AGE-DEPENDENT EFFECTS OF CHRONIC $GABA_A$ RECEPTOR BLOCKADE IN BARREL CORTEX

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$GABA_A$ receptor binding is transiently increased in rat whisker barrels during the second postnatal week, at a time when neurons in the developing rat cortex are vulnerable to excitotoxic effects. To test whether these $GABA_A$ receptors might serve to protect neurons from excessive excitatory input, polymer implants containing the $GABA_A$ receptor antagonist bicuculline were placed over barrel cortex for a 4-day period in young (postnatal days 8 – 12) and adult rats. In the cortex of young, but not adult rats, the chronic blockade of $GABA_A$ receptors resulted in substantial tissue loss and neuron loss. The greater loss of neurons in young rats supports the hypothesis that a high density of $GABA_A$ receptors protects neurons from excessive excitatory input during a sensitive period in development.

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CHAPTER I

INTRODUCTION

GABA is the major inhibitory neurotransmitter in the cortex. During the second postnatal week, there is a transient peak in $GABA_A$ receptor binding in rat whisker barrels. To investigate the role of transient increase in $GABA_A$ receptor binding, the barrel cortex of both developing and adult rats was exposed to polymer implants that release bicuculline, a specific $GABA_A$ receptor antagonist. Effects of bicuculline versus control implants were analyzed in Nissl-stained sections. In young but not adult rats, bicuculline administration was associated with significant tissue and neuron loss. In this first investigation of a chronic *in vivo* blockade of $GABA_A$ receptors in rat cortex, the results suggest a neuroprotective role of $GABA_A$ receptors in the developing rat cortex.

Development of Barrel Cortex

The posteromedial barrel field of rat somatosensory cortex, because of its transient increase in $GABA_A$ receptor binding, serves as a good model for studying developmental roles of cortical $GABA_A$ receptors. In the well defined whisker-to-barrel pathway, facial vibrissae correspond somatotopically to a specific clustering of cells called barrelettes in the brain stem, barrelloids in the thalamus, and barrels in cortex (Woolsey, 1967; Woolsey et al., 1975; Welker, 1976). At approximately postnatal day 3

(P3), cells in cortical layer IV begin to aggregate in barrel-like patterns imposed by the spatial pattern of the thalamocortical axons. During second postnatal week in the development of the somatosensory cortex, there is dramatic branching of dendrites and synaptogenesis within layer IV (Rice, 1985; Rice et al., 1985; Micheva and Beaulieu, 1996). Around P8-P12, $GABA_A$ receptor binding is transiently increased in cortical whisker barrels to almost two times that of the newborn or adult (Fuchs, 1995).

GABA and $GABA_A$ Receptor Development in the Cortex

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the cortex. From embryonic day 18 until the first part of the second postnatal week, GABAergic synapses in the rat cortex can generate membrane depolarization (Cherubini et al., 1990; Luhmann and Prince, 1991). GABA-induced depolarization may be due to the depolarized Cl^- equilibrium potential found in young neurons (Cherubini et al., 1990), but it may also be due to an under-developed Cl^- "transport system" (Plotkin et al., 1997) which allows an influx, rather than efflux, of chloride ions into the cell. Maturity of the inhibitory system associated with the GABAergic transition from depolarizing to hyperpolarizing activity may also depend on the establishment of complex cortico-cortical synapses (Lund and Harper 1991), or on the developmental changes in receptor subunits (Cherubini et al., 1998; Soldo et al., 1998). By the end of the first postnatal week, GABAergic responses are inhibitory (Plotkin et al., 1997; Luhmann and Prince, 1991).

In autoradiographic studies of the adult rat parietal cortex, approximately 70% of [^{3}H]GABA binding is to the $GABA_A$ site (Bowery et al., 1987; Chu et al., 1990). In the

whisker barrel of the somatosensory cortex of rats, [^{3}H]muscimol binding to $GABA_A$ receptors is relatively low from P0-P4. Around P8-P12, GABA receptor binding is transiently increased in cortical whisker barrels to almost two times that of the newborn or adult (Fuchs, 1995).

In postnatal cortical development, spatiotemporal patterns of neurotransmitter receptor binding generally change (Murrin et al., 1985; Dam et al., 1988; Palacios et al., 1988; Fuchs, 1995). In several cases, neurotransmitter receptor binding increases transiently during brain development (see Table 1). As with muscimol binding, the transience typically occurs in areas that receive direct thalamic input, such as in layer IV of the primary somatosensory or visual cortex (Insel et al., 1990; Fuchs, 1995). While the purpose of transient increases in receptors is not known, perhaps the developmental changes in receptors assist in cortical organization, maturation, or even neuronal survival (review: Zilles et al., 1991; Fuchs, 1995).

Development of Glutamate Receptors and Excitotoxicity

Glutaminergic (Kaneko and Mizuno, 1988; Kharazia and Weinberg, 1994) thalamic afferents terminate in layer IV in the barrel cortex (Chmielowska et al., 1989). These receptors, functional from birth (Kim et al., 1995), play an important role in synaptic maturation (reviews: McDonald and Johnston, 1990; Kaczmarek et al., 1997; Anwyl, 1999). Glutamate receptors in barrels also demonstrate transience during development (Insel et al., 1990; Kossut et al., 1993; Monyer et al., 1994; Blue and Johnston, 1995; Glazewski et al., 1995; Blue et al., 1997). For example, in the rat barrel field cortex, [^{3}H]glutamate binding to NMDA receptors increases to a peak at P21 (Blue

and Johnston, 1995), while [^{3}H]glutamate binding to quisqualate receptors peaks in the barrel centers at approximately P10 and then decline to adult levels by P21 (Blue and Johnston, 1995).

Excitotoxicity, which can result from an over-activation of excitatory amino acid receptors (Choi, 1992; Olney, 1994), may initiate a cascade of cellular events that produce neuronal injury and death in the developing rat brain (McDonald et al., 1988). Perhaps due to the slower maturation of the inhibitory system (Burgard and Hablitz, 1993), there is a transient vulnerability of cortical neurons to seizure activity (Kaminogo, 1983; Luhmann and Prince, 1991; Agmon and O'Dowd, 1992). In the second postnatal week, there is also a transient sensitivity to excitotoxic injury and cell death by AMPA (McDonald et al., 1992) and NMDA (McDonald et al., 1988; McDonald and Johnston, 1990; Carmignoto and Vicini, 1992; Deisz and Luhmann, 1995; Johnston, 1994, 1995). Given the coincident timing of increased glutamate sensitivity and the high levels of $GABA_A$ receptors during the second postnatal week, it is conceivable that the temporary increases in $GABA_A$ receptors serve to protect cortical neurons from glutamate receptor mediated excitotoxicity.

$GABA_A$ Receptor Blockade

Small changes in the balance between cortical excitation and inhibition during development can lead to changes in cortical structure and function (Charpier and Deniau, 1997; Wallace and Fox, 1999). GABA, through its hyperpolarizing effects on neurons, serves to balance excitatory neurotransmission, and contributes to the overall level of cortical activity (Tasker and Dudek, 1991; Bernardo and Wong, 1995; Burgard and

Hablitz, 1993; Douglas et al., 1995). Both development and maintenance of inhibitory circuits contribute to neuronal survival (Mattson and Kater, 1989; Ikeda et al., 1997).

Bicuculline can block the inhibitory effects of $GABA_A$ receptor mediated activity (Curtis et al., 1971). Blockade of cortical $GABA_A$ receptors can lead to increased activity and epileptiform activity, both *in vivo* (Pernberg et al., 1998) and *in vitro* (Chagnac-Amitai and Connors, 1989; Burgard and Hablitz, 1993). Blockade of $GABA_A$ receptors can also influence NMDA receptor activity (Luhmann and Prince, 1990; McCormick et al., 1993). Beginning at approximately P6-P8, bicuculline can increase excitatory postsynaptic potentials by electrical stimulation (Burgard and Hablitz, 1993) and lead to epileptiform bursting by P9 (Burgard and Hablitz, 1993).

In vivo, ionophoretic application of bicuculline increases the electrophysiological activity of cortical neurons (Hicks and Dykes, 1983; Kyriazi et al., 1998). Locally applied bicuculline has also been used to induce focal seizures (Soukupova et al., 1993; Stein et al., 2000). Cell injury following bicuculline-induced seizures has been demonstrated in rats (Soderfeldt et al., 1981, 1983; Turski et al., 1985; Sasahira et al., 1995). To block $GABA_A$ receptors, intraperitoneal (i.p.) bicuculline injections have often been employed. Developing rats show epileptiform seizures including shaking, rearing, and alternating muscle contraction and relaxation, known as clonus following i.p. injections of bicuculline (Turski et al., 1985; Zouhar, 1989; Baram and Snead, 1990). However, bicuculline injections are also accompanied by pulmonary edema and decreased cerebral oxygenation (Kiessling et al., 1981; Soderfeldt et al., 1983; Kreisman et al., 1987). By applying the $GABA_A$ receptor antagonist, bicuculline methiodide (BMI)

(Olsen, 1975), directly to the cortex, one can avoid the complications of systemic application.

Methodology

The implant material used was an ethylene vinyl acetate copolymer (Evatane, Elf Atochem North America, Philadelphia, PA) similar to Elvax 40P (Dupont). These polymers have been used to deliver drugs locally, bypassing systemic interaction and degradation. They also have an advantage over osmotic pumps and iontophoresis that are not designed for chronic applications in small mammals like the developing rat. *In vivo*, Elvax and similar polymer implants have been successfully used to deliver insulin, growth factors, immunoglobulins, neurotransmitters, or their antagonists (Table 2). Release of drugs within hours of implantation has been demonstrated *in vivo* (TTX: Chiaia et al., 1992; [^{3}H]APV: Fox et al., 1996) for a period of one week (Schlagger et al., 1993) and longer (Schnupp et al., 1995; Smith et al., 1995; Mooney et al., 1998; Prusky and Ramoa, 1999).

Table 1.

Examples of Receptors Displaying Transience During Rat Cortical Development

Receptor Type	**Method**	**Reference**
$GABA_A$	A	Schliebs and Roth, 1988; Kumar and Schliebs, 1993; Fuchs, 1995
$GABA_B$	A	Turgeon and Albin, 1994
Benzodiazepine	A	Schliebs et al., 1986
NMDA	A	Insel et al., 1990; Blue and Johnston, 1995
	A, I	Brennan et al., 1997
Kainate / Quisqualate	A	Insel et al., 1990; Miller et al., 1990
	A, I	Blue and Johnston, 1995; Brennan et al., 1997
Metabotropic glutamate	A	Blue et al, 1997
Nicotinic acetylcholine	A	Fuchs, 1989
Muscarinic acetylcholine	A	Kumar and Schliebs, 1992
	I	Buwalda et al., 1995

A = Receptor autoradiography; I = Immunohistochemistry

Table 2.

Examples of *In Vivo* Use of Polymer Implants in Rat

Reference	**Substance Delivered**	**Age**	**Exposure Periods**
Aamodt et al., 2000	NMDA; Bicuculline; NMDA with Bicuculline	P8	2 - 12 days
Boison et al., 1999	Adenosine	Adult	1 - 14 days
Brown et al., 1986	[^{3}H]Insulin	Adult	100 days
Chiaia et al., 1992; 1994	TTX	P0	6 - 11 days
Doulazmi et al., 1998	Immunoglobulins	P4 – P5	4 - 5 days
Fox et al., 1996	APV; [^{3}H]APV	P0	3 - 6 weeks
Graber and Prince, 1999	TTX	P28 – P30	10 - 15 days
Kokaia et al., 1994	GABA; Noradrenaline	Adult	2 - 14 days
Penschuck et al., 1999	BDNF; MK-801; [^{3}H]MK-801	P0	6 - 14 days
Persico et al., 1997	p-Chloroamphetamine (PCA)	P0	6 days
Rhoades et al., 1996; 1998	TTX	P0	6 days
Rozas et al., 1996	GABA	Adult	1 - 14 days
Schlaggar et al., 1993	APV	P0	8 days
Simon et al., 1992	APV; MK-801; dihydro-B-erythroidine	P0	19 days

CHAPTER II

MATERIALS AND METHODS

Polymer Implants

A 10% solution of ethylene-vinyl acetate copolymer beads (Evatane, Elf Atochem North America, Philadelphia, PA, N. 24937-78-8) in methylene chloride was prepared using methods previously described (Rhine et al., 1980; Silberstein and Daniel, 1982). Evatane (0.049 g) was dissolved in methylene chloride (0.44 ml) and thoroughly mixed with 0.01 g BMI, yielding a 40 mM bicuculline methiodide (BMI) solution (approximately 200 mg per g polymer). Implants prepared without a drug were used as controls. The suspension was vortexed for 4-5 min, poured into a pre-cooled glass plate or metal mold (6.0 cm X 6.0 cm X 0.3 cm), and kept covered at -80°C for 10 min. The frozen polymer was removed from the metal mold, placed on ParaFilm (American National Can, Greenwich, CT), wrapped in foil and sealed in a plastic freezer bag. The polymer remained in the -80°C freezer for 4 days and then was transferred to a -20°C freezer for an additional 4 days. Over the first four days, the polymer block was periodically removed from the bag and vented under a fume hood. The polymer was sectioned at 100 μm thickness using a sliding mictrotome (Microm Heidelberg, Zeiss), and a stereomicroscope was used to aid in cutting the polymer sheets into 2 mm X 2 mm implants. All implants were wrapped in foil and stored at –20°C. Prior to use, each

implant was presoaked overnight (12-16 hours) in 0.9% saline to avoid a high initial rate of release (Reh and Constantine-Paton, 1985).

Subjects

The subjects were Long-Evans hooded rats (Simonsen, Gilroy, CA). Rats were used at P8, where P0 is the day of birth. Adult male rats at least 3 months of age, but less than 1 year old (268 ± 90 g, mean ± S.D.), were also examined. Animals were kept on a 12:12 light-dark cycle, had food and water available *ad libitum*, and were sacrificed between 9:00 and 16:00.

Surgery

Postnatal day 8 rat pups were anesthetized with inhaled methoxyflurane (Pitman-Moore, Inc., Mundelein, IL). A midline incision was made in the scalp, and the fibrous sheath of periosteum that covers the bone was removed by gentle scraping. A circular 2 mm diameter "window" of skull was removed over the barrel field on each side of the midline. The barrel field in the P8 rat was determined to be located approximately 1.2-2.1 mm posterior to bregma and 3.0-3.5 mm from midline, (Sherwood and Timiras, 1970). The dura mater over SI was removed with a fine-curved microhook, and the implant was placed over the exposed cortex. The BMI implant was placed on either the right or the left SI; the control implant was placed on the contralateral side (See Fig. 1). All implants were secured by gently tucking the corners of the implant under the skull. The incision was held together by alpha-cyanoacrylate and without sutures. While recovering from anesthesia, the animal was warmed in an incubator and returned to its

mother within 1 hour. During recovery and for the four days after implant surgery, there were no noticeable seizure-like behaviors (convulsions, whisker twitching).

Adult rats were anesthetized with ketamine (100 mg/ml solution, i.p.; 90 mg/kg) and xylazine (100 mg/ml solution, i.p.; 10 mg/kg). Under surgical anesthesia, the head was shaved and a midline incision made in the scalp. Using the same procedure as above, the periosteum and the musculature were dissected away from the barrel region. In adults, the boundaries of the posteromedial barrel field are located approximately 4.0-6.0 mm from midline and 0.2 mm anterior to and 2.7 mm posterior from bregma (Paxinos and Watson, 1986). After the implantation, the incision was sutured with 4-0 silk, and the animals were returned to their home cages and monitored for recovery.

Tissue Preparation

Four days after the implant surgery, animals were deeply anesthetized and perfused intracardially with 200 ml room temperature 0.9% saline solution, followed by chilled 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. P12 rat pups were anesthetized with inhaled Metofane (Pitman-Moore, Inc., Mundelein, IL), and adult rats were anesthetized with ketamine (100 mg/ml solution, i.p.; 90 mg/kg) followed by xylazine (100 mg/ml solution, i.p.; 10 mg/kg). Following a 24-48 hour postfixation, perfused brains were cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 1 to 2 days. Brains were frozen by submersion in –35°C isopentane for 5 minutes, followed by -80°C isopentane for 5 minutes, and then were wrapped in foil and stored at –80°C until use. Whole brains were mounted with O.C.T. (TissueTek, Inc. Elkhart, IN), cut at 50 μm thickness using a sliding microtome (Microm Heidelberg, Zeiss), and collected in 0.1 M

phosphate buffer. Coronal brain sections were mounted on gelatinized subbed slides and stained for Nissl substance with 0.25% thionin. The stained slides were then dehydrated in a series of alcohols, cleared in xylenes, and coverslipped with DPX (BDH Microscopy Material Ltd., England).

Data Analysis

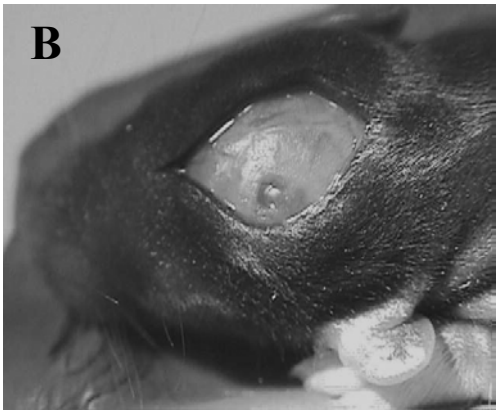
Drawings from each of the Nissl stained sections through the barrel field were made using a camera lucida. Four serial 50-μm coronal sections (a total of 200 μm anterior-posterior) were chosen to represent the area of maximum damage under the implant. Using a camera lucida at a final magnification of 40X, affected areas in each section were outlined and measured in mm^2 using a digitizing data tablet.

For each section, effects of bicuculline were calculated as the mean affected area on the BMI side minus that on the control side. Statistical analysis was performed using Jandel SigmaStat software. Effects of age and treatment were assessed with Student's *t*-tests and analyses of variance (ANOVAs) followed by Tukey's or Dunnett's post-hoc analysis.

Electrophysiology

To assess the effectiveness of bicuculline release from the implant, cortical electrophysiological recordings were made under both the control and bicuculline implants. These recordings were performed four days post-implantation (in three P12 rats), or one day post-implantation (in two P18 rats). Animals were surgically anesthetized with urethane (1.0 g/kg, i.p.; 20% solution) and mounted in a stereotaxic

instrument. After removal of the skin from the surgical area, the implants were removed. Within 20 min of implant removal, the first electrical recordings were made by lowering a 10 Ω tungsten recording electrode with a 5-μm tip into the center of the implant site. Electrical activity was recorded by multiple passes beginning at the surface of the cortex and at 100 μm intervals throughout the depth of the cortex.



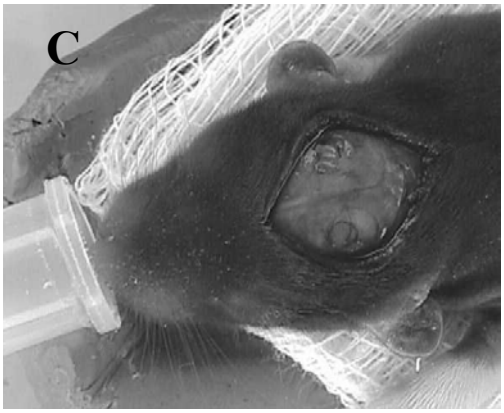


Figure 1. Surgical procedure in a P8 rat. (A) Anesthetized animal with inhaled Metofane. (B) Window in skull over posteromedial barrel field. (C) Polymer implants placed over both cortices prior to incision closure.

CHAPTER III

RESULTS

Drug Release from Implants

The coloring of the polymer implant that accompanied the doping of the implant indicated that the drug was distributed throughout the polymer. For example, when the polymer was mixed with methylene blue, the implant appeared light to deep blue depending on concentration. The introduction of the 40 mM BMI changed the normally clear polymer to a golden yellow. *In vivo* and *in vitro* release of drug from the polymer was also assessed by visual inspection. When implants were retrieved from the cortex post-termination, methylene blue implants were lighter in color; BMI implants had changed to a pale translucent yellow shade. These changes in implant color intensity were similar to studies in this laboratory, when doped polymer implants were placed in saline for four days (unpublished observations).

Further evidence of drug release *in vivo* was noted in electrophysiological studies following the one or four days of exposure to cortical implants. Observations of multiunit recordings in the P18 animals after the one-day of exposure revealed different activity levels under the bicuculline and control implants. Periodic electrophysiological bursting was observed in the cortex under the bicuculline implant, but was not quantified. Acute delivery of 40 mM bicuculline methiodide solution to the surface of the cortex in one P18 animal resembled the electrophysiological activity after one-day exposure to the

bicuculline implant. There was less bursting under the control implant compared to the bicuculline implant in these experimental animals.

In the P12 animals following 4 days of implant exposure, both driven and spontaneous electrophysiological activity were increased under the bicuculline implant compared to the control implants. As seen in the example presented in Figure 2, spontaneous activity appeared slightly greater under the bicuculline implant than under the control implant from the cortical surface to depths of 1200 – 1400 μm. In the developing cortex, these depths correspond approximately to layers I-IV. In a few animals, bicuculline-associated increase extended down to layers V and VI. The increase in electrophysiological spontaneous activity under the bicuculline implant compared to the control implant suggests $GABA_A$ blockade was still effective after four days *in vivo*. Evidence of sustained *in vivo* drug release is in agreement with results from other studies utilizing ethylene vinyl copolymer methodology (Mooney et al., 1998; Penschuck et al., 1999).

Histologic Observations

As with other studies using implants (Jablonska et al., 1999; Penschuck et al., 1999) brains with hematomas or gross surgical trauma, or whose implants had shifted from the barrel region, were not included in the study. Macroscopically, slight depressions remained over the barrel cortex where either implant had been placed. Microscopic examinations of sections under the implants suggested three categories of cytoarchitectural effects (see Figs. 3 and 4 and 5).

Tissue loss. Areas with a complete absence of tissue were measured from an interpolated cortical surface. The loss of tissue was generally conical in shape with the widest area being directly under the implant, occasionally tapering through all layers of cortex.

Neuron loss. Nissl-stained sections also contained areas with an apparent loss of neurons. Neurons, which are characteristically large cells with pale cytoplasm, a visible nucleus, and darkly staining nucleoli (Vaughan, 1984), were not present in this area, but glial cells were seen. Neuron loss was often seen adjacent to areas of tissue loss.

Histological disruption. In addition to tissue and neuron loss under implants, some areas contained disrupted lamination or cells with abnormal morphology. Damaged cells appeared similar to those in rat cortex following systemic bicuculline administrations, as described by Soderfeldt (1981, 1983). For example, many neurons were shrunken in size and/or had dark staining cytoplasm. These neurons however, were still relatively larger than glia. There were also degenerating neurons, cells with large cytoplasmic vacuoles, and cells containing membrane-bound apoptotic bodies. Such affected cells were not restricted to a specific layer, and were often found adjacent to the area of neuronal loss. Also in this category, there were areas with disturbances in numerical cell density and cortical lamination. Often there was a blurring of the normally distinct boundaries of layers IV and V, or general loss in the clarity of the barrel cytoarchitecture. Occasionally, cells and laminae shifted and extended above the interpolated normal cortical surface. This area of "tissue gain" was subtracted from the calculated tissue loss for an area of net tissue loss.

Summed Effect. The total area of cortex affected by the implant was calculated as the sum of the areas of tissue loss, neuron loss, and histological disruption.

Data Analysis

For the pups exposed to implants from P8-P12, effects of implants containing bicuculline methiodide were greater than those of the control implant (Table 3). The area of tissue loss was greater by 0.32 mm^2 ($P < 0.001$) and that of neuron loss was greater by 0.28 mm^2 ($P < 0.003$) (Table 4, Fig. 6). The area of histological disruption under bicuculline implants was not significantly greater than under the control implant (0.90 mm^2, $P < 0.07$). The summed effect was significantly greater under the bicuculline than control implant (1.50 mm^2, $P < 0.001$; Fig. 6). For adults, however, no significant difference was observed in any of the categories (Tables 3 and 4; Fig. 6). The results of a two-way ANOVA suggested that tissue loss was affected by treatment (bicuculline vs. control) ($F_{1,44} = 5.60$, $P < 0.02$), but not age ($F_{1,44} = 3.02$, $P < 0.09$; Table 5). There was not a significant interaction between the two ($P < 0.145$; Fig. 7A). For neuron loss, there was a significant effect of both age ($F_{1,44} = 9.69$, $P < 0.003$) and treatment ($F_{1,44} = 7.38$, $P < 0.01$). Again, there was no significant interaction between age and treatment ($P < 0.07$, Table 5; Fig. 7B). In the category of histological disruption there was a significant effect of age ($F_{1, 44} = 55.33$, $P < 0.001$) but not of treatment ($P < 0.07$). There was significant interaction between age and treatment ($F_{1,44} = 4.17$, $P < 0.05$, Table 5; Fig. 7C). When all categories were combined for the summed effect, there were effects of both age ($F_{1,44} = 39.34$, $P < 0.001$) and treatment ($F_{1,44} = 4.05$, $P < 0.05$), and a significant age by treatment interaction ($F_{1,44} = 5.68$, $P < 0.02$, Table 5; Fig. 7D).

The effects of the control implant were significantly greater in young than adult cortex in tissue loss ($F_{1,23} = 9.51$, $P < 0.005$; Table 6), histological disruption ($F_{1,23} = 20.61$, $P < 0.0002$), and summed effect ($F_{1,23} = 11.17$, $P < 0.003$; Table 6). The effects of the bicuculline implant were significantly greater in young than adult cortex in neuron loss (($F_{1,23} = 8.23$, $P < 0.01$; Table 6), histological disruption ($F_{1,23} = 34.75$, $P < 0.0001$; Table 6), and summed effect ($F_{1,23} = 28.32$, $P < 0.0001$; Table 6).

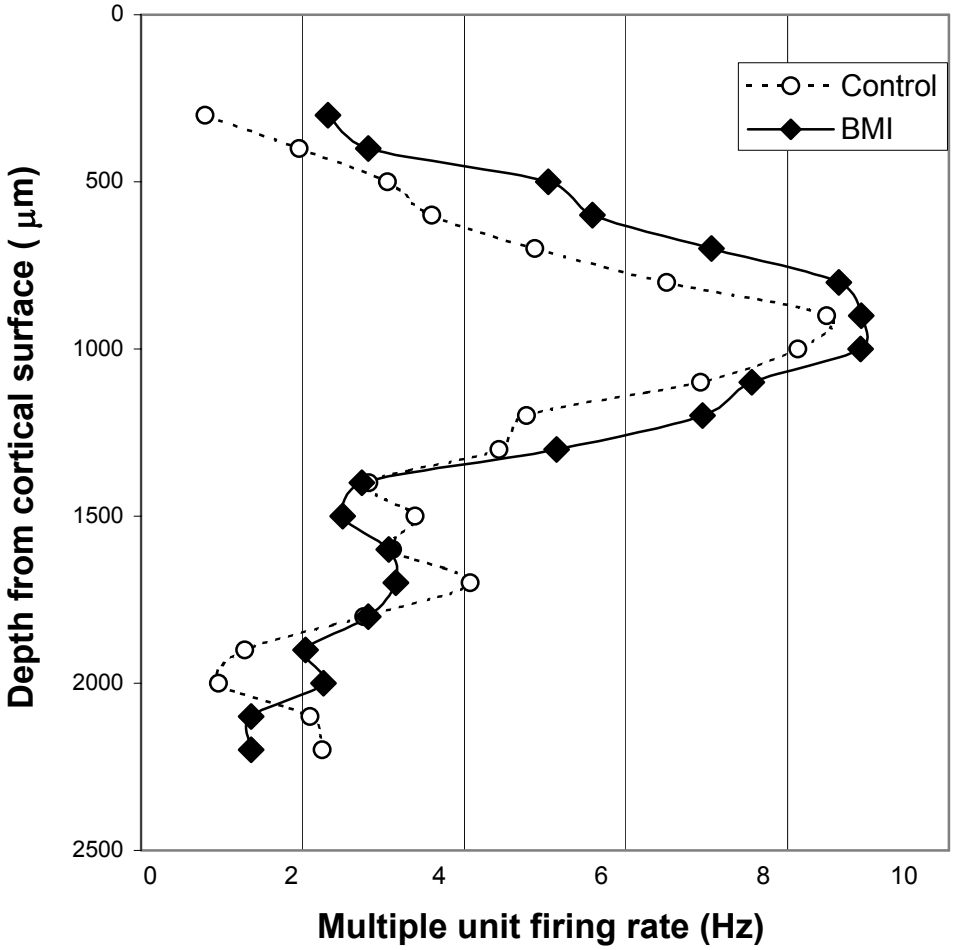


Figure 2. Spontaneous activity under cortical implants. Shown are data from barrel cortex of a P12 rat, 4 days after placement of the implants. In this example, spontaneous activity appears slightly increased under the bicuculline implant than under the control implant from the cortical surface to a depth of 1300 μm. Electrophysiological data were box-car averaged over three points.

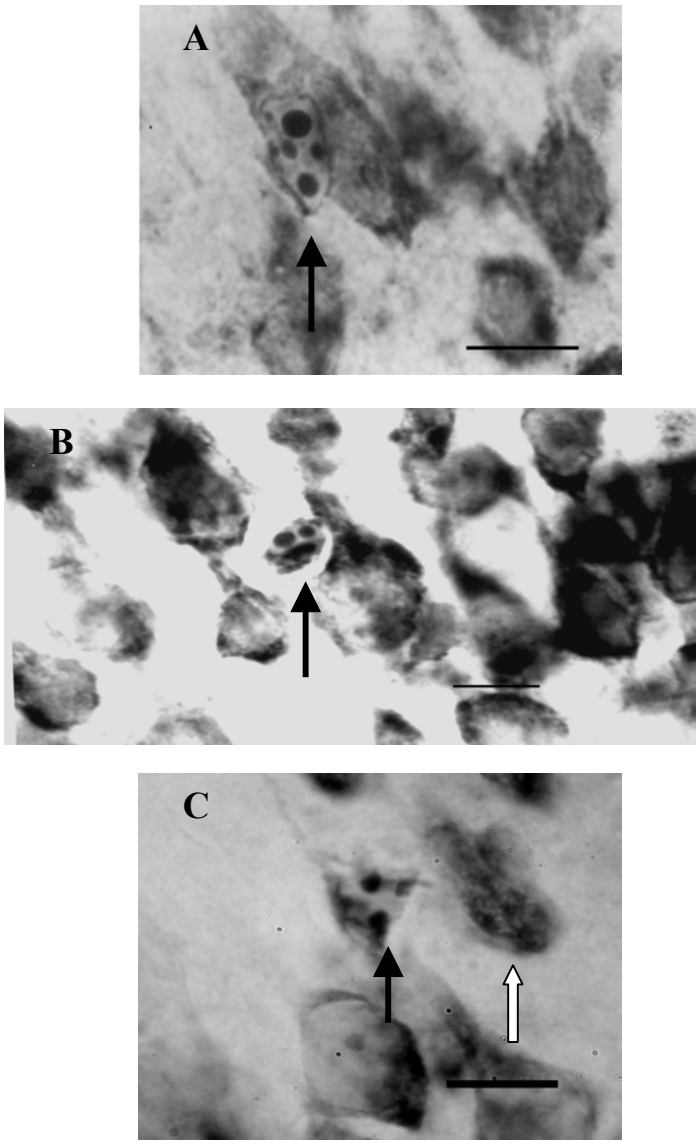


Figure 3. Examples of thionin-stained cells within the area of histological disruption, including apoptotic neurons (A and C, *closed arrows*), apoptotic glial cells (B, *closed arrow*), and degenerating neurons (C, *open arrow*). Scale bars, 10 μm.

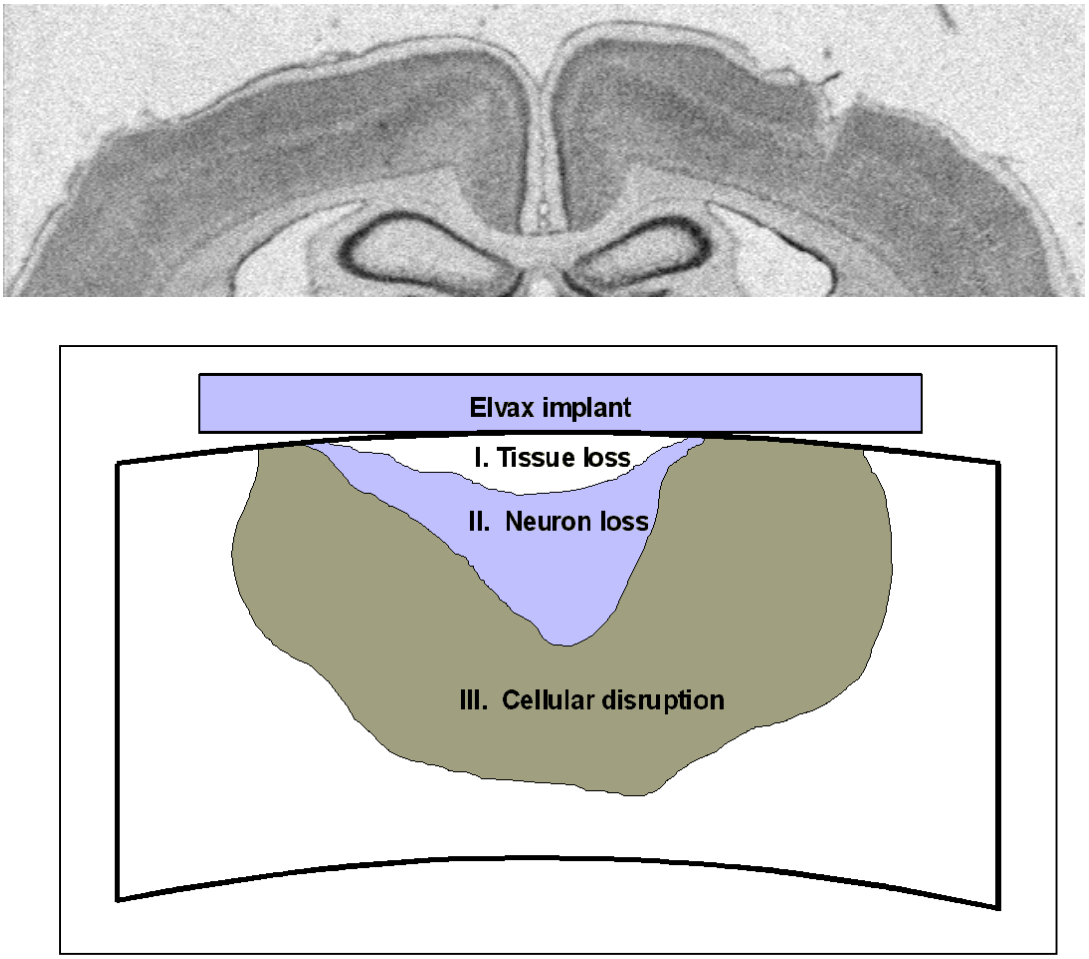


Figure 4. Tissue disruption under a polymer implant. (A) Photograph of thionin-stained section after a four-day exposure to a control implant on the left and bicuculline implant on the right. (B) Schematic reconstruction to illustrate defined areas of effects under an implant.

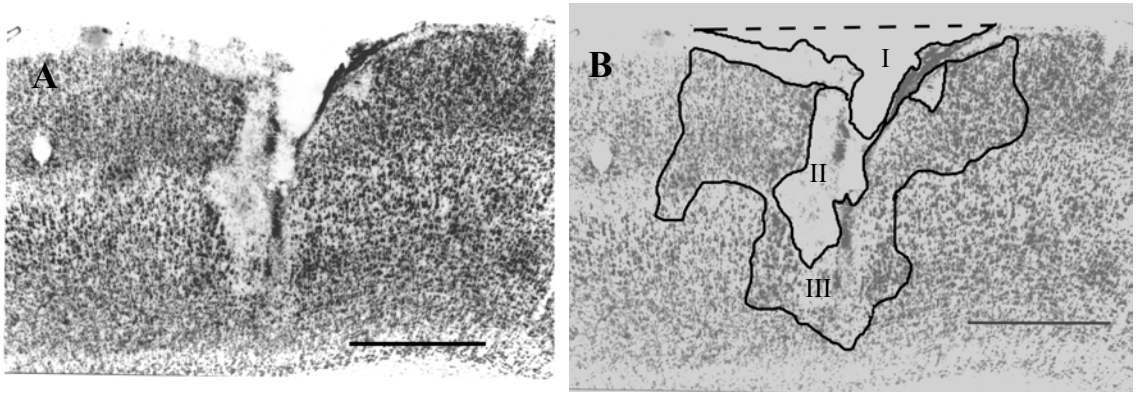


Figure 5. Affected areas under the implant. (A) Photograph of cortex under a bicuculline implant on a P12 rat. (B) Boundaries of areas of cortical effects are superimposed over the image. Numbers refer to (I) tissue loss, (II) neuron loss, and (III) histological disruption. Scale bars, 1 mm.

Table 3.

Effects of Bicuculline and Control Implants in Rats

		Tissue Loss	**Neuron Loss**	**Histological Disruption**	**Summed Effect**
PUPS (n = 13)	Bicuculline	0.30 ± 0.07	0.38 ± 0.09	3.10 ± 0.40	3.77 ± 0.45
	Control	-0.03 ± 0.06	0.10 ± 0.05	2.20 ± 0.26	2.27 ± 0.29
	(B minus C)	0.32 ± 0.08	0.28 ± 0.09	0.90 ± 0.23	1.50 ± 0.27
	One-Way ANOVA	$P < 0.001$; Post-hoc $P < 0.05$	$P < 0.003$ (KW); Post-hoc $P < 0.05$ (Dunnett's)	$P < 0.07$	$P < 0.01$; Post-hoc $P < 0.05$ (Tukey 's)
ADULTS (n = 11)	Bicuculline	0.32 ± 0.13	0.08 ± 0.05	0.42 ± 0.12	0.82 ± 0.28
	Control	0.24 ± 0.07	0.03 ± 0.03	0.68 ± 0.20	0.95 ± 0.26
	(B minus C)	0.07 ± 0.10	0.05 ± 0.06	-0.25 ± 0.13	-0.13 ± 0.14
	One-Way ANOVA	$P < 0.82$ (KW)	$P < 0.48$ (KW)	$P < 0.38$ (KW)	$P < 0.74$

Effects were calculated in mm^2 for each section as mean area of affected tissue under the implant on the bicuculline side minus that on the control side (B minus C). Shown are the mean ± S.E.M. (KW = Kruskal-Wallis).

Table 4.

Cortical Effects of Bicuculline

	Pups (n = 13)	**Adults (n = 11)**	**Pups Minus Adults**
Tissue Loss	0.32† ± 0.08 $P < 0.001$	0.07 ± 0.10 $P < 0.82$	0.25 $F_{1,22} = 3.98$ $P < 0.06$
Neuron Loss	0.28 ± 0.09 $P < 0.003$	0.05 ± 0.06 $P < 0.48$	0.23 $F_{1,22} = 4.01$ $P < 0.06$
Histological Disruption	0.90 ± 0.23 $P < 0.07$	-0.25 ± 0.13 $P < 0.38$	1.15 $F_{1,22} = 17.36$ $P < 0.001$
Summed Effect	1.50 ± 0.27 $P < 0.01$	-0.13 ± 0.14 $P < 0.74$	1.63 $F_{1,22} = 23.12$ $P < 0.001$

†Areas (in mm^2) of affected tissue under the implant for each section (bicuculline side minus control side). Shown are the mean ± S.E.M.

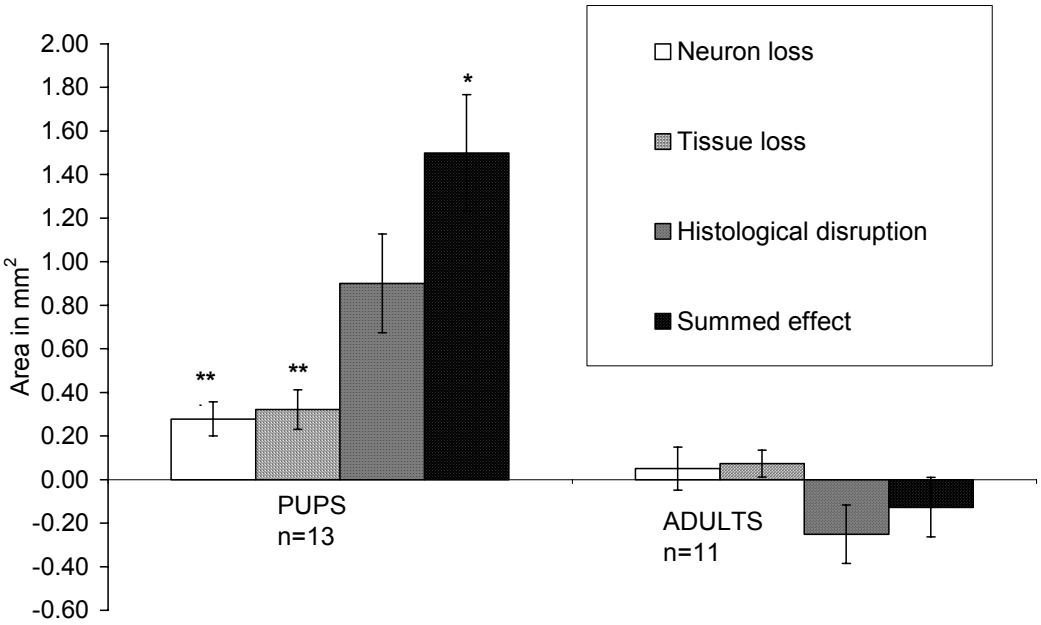


Figure 6. Effects of bicuculline. Shown are mean areas per section of neuron loss, tissue loss, histological disruption, and summed effect under the bicuculline minus the control implant. In cortex of pups, but not adults, bicuculline was associated with significant neuron loss, tissue loss, and summed effect (one-way ANOVA ** $P < 0.003$, *** $P < 0.001$, and, * $P < 0.01$, respectively). Each value represents the mean ± S.E.M.

Table 5.

Results of Two-Way ANOVA in Rat Barrel Cortex Following Bicuculline Implants

		Tissue Loss	Neuron Loss	Histological Disruption	Summed Effect
Two -Way ANOVA	**Age**	F = 3.02	F = 9.69	F = 55.33	F = 39.34
		$P < 0.09$	$P < 0.003$	$P < 0.001$	$P < 0.001$
	Treatment	F = 5.60	F = 7.38	F = 1.33	F = 4.05
		$P < 0.02$	$P < 0.01$	$P < 0.26$	$P < 0.05$
	Age X Treatment	F = 2.21	F = 3.55	F = 4.17	F = 5.68
		$P < 0.15$	$P < 0.07$	$P < 0.05$	$P < 0.02$

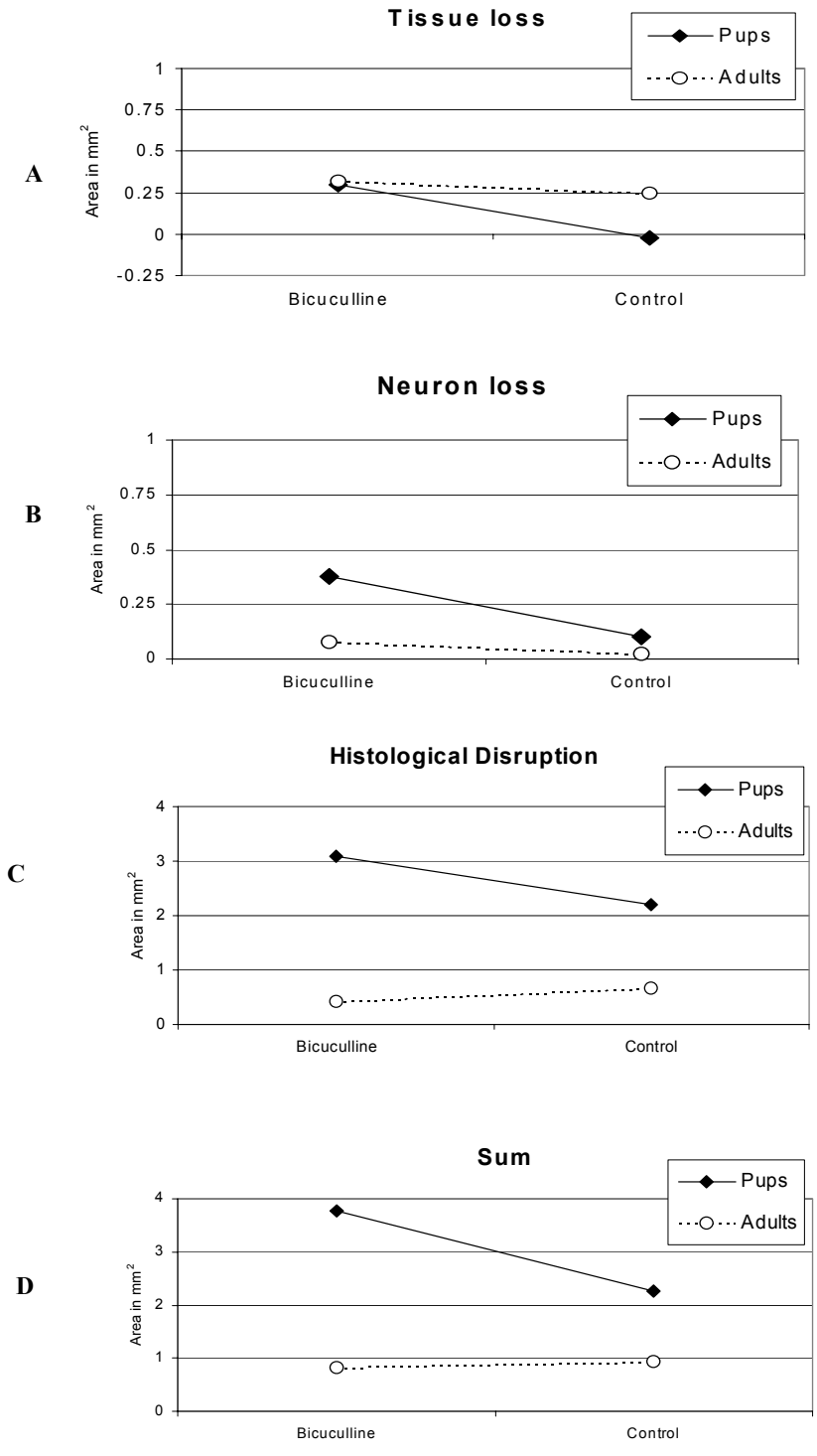


Figure 7. Graphs of means tested for effects of age, treatment, and age-by-treatment interactions (see Table 5).

Table 6.

Effects of Cortical Implants

		Tissue Loss	Neuron Loss	Histological Disruption	Summed Effect
One -Way ANOVA	**Control**	F = 9.51	F = 1.56	F = 20.61	F = 11.17
		$P < 0.005$	$P < 0.22$	$P < 0.0002$	$P < 0.003$
	Bicuculline	F = 0.02	F = 8.23	F = 34.75	F = 28.32
		$P < 0.88$	$P < 0.01$	$P < 0.0001$	$P < 0.0001$

CHAPTER IV

DISCUSSION

The developmental role of $GABA_A$ receptors was explored in rat whisker barrel cortex. Blockade of $GABA_A$ receptors in young (P8-P12), but not adult cortex, resulted in significant neuron loss and tissue loss. In addition, histological disruption was greater in young than adult cortex. The results of this study suggest there is a neuroprotective role of $GABA_A$ receptors in developing cortex.

GABA can act as a trophic signal that can accelerate growth and facilitate synapse formation during development (Belhage et al., 1988). Increased synaptogenesis occurs during the second postnatal week (Kristt, 1978; White et al., 1997), and a marked increase in GABAergic synapses occurs between P10 and P15 (Micheva and Beaulieu, 1996). Blocking $GABA_A$ receptors at such a critical time in development could lead to a loss of GABAergic synapses, and possibly a consequent loss of neurons, particularly GABAergic neurons.

Decreasing inhibition by $GABA_A$ receptor blockade could tip the balance in favor of increased cortical activity and release of glutamate. The loss of neurons resulting from $GABA_A$ receptor blockade in young animals was coincident with a time when the cortex is transiently vulnerable to excitotoxic influences (Johnston 1995, 1996) mediated by glutamate receptors (McDonald et al., 1988; McDonald and Johnston, 1990, 1993).

Although specific characteristics of neurons lost in the present study were not determined, other studies have described populations of neurons with a selective vulnerability to excitotoxic injury (Tecoma and Choi 1989; Weiss et al., 1990; Storey et al., 1992; Young et al., 1999).

Although $GABA_A$ receptors are not present on glia *in vivo* in barrel cortex (Fritschy et al., 1994; Lin et al., 1994), application of bicuculline in the developing rat cortex resulted in a significant loss of tissue, apparently including glia. It is known that excitatory amino acids can also cause swelling and destruction of astrocytes (Kimelberg, 1996). Furthermore, the excitotoxic damage can become cyclic as the response of the swelling of the astrocytes causes the release of more glutamate (Choi and Rothman, 1990; Choi, 1992).

The mechanical insult of the surgery or implant contributed more to neuron loss and disruption in cortex of the pup than in the adult. Cortical insults may result in loss of cells through temporary hypoxia, disturbances of the blood-brain barrier, or secondary apoptotic mechanisms (Wyszynski et al., 1989; Persico et al., 1997). However, surgical procedures of the two implant sites did not differ, and trauma effects would have been comparable within animal. Nevertheless, the effects of bicuculline in these experiments might be amplified by the perturbation of cortical surgery. Furthermore, bicuculline methiodide may act on sites other than the $GABA_A$ receptor complex (Olsen et al., 1976; Mestagh and Wulfert, 1999, Seutin and Johnson, 1999). Additionally, there may be variability in the distribution of bicuculline through the cortex as dictated by differences in cortical density between the two age groups.

The age-dependent effect of bicuculline on histological disruption suggests that $GABA_A$ receptors may also be necessary for proper cortical organization during development. Perhaps due to the substantial tissue and neuron loss, the surviving cells expand and/or shift to the newly vacated space, altering the cytoarchitecture. Cytoarchitectural remodeling following injury has been described previously (Isacson and Sofroniew, 1992; Ferrer, 1993; Redecker et al., 1998). Activation of microglia (Streit, 1996), or an increase in the replication or migration of astrocytes (Norton et al., 1992; Burtrum and Silverstein, 1993; Norenberg, 1996) may have also contributed to disruption of the normal cortical architecture.

The results presented here indicate a significant loss of neurons and tissue in the developing rat cortex following a chronic application of bicuculline methiodide. It is unclear if these effects are due to increased glutamate-receptor mediated activity or if the receptor blockade led to a loss of functional synapses and a subsequent loss of neurons. These results are consistant with the current theory that during the second postnatal week, cell survival requires an appropriate balance between excitatory and inhibitory systems (Ohkuma, et al., 1994; van den Pol et al., 1996; Ikonomidou et al., 1999, 2000). Furthermore, the neuroprotective role of $GABA_A$ receptors proposed here adds to the current view that GABA contributes to cortical development (Lauder et al., 1986, 1998; Belhage et al., 1988; LoTurco et al., 1995; Berninger et al., 1995; Kellogg 1998; Owens et al., 1999).

APPENDIX A

ENZYME HISTOCHEMISTRY

ENZYME HISTOCHEMISTRY

Background

Cytochrome oxidase (CO) staining is used as a marker of mitochondrial oxidative metabolism. Cytochromes are responsible for electron transport and oxidative phosphorylation that yield ATP. It has been hypothesized that the more active the neuron, the greater the need for ATP, and the greater the cytochrome oxidase activity (Wong-Riley, 1979). This link between metabolic processes and neuronal activity has been proposed in the somatosensory cortex (Wong-Riley and Welt, 1980, Land and Simons, 1985a). CO staining is decreased with deafferentation (Land and Simons, 1985b; Mjaatvedt and Wong-Riley, 1991) and other forms of sensory deprivation (Land and Akhtar, 1987; Wong-Riley and Welt, 1980; Rhoades et al., 1993; although see Garraghty et al., 1991). Conversely, CO staining increases in the cortex following epilepsy (Gerebtzoff et al., 1979) or injury (Martin et al., 1997; Valla et al., 1999). The predicted response in this study was greater CO staining in the cortex under the bicuculline than control implants, due to disinhibited activity.

Methods

Data presented here were obtained from some of the animals used for the experiment described in the main thesis. Sections from 10 pups and 4 adults were used for CO staining. Based on the procedure previously described (Wong-Riley, 1979), sections were stained for CO activity. The slides were then dehydrated in a series of

alcohols, cleared in xylenes, and then coverslipped with DPX. Qualitative comparison of staining was done within section.

Results and Discussion

Under both bicuculline and control implants, there was a decrease in cytochrome oxidase in areas with neuron loss (Fig. 8). Occasionally there was an increase in cytochrome oxidase activity in areas adjacent to the site of neuron loss. The decrease in staining is consistent with reduced CO activity in atrophic neurons following a cortical stab wound (Al Ali and Robinson, 1984). The increased metabolic activity in the surrounding tissue may be due to reduction of GABAergic inhibition from adjacent cortex.

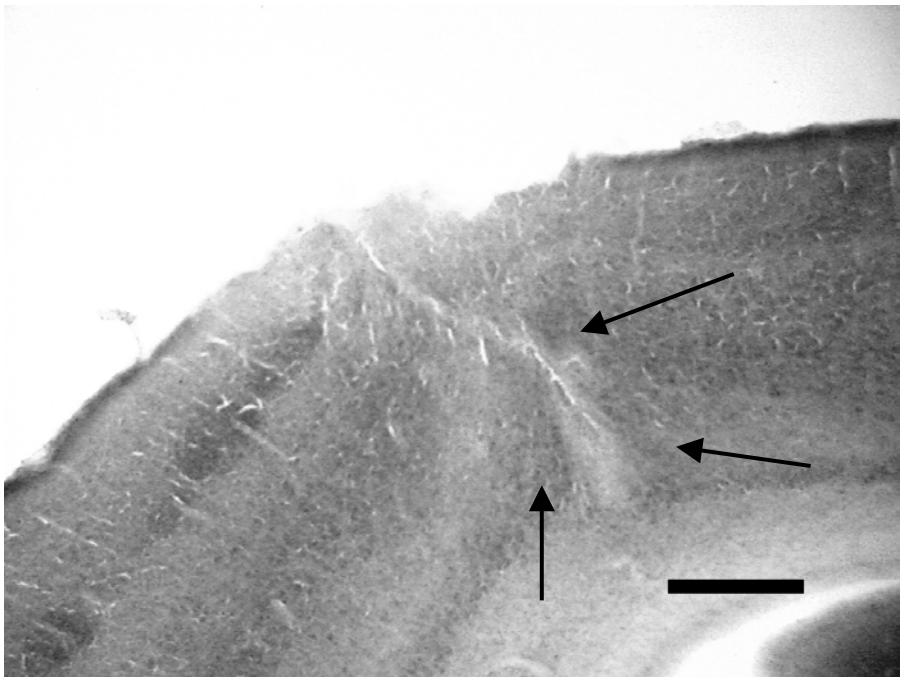


Figure 8. Photograph of cytochrome oxidase staining in a P12 rat cortex that was exposed to a bicuculline polymer implant. Increased CO staining (*arrows*) can be seen adjacent to areas with neuron loss. Scale bar, 500 μm.

APPENDIX B

$GABA_A$ RECEPTOR BINDING

$GABA_A$ RECEPTOR BINDING

Background

$GABA_A$ receptor binding has been shown to be regulated with sustained changes in neural activity. For example, following sensory deprivation, $GABA_A$ receptor binding is reduced in rat cortex (visual: Gordon et al., 1997; somatosensory: Skangiel-Kramska et al., 1994; Fuchs and Salazar, 1998). The changes in activity-dependent receptor regulation may also depend on age. For example, in response to increased activity, $GABA_A$ receptors are down-regulated in juvenile (P20-30) and up-regulated in adult rat slices (Shaw and Scarth, 1992). In the present experiment, receptor autoradiography was used to estimate changes in $GABA_A$ receptor binding following chronic localized bicuculline application.

Methods

Data presented here were obtained from some of the animals used for the experiment described in the main thesis. Sections from three pups and three adults were used for these experiments. The procedure for [^{3}H]muscimol binding to $GABA_A$ was based on the methods of Mower et al. (1986). Sections were preincubated in 0.31 M Tris-citrate (pH 7.1, 4ºC) solution for 20 min to remove endogenous GABA, then incubated in 10 nM [^{3}H]muscimol (specific activity = 20 Ci/mmol; New England Nuclear, Boston, MA) at 4ºC for 40 min. The sections were rinsed twice for 30 sec each in 4ºC Tris-citrate, followed by a brief dip in distilled water. GABA (final concentration

1 mM) was added to the incubation solution to determine nonspecific binding. Excess liquid from the rinses was removed quickly by aspiration and the sections were dried with a fan. The autoradiography sections and tritium standards were exposed to tritium-sensitive film (^{3}H-Hyperfilms, Amersham, Arlington Heights, IL) in X-ray cassettes at 4ºC for approximately 10 wk.

A qualitative analysis of [^{3}H]muscimol binding under each implant site was performed using a video-based computerized analysis system (MCID, Imaging Research, St. Catherines, Ont. Canada).

Results and Discussion

In this study, [^{3}H]muscimol binding was reduced in areas of neuron and tissue loss under both bicuculline and control implants. There appeared to be no obvious changes in [^{3}H]muscimol levels within the laminae and cortical barrels of the area of histological disruption between the bicuculline and control sides. $GABA_A$ receptor binding in relation to neuron density might reveal a bicuculline-induced change.

APPENDIX C

SYSTEMIC APPLICATION OF BICUCULLINE

SYSTEMIC APPLICATION OF BICUCULLINE

Background

In order to avoid the potential interaction between $GABA_A$ receptor blockade and the effects of the surgical implant procedure, bicuculline was systemically administered to developing rats. Evidence of neuronal damage following systemic application of bicuculline in adult rat cortex has been demonstrated in another lab (Soderfeldt et al., 1981, 1983). Doses of 1.0 – 8.0 mg/kg bicuculline (i.p.) in developing pups can lead to epileptiform seizures including shaking, rearing, and alternating muscle contraction and relaxation, known as clonus (Turski et al., 1985; Zouhar, 1989; Baram and Snead, 1990). As described in the main text, significant neuron loss under the bicuculline implant was present after four days in the developing cortex. By using cortical thickness as an index of tissue volume, substantial loss of neurons in the cortex following four days of systemic bicuculline administration might be uncovered.

Methods

The solution of $GABA_A$ receptor antagonist was prepared by dissolving bicuculline (Sigma Chemical Co., St. Louis, MO) in 0.1 M HCl, buffered to pH 5.0 with 0.1 M NaOH. The control solution was vehicle alone. In trial experiments, a single dose of 2 mg/kg i.p. was sufficient to cause seizures, and often death within minutes. A concentration of 1.5 mg/kg was found to induce seizures, with no mortality. Death from systemic bicuculline application is possibly due to pulmonary edema (Kiessling et al., 1981; Soderfeldt et al., 1983; Kreisman et al., 1987).

For 4 days, beginning on P8, unanesthetized rats were given a 0.1 ml daily injection of either the bicuculline solution (1.5 mg/kg i.p.; n = 3) or the control solution (n = 4). After injections, the pups were monitored for 1 hr before they were returned to the mother.

Tissue was prepared as previously described in the main thesis. Drawings from Nissl stained somatosensory cortex were made using a camera lucida at a final magnification of 20X. The barrel field in the P8 rat was determined to be located approximately 1.2-2.1 mm posterior to bregma and 3.0-3.5 mm from midline (Sherwood and Timiras, 1970). Cortical thickness from upper white matter through layer I was measured in mm using a digitizing data tablet. Thirty-two measurements per animal were chosen to represent changes in cortical thickness (4 serial coronal sections in the posteromedial barrel field, 8 measurements from each hemisphere). The thickness of the cortex from the two groups of animals was compared using a Student's *t*-test.

Results and Discussion

In this experiment, the somatosensory cortical thickness with bicuculline injections was 1.41 ± 0.02 mm and with control injections, 1.42 ± 0.01 mm. There was no significant difference ($P < 0.50$) in somatosensory cortical thickness between the control and bicuculline injections. Perhaps the lack of significant loss in tissue volume may be due to a daily single exposure to the $GABA_A$ receptor rather than the chronic blockade provided by the implant. However, the possibility of neuron loss from systemic bicuculline application cannot be rejected and could be further examined using stereological counts of neurons in semithin sections.

APPENDIX D

BDNF AND CORTICAL ACTIVITY

BDNF AND CORTICAL ACTIVITY

Background

Neurotrophins have been associated with trophic support in the developing nervous system. Recent literature suggests that brain-derived neurotrophic factor (BDNF) may go beyond this traditional role. BDNF in adults appears to play a role in regulation of cortical activity and the GABAergic system.

BDNF is upregulated by increased electrical activity. For example, while BDNF mRNA levels are barely detectable in barrels normally, whisker stimulation increases it markedly (Rocamora et al., 1996). It has been proposed that the up-regulation of BDNF is by the excitatory glutaminergic neurons (Zafra et al., 1992). The rise in BDNF can also be demonstrated following periods of hyperexcitability (Zafra et al., 1990; Ernfors et al., 1991) and kainic acid seizure-induced activity (Zafra et al., 1990; Ballarin, 1991; Gall et al., 1991). There is also an increase in BDNF in areas adjacent to electrolytic (Rocamora et al., 1992) and chemical (Kokaia et al., 1993) lesions. Additionally, reduction of BDNF by antisense to BDNF mRNA in the developing brain increases neuronal loss following kainic acid seizures (Tandon et al., 1999).

The barrel cortex, where there is a large concentration of GABAergic neurons and synapses, provides a good model to examine the relationship between BDNF and GABA. The disinhibition of the GABAergic system, as in the chronic application of bicuculline, may result in an increase in BDNF, whose role may also be one of neuroprotection.

Methods

Immunohistochemical methods were based on the protocol of Conner et al. (1997). Implanted pups (n = 5) were prepared for antibody staining by perfusion with 2% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4. Coronal sections (50-μm thick) were obtained using a sliding microtome and were stored free-floating in 0.1 M Millonig's buffer for no more than 24 hr, until processed.

Sections were washed for 10 min in Tris-buffered saline (TBS) followed by a 20 min incubation in TBS containing 0.25% Triton X-100. The sections were then blocked in 5% goat serum and 2% bovine serum albumin in TBS for 60 min. The free-floating sections were incubated with the primary anti-BDNF (50 ng/ml) provided by Dr. Qiao Yan (Amgen, Yan et al., 1997), containing 0.25% Triton X-100 and 5% goat serum in TBS. Sections were incubated for 40-64 hr at 4ºC on a slow moving shaker. The sections were then incubated with 1.5 μg/ml biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) containing 0.25% Triton X-100 and 5% goat serum in TBS for 3 hr, followed by incubation with avidin-peroxidase complex (Elite ABC kit, Vector Laboratories, Burlingame, CA) for 90 min at room temperature. The sections were rinsed with three changes of 0.1 M Tris-HCl buffer (15-30 min per rinse). The sections were then mounted and air-dried. The sections were developed with 0.4% diaminobenzidine tetrahydrochloride, 0.06% nickel chloride, and 0.06% H_2O_2 in 0.1 M Tris-HCl buffer (pH 7.4), then rinsed two times in 0.1 M phosphate-buffered saline (PBS). After being dehydrated in a series of ethanol and cleared in xylenes, the sections were mounted in DPX.

Results and Discussion

BDNF immunoreactivity increased under both control and bicuculline implant sites, with a slightly greater increase in staining within the area of histological disruption of the bicuculline than the control side (Fig. 9). These results are consistent with demonstrations of BDNF upregulation following addition of the $GABA_A$ receptor antagonist bicuculline (Metsis et al., 1993) or traumatic brain injury (Mattson and Scheff, 1994). However, this is the first reported example of injury or bicuculline-induced BDNF upregulation in developing rat cortex.

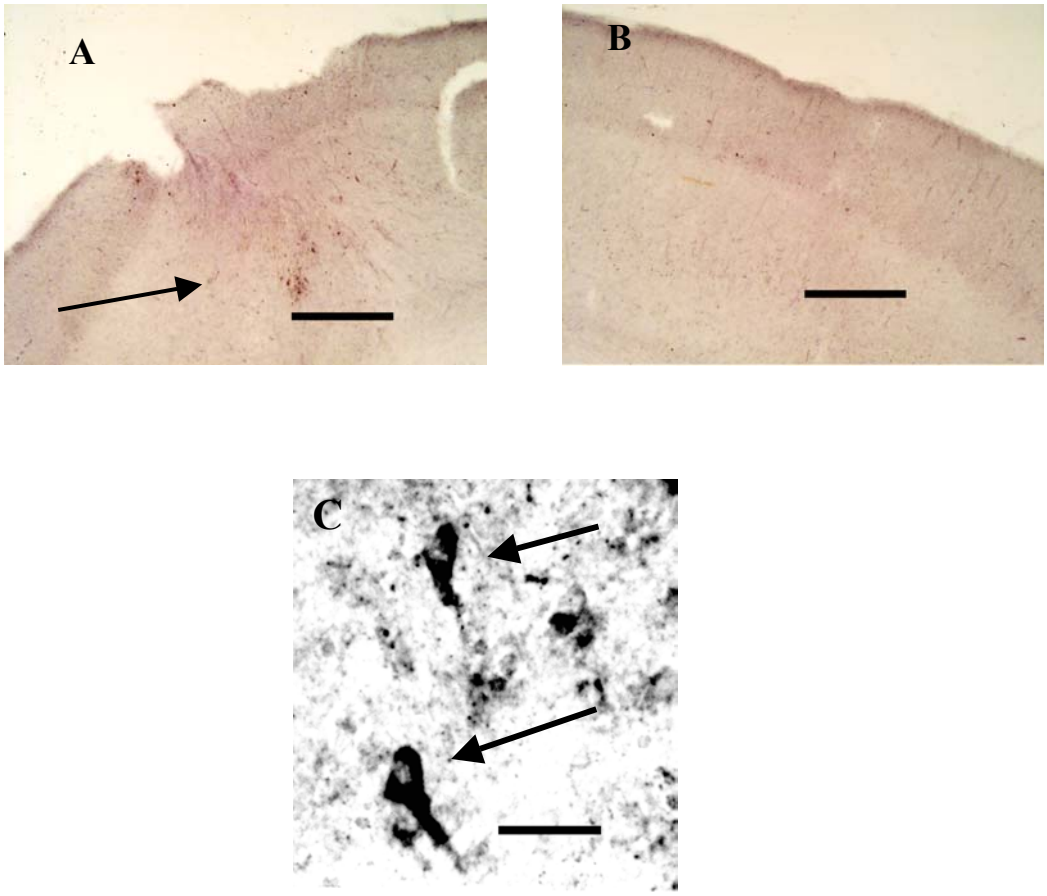


Figure 9. Photograph of anti-BDNF staining under control implants in a P12 rat. Increased BDNF antibody staining is greater (arrow) under the bicuculline implant (A) than under the control implant (B). Higher magnification (C) of cells positive for BDNF antibody (arrows). Scale bars for A and B, 500 μm; C, 25 μm.

APPENDIX E

SELECTIVE VULNERABILITIES OF NEURONAL AND GLIAL POPULATIONS

SELECTIVE VULNERABILITIES OF NEURONAL AND GLIAL POPULATIONS

Background

Apoptosis or active programmed cell death, can be seen during the first month of normal cortical development (Raff et al., 1993; Ferrer et al., 1994), as well as after excitotoxic damage (Portera-Cailliau, 1995; Bennett et al., 1998; Huang et al., 1999; Zipfel et al., 1999). Apoptotic cells can also be seen following traumatic injury (Liu et al., 1997; Newcomb et al., 1999; Pohl et al., 1999; Raghupathi et al., 2000).. The purpose of this study was to examine the time course and selective vulnerabilities of the neuron and glia populations following implant surgery.

Methods

Eleven rat pups (P8) were implanted bilaterally on P8 as described in the main thesis. After 1 day (n = 4), 2 days (n = 4), and 4 days (n = 3), the animals were perfused and brains cryoprotected as previously described. Sections were cut at 40 μm thickness and stained, dehydrated, and coverslipped.

Lange et al. (1999) described a method for identifying apoptotic cells in Nissl-stained sections following injury using dark-field microscopy. In this study, low-magnification dark-field was only slightly useful in screening for apoptosis. In dark-field microscopy, darkly stained structures like apoptotic bodies diffract the light rays and become apparent. Due to the thickness of the sections and the large numbers of pyknotic cells, many areas were bright under dark-field magnification. High-magnification bright-

field was then necessary for confirmation of apoptosis. Drawings from each of the Nissl stained sections through the barrel field were made using a camera lucida. Four serial coronal sections (a total of 160 μm A-P in thickness) were chosen to represent the area of maximum damage under the implant. Using a camera lucida at a final magnification of 40X, affected areas in each section were outlined and measured in mm^2 using a digitizing data tablet. At a final magnification of 400X, apoptotic neurons and apoptotic glia were counted within three 450 μm X 450 μm regions in the area of histological disruption. Apoptotic neurons, were distinguished from the smaller glial cells, by their characteristically large size and pale cytoplasm (Vaughan, 1984).

The proportions of apoptotic neurons to apoptotic glia were then calculated on each side. Statistical analysis was performed using Jandel SigmaStat software. Effects of treatment and exposure time to implants were assessed with analysis of variance (ANOVA) followed by Tukey's *post-hoc* analysis.

Results and Discussion

Apoptotic cells were identified by their shrunken cytoplasm and spherical apoptotic bodies within the cell (Portera-Cailliau et al., 1997). Apoptotic neurons in the developing rat brains have been seen within 24 hours following excitotoxic damage (Portera-Cailliau et al., 1997; Ishimaru et al., 1999) and traumatic brain injury (Bittigau et al., 1999; Pohl et al., 1999). In this study, apoptotic neurons and apoptotic glia were present in all animals, although it should be noted that the incidence of apoptotic cells was very low over the 4-day period (Table 7; Fig 10). The cortical apoptotic neuron:glia ratio between the control and bicuculline sides was not significantly different after 1, 2, or 4 days of

exposure (Table 7, Fig. 11). In addition, the neuron:glia ratio between the bicuculline and the control implants was not time-dependent ($F_{1, 8} = 2.26$, $P < 0.17$).

Table 7.

Effects of Bicuculline Implants on Numbers of Apoptotic Cells

	Neurons			Glia			Neuron: Glia Ratio
Duration of exposure to implant	Bicuculline	Control	Bicuculline minus Control	Bicuculline	Control	Bicuculline minus Control	
1 day (n = 4)	27.8 ± 8.9	11.0 ± 4.4	16.8 ± 2.2	8.0 ± 3.0	2.2 ± 1.0	5.8 ± 2.2	2.1 ± 0.8
2 day (n = 4)	18.0 ± 2.1	9.0 ± 2.1	9.0 ± 2.7	4.25 ± 1.6	1.2 ± 0.5	3.0 ± 1.5	3.6 ± 2.2
4 day (n = 3)	7.7 ± 2.2	4.0 ± 1.5	3.7 ± 3.3	0.0	2.0 ± 0.6	-2.0 ± 0.6	-1.6 ± 1.8

Numbers of apoptotic cells under the implant for each section on the bicuculline side minus that on the control side. Areas represent averages within three 450 X 450 μm boxed regions in the area of histological disruption. Shown are the mean ± S.E.M.

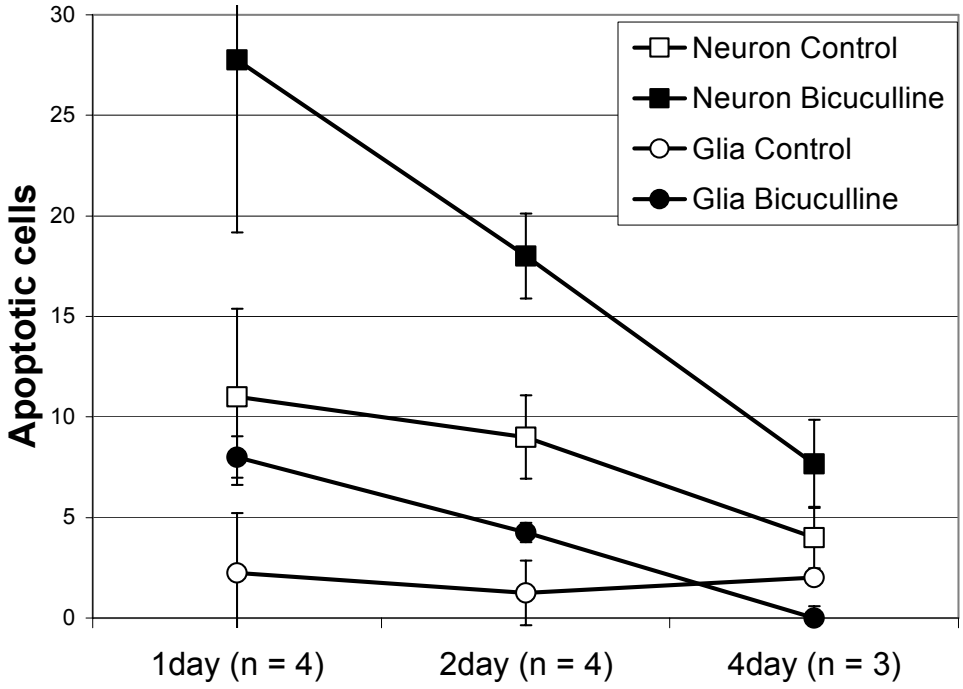


Figure 10. Effects of implants on apoptotic cells. Average numbers of apoptotic cells under the implant for each section.

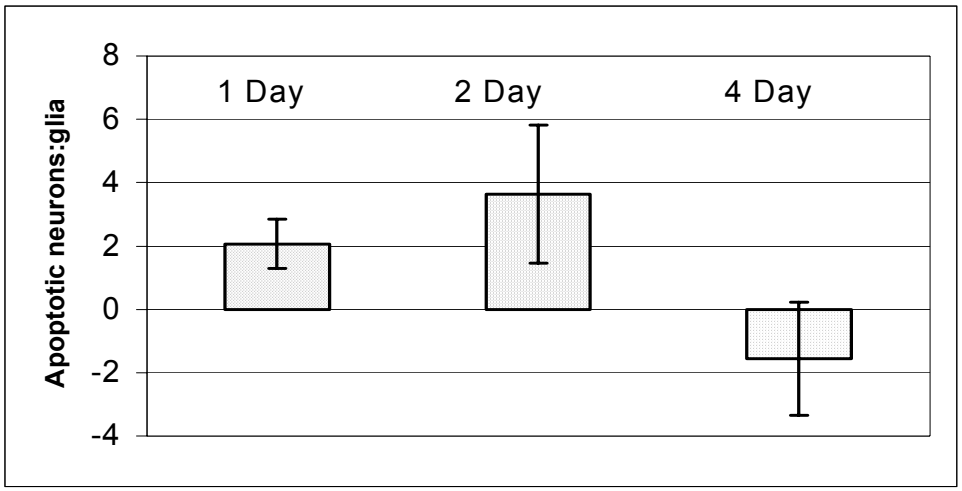


Figure 11. Apoptotic cells following cortical bicuculline implant. Effects were calculated as the ratio of apoptotic neurons to apoptotic glia on the bicuculline side minus that on the control side. Each value represents the mean ± S.E.M.

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