Intrinsic Circuitry of the Superior Colliculus: Pharmacophysiological Identification of Horizontally Oriented Inhibitory Interneurons

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Meredith, M. Alex and Ary S. Ramoa. Intrinsic circuitry of the superior colliculus: pharmacophysiological identification of horizontally oriented inhibitory interneurons. J. Neurophysiol. 79: 1597–1602, 1998. Much of what is known about the organization of the superior colliculus is based on the arrangement of its external connections. Consequently, there is little information regarding pathways that remain intrinsic to it, even though recent data suggest that a horizontally oriented local circuit may mediate the functional reciprocity among fixation and saccade-related neurons. Therefore, the present experiments sought physiological evidence for neurons intrinsic to the superior colliculus that might participate in a horizontally oriented local circuit. Parasagittal slices of the ferret superior colliculus were prepared for in vitro recording, and 125 intermediate/deep layer neurons were examined in response to electrical stimulation rostral or caudal to the recording site. A substantial proportion (37%) of neurons responded with a prolonged period (means = 59.3 ± 30 ms) of poststimulus suppression of spontaneous action potential activity. Of the suppressed neurons, most (53%) were disinhibited when the excitatory amino acid receptor antagonists d-2-amino-5-phosphonovaleric acid (d-APV) and 6-nitro-7-sulphamoylbeno[f]quinoxaline-2,3-dione (NBQX) were administered, indicating that excitatory input to inhibitory interneurons was blocked. Of the neurons that received inputs from inhibitory interneurons, all had their suppressive responses decreased or eliminated by the γ-aminobutyric acid antagonist, bicuculline. Finally, severing the superficial layers from the slice had no effect on intermediate layer responses to intrinsic stimulation. These data provide physiological evidence for the presence of horizontally oriented inhibitory interneurons in the superior colliculus. Furthermore, these findings are consistent with the hypothesis that an intrinsic circuit, routed through interneurons, might account for the reciprocal inhibition observed among fixation and saccade-related neurons.

INTRODUCTION

The intermediate and deep layers of the superior colliculus have long been regarded as a critical sensorimotor site where visual, auditory, and somatosensory inputs are transformed into motor commands that drive the eyes, pinnae, and head toward the location of the stimulus (for review, see Sparks 1986). Although a great deal of attention has been directed toward understanding the afferent and efferent connections that underlie this process, surprisingly little is known about connections that are intrinsic to the colliculus.

Recently, a horizontally oriented intrinsic collicular circuit has been proposed through which fixation (e.g., neurons that discharge when the eyes are stationary but halt their activity during saccades) and saccade-related neurons (e.g., neurons that discharge before rapid, conjugate eye movements toward a real or remembered target) might mutually inhibit one another (Munoz and Guitton 1989; Munoz and Wurtz 1993a, 1995; Munoz et al. 1991). The identity of the neurons subserving the intrinsic portion of this theoretical circuit is not known. One possibility is that the mutual inhibitory effects might be due to recurrent axon collaterals of fixation and saccade neurons themselves. However, fixation and saccade neurons are presumed to produce excitatory postsynaptic effects on their efferent targets (Pare and Guitton 1994; Raybourn and Keller 1977), and they probably correlate with a specific class of output neuron (X-type) (Munoz and Wurtz 1995) that exhibits few recurrent axon collaterals (at least for cat) (see Moschovakis and Karabelas 1985). Therefore, fixation and saccade neurons themselves appear poorly suited for subserving a robust, bidirectional inhibitory effect that spans the rostrocaudal extent of the colliculus. On the other hand, anatomic studies have identified numerous putative interneurons on the basis of their small size and/or inhibitory neurotransmitter content (Behan and Kime 1996a; Mize 1992; Okada 1992). Inclusion of interneurons in the proposed intrinsic circuit has the advantage of conveying the appropriate sign: inhibition. Furthermore, Behan and Kime (1996a) have shown that the network of intrinsic collicular connections is so extensive that “it is difficult to imagine how a given region in the colliculus can potentially influence any other region.” Thus the quantity of interneurons and their putative inhibitory function is appropriate for the proposed horizontal intrinsic circuit. However, because physiological examinations of the intrinsic circuitry of the superior colliculus have dealt almost exclusively with vertical connections between the superficial and deep layers (see Behan and Appell 1992; Grantyn et al. 1984; Lee and Hall 1995; Mooney et al. 1988), little functional information is available regarding interneurons within the layers relevant to the control of fixation and saccadic activity. Therefore, the present study was initiated to seek physiological evidence for horizontally directed interneurons within the intermediate/deep layers of the superior colliculus. Abstracts of this work have been presented previously (Meredith and Ramoa 1996, 1997).

METHODS

All procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23) and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Young, adult ferrets (n = 14, weight range 600–900 g) of either sex were anesthetized deeply (80 mg/kg pentobarbital sodium)
and euthanized by decapitation. The brain stem was exposed quickly, and a block of tissue containing the superior colliculus was removed and submerged in 3°C modified (sodium free, 10 mM glucose) Ringer solution. The block was transected longitudinally on the midline (cutting commissural inputs), trimmed rostrally through the pretectum (cutting descending afferents), caudally through the inferior colliculus (cutting ascending afferents), and ventrally through the periaqueductal gray (cutting ascending inputs). Subsequently, parasagittal slices (400-µm thick) were taken using a Vibratome, and these sections represented superior colliculi that were isolated from all known external inputs. Slices were transferred to an interface-type recording chamber where they were incubated in a buffered solution [which contained (in mM) 126 NaCl, 2.5 KCl, 1.2 MgSO₄, 26 NaHCO₃, 1.25 NaHPO₄, 2 CaCl₂, and 10 dextrose, pH 7.4] with 95% O₂-5% CO₂ at 33–35°C.

Glass-insulated tungsten electrodes (~1 MΩ at 1 kHz, tip exposure 20–30 µm) were used for recording. Because the lamination of the superior colliculus was easily apparent when viewed through a dissecting microscope, recording and stimulating electrodes could be placed reliably within a desired layer and neurons in the upper half of the intermediate layers were specifically targeted for study. Once a neuron was isolated, its electrical activity was amplified, routed to an oscilloscope and audio-monitor, and stored on tape. Next, the superior colliculus was stimulated electrically [concentric bipolar electrode, 0.1-ms duration, 25–250 µA stimulus intensity (1.5–2 times threshold); 50 presentations, 2.5-s interstimulus interval] through an electrode positioned within the intermediate layers ±1 mm from the recording site in either the rostral or caudal third of the superior colliculus. Although current spread was not a factor in this essentially isolated preparation, the lowest effective stimulus current always was sought.

Neuronal responses to intrinsic electrical stimulation were recorded as well as their responses to stimulation during local or bath delivery of pharmacologic agents. For the most part, neurotransmitter antagonists were applied focally using a picospritzer, which pulsed ~10–100 pl of antagonist through a glass pipette (tip diameter 2–5 µm). Antagonists were picospritzed in a nearly continuous fashion (3–4 pulses/min at regular intervals) for the duration of the trial. Bath application of antagonists, whereby accurate measures of drug concentration at equilibrium could be determined, was used to confirm results from local applications. For tests using bicuculline methiodide (30–50 µM), a 60-s delay between application and testing was allowed to permit the effect to stabilize. Tests involving local application of a cocktail of D(-)-2-amino-5-phosphonovaleric acid (D-APV, 40 µM) and 6-nitro-7-sulfaphenylbenzof[1]quinoxaline-2,3-dione (NBQX, 10 µM) required a substantially longer period (8–12 min) before full effect was achieved. Whenever possible, responses to intrinsic electrical stimulation were tested before drug application, during a drug-induced effect, and after washout and recovery. Taped spike records were played back through a computer that used a waveform templating program (Spike2, CED) to identify and digitize spikes (25-KHz sampling rate). Data analysis consisted computer construction of peristimulus histograms and statistical comparisons (2-tailed paired t-test) of spike counts taken within the suppressive period after electrical stimulation for the same epoch during stimulation and pharmacological treatment and for the same duration during spontaneous activity. Criterion for an effect was a significant (P < 0.05) change in activity between the different treatments. Routine histological procedures (75-µm frozen sections, cresyl violet stain) were used to confirm the position of recording and stimulation sites in selected slices.

RESULTS

A total of 125 neurons were examined. All histologically confirmed recording sites were located in the dorsal half of the intermediate gray layer. Each neuron was tested for response to electrical stimulation (0.1-ms duration, 25–250 µA) through a concentric bipolar electrode placed within the same layer at a distance of ±1 mm, either rostral or caudal to the recording site (see Fig. 1A). A large proportion (n = 43, 34%) of neurons responded to the stimulus with excitation, which was usually observed as a facilitation of spontaneous activity. A smaller proportion (n = 34, 27%) exhibited a suppression of ongoing activity (as shown in Fig. 1B), while a few neurons (n = 12, 10%) showed post- excitatory suppression. The duration of suppression (for suppression and postexcitatory suppression) ranged from 20 to 160 ms [mean, 59.7 ± 30 (SD) ms]. Neurons unresponsive to intrinsic stimulation constituted the remainder (n = 36, 29%) of the sampled population. As indicated in Table 1, there did not appear to be clear evidence for polarity because suppression or postexcitatory suppression could be evoked by rostrally (82%) or caudally (59%) placed stimulating electrodes, although suppression was infrequently evoked (18%) exclusively from a caudal stimulation site.

Anatomic studies have identified γ-aminobutyric acid (GABA)-containing neurons in the superior colliculus and suggested that these cells function as interneurons (Mize 1992; Okada 1992). Because GABAergic connections induce inhibitory postsynaptic effects, we examined suppressive responses for the presence of the GABAₐ receptor subtype using its specific antagonist, bicuculline methiodide. Of the suppressed neurons tested (n = 18), bicuculline methiodide (30–50 µM, picospritzed) reduced or eliminated the suppressive effect in 14 (78%). An example is shown in Fig. 1C, in which intrinsic stimulation suppressed the spontaneous activity of an intermediate layer neuron, which, in turn, was disinhibited when bicuculline methiodide was administered.

Excitation of an inhibitory interneuron will produce suppression in a postsynaptic neuron, and pharmacological blockade of an interneuron’s excitatory inputs will block that suppressive effect. Therefore, antagonists to the excitatory transmitter glutamate (D-APV, 40 µM; NBQX, 10 µM) were combined and picospritzed between the stimulation and recording sites of 16 suppressed intermediate layer neurons. Eight (8/16; 50%) of the neurons had their suppressive responses blocked by picospritzed application of D-APV/NBQX (see Fig. 1D). In addition, in a separate experiment, when these same glutamate antagonists were applied at low concentrations to the bath (D-APV, 25 µM; NBQX, 6 µM final concentration), an additional neuron was tested and observed to lose its suppressive response. As summarized in Fig. 2, 9 (of 17; 16 picospritzed + 1 bath) suppressed neurons lost their suppressive responses when excitatory neurotransmitter antagonists were present. Figure 2 also shows that after an appropriate recovery period from excitatory blockade (usually 60–75 min), stimulation-induced suppression returned to pre-antagonist levels. Furthermore, in all (5/5) of the neurons recovered from D-APV/NBQX treatment, the stimulation-induced suppressive period was disinflicted by the application of the GABAₐ antagonist, bicuculline methiodide.

For neurons with demonstrated interneuron input, excitatory antagonist blockade of suppression was complete in most instances (5/9) and poststimulus activity under these
FIG. 1. A: intrinsic stimulation of excitatory afferents (+) to an intrinsic inhibitory neuron (●), the axon of which makes inhibitory contact (−) with an output neuron (○), will inhibit the activity of the latter, as depicted in this schematic of a parasagittal slice through the dorsal midbrain. An example is provided in B, where spontaneous activity (top: 16 overlapped trials) of a neuron (large spike) is suppressed by intrinsic stimulation (bottom: 16 overlapped trials; 200 μA, 0.1-ms stimulus at ▲) of the intermediate gray layer. Note, a 2nd neuron (smaller spike) was excited at a short latency by the stimulus. SGS, stratum griseum superficiale; SGI, stratum griseum intermediate; SGP, stratum griseum profundum; PT, pretectum; IC, inferior colliculus; PAG, periaquaductal gray. C: inhibitory afferents to an intermediate layer neuron could be blocked using the γ-aminobutyric acid-A (GABA_A) antagonist, bicuculline methiodide. Top: electrical stimulation (▲: 250 μA, 0.1 ms, caudal to the recording site) of the intermediate layers suppressed ongoing activity in this neuron for ~50 ms. Middle: when the bicuculline methiodide (30 μM) was picospritzed between the stimulation and recording site, poststimulus suppression was replaced by a profound increase in excitatory activity. Bottom: after a 10-min recovery period, poststimulus suppression returned to near pretest levels. D: when excitatory transmission was blocked by the application of excitatory antagonists, stimulation-induced suppression was blocked as well, indicating the presence of an inhibitory interneuron. Top: stimulation (75 μA, 0.1 ms, rostral to recording site) of the intermediate layers significantly (P < 0.05, paired t-test) suppressed the spontaneous activity of this neuron for ~70 ms. Middle: local application of the glutamate antagonists D-2-amino-5-phosphonovaleric acid (D-APV; 40 μM) and 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX; 10 μM) resulted in the loss of suppression (not significantly different from spontaneous levels, paired t-test). Bottom: after a 70-min recovery period, the poststimulus suppressive period returned to near pretest levels. E: superficial layers of the superior colliculus are not required for inhibition induced by stimulation of the intermediate layers. Top: ongoing activity in this intermediate layer neuron was significantly suppressed (P < 0.05, paired t-test) by stimulation (125 μA, 0.1 ms, caudal to recording site) of the intermediate layers and, in the middle histogram, this suppression was disinhibited by the excitatory antagonists D-APV (40 μM) and NBQX (10 μM). Bottom: during the recovery and washout period, the superficial layers were cut from the intermediate layers by longitudinally transecting the stratum opticum with a small scalpel. Despite the loss of connectivity with the superficial layers (postlesion superficial stimulation results not shown), intrinsic stimulation of the intermediate layers still suppressed the activity of the neuron.
TABLE 1. Intrinsically evoked suppression and its relation to rostral or caudal sites of stimulation

<table>
<thead>
<tr>
<th>Stimulus Location</th>
<th>Suppression</th>
<th>Postexcitatory Suppression</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Rostral only</td>
<td>5 (15)</td>
<td>2 (6)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Caudal only</td>
<td>3 (9)</td>
<td>0</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Both rostral and</td>
<td>4 (12)</td>
<td>3 (9)</td>
<td>7 (20)</td>
</tr>
</tbody>
</table>
| caudal

Data are from 34 neurons tested from both rostral and caudal stimulation sites. The total from affected from rostral sites is 82%; total from caudal sites is 59%. Data in parentheses are percent of sample.

These results physiologically document the presence of inhibitory interneurons, the effects of which are expressed horizontally within the intermediate layers of the superior colliculus. This conclusion is based on: intrinsic electrical stimulation at rostral or caudal locations in the intermediate layers suppressed the activity of neurons within those same layers, application of excitatory neurotransmitter antagonists eliminated suppressive responses to intracollicular stimulation (indicating the presence of an excitatory synapse on an inhibitory interneuron), and suppression induced by intrinsic stimulation persisted after dissecting away the superficial layers. Thus both pre- and postsynaptic effects were manifest within intermediate layers the superior colliculus. Although these observations might be attributable to the recurrent portion of axon collaterals from colliculus projection neurons, this possibility is unlikely. Most superior colliculus projection neurons are known to be excitatory (Mooney et al. 1990; Pare and Guitton 1994; Raybourn and Keller 1979) and excitatory recurrent collaterals could not elicit the bicuculline-sensitive postsynaptic suppressive effects that were observed. In addition, although some neurons projecting to the opposite colliculus (T-type, or commissural) are GABAergic (Appell and Behan 1990) and may exhibit recurrent axon collaterals, T-type neurons are dis-

D I S C U S S I O N

conditions was virtually equivalent to spontaneous levels. However, in four neurons, a substantially abbreviated (10- to 20-ms duration), short latency (1–2 ms) inhibitory period remained despite the presence of the excitatory antagonists. The most reasonable explanation for this residual suppression is that, in these few cases, inhibitory axons, perhaps from the distal ends of severed fibers arising external to the superior colliculus, were stimulated in passage.

Given that vertical connections between the superficial and deep layers are well established (Behan and Appell 1992; Grantyn et al. 1984; Lee and Hall 1995; Mooney et al. 1988), it seemed possible that activation of inputs to the superficial layers might account for the observed, suppressive effects. To control for this possibility, the following tests were conducted. A suppressed neuron was isolated and the inhibitory period subsequently was blocked by application of excitatory antagonists (d-APV 40 μM; NBQX 10, μM, picospritzed, see Fig. 1E). Next, under visual guidance, a fine-tipped scalpel was used to cut the stratum opticum longitudinally from its rostral to caudal extent, thereby severing the superficial from the intermediate layers. In two instances, the neuron under examination remained well isolated during and after this procedure. In both of these instances, after the effects of the d-APV/NBQX had worn off, intermediate layer stimulation still suppressed neuronal activity even though the superficial layers had been removed functionally (see Fig. 1E, bottom). Furthermore, although stimulation of the superficial layers before the transection elicited excitatory responses from the neuron under examination, no response to superficial stimulation was effected after transection. These data, therefore, indicate that the source of the observed suppression does not originate in or pass through the superficial layers.

Fig. 2. Pharmacophysiological evidence for inhibitory interneurons in the superior colliculus: a summary of intrinsic stimulation-induced suppression in the presence excitatory and inhibitory antagonists. Spontaneous activity was significantly (P < 0.05, paired t-test) reduced in each of 9 neurons by 50–97% by intrinsic electrical of the superior colliculus. However, stimulation-induced suppression was itself reversed to near spontaneous levels when the excitatory antagonists d-APV and NBQX were introduced, indicating the presence of an inhibitory interneuron. After a 60- to 70-min washout, responses to intrinsic stimulation returned to pretreatment levels. Finally, when the GABA_a antagonist, bicuculline methiodide was introduced, suppression also was blocked, indicating the interneuronal source of inhibition was GABAergic. Extremely high values (507%, 307%) elicited during bicuculline/stimulation were not plotted on the chart to better display the inhibitory effects. The heavy line indicates the mean value for all 9 of the neurons for each of the treatments.
tributed only within the rostral two-thirds of the colliculus (Behan and Kime 1996b) and, therefore, are not in position to mediate caudal-to-rostral effects. Moreover, reconstructions of these and other types of output neurons (Moschovakis and Karabelas 1985; Moschovakis et al. 1988a,b) indicate that recurrent axonal collateralization occurs within only a few hundred microns of the soma, which is insufficient to mediate effects that supposedly span the rostrocaudal extent of the colliculus.

Unlike the previous studies that examined vertical connections between the superficial and deep layers of the superior colliculus (Behan and Appell 1992; Grantyn et al. 1984; Lee and Hall 1995; Mooney et al. 1988), the effects observed here rely on horizontal connections between the rostral and middle or caudal and middle portions of the intermediate layers. Such horizontally oriented intrinsic connections are consistent with predictions based on physiological studies (Keller and Edelman 1994; Munoz and Guitton 1989; Munoz and Wurtz 1993a, 1995), as well as on recent anatomical data (Behan and Kime 1996a). Fixation and saccade-related zones have been identified in both cat (Munoz and Guitton 1989) and monkey (Munoz and Wurtz 1993a, 1995), and neurons in one of these areas may be inhibited by inputs from the other (and vice versa) to account for their reciprocal activity profiles (Munoz and Wurtz 1993b). Anatomic evidence from cats extends this prediction even further by showing that intermediate/deep layer neurons project intrinsic axons to any other region of the intermediate/deep layers without regard to rostrocaudal or mediolateral orientation or even laminar boundaries (Behan and Kime 1996a). The present experiments affirm this lack of polarity among intrinsic projections because stimulation-induced suppression was observed in response to both rostral and caudal electrical stimulation. Furthermore, given the dense network of anatomically identified intrinsic connections (Behan and Kime 1996a), the proportion (53%) of neurons identified as receiving inhibitory inputs from interneurons may actually represent an underestimation of these effects.

In the majority of the neurons tested, the suppressive effect of intrinsic stimulation was reduced or eliminated by the application of the GABAA antagonist, bicuculline methiodide. This result was expected because anatomic studies have shown that GABAergic neurons are plentiful in the superior colliculus and are distributed throughout its rostrocaudal and mediolateral extent (for review, see Mize 1992). However, only 13% of GABAergic collicular neurons are found in the intermediate layers and are the smallest proportion in all of the layers (Mize et al. 1991). Nevertheless, the large ratio of intermediate layer neurons shown to receive intrinsic inhibitory input as well as the relatively high levels of GABA in the intermediate layers (Arakawa and Okada 1988) suggests that the GABAergic neurons that are present in the intermediate layers may be very highly branched.

Application of the excitatory antagonists D-APV and NBQX was not entirely effective in blocking intrinsically evoked suppression in all cases, and the suppressive periods that remained were characteristically very brief (<20 ms) and occurred at a very short latency. In these instances, it seems likely that intrinsic stimulation activated inhibitory fibers of passage that may have originated further away in the colliculus or may even have arisen extrinsically. If this explanation is true, its relative infrequency is somewhat surprising, given the volume of inhibitory inputs the superior colliculus receives from the substantia nigra (Araki et al. 1984; Ficalora and Mize 1989), the zona incerta (Araki et al. 1984; Ficalora and Mize 1989), the contralateral superior colliculus, the pretectal complex, perihypoglossal nucleus, the cuneiform nucleus, nuclei of the lateral lemniscus, and the inferior colliculus (Appell and Behan 1990). The incidence of short latency/duration inhibition may be due to a low proportion of intrinsic neurons with long axons (Behan and Kime 1996a). Alternatively, the configuration of stimulating and recording electrodes might not have been optimal for activating the intrinsic trajectory of inhibitory fibers that arrive from external sources.

The functional consequences of the horizontally oriented inhibitory interneurons identified here cannot be determined at this time, especially because little is known regarding the sensorimotor character of the ferret superior colliculus. However, a recent report (L. F. Miller, A. S. Ramoa, M. Behan, and M. A. Meredith, unpublished data) indicates that the anatomic organization of the ferret superior colliculus appears to resemble that of other members of its phylogenetic family, such as cats. Given that the superior colliculus of cats, as well as that of primates, are known to contain fixation as well as saccade-related neurons that are reciprocally active (Munoz and Guitton 1989; Munoz and Wurtz 1993a, 1995), it is tempting to postulate that the inhibitory interneurons identified here provide the critical substrate by which the opposing functions of fixation and eye movement mutually inhibit one another. However, although functional properties of neurons postsynaptic to inhibitory interneurons are obviously crucial to this question, their identity is not yet known. Nonetheless, the physiological documentation of horizontally oriented interneurons in the superior colliculus extends the range of possibilities of collicular function beyond the sum of its input/output connections and offers the opportunity to examine the multisensory, multimotor role of this complex structure from a new perspective.

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