks activity at synapses (Malpeli 1999) but does not disrupt the activity of fibers of passage.

The development of this polysynaptic intracortical pathway through dysgranular cortex could result from one of two mechanisms. First, this circuit could represent the maintenance or amplification of an intracortical pathway normally formed during development (Hirsch and Gilbert 1993; Jenkins et al. 1990; Kolb et al. 1994; Li and Waters 1996; Li et al. 1996). Conversely, this pathway might reflect the emergence of a new connection as a result of peripheral nerve injury, perhaps
Although the role of VPL in hindlimb expression in the S-I hindlimb representation involves axon collateral sprouting (Darian-Smith and Gilbert 1994; Florence et al. 1998). The present results suggest that the intracortical circuit involved in transmitting hindlimb inputs to the S-I forelimb-stump representation of neonatally amputated rats reflects the strengthening or amplification of a circuit that is normally present between the S-I hindlimb and forelimb-stump fields. This view is supported by the data from the three normal rats in which the dysgranular cortex was inactivated with CoCl₂, causing the hindlimb inputs to the few sites within the S-I forelimb field of normal rats revealed during GABA receptor blockade to be completely abolished. Our physiologic data indicate that a polysynaptic pathway from S-I hindlimb to dysgranular cortex to S-I forelimb is present in both normal and neonatally amputated rats. This conclusion is further supported by the results of tracing experiments that showed that the S-I hindlimb representation projects to dysgranular cortex and dysgranular cortex sends axons to the S-I forelimb representation in both normal and neonatally amputated rats (Chapin et al. 1987; Fabri and Burton 1991; Stojic et al. 1998).

Role of cortical and subcortical change in the development of hindlimb inputs in the S-I forelimb-stump representation of neonatally amputated rats

The degree to which experience-induced functional changes at the cortical level can be explained by subcortical reorganization has been a long standing issue in plasticity research (e.g., Darian-Smith and Gilbert 1994; Faggin et al. 1997; Florence and Kaas 1995; Kiss et al. 1994; Panetos et al. 1995; Rasmussen and Northgrave 1997; Tinazzi et al. 1997). Many studies have documented the capacity for functional reorganization in the brain stem (Jain et al. 2000; Kalaska and Pomeranz 1979, 1982; Northgrave and Rasmusson 1996; Panetos et al. 1995; Waite 1984), thalamus (Alloway and Aaron 1996; Chiaia et al. 1992; Davis et al. 1998; Florence et al. 2000; Garraghty and Kaas 1991; Jones and Pons 1998; Kiss et al. 1994; Nicolelis et al. 1991, 1993; Rasmussen 1996; Rhodes et al. 1987; Verlay and Onnen 1981), and cortex (Aglioti et al. 1994; Calford and Tweedale 1991; Diamond et al. 1993; Florence et al. 2000; Fox 1994; Halligan et al. 1993, 1994; Kelahan et al. 1981; Mezernich et al. 1983; Pascual-Leone and Torres 1993; Pons et al. 1991; Rasmussen and Turnbull 1983; Rasmussen et al. 1982; Recanzone et al. 1992a,b) in response to changes in sensory experience. The relationship between changes occurring at subcortical levels to rearrangements in cortex is not well understood. The data presented in this study, along with the previous work (Lane et al. 1995, 1997, 1999; Stojic et al. 1998) strongly suggest that reorganization of the S-I forelimb-stump representation in neonatally amputated rats is principally the result of intrinsic cortical changes, and to a far lesser degree, a reflection of changes occurring at subcortical levels of the dorsal column-medial lemniscal pathway. The data presented here, and the study of Lane et al. (1999) indicate that most (>80%) of the hindlimb inputs expressed in the S-I forelimb-stump field are derived from a polysynaptic intracortical pathway between the S-I forelimb-stump and hindlimb representations involving dysgranular cortex. The remaining hindlimb responsive sites (<20%) are likely to reflect inputs derived from VPL neurons that express dual forelimb-stump and hindlimb neurons (Stojic et al. 1998). Although the role of VPL in hindlimb expression in the S-I forelimb-stump field has not been directly tested, recent work by Stojic et al. (1999) indicates that the hindlimb inputs that remained following lesioning of the S-I hindlimb representation reflect dual forelimb-stump and hindlimb inputs transmitted from the reorganized cuneate nucleus to the S-I forelimb-stump field via the thalamus.

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