Chronic NMDA Exposure Accelerates Development of GABAergic Inhibition in the Superior Colliculus

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Aamodt, Sandra M., Jian Shi, Matthew T. Colonnese, Wellington Veras, and Martha Constantine-Paton. Chronic NMDA exposure accelerates development of GABAergic inhibition in the superior colliculus. J. Neurophysiol. 83: 1580–1591, 2000. Maturation of excitatory synaptic connections depends on the amount and pattern of their activity, and activity can affect development of inhibitory synapses as well. In the superficial visual layers of the superior colliculus (sSC), developmental increases in the effectiveness of γ-aminobutyric acid (GABA) receptor-mediated inhibition may be driven by the maturation of visual inputs. In the rat sSC, GABAβ receptor currents significantly jump in amplitude between postnatal days 17 and 18 (P17 and P18), approximately when the effects of cortical inputs are first detected in collicular neurons. We manipulated the development of these currents in vivo by implanting a drug-infused slice of the ethylene-vinyl acetate copolymer Elvax over the superior colliculus of P8 rats to chronically release from this plastic low levels of N-methyl-D-aspartate (NMDA). Sham-treated control animals received a similar implant containing only the solvent for NMDA. To examine the effects of this treatment on the development of GABA-mediated neurotransmission, we used whole cell voltage-clamp recording of spontaneous synaptic currents (sPSCs) from sSC neurons in untreated, NMDA-treated, and sham-treated superior colliculus slices ranging in age from 10 to 20 days postnatal. Both amplitude and frequency of sPSCs were studied at holding potentials of +50 mV in the presence and absence of the GABAβ receptor antagonist, bicuculline methiodide (BMI). The normal developmental increase in GABAβ receptor currents occurred on schedule (P18) in sham-treated sSC, but NMDA treatment caused premature up-regulation (P12). The average sPSCs in early NMDA-treated neurons were significantly larger than in age-matched sham controls or in age-matched, untreated neurons. No differences in average sPSC amplitudes across treatments or ages were present in BMI-insensitive, predominantly glutamatergic synaptic currents of the same neurons. NMDA treatment also significantly increased levels of glutamate decarboxylase (GAD), measured by quantitative western blotting with staining at P13 and P19. Cell counting using the dissector method for MAP 2 and GAD67 at P13 and P19 indicated that the differences in GABAergic transmission were not due to increases in the proportion of inhibitory to excitatory neurons after NMDA treatment. However, chronic treatments begun at P8 with Elvax containing both NMDA and BMI significantly decreased total neuron density at P19 (~15%), suggesting that the NMDA-induced increase in GABAβ receptor currents may protect against excitotoxicity.

INTRODUCTION

During the final stages of neural development, the temporal and spatial patterning of synaptic activity can regulate neuron number, synapse position, and synaptic strength, thereby assuring that developing circuitry adapts the growing brain to its environment (Constantine-Paton and Cline 1998). In many systems, N-methyl-D-aspartate (NMDA) receptor activation during a sensitive period is required for appropriate synaptic maturation, because NMDA receptor blockade causes topographically incorrect positioning of synapses (Bear et al. 1990; Cline and Constantine-Paton 1989; Hahm et al. 1991; Rabacchi et al. 1992; Schlaggar et al. 1993; Schnupp et al. 1995; Simon et al. 1992).

In theory, appropriate neuronal responsiveness could develop by adjusting excitation and/or inhibition, based on environmental input, to maintain activity levels within an effective range. This balance is critical because small decreases in GABAergic inhibition can lead to seizures (Chagnac-Amitai and Connors 1989; Kriegstein et al. 1987). For technical reasons, most research on activity-dependent synaptic plasticity has focused on excitatory neurons with extrinsic projections. However, inhibitory neural development is also modulated by activity, as shown in dissociated central neurons (Memo et al. 1991; Rutherford et al. 1997; Seil and Drake-Baumann 1994; Zhu et al. 1995) and by sensory deprivation in vivo (Benevento et al. 1995; Hendry and Carnder 1992; Micheva and Beaulieu 1997). Nevertheless, in many CNS regions, excitatory inputs develop before hyperpolarizing or shunting GABA-mediated synaptic events are detected (Kirkwood and Bear 1994; Lator et al. 1991; Luhmann and Prince 1990). Consequently it is possible that inhibition in vivo is regulated to balance excitation during the early stages of synapse formation.

In this study, we chronically applied low levels of NMDA to the developing superior colliculus beginning at postnatal day 8 (P8), before functional GABAergic inhibition normally appears. The treatment produced early potentiation of GABAβ receptor currents and increased glutamate decarboxylase (GAD) protein levels in the superficial visual layers of the superior colliculus (sSC). The up-regulation of GABAβ receptor currents occurred 4 days after the onset of treatment and was not associated with an increase in the ratio of inhibitory to excitatory neurons. However, when bicuculline methiodide (BMI) was applied along with NMDA to P8 colliculi, a significant drop in total neuron number was detected at P19. Thus the NMDA-induced increases in GABAβ receptor currents appear to protect against some cell loss that could potentially result from tonic activation of NMDA receptors.

Our observations suggest that the immature GABAβ receptor system in the sSC in vivo is poised to dampen potentially damaging overexcitation as the visual inputs develop. Such a
method could also moderate afferents that, by chance, arrive earlier or in larger numbers than other inputs destined to contribute to sSC function. Similar latent inhibitory circuitry residing throughout the CNS could play a significant role in buffering the normal adaptive pattern of circuit formation against developmental perturbations in the onset, source or amount of excitatory activity.

**METHODS**

**Animals and surgery**

Timed pregnant Sprague-Dawley female rats were purchased from Camm, and their litters were used for all experiments. The day of birth was counted as *postnatal day 0 (P0)*. Chronic local drug treatment was begun at P8 by surgically implanting a 180-µm-thick slice of the inferior ethylene-vinyl acetate copolymer Elvax directly over the superficial layers of the superior colliculus, as previously described (Simon et al. 1992). Briefly, rat pups were anesthetized under a halothane vaporizer. A small incision was made over the sagittal sinus, and a slit was opened in the skull to allow insertion of a slab of Elvax. Three or four sutures were used to close the incision, antibiotic ointment was applied, and pups were returned to the mother after they recovered from anesthesia.

The Elvax contained a final concentration of 100 µM NMDA in water (NMDA treatment). This concentration was estimated to release molecules of the size of NMDA in the range of hundreds of nanomoles/day (Cline and Constantine-Paton 1989, 1990; Simon et al. 1992; Smith et al. 1995). Elvax was also prepared to contain an equivalent volume (20 µl) of water (sham treatment), 500 µM BMI in 20 µl chloroform (bicusculline treatment) or both (NMDA/biscusculline treatment) in 20 µl chloroform.

**Electrophysiology**

For electrophysiology, NMDA- or sham-treated pups, ages P10 to P20, were anesthetized with ether and killed by decapitation. The diencephalon and midbrain were rapidly dissected and placed in ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 117 NaCl, 3 MgCl2, 4 KCl, 3 CaCl2, 1.2 NaHPO4, 26 NaHCO3, and 16 mM glucose, saturated with 95% O2-5% CO2 to a final pH of 7.4. Parasagittal slices of the midbrain were cut at 300–400 µm from a Vibratome (Oxford) and secured in the recording chamber. The slices were maintained at room temperature (22–24°C) and perfused with ACSF at 4 ml/min. Recording began 2 h later, to allow slices to recover from anesthesia and cutting and to assure wash out of chronically applied NMDA.

Recording procedures have been presented previously (Shi et al. 1997). Briefly, borosilicate glass (World Precision Instruments) patch electrodes with tip resistances of 5–10 MΩ were filled with (in mM) 122.5 Cs-glucuronate, 17.5 CsCl, 10 HEPES (CsOH), 0.2 NaEGTA, 2 MgATP, 0.3 NaGTP, 8 NaCl, and 0.2% biocytin at pH 7.3. Amplifier and electrode offsets were zeroed in ACSF before obtaining a patch, and recorded voltages were adjusted for a liquid junction potential and electrode offsets were zeroed in ACSF before obtaining a patch.

**Molecular analyses of biocytin-filled neurons**

To determine the range of cell types and the location of cells analyzed physiologically, we identified cells by labeling them with biocytin in the recording electrode. Slices were fixed in 4% Formalin overnight, and some were cut at 90 µm on dry ice with a sliding microtome, followed by a 4% Formalin postfix before staining with Texas Red-conjugated streptavidin (Jackson Labs). In later experiments, equally good morphology was obtained with unsectioned slices examined using a BioRad 60 or a BioRad 1024 confocal microscope. Cell type was categorized by an investigator blind to the treatment the animal had received.

**Molecular analyses**

For biochemical experiments, rats were killed at P13 or P19 by carbon dioxide followed by cervical dislocation, and the superficial layers of the superior colliculus were rapidly dissected. For protein...
extraction, tissue was homogenized immediately in 20 volumes of buffer (10 mM phosphate buffer, pH 7.0, 5 mM EGTA, 5 mM EDTA, 1 mM DTT and Complete protease inhibitor, Boehringer Mannheim) and then either fractionated or frozen in liquid nitrogen and stored at −80°C until needed. Freezing did not affect results in preliminary experiments. Crude fractionation was done with a modification of the Yip and Kelly (1989) procedure. Rapidly thawed homogenate was centrifuged for 10 min at 4°C at 16,000 × g, and the supernatant (crude soluble fraction) was collected and placed on ice. The pellet was resuspended in ¼ volume of 2 mM HEPES (pH 7.2) and centrifuged for 10 min at 4°C at 11,000 × g. The supernatant was discarded, and the pellet was resuspended in 0.5 mM HEPES (pH 7.3) containing 0.32 M sucrose and centrifuged for 8 min at 450 × g. The supernatant from this spin (crude particulate fraction) and the crude soluble fraction were placed in Laemmli buffer, heated to 90°C for 5 min, and then frozen in aliquots at −80°C.

Immunoblotting of proteins was done with primary antibodies to GAD, both the 65-kD isoform found mainly at synapses (GAD65, 1:2,000, Boehringer-Mannheim) and the 67-kD isoform found mainly (Kaufman et al. 1991), and to the two GABA transporters found Thirty micrometers was chosen for section thickness after several


dissector method (Gundersen 1986). We counted six fields (65 µm square for P13, 75 µm ×100 µm for P19), consistently spaced across the mediolateral extent of the sSC, on each section. Control sections in which the primary antibody was not added showed only very light DAB staining within the neuropil.

RESULTS

Electrophysiology

The functional effects of chronic NMDA treatment were studied by whole cell patch-clamp recording. We have previously shown that the frequency of spontaneous excitatory post synaptic currents (EPSCs) is high in the young postnatal rat sSC and that the development of mature GABAergic inhibition is associated with an abrupt reduction in sEPSC frequency after P17, which is sensitive to bath-applied BMI (Shi et al. 1997). GABA is the dominant inhibitory transmitter in the mature sSC, and all GABAergic contacts in these visual layers derive from intrinsic GABAergic interneurons (Mize 1992).

To directly examine the fast synaptic currents through GABA_A receptors, we recorded postsynaptic currents in the sSC at a holding potential of +50 mV in the presence of 2 mM Mg2+ in normal, untreated sSC slices (n = 41 neurons from 21 animals) and in sSC slices after in vivo NMDA (n = 38 neurons from 21 animals) or sham treatment (n = 44 neurons from 22 animals).

Recordings were obtained between P10 and P20. However, it is likely that a residual effect of surgery affected most recordings made shortly after Elvax implantation. Neurons in both sham- and NMDA-treated animals were difficult to record from on P10 and had somewhat smaller GABA current amplitudes compared with untreated animals on P10 and P11.

When held at +50 mV, sSC neurons P18 and older had spontaneous currents composed of two types of outward current: larger-amplitude, BMI-sensitive currents that reversed around −40 mV, and smaller-amplitude, BMI-insensitive currents that reversed around 0 mV. The latter population was sensitive to the glutamate receptor antagonists CNQX and AP5 and was detected in neurons from slices of all ages (P10–P20) and treatment groups. The two populations of currents can be seen in Fig. 1 in the traces from P19 neurons from all treatment groups. At P12, BMI-sensitive currents were relatively small in both untreated and sham-treated neurons. Neurons in slices from NMDA-treated colliculi, however, showed large, BMI-sensitive currents as early as P12.

Changes in BMI-sensitive and BMI-insensitive currents across age and treatment groups were examined quantitatively by measuring the amplitudes (Fig. 2) and frequencies (Fig. 3) of the sPSCs (see METHODS for sPSC selection criteria) for each neuron without BMI. BMI was subsequently applied to each slice, and the amplitudes and the frequencies of the remaining events were measured 6–7 min later. Only neurons for which stable recording conditions were maintained over the course of the experiments were included in this analysis. Data for BMI-insensitive currents were not included if large outward currents were not recovered on wash out of BMI.

The scatterplots in Fig. 2, A, C, and E show the average sPSC amplitudes for each of the neurons studied both in the absence and in the presence of BMI. The amplitude and frequency data from these cells was averaged across 2-day inter-
The developmental patterning of sPSC amplitudes was different after NMDA treatment, however (Fig. 2, F vs. D). Large-amplitude sPSCs appeared in NMDA-treated neurons at P12, and they maintained this large size throughout the period studied. These amplitudes were significantly higher than in sham-treated neurons in the P12–P13 and the P16–P17 interval and significantly higher than in untreated neurons in the P12–P13 interval (Tukey post hoc test, *P* = 0.005). As before, the size of the BMI-insensitive currents remained unchanged throughout the period studied.

Frequencies of the spontaneous currents at ±50 mV were also compared among untreated, sham- and NMDA-treated neurons (Fig. 3). The two-way ANOVA over all ages and treatments without BMI showed no significant differences between treatment groups (df = 2; *F* ratio = 2.8; *P* = 0.067) but some difference between ages (df = 5; *F* ratio = 12.2; *P* < 0.001) within treatments. There was a tendency for frequencies to increase in all older slices. Frequencies of sPSCs were significantly higher in the P18–P20 range than in the P10–P17 range in untreated neurons. In sham-treated neurons, sPSC frequency was significantly different at P20 from the frequencies in the P10–P17 age range. In NMDA-treated slices, the rise in sPSC frequency appeared slightly delayed. There were no significant differences in frequency in the P10–P19 range, but frequencies were significantly increased at P20.

For the sPSC frequency data obtained with BMI, which predominantly reflect glutamatergic currents, the ANOVA revealed significant differences both between treatments and across ages within treatment. Thus there was a tendency for sPSC frequency to increase in older neurons in both Elvax-treated groups. However, this was significant relative to normal neurons only for sham-treated neurons in the P18–P19 age interval. Within treatments, normal neurons showed no frequency changes across all ages. However, sham-treated neurons showed a significant frequency increase between the early (P12–P17) and the late (P18–P19) interval, and NMDA-treated neurons did not show a significant frequency increase until P20.

**Morphology of biocytin-filled cells**

Cells in sham-treated and NMDA-treated colliculi were categorized according to cell body shape and the organization and orientation of dendritic branches, using previous Golgi descriptions of neurons in both developing (Labriola and Laemle 1977) and mature (Langer and Lund 1974) rat sSC. Figure 4 contains confocal z-series projections illustrating this classification with typical biocytin-filled, narrow-field vertical neurons, distinguished by their pyramidal cell body and narrowly arborizing, dorsally directed dendrites, and typical horizontal neurons, distinguished by their large, laterally extending principal dendrites and horizontally oriented elliptical cell bodies. These relatively broad morphological characteristics are distinct in both sham- and NMDA-treated colliculi. Only a few cells failed to meet the criteria of a particular cell class, and these were classified as ambiguous. Complexity or size of dendritic or axon arbors were not measured because these characteristics varied with the efficiency of biocytin filling. However, no qualitative differences across treatment groups were found when GABA<sub>A</sub> receptor currents were blocked with BMI, indicating that it reflected a selective increase in these events.

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were apparent in any of these parameters or in the size of somata within any cell class. Furthermore, there was no correlation between any particular cell type and unusually large or small BMI-sensitive currents.

Five of the six major cell categories described in the superficial layers of the colliculus were sampled with our blind whole cell patch-clamping technique (Table 1), the exception being a small inwardly oriented cell, the marginal cell, located in the immediate subpial region. A few cells of this morphology were observed in several slices, but they were classified as

![Figure 2](image-url)

**Figure 2.** Quantitative analysis of the amplitudes of sPSCs like those shown in Fig. 1. Left: scatterplots of the average amplitudes of sPSCs in individual neurons of the superficial visual layer of the superior colliculus (sSC) against postnatal age in untreated (A), sham-treated (C), and NMDA-treated (E) tissue. Right: (B, D, and F) averages (±SE) of the data for the same neurons grouped in 2-day bins across the P10–P20 interval. For all data, filled symbols represent sPSCs that include GABA<sub>A</sub> receptor chloride currents, whereas open symbols represent data taken from the same neurons in the presence of 2 μM BMI to reveal the developmental pattern of the glutamatergic currents. Glutamatergic currents are generally smaller than the GABA<sub>A</sub> receptor currents in these data because the neurons were voltage clamped −50 mV above the glutamatergic channel reversal potentials of 0 mV but −90 mV above the chloride reversal potential. Both untreated and sham-treated neurons show a significant up-regulation of the GABA<sub>A</sub> receptor–mediated potentials between P17 and P18. In NMDA-treated neurons, this up-regulation occurs at P12 and remains high. See text for the statistical analyses of these data.
ambiguous because they did not appear to have a subpial position. The proportions of the remaining five cell classes differed between untreated, sham-treated and NMDA-treated slices (Pearson $\chi^2 = 38.88, \text{df} = 8$), and most of this difference was due to changes in two cell classes. First, wide-field vertical neurons were much more heavily represented in untreated colliculus slices than in either Elvax-treated group. It seems likely, however, that this difference is related to age, not treatment. The excitatory wide-field vertical neurons (Mize et al. 1982) are the largest cells in the sSC, and a retrospective analysis of the age distribution of this cell type indicated that, across all treatment groups, they were more frequently encountered in younger tissue ($P14$ and below). The blind whole cell patching technique is likely to be biased toward these larger cells, particularly in young slices. The soma size of all sSC neurons increases with age (Labriola and Laemle 1977), which would reduce this bias in older tissue. We analyzed a larger number of cells from younger slices in untreated animals compared with either experimentally treated group (61% between $P10–P13$ for untreated as compared with 50 and 46% from NMDA- and sham-treated neurons, respectively), which may account for the overrepresentation of wide-field vertical cells in untreated tissue.

The second large difference in cell type frequency was observed in horizontal cells. This morphologically distinctive, superficially located class is one of the major GABAergic cell types in the sSC (Mize 1992; Sterling and Davis 1980). This cell type, represented by 16% of the neurons from NMDA-treated sSC, was less prevalent in untreated (2%) and sham-treated (10%) populations. Horizontal cells have been described in Golgi studies of the sSC as early as the first week (Labriola and Laemle 1977). However, they receive most of their inputs from the cerebral cortex (Mize et al. 1982), whose inhibitory GABA$_A$ receptor–mediated effects on collicular neuron receptive fields (Binns and Salt 1997b) are first observed around $P18$ in the rat (Binns and Salt 1997a). It is tempting to speculate that activation of horizontal cells may accelerate their differentiation and thus make them easier to patch, because eight of the nine NMDA-treated horizontal neurons were from slices $P16$ or younger. Only two of the five horizontal neurons in sham-treated tissue were found in this young age range, and the only horizontal neuron found in untreated tissue was in a $P20$ slice. Unfortunately, these numbers are small and complicated by the largely unknown determinants of the age distribution of this cell type.
minimants of stable whole cell patch clamping in young tissue. Thus any convincing statement concerning the early NMDA-dependent maturation of a particular inhibitory cell type will require considerably more study.

Biochemistry and immunocytochemistry

Levels of both GAD isoforms (GAD_{67} and GAD_{65}) and the two neuronal GABA transporters (GAT-1 and GAT-3) rise dramatically during the second postnatal week in normal sSC, providing a biochemical index of GABA differentiation that corresponds closely to the increased effectiveness of BMI in normal neuropil at this age (Shi et al. 1997). Thus we compared levels of both GAD isoforms and GABA transporters between sham- and NMDA-treated tissue by normalizing protein levels in each group to normal age-matched tissue measured from the same gels. This comparison was unambiguous at P19, when relatively high levels of GAD were present in tissues from all treatment groups. Despite our inability to detect differences in GABA_{A} receptor currents between treatment groups in the P18–P19 interval, both GAD isoforms were significantly elevated in NMDA-treated tissue over sham-treated tissue at P19 (P = 0.02 for GAD_{65}, n = 3 animals, 9 immunoblots; P = 0.03 for GAD_{67}, n = 3 animals, 3 immunoblots; paired Student’s t-test). No significant difference between NMDA- and sham-treated tissue was found in the levels of the two GABA transporters, GAT-1 and GAT-3, at this age (Fig. 5). In P13 sSC, when significant physiological changes between treatment groups were observed, quantitative biochemical comparisons were difficult because of the low levels of all the GABA-associated proteins that we measured. Thus GABA transporters could not be examined at P13, and no significant differences in levels of the GAD_{67} isoform were detectable at this age between NMDA- and sham-treated sSC (P = 0.2, paired Student’s t-test, n = 2 sham- and 2 NMDA-treated animals, 3 immunoblots), possibly because of the relatively high levels of background staining. Nevertheless, GAD_{65} levels in NMDA-treated sSC were increased compared with sham-treated sSC (P = 0.04, paired Student’s t-test, n = 5 sham-treated and 5 NMDA-treated animals, 11 immunoblots) at this age.

Biochemical analysis also suggested that levels of the GAD_{67} enzyme were lower in sham-treated as compared with untreated sSC at both ages examined. The GAD_{67} levels in sham-treated tissue relative to untreated lanes of the same gels produced values less than one (Fig. 5). Optical density measurements for sham and normal tissue run on the same gel and taken at P13 and P19 were significantly different when specifically tested for such a decrease using a paired-sample Student’s t statistic. The decrease in GAD_{67} level is consistent with the tendency toward decreased GABA function in sham-treated versus untreated sSC that we detected in the physiological analyses (Fig. 2, D and B). Levels of the other isoform, GAD_{65}, were not consistently affected. The stronger effect of sham treatment on GAD_{67} over GAD_{65} may reflect differences in the control of the two enzymes by activity. GAD_{67} is presumed to represent most of the constitutively active GAD in the brain because it is almost entirely saturated with the activating cofactor pyridoxal phosphate (PLP), whereas the GAD_{65} enzyme is more immediately controlled by activity through transient associations with PLP (Erlander et al. 1991). Thus tonic decreases in GABA synaptic activity might be expected to involve decreases in levels of the GAD_{65} enzyme, whereas the need to change levels of GAD_{65} could be minimized by changes in cofactor association.

Cell density analyses

Cell densities were estimated in sham- and drug-treated sSC to answer two questions: did the increase in GABA_{A} receptor currents in NMDA-treated tissue reflect a differential loss of excitatory neurons relative to GABAergic neurons, and could the increase in GABA_{A} receptor currents represent an early onset of inhibition that could protect the neurons of the young sSC from over-excitation? It was important to address the latter question, because some early postnatal GABAergic currents are excitatory (Ben-Ari et al. 1997), and voltage-clamp recordings do not permit identification of chloride currents as either depolarizing or hyperpolarizing.

For cell counting, anti–MAP-2 staining was used to identify all neurons, and GAD immunohistochemistry, as a marker for GABAergic neurons (Houser et al. 1983), was used on adjacent sections. We stained for GAD_{67}, which is primarily localized to cell bodies (Hendrickson et al. 1994), instead of the predominantly synaptic GAD_{65} in this analysis because the dense local GABAergic innervation in the sSC (Mize 1992; Okada 1992) resulted in high levels of neuropil staining that obscured cell bodies.

Immunohistochemistry for both GAD_{67} and MAP-2 was qualitatively similar in all treatment conditions, and no histological or cytoarchitectonic differences were observed (Fig. 6). Although light staining of the entire sSC neuropil was present with the GAD_{67} antibody, GAD-positive somata were easily identified by granular deposits in the cytoplasm (columns 1 and 3, Fig. 6). MAP-2 staining of somata and processes was always pronounced, with no visible background (columns 2 and 4). Neuronal cell bodies in the center of each 30-µm section were discernible with both antibodies. However, unambiguous iden-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neuronal Cell Type</th>
<th>Stellate</th>
<th>Piriform</th>
<th>Narrow-field vertical</th>
<th>Wide-field vertical *</th>
<th>Horizontal *</th>
<th>Ambiguous</th>
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<td>10</td>
<td>8</td>
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<tr>
<td>NMDA</td>
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<td>58</td>
<td>28</td>
<td>24</td>
<td>16</td>
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Values in Neuronal Cell Type are percent of total. n is number of neurons. NMDA, N-methyl-D-aspartate. * Cell types where major differences were observed across treatments.
tification of particular cell types was not possible because of the thinness of the section.

The optical dissector method (Gundersen 1986) was chosen to quantify density, because it is robust to changes in cell size. At P13, when there were pronounced differences in GABAergic function between NMDA- and sham-treated littermates, we found no differences between their cell densities (Fig. 7). Both the total neuron density and the ratio of GABAergic neuron density to total neuron density were the same in both treatment groups (sham, n = 4 animals; NMDA, n = 3 animals).

Next, in P19 tissue, we determined whether the early increases in GABAergic currents detected physiologically could be facilitating cell survival in the continuous presence of a glutamate analogue. P19 was chosen to allow a longer interval for treatment-induced changes to appear and because, at this age, sSC neurons were well past their normal period of neuron death, which ends at P11 (Arees and Aström 1977; Giordano et al. 1980). For this analysis, in addition to the sham- and NMDA-treated groups, the densities of neurons positive for GAD<sub>67</sub> and MAP-2 were also examined in sSC where GABA<sub>A</sub> receptor–mediated responses were chronically antagonized with BMI. Nine litters were each divided into two or three of the four treatment conditions: sham, BMI, NMDA, or NMDA with BMI. Neurons positive for GAD<sub>67</sub> and MAP-2 were counted in the sSC of 17 sham-treated, 17 NMDA-treated, 6 BMI-treated, and 7 BMI- and NMDA-treated pups.

Cell densities at P19 were lower than the cell densities at P13 in all treatment groups, probably because of an overall expansion of the sSC neuropil due to axonal, dendritic, and glial maturation. When NMDA and BMI were applied simultaneously, sSC neuron density was reduced by 15% compared with sham, BMI, or NMDA treatment (mANOVA, df = 3, F ratio = 13.6, P < 0.001 by Tukey post hoc analysis), suggesting that the increases in GABAergic currents detected after NMDA treatment may act to mitigate any increased excitation and therefore protect young sSC neurons from excitotoxic cell death (Choi 1992). In addition, GABAergic neuron density in the NMDA-treated group and in the group treated with both BMI and NMDA was decreased slightly relative to sham-treated sSC (mANOVA, df = 3, F ratio = 5.6, P < 0.05 by Tukey post hoc analysis; Fig. 8). The latter effect suggests a greater sensitivity of the sSC GABAergic interneurons to early over-activation, similar to results in developing hippocampus (Geary et al. 1996; Houser and Esclapez 1996). Nevertheless, decreases in GABAergic neuron number are in the wrong direction and occur at the wrong age (P19) to account for the early (P13) up-regulation of GABA<sub>A</sub> receptor currents that we observed.

**DISCUSSION**

In the superficial layers of the superior colliculus, all GABA-mediated inhibition is generated by local interneurons (Mize 1992), and most sSC neurons respond to optic tract stimulation with short-latency excitation followed by GABA<sub>A</sub>...
receptor–mediated shunting inhibition. In addition, GABA_\text{A} receptor–mediated surround inhibition, driven largely by descending input from visual cortex, shapes response properties of these neurons (Binns and Salt 1997b). In a previous study, we found that GABA_\text{A} receptor–mediated inhibition of excitatory activity within sSC slices appears abruptly at P18 in rats; 4 days after the eyes open. This timing corresponds closely to the refinement of descending cortical projections (Thong and Dreher 1986) and the onset of visual cortical effects on individual collicular neuron response properties in vivo (Binns and Salt 1997a), suggesting that the differentiation of these inhibitory inputs might be driven by visual cortical activity.

To test the hypothesis that glutamatergic activation of GABAergic interneurons facilitates the differentiation of GABA_\text{A} receptor–mediated responses in this neuropil, we studied the development of BMI-sensitive currents and GAD expression in the sSC in normal tissue and in tissue treated chronically with low levels of NMDA. The present voltage-clamp analysis of synaptic currents in both normal and sham-treated sSC neurons supports our earlier observation (Shi et al. 1997) of a rapid up-regulation of GABA_\text{A} receptor current amplitudes between P17 and P18. This up-regulation is not observed in the presence of BMI and is therefore selective for
this rapid up-regulation of GABA_A receptor currents occurred at P12, 4 days after the initiation of NMDA treatment, and significantly earlier than in normal or sham-treated neurons (Fig. 2). Although it may be coincidental that 4 days elapse between eye-opening and the abrupt increase in inhibition in untreated animals, and between NMDA treatment and the early onset of inhibition in experimental animals, these data do suggest that NMDA receptor activity may lead to GABAergic synaptic maturation in this system. It remains to be determined whether the effect is a direct result of NMDA receptor activation on the GABAergic neurons themselves or an indirect effect of activating other neurons in the sSC, as has been suggested for activity-dependent changes in cortical GABAergic neurons in culture (Rutherford et al. 1997, 1998).

The frequency of synaptic events studied at +50 mV was relatively unchanged by chronic NMDA treatment. During the P10–P20 interval studied, sPSCs frequencies in all three groups increased at, or shortly after, P18–P19. This late frequency increase was observed in the predominantly glutamatergic responses remaining after BMI application from both NMDA- and sham-treated neurons. The late frequency increase was not observed in the BMI-insensitive currents from untreated neurons. These late differences in frequency were small, however, and differences across treatments were only statistically significant between the sham and untreated neurons in the P18–P19 interval (Fig. 3).

In many regions of the mature and developing CNS, changes in GAD levels seem to reflect changes in the amount of activity carried by the particular pathway (Benevento et al. 1995; Hendry and Carder 1992; Hendry and Jones 1988; Micheva and Beaulieu 1997). Our initial study of synaptic differentiation in the sSC suggested a close correlation between developmental increases in GAD_65, GAD_67, GAT-1, and GAT-3 protein levels and the increased effectiveness of GABA_A receptor-mediated inhibition (Shi et al. 1997). Thus we measured these biochemical indices of GABAergic differentiation in sham- and NMDA-treated sSC to determine whether they changed in association with the functional changes in inhibition after NMDA treatment. The present data suggest that the relationship may be more complicated. At P13, when the amplitudes of GABA_A receptor currents were significantly different between NMDA-treated and control animals, there were significant increases in GAD_65 levels in NMDA-treated sSC, but no significant changes in GAD_67 levels. This lack of effect may result from high background staining with the anti-GAD_67 antibody and relatively low levels of GAD_67 protein at this age, but these concerns do not apply to the P19 data. At P19, when no significant differences in the GABA_A receptor currents were apparent among the groups, immunoblotting showed significantly increased levels of both GAD isoforms in NMDA- as compared with sham-treated tissue (Fig. 5).

These observations suggest that parameters other than levels of the GABA synthetic proteins are important in determining the efficacy of GABAergic synapses under conditions of chronic low-level exposure to NMDA. The most likely candidates for such an effect are the GABA_A receptors themselves and/or changes in the efficacy of synaptic release. Indeed a number of investigators have documented subunit changes in GABA_A receptors during development (Fritschy et al. 1994; Oh et al. 1995), and some GABA_A receptor changes are correlated with activity changes early in development (Harris et al. 1995; Kumar and Schiehs 1993). In adults, the potentiation of hippocampal inhibitory synapses during the increased activity associated with kindling involves increased GABA_A receptor number (Nusser et al. 1998). Furthermore pre- and postsynaptic GABAergic up-regulation following chronically increased activation have been observed in tissue culture (Meno et al. 1991; Ramakers et al. 1994; Turrigiano et al. 1998; Zhu et al. 1995).

The tendency toward depression of GABA_A receptor current amplitude in sham-treated tissue relative to normal (which only reached physiological significance at P18–P19) was paralleled in the biochemical data at both P13 and P19 by a decrease in GAD_67 levels (Fig. 5). This was surprising, given the loose association between increased GAD levels and increased synaptic current in our data, but decreases in activity have been correlated with decreased GAD expression by previous investigators (Bear et al. 1985; Benevento et al. 1995; Hendry and Carder 1992; Micheau and Beaulieu 1997). Elvax may produce a pressure blockade of some axons in the young sSC and thus mildly decrease the overall level of activity relative to untreated animals. Evidence consistent with a similar sham effect from Elvax has been previously reported in frog tecta (Hickmott and Constantine-Paton 1997).

When NMDA and BMI were applied simultaneously, sSC neuron density was reduced by 15% compared with sham, BMI, or NMDA treatment. Decreases in cell density with NMDA treatment alone were not observed at P13 (Fig. 7), and at P19 (Fig. 8) they were small and restricted to the GAD-positive neuron population, indicating that the physiologically and biochemically observed increases in GABAergic indexes were not due to an increased proportion of inhibitory to exci-
tatory neurons. In addition, simultaneous exposure to BMI and NMDA did not appear to exacerbate the small density decreases in the GAD-positive population. These observations suggest that the observed GABA currents are associated with a protective effect on young collicular neurons and are therefore likely to shunt or counteract any excitation that is produced by the continuous presence of a glutamate analogue. They also suggest that any protective effects of the early increases in GABA receptor currents are not exerted on the GABA neurons themselves.

Interpretation of the results of chronic treatment of neuropil with neurotransmitter receptor agonists and antagonists can clearly be complicated by the compensatory adjustments of multiple neurotransmitter receptors. It is also difficult to extrapolate the actual level of excitation in intact, chronically treated neuropil from assays of transmitter alterations in a brain slice. Nevertheless, a parallel study of the development of glutamatergic currents in sSC neuropil similarly treated with NMDA suggests that NMDA receptor currents remain exceptionally potent in this tissue, whereas the development of non-NMDA ionotropic currents is retarded. These glutamatergic receptor changes reduce spontaneous activity in slices from NMDA-treated tissue whenever NMDA channels are blocked (Shi et al., unpublished observations). Presumably this condition helps to counteract increased activity in collicular neurons caused by the low levels of NMDA continuously present in the treated animals, and these compensations could account for the relatively small amount of cell loss observed under any of the treatment conditions.

Conclusions

In normal rat sSC development, the onset of pronounced GABA_A receptor–mediated inhibition occurs 4 days after eye opening. In this interval, pattern vision helps to organize collicular circuitry (Binns and Salt 1997a), and increased synaptic activity is observed in the sSC (Shi et al. 1997). The establishment of descending inhibition from the cortex onto sSC neurons at this time may therefore serve an important homeostatic function. In the sSC, inhibition generally appears after excitatory increased. During normal development of many CNS synapses become more potent when excitation is experimen-

tally potent in this tissue, whereas the development of non-NMDA ionotropic currents is retarded. These glutamatergic receptor changes reduce spontaneous activity in slices from NMDA-treated tissue whenever NMDA channels are blocked (Shi et al., unpublished observations). Presumably this condition helps to counteract increased activity in collicular neurons caused by the low levels of NMDA continuously present in the treated animals, and these compensations could account for the relatively small amount of cell loss observed under any of the treatment conditions.

Conclusions

In normal rat sSC development, the onset of pronounced GABA_A receptor–mediated inhibition occurs 4 days after eye opening. In this interval, pattern vision helps to organize collicular circuitry (Binns and Salt 1997a), and increased synaptic activity is observed in the sSC (Shi et al. 1997). The establishment of descending inhibition from the cortex onto sSC neurons at this time may therefore serve an important homeostatic function in addition to its role in patterning collicular neuron responses. Our findings indicate that a mechanism previously identified in mature cortex exists in vivo, namely that GABAAergic synapses become more potent when excitation is experimentally increased. During normal development of many CNS regions, as in the sSC, inhibition generally appears after excitation (Kirkwood and Bear 1994; LoTurco et al. 1991; Luhman and Prince 1990) and could be similarly facilitated by the increasing effectiveness of excitatory transmission. Thus maturation of GABA_A receptor–mediated inhibition by excitation may be an important developmental mechanism for assuring that young, and highly effective, glutamatergic currents are susceptible to tight control even before inhibitory circuitry normally appears.

The authors thank Dr. Sandra Hill-Felberg, K. Lee, and S. Gian for assistance in the anatomic preparation and analysis of biocytin-filled neurons. This work was supported by National Institutes of Health Grants NS-32290 and EY-06039 to M. Constantine-Paton and NS-09569 and MH-11535 to S. M. Aamodt.

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Received 15 June 1999; accepted in final form 12 November 1999.

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