Local GABA Receptor Blockade Reveals Hindlimb Responses in the SI Forelimb-Stump Representation of Neonatally Amputated Rats

Charles P. Pluto, Richard D. Lane, and Robert W. Rhoades
Department of Anatomy and Neurobiology, Medical College of Ohio, Toledo, Ohio 43614
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INTRODUCTION

Peripheral and CNS lesions can lead to structural and functional changes at spinal, brain stem, thalamic, and cortical levels of the mammalian somatosensory system (for reviews, see Dykes 1997; Jones 2000a; Kaas 2000; Wall et al. 2002). The age of the animal and the type of injury are important factors in the nature and extent of reorganization (Bowles et al. 2003; Kalaska and Pomeranz 1979; McKinley and Smith 1990; Pluto et al. 2003). Adult rats with one forelimb amputated on the day of birth exhibit sprouting of sciatic nerve fibers from the gracile into the deafferented cuneate nucleus, and >40% of cuneate neurons respond to stimulation of the stump as well as the ipsilateral hindlimb (Lane et al. 1995). The incidence of stump/hindlimb responsive sites in the contralateral ventroposterolateral thalamus is 19%, whereas in the primary somatosensory cortex (SI) forelimb-stump representation, it is only 5% (Stojic et al. 1998). However, when cortical GABA\(_{A+B}\) receptors are blocked (GRB), nearly 40% of SI forelimb-stump sites are stump/hindlimb responsive (Lane et al. 1997). Thus cortical GABA receptors actively suppress hindlimb inputs from being expressed by neurons in the reorganized SI forelimb-stump representation. The location where GABA acts to suppress this hindlimb-to-forelimb-stump circuit could not be determined in our previous studies, since GABA antagonists were applied topically to all of SI (global-GRB). Under conditions of global-GRB, electrolesioning the SI hindlimb representation and inactivating synapses in the intervening dysgranular cortex with cobalt chloride both significantly reduce the number of stump/hindlimb responsive sites (Lane et al. 1999; Stojic et al. 2001). This suggests that a polysynaptic, intracortical pathway conveys inputs from the SI hindlimb representation, to dysgranular cortex, to the SI forelimb-stump representation. It also leaves open the possibility that suppression of hindlimb inputs could occur either at, or some distance from, stump/hindlimb responsive neurons (Stojic et al. 2000).

In this study, localized injections of a GRB solution were targeted at three distinct cortical areas involved in the hindlimb-to-forelimb-stump circuit. Receptive fields of forelimb-stump neurons were assessed under normal conditions, during global-GRB, and sequentially during local GRB at 1) the SI forelimb-stump recording site, 2) the dysgranular cortex between the hindlimb and forelimb-stump representations, and 3) the SI hindlimb representation.

METHODS

Neonatal forelimb amputation

Neonatal forelimb amputations were carried out using methods previously described (Lane et al. 1995). Within 12 h of birth, rat pups were anesthetized by hypothermia until immobile. The left forelimb was amputated just distal to the shoulder with iridectomy scissors, and the brachial artery was sealed by electrocautery. The stump was infiltrated with 0.7% bupivicaine, and the skin closed with cyanoacrylate adhesive. The pups were re-warmed, returned to their mothers, and allowed to reach ≥60 days of age before being used in terminal recording experiments.

Receptive field mapping in SI

Rats were anesthetized with 60 mg/kg ketamine hydrochloride and 15 mg/kg xylazine administered intraperitoneally and prepared for recordings as previously described (Lane et al. 1995, 1997, 1999). The trachea was cannulated before the animal was placed on a thermoregulatory blanket, its head fixed in a stereotaxic holder. Mechanical ventilation was set at 65-75 breaths/min, and heart rate was monitored periodically. A state of light anesthesia was maintained in which the eyeblink reflex could be triggered by touching the lateral cornea with a plastic pipette tip. When the eyeblink reflex was more sensitive and
could be triggered by a puff of air or drop of saline on the lateral cornea, supplemental 1g/kg urethane was administered intramuscularly. Urethane was used in addition to ketamine/xylazine because it maintains a long-lasting, relatively light level of anesthesia. The cisterna magna was opened to drain cerebral spinal fluid, a midsgittal incision was made over the skull, and a craniotomy was performed over the cerebral cortex contralateral to the amputated forelimb. The dura and arachnoid mater were incised and reflected. Warmed neurobasal culture medium (Gibco-BRL) was applied to the cortical surface periodically to prevent desiccation. The surface of the cortex was digitally photographed and magnified (×20) to mark the placement of recording electrode penetrations during mapping. Multi-unit neuron clusters were recorded with varnish-coated tungsten electrodes (0.9-1.1 MΩ). Electrode penetrations were spaced 250-300 μm apart, and activity was recorded at depths of 600-800 μm below the pial surface (the approximate depth of layer IV). Tactile stimuli to the stump, whisker pad, lower jaw, trunk, and hindlimb were delivered separately by lightly tapping the body surface with a modified artist’s brush. In addition to activating superficial touch receptors (e.g., Meissner, Merkel, Pacinian, Ruffini), this type of “cutaneous” stimulation could also, to some degree, activate deeper proprioceptive receptors (e.g., muscle spindle, joint, golgi tendon organs). In animal 13 (Fig. 4), bipolar stimulating electrodes were placed on the exposed sciatic nerve and brachial plexus. These peripheral nerves were stimulated (0.1-ms pulses ranging from 2.5 to 10 V), and responses were monitored from a single stump/hindlimb responsive SI recording site. The SI forelimb-stump and hindlimb representations were mapped, and the unresponsive dysgranular zone separating these representations was identified for subsequent injections into this region. This initial mapping took 2-3 h to complete.

Global GRB

After the initial receptive field mapping had been completed, 30 μl of a solution containing 50 μM bicuculline methiodide (BMI) and 50 μM saclofen (SAC; Research Biochemicals International) was applied to the surface of the cortex as previously described (Lane et al. 1997). The forelimb-stump region was remapped 10-15 min later, when increased neuronal bursting activity indicated that GRB had taken effect. The bursting pattern induced by GRB was used to monitor the level of receptor antagonism, and tactile stimuli were delivered between periods of intense bursting. All sites were retested during global-GRB (×1 h); additional 30 μl drug applications were employed if needed. Evoked responses were considered significant if ≥5 sets of action potentials were elicited in response to a train of 10 light taps delivered to the body surface at a frequency of ~3 Hz. After completion of the second map, the BMI/SAC solution was pipetted from the cortex, fresh neurobasal medium was generously washed over the area, and 60 min were allowed to pass before proceeding to local injections. Spontaneous activity consistently returned to baseline levels within the first 30 min of the drug washout period. After global-GRB, responses were tested w/BMI/SAC injected at selected recording sites, w/BMI/SAC injected in the dysgranular cortex, and w/BMI/SAC injected in the SI hindlimb cortex. At least one hour elapsed between series of tests. In animal 13, the electrode was maintained in one position throughout GRB testing.

Localized GRB

A Stoeiing Microelectrode Puller was used to prepare a glass micropipette with a tip diameter of 20 μm. This pipette was attached to a microdrive injector (Sutter Instrument) and filled with the same solution (50 μM BMI/SAC) that was used to induce global-GRB. The recording electrode was used to resample several sites that were stump/hindlimb responsive during global-GRB to confirm that they were stump-only responsive (due to GRB washout). With the electrode in recording position, the pipette was lowered so that its tip was immediately adjacent to the electrode, and 100 nl of BMI/SAC was injected just below the pial surface. The site was retested for receptive fields, and if no hindlimb response was noted within 5 min, the electrode and pipette were advanced to the next site. This sequence was repeated until all sites that were stump/hindlimb responsive during global-GRB had been retested (~1 h). During localized injec-ions, the cortical surface was kept relatively dry so that placement and possible surface spread of drug could be monitored visually. Neurobasal medium was periodically applied to prevent desiccation. Prior to application of BMI/SAC excess fluid was removed from the cortex. When injections were made in the dysgranular and SI hindlimb cortices, 1-2 μl of BMI/SAC solution was injected at each of 8-12 sites, depending on the area of the region. This was done so that a specific cortical area would be disinhibited long enough for the retesting of all stump/hindlimb sites (~45 min). Increased spontaneous activity in the injected region was noted and used to monitor the effectiveness of GRB.

Following the recording session, the animal was given a lethal dose of carbon dioxide and perfused with heparinized saline followed by 4% paraformaldehyde dissolved in sodium phosphate buffer (pH 7.4). The brain was postfixified overnight, and the cortex was removed, flattened, and tangentially cut into 50-μm sections on a freezing microtome. Tissue sections were processed for cytochrome oxidase (Wong-Riley 1979).

Data analysis

Multi-unit recording sites were characterized under normal conditions and separately during global-GRB, site-GRB, dysgranular-GRB, and SI hindlimb-GRB. Sites were defined as responsive to cutaneous stimulation of the stump only, stump/hindlimb, stump/face (vibrissae and/or lower jaw), or stump/trunk. The χ² test was used to demonstrate significant overall differences in the frequency of receptive field subtypes under pre-GRB versus global-GRB conditions. Specific comparisons of pre-GRB versus global-GRB receptive field frequen-cies were analyzed by the t-test for dependent samples (Fig. 1). All sites that were identified as stump/hindlimb responsive during global-GRB were retested during site-GRB, dysgranular-GRB, and SI hindlimb-GRB for the presence of hindlimb receptive fields. Figure 2 illustrates this sequence of testing in one animal. Frequency data were compared by one-way ANOVA to test for the relative persistence of hindlimb responses under each GRB condition (Fig. 3). The Scheffe post hoc test was used to determine significant differences in individual group means. The accepted level of significance for all statistical analyses was P < 0.05.

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as prescribed by the National Research Council, and were approved by the Institutional Animal Care and Use Committee (IACUC).

RESULTS

The SI forelimb-stump and hindlimb representations were mapped in 10 adult (>60 days) Sprague-Dawley rats that had sustained forelimb removal on the day of birth. Evoked responses were recorded from a total of 573 SI forelimb-stump and 207 SI hindlimb sites. Animal ages ranged from 2 to 18 mo at the time of mapping, and results were consistent across these ages. Locations of cutaneous receptive fields for SI forelimb-stump recording sites under normal conditions and during global-GRB are shown in Fig. 1. Under normal conditions, 87 ± 11% (SD) of sites responded to stimulation of the stump only, while the remaining sites were stump/hindlimb (6 ± 4%).
stump/face (vibrissae and/or lower jaw; 6 ± 3%), or stump/trunk (2 ± 2%) responsive. Shortly after global-GRB was induced by topically applying BMI/SAC to cortex, remapping revealed that significantly fewer sites (48 ± 13%; \(P = 0.0003\)) were stump only, because significantly more sites (31 ± 9%; \(P = 0.0001\)) were stump/hindlimb responsive. The frequency of stump/face sites did increase slightly to 12 ± 5% (\(P = 0.077\)) during GRB; the frequency of stump/trunk responses did not change. Occasional sites were responsive to three body regions during GRB.

Following global-GRB mapping, the cortical surface was washed liberally with neurobasal medium. Bursting activity and hindlimb responses were monitored in several recording sites to determine when GRB was no longer effective. Sites that were stump/hindlimb responsive during global-GRB (80 ± 11%) were also stump/hindlimb responsive following site-GRB. In fact, the frequency of stump/hindlimb responses elicited by site-GRB was not significantly different from that recorded during global-GRB (\(P = 0.3\)). Hindlimb responses were noted on average 35 s (range, 10-125) from the time of the site-GRB injection.

Dysgranular-GRB resulted in hindlimb responses in 19 ± 8% of stump/hindlimb responsive sites. Over two-thirds of these (16/23) were “border sites” located ≤300 μm from the dysgranular zone, and therefore hindlimb responses in these sites could have resulted from the spread of relatively large (1-2 μl) BMI/SAC injections into the nearby recording site.

SI hindlimb-GRB elicited hindlimb responses in 13 ± 6% of stump/hindlimb responsive sites. Nearly one-half of these (7/17) were located ≤300 μm from a hindlimb BMI/SAC injection site. The horizontal dashes in Fig. 3 separate border from nonborder sites; border sites are represented above the dashes.

In animal 13 (Fig. 4), the recording electrode was kept in one position for the duration of the recording session to avoid possible fluctuations in the quality of the recordings associated with not recording from exactly the same location every time a site is tested. The pattern of cortical responses evoked by electrically stimulating the sciatic nerve and brachial plexus during global-, site-, dysgranular-, and hindlimb-GRB was similar to that for cutaneous stimulation in the same animal, and similar to the overall group data. Note, however, that there is a small degree of activation in response to electrical hindlimb stimulation during dysgranular GRB (Fig. 4B). This response is consistent with the ~10% (not including border sites) of stump/hindlimb sites that did respond to hindlimb stimulation following dysgranular or SI hindlimb injections. These findings leave open the possibility that GABAergic neurons in these areas are also involved (though minimally) in suppressing hindlimb inputs.

**DISCUSSION**

In this study, local injections of BMI/SAC to recording sites (site-GRB) in the SI forelimb-stump representation of neonatally amputated rats were nearly as effective in eliciting hindlimb responses at these sites as was topical application of BMI/SAC to the cortical surface (global-GRB). Of all sites identified as stump/hindlimb responsive during global-GRB, 80% were also stump/hindlimb responsive following site-GRB. In contrast, injections of BMI/SAC delivered to the SI hindlimb region and to the dysgranular cortex between the hindlimb and forelimb-stump regions were minimally effective in eliciting hindlimb responses. This indicates that GABAergic synapses located primarily within the SI forelimb-stump representation suppress reorganized hindlimb inputs to these neurons, whereas GABAergic synapses located in the hindlimb region and the intervening dysgranular cortex probably play only minor roles in suppressing these inputs.
FIG. 2. Electrophysiological maps show experimental sequence and results of receptive field mapping in one animal (11). A: pre-GRB, sites in the SI forelimb-stump and hindlimb representations respond almost exclusively to stimulation of the “appropriate” body region and are divided by a thin strip of unresponsive dysgranular cortex. B: global-GRB, 23 of 84 sites (27%) are stump/hindlimb responsive. One hour was allowed for drug washout in between each GRB mapping experiment. C: site-GRB (typical injection shown in c) resulted in hindlimb responses in 20 of the 23 sites (87%). One of the remaining sites remained stump-only, while 2 were stump/hindlimb and 3 were stump/vibrissae responsive before site injection (likely due to drug spillover from previous nearby injections). D: dysgranular-GRB (typical injection shown in d) resulted in hindlimb responses in 6 of the 23 sites (26%); 5 of these are border sites located within 300 μm of a dysgranular injection site (4% if border sites omitted). E: hindlimb-GRB results in hindlimb responses in 4 of the 23 sites (17%); 2 of these are border sites (9% if border sites omitted).
Technical limitations

Although injecting 100 nl of BMI/SAC solution at the site of the recording electrode was relatively straightforward, it was more challenging to block GABA receptors in the larger SI hindlimb and dysgranular areas with multiple 1- to 2-μl injections without having some lateral spread of the antagonists into adjacent (forelimb-stump) regions. Such spread may account for the emergence of stump/hindlimb responsive sites near (≤300 μm) BMI/SAC injection sites in the dysgranular or SI hindlimb zones.

Excitation

Ascending thalamocortical projections from the lateral ventroposterior thalamus transmit afferent sensory information from the body and limbs to the cortex (see Fig. 5). This input is relayed to neurons in the granular layer IV of SI, which is
organized into cytoarchitectural units and stains densely with histochemical markers for postsynaptic activity such as cytochrome oxidase (Wong-Riley 1979; Woolsey and Van der Loos 1970). Layer IV neurons are connected vertically to supragranular and infragranular pyramidal neurons, thus forming functional cortical columns (Bender et al. 2003; Feldmeyer et al. 2002; Jones 2000b; Lubke et al. 2000; Mountcastle 1997; Petersen and Sakmann 2001; Schubert et al. 2003). Axons of these pyramidal neurons project horizontally and convey excitation from one column to other areas of cortex (Goldreich et al. 1999; Gottlieb and Keller 1997; Huang et al. 1998; Laaris and Keller 2002). Areas of dysgranular cortex surround and divide granular regions, stain weakly for cytochrome oxidase, and receive innervation from the posteromedial thalamus (Chapin and Lin 1984; Killackey and Sherman 2003; Koralek et al. 1988).

Inhibition

Inhibitory synapses depress spontaneous and evoked cortical activity and play a major role in shaping neuronal receptive fields (Chowdhury and Rasmusson 2003; Dykes et al. 1984; Gupta et al. 2000; Kaneko and Hicks 1988; Kyriazi et al. 1996a,b; Li et al. 2002; Micheva and Beaulieu 1997). Immunocytochemical studies have demonstrated that GABAergic synapses are present on dendritic spines and shafts in all six layers of SI, with higher densities in layers II-V (Chmielowska et al. 1988; de Blas et al. 1988; Gutierrez et al. 1994; Keller and White 1986, 1987; Lin et al. 1985; Pirker et al. 2000; Taguchi et al. 1989). Many inhibitory neurons at the layer IV-V boundary of barrel cortex are activated by peripheral whisker stimulation (McCasland et al. 1997). Altered GABA neurotransmission may be an important factor in lesion-induced plasticity, because significant changes in levels of cortical GABA, its synthesizing enzyme, and its receptors have been reported in SI and cat visual cortex following various sensory deprivations (Akhtar and Land 1991; Hendry and Jones 1988; Land et al. 1995; Levy et al. 2002; Rosier et al. 1995). Although hindlimb inputs to the forelimb-stump representation are increased after amputation in the rat, these inputs are still largely suppressed by the GABAergic system. This suppression of “inappropriate” hindlimb inputs to neurons that normally only express a receptive field on the stump may play a role in maintaining somatotopic organization in SI (Stojic et al. 2000).

Reorganization

Forelimb amputation deprives dorsal root ganglia, the cuneate nucleus, the ventroposterolateral thalamic nucleus, and the SI forelimb-stump representation of a large portion of normal sensory input. This, and other peripheral manipulations, can lead to short- and long-term changes in somatotopic maps and receptive fields for individual neurons (reviewed in Buonomano and Merzenich 1998; Calford 2002; Chen et al. 2002; Donoghue 1995). Electrical and chemical cortical lesioning experiments in neonatal amputees indicate that hindlimb inputs originate in the SI hindlimb region, synapse in the
intervening dysgranular cortex, and terminate on SI forelimb-stump neurons (Lane et al. 1999; Stojic et al. 2001). Since our previous studies have employed global-GRB to detect hindlimb responses, the precise location along the hindlimb-to-forelimb-stump pathway where inhibitory synapses suppress this circuit has remained undetermined. In the current study, hindlimb responses were elicited following localized, site-GRB, indicating that those GABA synapses responsible for suppressing hindlimb inputs are located within the forelimb-stump region.

As depicted in the proposed circuit model (Fig. 5), afferent hindlimb information is relayed to layer IV spiny stellate cells in the SI hindlimb representation and conveyed vertically to presumed pyramidal neurons in supra- and infra-granular layers. Horizontal connections transmit this excitation to dysgranular neurons and to layer IV neurons within the forelimb-stump representation. At this location, GABA interneurons would be appropriately positioned to modulate inputs to layer IV. Our results suggest that the receptive field response properties of layer IV cells in neonatally amputated rats are influenced by the inhibitory (symmetrical) synapses located on dendrites and soma of layer IV neurons (Keller and White 1987). While this simplified model provides an initial framework to aid our understanding of reorganized sensory processing, a number of specific points are yet to be clarified. For example, although the depicted neurons are phenotypically consistent with cytoarchitectural studies of SI, extracellular recordings provide no information about the morphology of stump/hindlimb responsive neurons. Also, multi-unit recording at a depth of 600-800 μm does not guarantee that our findings are specific to layer IV cells; the excitation of prominent apical dendrites of layer V pyramidal neurons, for instance, can be monitored at these depths with low resistance electrodes. Another question is whether the inhibitory GABAergic interneurons are tonically active or driven by feed-forward mechanisms, possibly by thalamocortical projections to the SI forelimb-stump region (black dashed arrow in Fig. 5). Studies are currently underway to address these questions.

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J Neurophysiol • VOL 92 • JULY 2004 • www.jn.org
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