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CONTROL OF INHIBITION IN RAT NEOCORTEX

by

SOTIRIOS T. KEROS

A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2005

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Neurobiology

Name of Candidate Sotirios T. Keros

Committee Chair John J. Hablitz

Title Control of Inhibition in Rat Neocortex

The actions of the inhibitory neurotransmitter GABA are tightly regulated to ensure effective information processing as well as prevention of excess excitatory activity, which can lead to seizures and neurological disorders such as epilepsy. Drugs which enhance the effects of GABA are used as anesthetics, sedatives and anticonvulsants. Here, we utilize whole-cell patch-clamp techniques to investigate three mechanisms of GABAergic modulation in the rat neocortex. First, we demonstrate that the neuronal K^+-Cl^- cotransporter (KCC2) can extrude intracellular chloride from neurons despite the presence of a large chloride load and that physiologically relevant potassium elevations can reverse the transporter. Second, we show that, in a slice model of hyperexcitability induced by 4-aminopyridine (4-AP) and excitatory amino acid (EAA) blockers, layer I interneurons and layer II/III chandelier cells fire ectopic action potentials (EAPs) during propagating depolarizing GABA responses, whereas pyramidal cells do not. Increasing GABA release by inhibiting $GABA_B$ receptors increases the duration of GABA responses and increases the number of EAPs. We postulate a mechanism whereby potassium elevations increase intracellular chloride via KCC2, leading to GABA-dependent excitation and initiation of EAPs. Third, we examined the role of specific GABA transporter (GAT) subtypes in modulating tonic and phasic $GABA_A$ currents. We report that GAT-1 is concentrated at synapses and speeds the decay of evoked inhibitory postsynap-

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tic potentials (IPSCs), prevents desensitization of postsynaptic receptors and limits spill-over of GABA from the synapse. GAT-2/3 inhibition synergistically potentiates the effects of GAT-1 inhibition but alone has no effect. A tonically active $GABA_A$ current is unaffected by antagonists of either GAT-1 or GAT-2/3 alone, yet dramatically increases when both GAT-1 and GAT-2/3 are inhibited. Thus, GAT-1 and GAT-2/3 have distinct but overlapping roles in modulating GABA conductances. This suggests that better control of seizures may be possible by titrating the ratio of GAT-1- and GAT-2/3-selective drugs to best treat a specific seizure disorder phenotype. The studies in this dissertation provide new insights into the intracellular and intercellular regulation of normal GABAergic conductances in the neocortex via KCC2 and GATs.

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DEDICATION

This dissertation is dedicated to my wife, Beth. I am forever indebted to her for her immeasurable devotion, support, love, and friendship.

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TABLE OF CONTENTS

Page

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TABLE OF CONTENTS (Continued)

Page

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LIST OF TABLES

Table *Page*

SUBTYPE-SPECIFIC GABA TRANSPORTER ANTAGONISTS SYNERGISTICALLY MODULATE PHASIC AND TONIC $GABA_A$ CONDUCTANCES IN RAT NEOCORTEX

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LIST OF FIGURES

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LIST OF ABBREVIATIONS

$[K^+]_{i,o}$	intracellular, extracellular potassium concentration
4-AP	4-aminopyridine
A	amperes
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic
APV	D-aminophosphonovalerate
ATP	adenosine triphosphate
Cl^-	chloride
$[Cl^-]_i$	intracellular chloride concentration
$[Cl^-]_o$	extracellular chloride concentration
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
dNTP	nucleotide
EAA	excitatory amino acid
EAP	ectopic action potential
EC50	half-maximal effective concentration
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A
$GABA_AR$	γ-aminobutyric acid type A receptor

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$GABA_B$	γ-aminobutyric acid type B
GAT	γ-aminobutyric acid transporter
GTP	guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N*,-2-ethanesulphonic acid
IC50	half-maximal inhibitory concentration
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
K^+	potassium
$[K^+]_i$	intracellular potassium concentration
$[K^+]_o$	extracellular potassium concentration
KCC	potassium-chloride cotransporter
KCC2	type 2 potassium-chloride cotransporter
mRNA	messenger ribonucleic acid
n	number of experiments
NKCC	sodium-potassium-chloride cotransporter
P	postnatal day
PCR	polymerase chain reaction
psi	pounds per square inch
R_{in}	input resistance
Rs	series resistance
rt	reverse transcriptase
SD	standard deviation

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sem	standard error of the mean
TTX	tetrodotoxin

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INTRODUCTION

The precise balance and control of excitation and inhibition are critical for information processing in neural networks. Many mechanisms have evolved in the brain to ensure that inhibition stays within tightly controlled parameters. Disruption of the balance between inhibition and excitation can lead to a variety of human neurological pathologies, the most common of which is epilepsy (Sander, 2003), a disorder which is characterized by recurrent seizures. The principle inhibitory neurotransmitter in the brain is GABA, and epilepsy is generally treated with drugs that enhance the effects of GABA. There is evidence, however, which shows that it is an oversimplification to assume that GABA is purely inhibitory (Gulledge and Stewart, 2003; Cossart et al., 2005). In addition, the effects of GABA can vary depending on the region of the brain or developmental stage. Continuing efforts are needed to further understand the role of GABA in the central nervous system and the specific mechanisms by which it exerts its effects.

Inhibition is primarily mediated by synaptic release of GABA from interneurons onto chloride (Cl^-)-permeable postsynaptic GABA receptors. Cortical interneurons are an extremely morphologically diverse set of local circuit cells that can form synapses with both excitatory pyramidal and other interneurons. The regulation of GABA's effects in the brain can be broken down into three broad areas. First, regulation of intracellular Cl^- concentrations can alter the strength of postsynaptic GABA responses through changes in the chloride driving force. Second, synaptic parameters such as release of GABA, timing and concentration of GABA in the synapse, and GABA receptor location and identity

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are all sites of potential modulation. Third, GABA's effects can be regulated by the specific anatomy, morphology, and connectivity of GABAergic interneurons that determine their specific area of influence, including synchronization of interneurons and network properties. The experiments contained in this dissertation provide insight into inhibition within the neocortex from the perspective of the intracellular, intercellular and network properties of GABAergic signaling.

Chloride Regulation and the $GABA_A$ Receptor

Chloride homeostasis is important for both volume regulation and cell signaling (Kaila, 1994; Hoffmann and Dunham, 1995). GABA exerts its inhibitory effects primarily through activation of the Cl^--permeable $GABA_AR$ (Weiss and Hablitz, 1984; Howe et al., 1987; Seeburg, 1990; Sivilotti and Nistri, 1991), and mature neurons keep $[Cl^-]_i$s low relative to the extracellular level (Clayton et al., 1998; Lu et al., 1999; Rivera et al., 1999; DeFazio et al., 2000; Vu et al., 2000). This causes $GABA_AR$ activation to produce hyperpolarizing inhibitory postsynaptic currents (IPSCs) that act to prevent the postsynaptic neurons from reaching the threshold for firing an action potential (Krnjevic and Schwartz, 1967; Dreifus et al., 1969). Alternatively, $GABA_A$ currents can be minimal, yet the shunting effect of open $GABA_A$ receptors can still have a net inhibitory effect (Andersen et al., 1980; Staley and Mody, 1992). In contrast to mature neurons, immature neurons have an elevated internal chloride concentration that results in a depolarizing $GABA_A$ response (Cherubini et al., 1991; Ben Ari, 2001). Ultimately, it is the difference between the chloride equilibrium potential and the neuron's resting potential that dictates whether an IPSP will be depolarizing or hyperpolarizing.

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Since internal chloride concentrations in neurons are low relative to external chloride, changes of a few millimolar of internal chloride can result in large changes in the equilibrium potential, as predicted by the Nernst equation. Thus, small changes in $[Cl^-]_i$ can alter the function of the chloride-dependent IPSP, particularly since resting membrane potentials tend to be near the chloride equilibrium potential. Small GABA-dependent depolarizations can increase the probability of action potential initiation, despite chloride reversal potentials well below the action potential threshold voltage (Gulledge and Stuart, 2003). This observation stresses the importance of tight regulation of intracellular chloride.

Type 2 Potassium-Chloride Cotransporter Regulation of Intracellular Chloride

Electroneutral chloride transport is responsible for maintaining chloride gradients across the cell membrane, and there are two main chloride transporters that are responsible for chloride homeostasis (Payne et al., 2003). The Na^+-K^+-$2Cl^-$ cotransporter (NKCC) uses sodium and chloride gradients to move chloride into neurons (Geck et al., 1980; Geck and Heinz, 1986; Kakazu et al., 1999), wheras the K^+-Cl^- cotransporter (KCC) uses the potassium gradient to generally move chloride out of the cell as potassium moves into the cell (Payne, 1997). Both NKCC and KCC are furosemide sensitive (Misgeld, 1986; Gillen et al., 1996; Payne, 1997). KCC is located in a wide variety of cell types, especially kidney cells; however, a neuron-specific form of this transporter, type 2 K^+-Cl^- cotransporter (KCC2), has been recently characterized (Payne, 1997; Rivera et al., 1999). KCC2 expression increases during development and is believed to be responsible for developmental reductions in intracellular chloride levels (Rivera et al., 1999; DeFazio et al., 2000). The NKCC transporter pumps chloride into the cell over a

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very wide range of ionic environments, whereas the KCC2 transporter normally pumps chloride out of the cell (based on equations from Stein, 1990). However, unlike NKCC, KCC2's direction is sensitive to potassium and chloride concentrations (Kakazu et al., 1999) and has the potential to operate in reverse, as predicted by Payne (1997). Based on thermodynamic considerations, at a hypothetical $[Cl^-]_i$ of 7 mM and $[K^+]_o$ of 5 mM, KCC2 is predicted to pump chloride out of the neuron. If $[K^+]_o$ rises to 10 mM, however, the pump is predicted to reverse direction and pump both potassium and chloride inward. Large potassium increases have been observed to occur during seizure-like activity *in vitro* (Benninger et al., 1980; Swann et al., 1986; Hablitz and Heinemann, 1989) and *in vivo* (Lux et al., 1974; Xiong and Stringer, 1999). In addition to chloride regulation, KCC2 could play a role in maintaining $[K^+]_o$ by facilitating the movement of potassium out of the extracellular space (see Xiong and Stringer, 1999). However, because of KCC2's coupling of potassium and chloride, increased entry of potassium would also lead to the accumulation of $[Cl^-]_i$, possibly contributing to a depolarizing effect of $GABA_A$ activation. In this way, the predicted excitatory effect of increased $[K^+]_o$ could lead to additional excitation through elevated $[Cl^-]_i$.

KCC2 expression levels are known to dramatically increase during the 2nd week of life and coincide with the depolarizing to hyperpolarizing switch in $GABA_A$ responses (Clayton et al., 1998; Lu et al., 1999; Rivera et al., 1999; DeFazio et al., 2000). Recent experiments in acute hippocampal slices demonstrated that KCC2 was necessary for hyperpolarizing $GABA_A$ responses in mature animals (Rivera et al., 1999) and that KCC2 might be responsible for establishing a chloride gradient between the soma and dendrites of cultured midbrain neurons (Jarolimek et al., 1999).

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The first article in this dissertation demonstrates the strength of KCC2 in regulating intracellular chloride levels despite heavy chloride loads. The second article explores a brain slice model of epilepsy in which GABAergic activity is excitatory; in this article, we postulate a contribution from KCC2 in elevating intracellular chloride concentrations as a result of increases in extracellular potassium levels.

Interneurons and Inhibitory Networks

Unlike the relatively stereotypical excitatory principal neurons, interneurons exhibit profound heterogeneity and are not easily classified (Connors and Gutnick, 1990; Thomson and Deuchars, 1997; Kawaguchi and Kubota, 1998). Attempts to group interneurons by morphology, calcium binding proteins, neuromodulators, and function have been difficult and have failed to yield meaningful results with predictive value. A classification of interneurons based on their synaptic effects on target neurons has been suggested (Gupta et al., 2000). In this scheme, three types of synapses were described where test IPSPs were facilitated, depressed, or unchanged in response to a train protocol. This information could be combined with interneuron morphology and firing properties to create unique interneuron classifications. However, it is unlikely that any one scheme will be sufficient to fully categorize the diverse interneuron populations (Maccaferri and Lacaille, 2003).

The neocortex has six layers. Interneurons and pyramidal cells coexist in layers II-V, but layer I is a thin, sparsely populated layer (DeFelipe and Jones, 1988) that contains the cell bodies of neurons that are mostly if not exclusively interneurons (Li and Schwark, 1994). Layer I is thought to be important for fetal development (Marin-Padilla, 1984), and layer I of the visual cortex has been shown to be quantitatively important for

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information processing (Beaulieu et al., 1994). Although the somata of layer I interneurons vary in size and shape, their dendrites tend to stay in layer I (DeFelipe and Jones, 1988; Marin-Padilla, 1988, 1990). Biocytin labeling of layer I interneurons reveals axonal arbors that can span 700 μM and often stay within layer I, but many layer I interneurons can send axons as deep as layer IV (Zhou and Hablitz, 1994). In addition, deeper layer pyramidal cells send both apical dendrites and axon collaterals into layer I, along with the axons of some deep interneurons (Marin-Padilla, 1992; Cowan and Wilson, 1994; Martin, 1984; Kawaguchi, 1993). Layer I interneuron activity can thus potentially affect all layers of the neocortex.

The density of neurons in layer I is so low that is has been called the cell-free layer and the molecular layer. This has delayed detailed characterization of layer I interneurons, and knowledge of layer I properties lags that of deeper layer's properties. Zhou and Hablitz have a series of studies that focus on the morphology, firing properties and voltage-dependent currents of layer I interneurons (Zhou and Hablitz, 1994, 1996a,b). Layer I interneurons exhibit fast-spiking behavior with little frequency adaptation, similar to many types of cortical interneurons. In addition, layer I interneurons have a large, fast afterhyperpolarization. Voltage-clamp experiments reveal that layer I interneurons have fast transient potassium currents, $I_{(A)}$, and delayed rectifier currents, $I_{(DR)}$, whose kinetics and pharmacology are similar to those in layer II/III pyramidal cells. A higher density of $I_{(A)}$ in layer I may contribute to maintaining a fast firing rate.

It is known that cortical neurons can exhibit highly synchronous firing (see McBain and Fisahn, 2001). One example is 30-80 Hz gamma oscillations (Bragin et al., 1995). These oscillations can be tightly correlated, even among neurons that are several millimeters apart (Whittington et al., 1995). It is difficult to imagine that relatively slow

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chemical synapses are responsible for maintaining time-locked high frequency oscillations. However, electrical synapses such as those that couple neurons via gap junctions (Llinas et al., 1974) are sufficiently fast to be able to synchronize a large population of neurons. Electrical coupling exists between interneurons in the hippocampus as well as the neocortex (Benardo, 1997; Galaretta and Hestrin, 1999; Fukada and Kosaka, 2000). It is now well accepted that interneurons play an important role in synchronous oscillatory firing of cortical neurons via gap junctions (Fukada and Kosaka, 2000; Whittington et al., 1995; Deans et al., 2001, Traub et al., 2001a,b). Thus, interneurons form tightly coupled networks and can influence large areas of cortex. This underscores the importance of understanding the specifics of interneuron signaling onto postsynaptic neurons.

A general theme of this dissertation is to add to our knowledge of layer I interneurons and explore differences between layer I interneurons and the principle neurons and interneurons of layer II/III.

Depolarizing GABA Responses

4-Aminopyridine (4-AP) is a potassium channel antagonist that has epileptogenic effects *in vitro*, and is also used in a model of interneuronal synchronization (Michelson and Wong, 1991; Muller and Misgeld, 1991; Perrault and Avoli, 1991). Application of 4-AP together with ionotropic glutamate receptor antagonists to hippocampal and neocortical slices induces interneurons to synchronously burst-fire, causing so-called giant or synchronous IPSPs in pyramidal cells (Aram et al., 1991; Avoli et al., 1994). In many cases, these IPSPs have both depolarizing and hyperpolarizing components (Perrault and Avoli, 1992; Lamsa and Kaila, 1997). The mechanism of synchronization of interneurons may involve dendritic and axonic electrical connections between neurons as well as

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recurrent axon collaterals (Benardo, 1997; Traub et al., 2001a). Under certain conditions, these responses are depolarizing and are blocked by the $GABA_A$ antagonist bicuculline (Aram et al., 1991; Avoli et al., 1994; DeFazio and Hablitz, 2005). In many cases, these depolarizing events occur spontaneously at ~1-min intervals but can also be evoked with extracellular stimulation. Neocortical voltage-sensitive dye imaging indicates that evoked events can slowly propagate across the slice and are referred to as depolarizing GABA responses (DeFazio and Hablitz, 2005). Throughout this dissertation the term *GABA response* will be refer to large spontaneous and evoked propagating events recorded from slices bathed in 4-AP and ionotropic glutamate receptor antagonists.

Although GABA is generally considered to be inhibitory, depolarizing effects of GABA in mature cortex have been described (Alger and Nicoll, 1979; Andersen et al., 1980; Lambert et al., 1991; Staley and Mody, 1992; Staley et al., 1995). Recent experiments using the gramicidin perforated-patch technique in layer V of the neocortex have shown that GABA responses are always depolarizing compared to the resting potential (Gulledge and Stuart, 2003). However, this depolarization can either suppress or facilitate action potential generation, depending on the location and timing of the GABA input relative to other inputs. Thus, excitatory GABA activity need not in itself be considered a criterion for abnormal GABA function.

Ectopic Action Potentials

Action potentials have been traditionally described as sodium-dependent events that originate from the axon hillock or initial segment. Once started, action potentials propagate orthodromically down an axon and away from the soma on their way to presynaptic terminals where they initiate transmitter release. However, action potentials can

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also be generated distally in the dendrites and axons and propagate toward the soma (Gutnick and Prince, 1972; Stasheff et al., 1993; for review see Pinault, 1995). Such events are referred to as ectopic action potentials (EAPs). Several models of epilepsy exhibit EAPs, and EAPs may be involved with the initiation or spread of seizure-like activity (Rosen and Vastola, 1971; Gutnick and Prince, 1972; Pinault and Pumain, 1985). EAPs can also be recorded during spontaneous and evoked giant IPSPs from slices treated with 4-AP. Perreault and Avoli (1989, 1991, 1992) concluded that these were EAPs on the basis of four generally recognized criteria: (1) EAPs occur at membrane potentials more negative than those required to elicit action potentials via current injection or EPSPs; (2) EAPs persist despite hyperpolarizing current injection; (3) EAPs were of variable amplitude and were almost always smaller than full action potentials; and (4) EAPs sometimes had inflections that resembled the initial segment-somatodendritic (IS-SD) fractionation. Further studies in the hippocampus led to the conclusion that EAPs originate in the axons and depend on synaptic activation of $GABA_A$Rs (Avoli et al., 1998; Traub et al., 2001b). Recent evidence in support of axo-axonal gap junctions (Schmitz et al., 2001; Traub et al., 2001b) suggest that EAPs in one axon could spread to axons of connected neurons, thus amplifying the network effects of EAPs.

Information about EAPs during giant IPSPs or GABA responses has been focused mainly on the hippocampus (Avoli et al., 1998; Lamsa and Kaila, 1997; Benardo, 1997; Traub et al., 2001a; Stasheff et al., 1993; Perreault and Avoli, 1992), where both pyramidal cells and interneurons display EAPs. Studies in the neocortex, however, indicate that interneurons are more likely to have EAPs (Benardo, 1997; DeFazio and Hablitz, 2005; Keros and Hablitz, 2002), especially layer I interneurons. Investigations of 4-AP induced IPSPs in the neocortex have revealed laminar differences (Barkai et al.,

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1995; Yang and Benardo, 2002). Specifically, only superficial layers (I and II) supported synchronized GABAergic activity in the presence of excitatory transmission blockers (Yang and Benardo, 2002). Furthermore, voltage-sensitive dye experiments reveal that depolarizing GABA responses propagate mainly along the superficial layers of the slice (DeFazio and Hablitz, 2005). Laminar differences in 4-AP-induced responses and evidence of the prevalence of EAPs in certain cell types warrant further investigation of EAPs in various neocortical layers and their role in 4-AP-induced GABA responses.

The second article in this dissertation describes the incidence of EAPs among various neuronal populations in superficial neocortical layers. Basic properties of GABA responses such as response amplitude, reversal potentials, and duration are examined together with EAP properties such as amplitude and number of EAPs per response.

IPSPs and Synaptic GABA Concentration

Many parameters of synaptic transmission are intertwined to ultimately determine IPSP kinetics. (Cherubini and Conti, 2001). For example, receptor activation and affinity will affect IPSP rise times (Colquhoun and Hawkes, 1983; Macdonald and Twyman, 1992), and IPSP amplitudes can be used to estimate the amount of neurotransmitter release or the number of postsynaptic receptors (Katz, 1969). Likewise, decay kinetics are influenced by various synaptic properties. The specific factors that contribute to the decay of the IPSP have been the subject of debate (Roepstorff and Lambert, 1994). If the time course of GABA in the synaptic cleft is rapid (Clements, 1996), then the decay is determined primarily by dissociation of transmitter from the receptor, as proposed by Magleby and Stevens (1972) for the neuromuscular junction, or by a combination of dissociation and desensitization (Clements et al., 1992; Lester et al., 1990; Jones and West-

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brook, 1995, 1996). If GABA concentration decays slowly or if low levels of transmitter are maintained, then receptor desensitization plays a larger role in the decay (Otis et al., 1996a,b; Overstreet et al., 2000). Of course, the decay can also be a complex function of deactivation, desensitization, and GABA concentration (Banks et al., 1998; Collingridge et al., 1984; Pearce et al., 1995; and for a related discussion of decay of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic (AMPA)-mediated EPSPs (see Glavinovic, 2001). This may explain the observation that some decays are best fit with more than one exponential.

GABA Transporter Expression and IPSP Kinetics

No enzyme exists to break down GABA in the synapse such as with acetylcholine. Along with diffusion, an important regulator of GABA concentration in the synapse is the GABA transporter (GAT). GATs use ionic gradients as energy to pump GABA, along with two sodium ions and one chloride ion, out of the synapse (Kanner 1983,1994). Inhibition of GATs in the hippocampus results in a dramatic prolongation of the IPSC decay (Dingledine and Korn, 1985; Draguhn and Heinemann, 1996; Isaacson et al., 1993; Roepstorff and Lambert, 1992, 1994; Thompson and Gähwiler, 1992). This is consistent with the idea that transporter inhibition causes GABA levels in the synapse to remain high for an increased time. In contrast, there is evidence that suggests that, instead of synaptic GABA concentration, receptor kinetics are the rate limiting step in the IPSC time course (Maconochie et al., 1994; Jones and Westbrook, 1995). It is possible that the magnitude and timing of GABA release determine whether GATs act to shape IPSC kinetics. The fact that some IPSC decays exhibit both slow and fast components (Pearce, 1993) indicates that different factors are responsible for various IPSC parame-

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ters. Thus, GATs may influence the IPSC only in cases where, for example, there exists a slow component.

At least three different transporters have been cloned from the rat central nervous system, and are named GAT-1, GAT-2, and GAT-3 (Krogsgaard-Larsen et al., 1987; Borden, 1996). A fourth GABA transporter, BGT-1, is a betaine transporter whose primary role is to help with osmoregulation. The high-affinity GAT-1 was the first to be cloned (Guastella et al., 1990), is the most abundant in the brain (Durkin et al., 1995), and was initially thought to be primarily restricted to neurons and some specialty glial cells such as the Bergman glia. Recent studies, however, have isolated GAT-1 currents from cortical layer II/III and layer V glial cells (Kinney and Spain, 2002). GAT-1 expression is heaviest at axon terminals, and GAT-1 immunoreactivity is seen in all layers of the neocortex, with less expression in layer I (Conti et al., 1998). GAT-2 expression is limited to the leptomeninges (Conti et al., 1999), whereas GAT-3 expression has traditionally been thought to be located primarily in glia (Ikegaki et al., 1994; Durkin et al., 1995). However, it is becoming increasingly clear that neurons can also express GAT-3 (Conti et al., 2004).

Tonic and Phasic $GABA_A$ Conductances and Desensitization

Neurotransmitter receptors are not all located across from agonist release sites (Herkenham, 1987; Mody, 2001; Farrant and Nusser, 2005). Receptors clustered at synaptic locations account for "wired transmission" or "phasic" conductances, whereas extrasynaptic receptors are responsible for "volume transmission" or "tonic" conductances (Mody, 2001). Large tonic $GABA_A$ conductances have been described in the hippocampus and cerebellum (Otis et al., 1991; Brickley et al., 1996; Stell and Mody, 2002) and

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are mediated primarily through high-affinity α6 containing $GABA_A$ receptors (Wisden, et al., 2002; Rossi and Hammann, 1998; Rossi et al., 2003). Tonic conductances in mature animals are action potential independent (Wall and Usowicz, 1997; Brickley et al., 1996). Extracellular GABA concentrations approach 1 μM (Lerma et al., 1986; Tossman et al., 1986), and the presence of tonic $GABA_A$ currents implies that extrasynaptic $GABA_A$ receptors are likely to be nondesensitizing at low GABA concentrations. GABA transporter antagonists, however, reduce IPSC amplitudes in hippocampal neurons (Overstreet et al., 2000) by blocking a slow desensitization caused by persistent low levels of GABA.

Spillover

Neurotransmitter spillover out of the synapse can activate receptors of neighboring synapses or activate extrasynaptic receptors (Kullmann et al., 1996; Barbour and Hausser, 1997; Asztely et al., 1997; Rossi and Hamann, 1998), although tonic $GABA_A$ conductances persist when exocytosis is blocked (Rossi et al., 2003). This implies that tonic conductances are determined by overall background GABA concentrations that can result from nonexocytotic sources. GABA transporters can regulate tonic conductances by limiting extracellular GABA concentrations (Attwell et al., 1993), and GAT-1 antagonism leads to increased tonic currents (Wisden et al., 2002; Nusser and Mody, 2002). Despite these studies, there are little data available from the neocortex on GAT-1 regulation of tonic $GABA_A$ conductances.

The third article in this dissertation examines GABA transporter modulation of tonic and phasic currents in layer I interneurons and layer II/III pyramidal cells. The recent availability of a selective GAT-2/3 antagonist, (S)-SNAP-5114 (SNAP), provides an

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opportunity to determine the role of GAT-2/3 in shaping inhibitory phasic and tonic currents.

This dissertation focuses on regulatory mechanisms by which the effects of GABA are controlled after release. The ionic dependence of KCC2 on potassium and chloride suggests that extracellular potassium elevations will also increase intracellular chloride, thus exacerbating hyperexcitation by shifting reversal potentials for $GABA_A$ currents. Potassium elevations are also implicated in the initiation of EAPs which may be responsible for supporting propagating depolarizing GABA responses across a network of interneurons. Finally, the kinetics of GABA currents are highly dependent on GATs, which are pharmaceutical targets for seizure control. It is hoped that the studies contained in this dissertation will contribute to our understanding of the modulation of GABA conductances, which when perturbed can give rise to hyperexcitability and seizure.

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POTASSIUM-COUPLED CHLORIDE COTRANSPORT CONTROLS INTRACELLULAR CHLORIDE IN RAT NEOCORTICAL PYRAMIDAL NEURONS

by

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Abstract

Chloride (Cl^-) homeostasis is critical for many cell functions including cell signaling and volume regulation. The action of GABA at $GABA_A$ receptors is primarily determined by the concentration of intracellular Cl^-. Developmental regulation of intracellular Cl^- results in a depolarizing response to GABA in immature neocortical neurons and a hyperpolarizing or shunting response in mature neocortical neurons. One protein that participates in Cl^- homeostasis is the neuron-specific K^+-Cl^- cotransporter (KCC2). Thermodynamic considerations predict that in the physiological ranges of intracellular Cl^- and extracellular K^+ concentrations, KCC2 can act to either extrude or accumulate Cl^-. To test this hypothesis, we examined KCC2 function in pyramidal cells from rat neocortical slices in mature (18-28 postnatal) and immature (3-6 days postnatal) rats. Intracellular Cl^- concentration was estimated from the reversal potential of whole-cell currents evoked by local application of exogenous GABA. Both increasing and decreasing the extracellular K^+ concentration resulted in a concomitant change in intracellular Cl^- concentration in neurons from mature rats. KCC2 inhibition by furosemide caused a change in the intracellular Cl^- concentration that depended on the concentration of pipette Cl^-; in recordings with low pipette Cl^-, furosemide lowered intracellular Cl^-, whereas in recordings with elevated pipette Cl^-, furosemide raised intracellular Cl^-. In neurons from neonatal rats, manipulation of extracellular K^+ had no effect on intracellular Cl^- concentration, consistent with the minimal KCC2 mRNA levels observed in neocortical neurons from immature animals. These data demonstrate a physiologically relevant and developmentally regulated role for KCC2 in Cl^- homeostasis via both Cl^- extrusion and accumulation.

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Introduction

The principal inhibitory neurotransmitter in the neocortex is GABA. Early studies *in vivo* demonstrated that fast IPSPs and responses to iontophoretically applied GABA have similar reversal potentials and ionic sensitivities (Krnjevic and Schwartz, 1967; Dreifuss et al., 1969). Subsequent studies *in vitro* showed that both GABA responses and IPSPs reverse near the expected chloride (Cl^-) equilibrium potential and are blocked by bicuculline, suggesting mediation by $GABA_A$ receptors ($GABA_ARs$), (Weiss and Hablitz, 1984; Howe et al., 1987). The main permeant ion of $GABA_A$ receptor channel complexes is Cl^-, although permeability to bicarbonate ions has been demonstrated (Bormann et al., 1987; Kaila et al., 1993). The membrane potential and the transmembrane gradients of permeant ions determine ionic flux through the $GABA_A$ receptor. In most mature neurons, the resting potential is close to the Cl^- equilibrium potential, and activation of $GABA_ARs$ results in shunting inhibition (Andersen et al., 1980); i.e., activation of $GABA_A$ receptors with minimal net ionic flux results in decreased excitability. If the neuron is depolarized relative to the Cl^- equilibrium potential, $GABA_A$ receptor activation results in an inward flux of Cl^- that hyperpolarizes the neuron. Immature neocortical neurons have an elevated $[Cl^-]_i$, and $GABA_AR$ activation results in a depolarizing response to GABA (Owens et al., 1996).

Electroneutral cotransport of Cl^- ions plays a critical role in $[Cl^-]_i$ homeostasis (see Alvarez-Leefmans, 1990; Kaila et al., 1993). One mechanism for accumulating Cl^- is the Na^+-K^+-2 Cl^- cotransporter (NKCC), (Kakazu et al., 1999). The primary Cl^- extrusion mechanism is K^+-Cl^- cotransport (Alvarez-Leefmans, 1990). A neuron-specific form of the ubiquitous K^+-coupled Cl^- cotransporter (KCC2) has been characterized recently (Payne, 1997; Rivera et al., 1999; Williams et al., 1999). The expression of this trans-

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porter increases with development and is believed to support the developmental changes in $GABA_AR$-mediated signaling (Lu et al. 1999; Rivera et al., 1999).

The direction of K^+-Cl^- cotransport is determined by the transmembrane K^+ and Cl^- gradients. Under conditions of low $[Cl^-]_i$ and elevated $[K^+]_o$, thermodynamic considerations suggest that KCC2 could operate in reverse to accumulate Cl^- (Payne, 1997; Jarolimek et al., 1999; see also Kakazu et al., 2000). A role for KCC2 in maintaining $[K^+]_o$, i.e., that KCC2 could lower $[K^+]_o$ by cotransport of K^+ and Cl^- ions into neurons, has also been proposed (Payne, 1997). A consequence of such a mechanism would be the accumulation of $[Cl^-]_i$, a phenomenon consistent with activity-dependent decreases in GABAergic inhibition (Thompson and Gähwiler, 1989a; Ling and Benardo, 1995).

In the present study, we tested the hypothesis that developmental changes in the expression of KCC2 result in the coupling of $[Cl^-]_i$ and $[K^+]_o$ via the activity of the furosemide-sensitive K^+-coupled Cl^- cotransporter. Our results demonstrate that manipulations of $[K^+]_o$ and furosemide altered $[Cl^-]_i$ in a manner consistent with either accumulation or extrusion of Cl^- via a K^+-coupled Cl^- cotransport mechanism. $[K^+]_o$ manipulations in immature neurons had no effect on $[Cl^-]_i$, consistent with the low expression of KCC2 mRNA detected in cytoplasm harvested from these cells. Expression of KCC2 results in lowered $[Cl^-]_i$ and translates physiological changes in $[K^+]_o$ to marked changes in $[Cl^-]_i$.

Materials and Methods

Brain Slice Preparation, Maintenance, and Electrophysiological Recording

Animals were housed and handled according to approved guidelines. Brain slices were prepared from postnatal day 3 (P3) to P6 and P18 to P28 animals. Rats were anesthetized with ketamine (100 mg/kg) before decapitation. The brain was rapidly removed and submerged in oxygenated (95% O_2/5% CO_2) ice-cold saline with no added

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and submerged in oxygenated (95% O_2/5% CO_2) ice-cold saline with no added calcium [containing in (mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, and 4 $MgCl_2$]. Coronal sections (300 μm) containing somatosensory cortex were cut with a Vibratome. Slices were stored in saline consisting of (in Mm): 125 NaCl, 5 KCl, 26 $NaHCO_3$, 10 D-glucose, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, bubbled with 95% O_2/ 5% CO_2.

Whole-cell voltage-clamp recordings were made from visually identified neocortical pyramidal cells in layer II/III. Cells were identified by their distance from the pial surface, pyramidal shape, and the presence of a prominent apical dendrite. Recordings were made at 30°C and at a holding potential of -70 mV. Pipettes were pulled from 1.5 mm glass capillaries (KG-33; Garner Glass Company). They had resistances of 2-4 MΩ when filled with the intracellular solution. Series resistance (Rs) was carefully monitored throughout each experiment by the use of a small hyperpolarizing voltage step applied before each acquisition (see Fig. *1A, inset*). The peak of the capacitative transient was used to estimate series resistance (equal to the instantaneous current divided by the voltage step: Rs = *V step/I peak*). Only recordings with stable Rs (<20 MΩ with <5 MΩ change in 20 min) were used in the analysis. The internal solutions contained (in mM): 135 K-gluconate, 5 EGTA, 10 HEPES, 2 MgATP, 0.2 NaGTP, and 0.2 $CaCl_2$. KCl was substituted for K-gluconate to arrive at the desired Cl^- concentration. The pH was adjusted to 7.3 with 1 mM KOH and HCl such that the final added Cl^- concentration equaled 1, 20, or 40 mM and the final $[K^+]_i$ was always 150 mM. Sucrose was added to achieve a final osmolarity of 300 mOsm. Liquid junction potentials for all solutions were measured, and all voltages reported are corrected values (Neher, 1992).

During recording, slices were continuously perfused with the storage saline listed above with the addition of 0.5 μM TTX (Calbiochem) to block Na^+-dependent action

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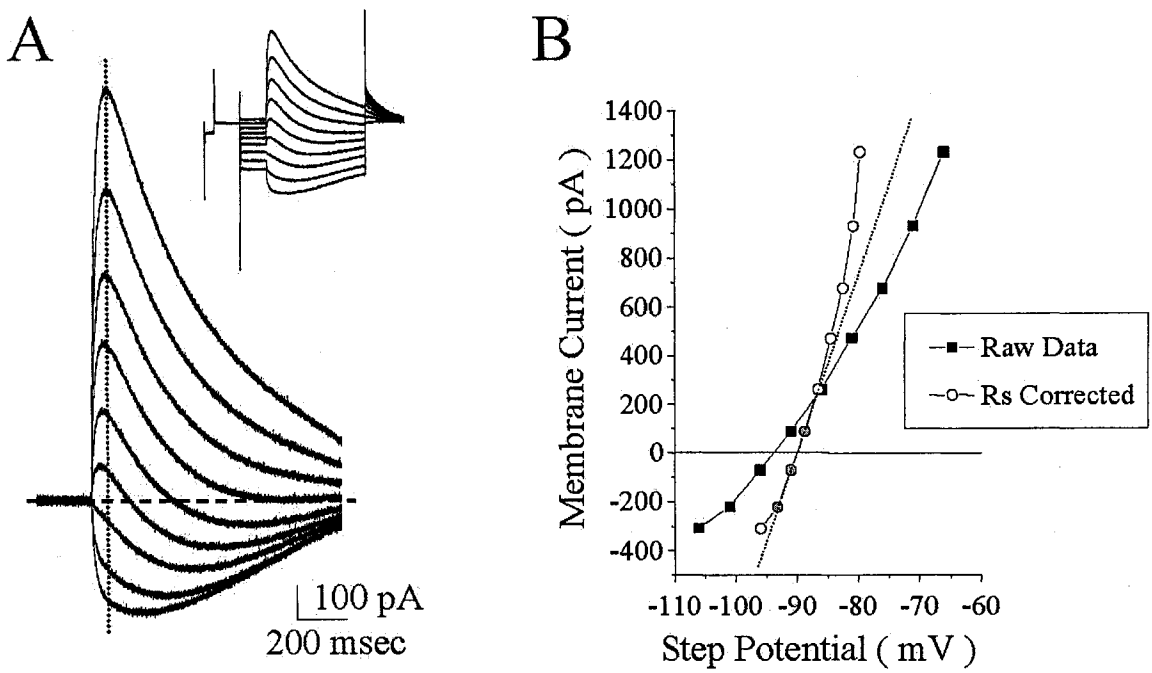

Figure 1. The reversal potential of the $GABA_A$ evoked current measured from the amplitudes of the responses at different step potentials. *A*, The baseline current at each step potential was subtracted from the *raw traces* (shown in the *inset*). *B,* Then the amplitude of the current at the *dotted line* was plotted as a function of step potential. *Squares* illustrate the amplitudes as a function of the command potential. *Circles* represent current amplitude versus membrane potential corrected for the series resistance (*Rs*) error. A linear fit (*dotted line*) to the current as a function of the *Rs*-corrected step potential was obtained from the three consecutive data points closest to zero current (*gray circles*). This recording was obtained from a P18-P28 neuron with 1 mM pipette Cl^-

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potentials, 2-5 mM kynurenic acid (Tocris) to block ionotropic glutamate receptors, and 10 μM SCH50911 (Research Biochemicals, Natick, MA) to antagonize $GABA_B$ receptors. Extracellular K^+ was varied by making K^+-free saline and adding 1, 3.5, or 10 mM KCl. Saline containing 1 mM furosemide was sonicated for 30 min and then oxygenated for at least 30 min before bath application. All drugs (except GABA) were bath applied, and each cell served as its own control. Bath temperature was maintained at 30°C by the use of an in-line heating unit (Warner Instruments). Whole-cell and excised patch recordings (Sakmann and Neher, 1995) were made with an Axopatch-1B amplifier. No series resistance compensation was used. Recordings were digitized at 5-10 kHz by the use of a Digidata 1200 data acquisition system and Clampex software (Axon Instruments). Data analysis was performed with custom scripts written for Origin Pro (Microcal Software). All averages are reported as the mean ± SEM. A Student's *t* test was used to determine significance ($p < 0.05$).

Pressure Application of GABA

Under direct visual guidance, GABA (250 μM or 1 mM) was pressure applied to the soma of the recorded neuron or to excised patches. Pipettes for pressure applications were fabricated in the same manner as patch electrodes described above. GABA was applied in a solution consisting of (in mM): 125 NaCl, 3.5 KCl, 20 HEPES, and 10 glucose; pH is 7.3 with NaOH. Pressure applications were controlled by the use of a Picospritzer II (General Valve, Fairfield, NJ). Pulses of 5-15 msec were delivered at 3-12 psi. These settings were kept constant during recording. Application of the pressure pipette solution without GABA did not evoke a detectable current ($n = 3$).

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Reverse Transcription-PCR

The methods used for determination of mRNA in single neurons have been described (Poth et al, 1997; Devay et al., 1999). In brief, cytoplasm and pipette solution (~6 μl) were reverse transcribed in a 20 μl reaction (1 hr at 37°C) containing 1 mM each dNTP, 100 pmol of 18-mer polyT, 20 U RNase inhibitor, and 40 U of AMV reverse transcriptase. Sets of specific primers were constructed such that the annealed products crossed at least one intron/exon boundary, excluding the possibility of amplification of genomic DNA. PCR reactions (50 μl) contained aliquots of the reverse transcriptase (rt) product, 1 mM dNTPs, 2.5 mM $MgCl_2$, 10 pmol of each forward and reverse primer, and 5 U of *Taq* polymerase. The PCR cycling parameters were: 5 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. The reaction products were then spun through CentriSep columns to remove excess primers and subjected to reamplification (one or two additional amplification steps) by the use of 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. Final reaction products were purified by phenol/chloroform extraction. The PCR products were subjected to analysis on 2% agarose gels. rt-PCR was performed without knowledge of the age of the animal or the contents of each centrifuge tube (control extracellular solution or intracellular harvest).

The primers for the experiments were as follows: actin (GenBank accession number L08165) sense, ATCTTTCTTGGGTATGGA, and antisense, ACATCTGCTGGAAGGTGG; KCC2 (Genbank accession number AJ011033) sense, GCAGAGAGTACGAT-GGCAGG, and antisense, CGTGCCAAGGATGTACATAGC.

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Intracellular Chloride Calculation

$[Cl^-]_i$ was calculated from the reversal potential of GABA-evoked currents. Cells were voltage-clamped to –70 mV and stepped to various test potentials. The series resistance (estimated from the peak transient during a 10 mV test pulse given before each trial) and the amplitude of the current (before baseline correction, see Fig. 1*A, inset*) 100-200 msec from the time of the pressure application were used to calculate the voltage error caused by uncorrected series resistance errors. The baseline current (taken 20 msec before the time of the pressure pulse) was subtracted from the absolute current amplitude (see Fig 1*A*, baseline-corrected *traces*). These values were plotted as a function of the series resistance corrected membrane potential (see Fig. 1*B*). The three consecutive data points whose sum was closest to zero current were selected for a linear fit. The *x*-axis intercept ($y = 0$) of this fit was verified by eye and referred to throughout this paper as the reversal potential (V_{rev}). $[Cl^-]_i$ was calculated from the reversal potential by the use of a derivation from the Nernst equation:

$$[Cl^-]_{in} = [Cl^-]_{out} \exp(-V_{rev}F / RT)$$

$[Cl^-]_o$ was set to the extracellular Cl^- concentration, corrected for activity (Robinson and Stokes, 1959) (e.g., in 3.5 mM $[K^+]_o$, 139.6 mM x 76% = 106.1 mM). Averaged changes in reversal potential and $[Cl^-]_i$ reflect the mean of the differences from the control condition (3.5 mM $[K^+]_o$) for each cell; thus, each cell served as its own control.

The Role of Bicarbonate and Other Anions

Bicarbonate is known to permeate $GABA_A$ receptors (Bormann et al., 1987). We did not account for a bicarbonate component of the $GABA_A$ current because our results

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suggest that bicarbonate does not contribute significantly to the whole-cell currents under our recording conditions. When 1 mM Cl^- was included in the pipette, the reversal potential of $GABA_A$-mediated currents was depolarized relative to the reversal potential obtained in excised patches (see Fig. 2*A*). This result is consistent with a bicarbonate efflux caused by 20 mM intracellular bicarbonate and a permeability ratio of 1:5 (HCO_3^-/Cl^-). However, lowering $[K^+]_o$ and cotransport antagonism with furosemide both lowered the reversal potential to values similar to those obtained in excised patches (see Results). Because these types of manipulations are not expected to affect intracellular bicarbonate, changes in V_{rev} are attributed to alterations in Cl^- and K^+-Cl^- cotransport.

Another source of error was the purity of the compound providing the primary anion in the internal solution. We obtained potassium-D-gluconate, "puriss" grade, from Fluka. Other sources of the potassium salts of isethionate (Eastman Kodak) and methylsulfate (J. T. Baker Chemical Company) resulted in reversal potential measurements indicative of 10-15 mM internal Cl^- when no Cl^- had been added to the pipette solution. This suggests that these compounds were contaminated with significant amounts of chloride (or another substance permeable through $GABA_A$ receptors). It has also been reported that gluconate can pass through $GABA_A$ channels (Barker and Harrison, 1988). Our results with excised patches demonstrated an effective concentration of pipette Cl^- of 2.06 ± 0.16 mM when only 1 mM KCl was added. Assuming that this difference from added Cl^- is caused solely by gluconate permeability, the relative permeability of gluconate to Cl^- is ~0.008.

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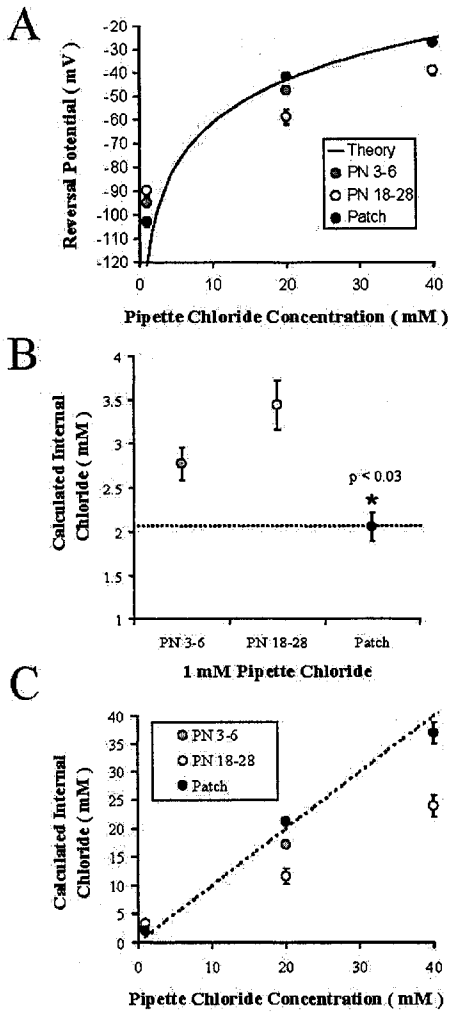

Figure 2. Reversal potentials for $GABA_A$ currents recorded with different pipette Cl^- concentrations support Cl^- accumulation and extrusion in neocortical pyramidal cells. *A*, Reversal potentials obtained at three pipette Cl^- concentrations in (postnatal days 3-6) PN 3-6, PN 18-28, and outside-out patches are plotted as a function of pipette Cl^- concentration. For comparison, the theoretical relationship between $[Cl^-]_i$ and reversal potential (assuming 103.4 mM $[Cl^-]_o$) is shown as a *solid line*. Reversal potentials obtained from outside-out patches were closest to theoretical. The reversal potentials from *PN 3-6* and *PN 18-28* were significantly different from outside-out patches at all pipette Cl^- concentrations ($p < 0.03$). *B*, Mean calculated $[Cl^-]_i$ was significantly lower in outside-patches (*) ($p<0.03$) and 1.1 mM higher than the added KCl concentration (1 mM). The differences in whole-cell $[Cl^-]_i$ from the excised patch values suggest a Cl^- accumulation mechanism in both neonatal and *PN 18-28* neurons. *C*, Calculated $[Cl^-]_i$ is plotted as a function of pipette Cl^- concentration. As in *A* both *PN 3-6* and *PN 18-28* neurons had significantly lower calculated $[Cl^-]_i$ compared with excised patches, suggesting the action of a Cl^- extrusion mechanism.

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Results

Disparity Between Pipette Chloride and Calculated Intracellular Chloride

We assessed the intracellular Cl^- concentration of neocortical pyramidal neurons by measuring the reversal potential of $GABA_A$ receptor-mediated currents. Responses to pressure application of 250 μM GABA are shown in Figure 1*A*. The pipette Cl^- concentration was 1 mM in this recording. The *inset* illustrates the currents evoked by the voltage step protocol and local pressure application of GABA. The currents are shown superimposed on a faster time base after zeroing the baseline currents. GABA response amplitude was measured 100-200 msec after the pressure pulse, at the time indicated by the *vertical dotted line*. Response amplitude is plotted as a function of membrane potential in Figure 1*B* (*squares*). After correction for the series resistance error (*circles*), the reversal potential was calculated from a linear fit to the three consecutive data points closest to zero current (*dotted line*). This indicated a V_{rev} of –89.9 mV, > 30 mV depolarized from the expected value of -121 mV calculated for 1 mM $[Cl^-]_i$.

The observed discrepancy in V_{rev} implies the existence of a homeostatic mechanism that regulates $[Cl^-]_i$. To test this hypothesis and to determine whether such a mechanism is developmentally regulated, measurements of V_{rev} were made in P3-P6 and P18-P28 animals at various pipette Cl^- concentrations. Reversal potentials determined for each of these experimental conditions, at a $[K^+]_o$ of 3.5 mM, are shown in Figure 2*A*. The mean reversal potentials for the three pipette chloride concentrations are shown for recordings from P18-P28 (*white circles*) and P3-P6 (*gray triangles*) neurons. This plot also shows the theoretical relationship between $[Cl^-]_i$ and the reversal potential (*solid line*). In whole-cell recordings with 1 mM pipette chloride, the reversal potential in both P3-P6 and P18-P28 neurons was always more depolarized than the value predicted from the

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Nernst equation. This implies the existence of a Cl^- accumulation mechanism in both groups. At higher pipette chloride concentrations (20 and 40 mM) the measured reversal potential was consistently below the predicted reversal potential, consistent with a Cl^- extrusion mechanism.

We also estimated reversal potentials in excised patches, reasoning that the large volume of the pipette solution should minimize the effects of Cl^- homeostatic mechanisms residing in the small patch of membrane in the pipette tip. The reversal potentials from excised patches were closer to values calculated from the expected pipette $[Cl^-]$ than those obtained in whole-cell recordings, as shown in Figure 2*A* (*black squares*). Also, the reversal potentials in excised patches were insensitive to manipulations that effected KCC2 function in whole-cell recordings (changes in $[K^+]_o$ and furosemide; data not shown). These data support the existence of homeostatic $[Cl^-]_i$ regulation involving a Cl^- accumulation mechanism at low pipette Cl^- concentrations and a Cl^- extrusion mechanism when pipette Cl^- is elevated.

Figure 2*B* plots the calculated $[Cl^-]_i$ for each group when the pipette contained 1 mM Cl^-. In P18-P28 neurons, $[Cl^-]_i$ was 3.73 ± 0.35 mM (n = 11), whereas it was significantly lower in P3-P6 cells (2.82 ± 0.16 mM; p <0.04, n = 5). Recordings from excised patches indicated a pipette Cl^- concentration of 2.10 ± 0.16 mM (n = 4). Estimates of $[Cl^-]_i$ in P3-P6 and P18-P28 neurons were significantly greater than that in excised patches ($p < 0.03$). If we assume that the reversal potential of currents in excised patches with 1 mM pipette Cl^- reflects the actual pipette Cl^- concentration, these results suggest that in 3.5 mM $[K^+]_o$ and with low pipette Cl^-, P3-P6 and P18-P28 neurons can accumulate 0.7-1.6 mM Cl^-.

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Figure 2*C* plots the calculated $[Cl^-]_i$ as a function of pipette chloride concentration. In 3.5 mM $[K^+]_o$ and elevated pipette Cl^-, the reversal potentials of whole-cell currents evoked by GABA were consistently lower than the expected values calculated from the Nernst equation. They were also lower than the values obtained in outside-out patches. With 20 mM pipette Cl^-, the calculated $[Cl^-]_i$ in P3-P6 neurons was 17.55 ± 0.71 mM (n = 5) whereas in P18-P28 neurons, $[Cl^-]_i$ was significantly lower (11.9 ± 1.1 mM, $p < 0.002$, n =11). $[Cl^-]_i$ determined in excised patches was significantly higher than either P3-P6 or P18-P28 neurons (21.69 ± 0.72 mM; n = 7; $p < 0.005$). Assuming that the reversal potential in excised patches reflects the actual pipette Cl^- concentration, our results imply that the neuronal Cl^- homeostatic mechanisms can extrude 4-9 mM Cl^- in 3.5 mM $[K^+]_o$. These data support a prominent role for Cl^- extrusion in determining $[Cl^-]_i$ in these neurons.

Alterations in $[K^+]_o$ Affect $[Cl^-]_i$

If K^+-coupled Cl^- cotransport is involved in the accumulation and extrusion of Cl^- demonstrated above, manipulations of $[K^+]_o$ should change the reversal potential of the GABA-evoked currents. Lowering $[K^+]_o$ should lower the Cl^- set point and thus enhance extrusion and/or retard accumulation. Raising $[K^+]_o$ should have the opposite effect by raising the Cl^- set point via impairment of extrusion and/or enhancement of accumulation.

Figure 3 illustrates the effect of decreasing $[K^+]_o$ on whole-cell responses to GABA. The recordings were obtained from a PN 18-28 neuron by the use of 1 mM Cl^- in the recording pipette. GABA responses in 3.5 and 1 mM $[K^+]_o$ are shown in Figure 3*A*. At a holding potentialof –85 mV, the GABA-evoked current changes from inward to

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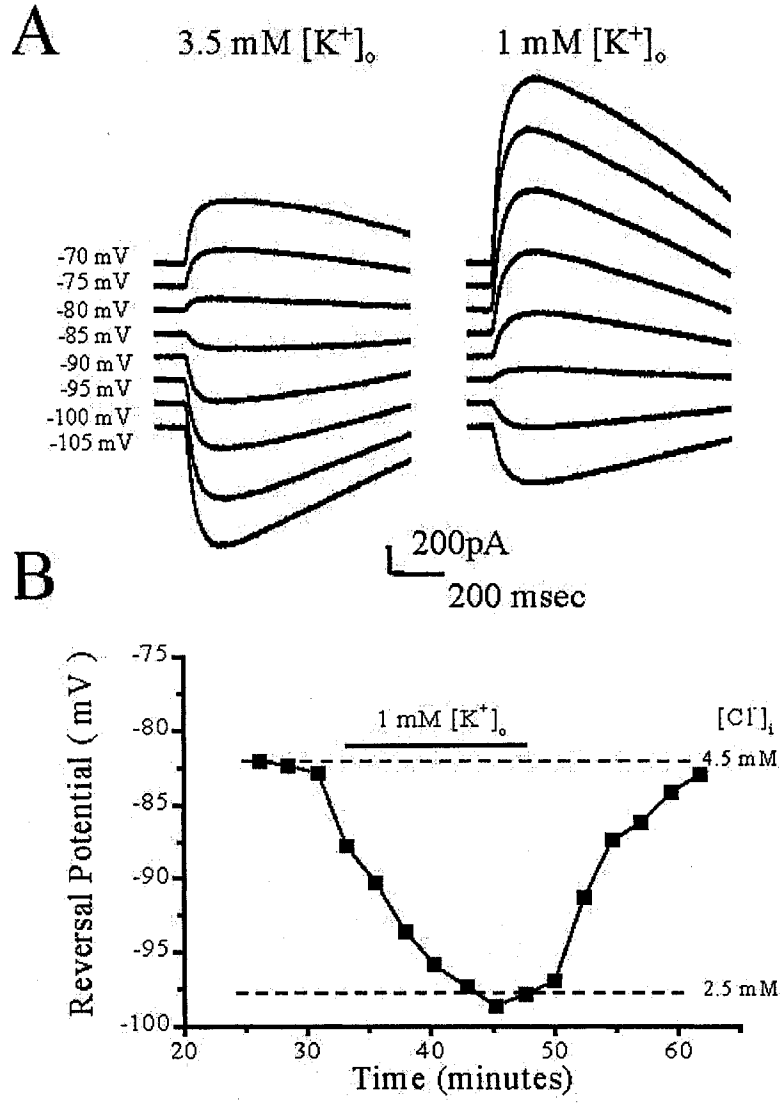

Figure 3. Lowering extracellular potassium lowers intracellular chloride. *A*, GABA (250 μM) was applied at 20 sec intervals to the soma during voltage steps to the indicated potentials. The pipette solution contained 1 mM added Cl^-. Relative to the currents evoked by GABA application in 3.5 mM $[K^+]_o$, a 15 mV drop in the reversal potential is apparent. *B*, A time course plot of the change in reversal potential is shown.

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outward when the $[K^+]_o$ was changed from 3.5 to 1 mM. Lowering $[K^+]_o$ consistently shifted the reversal potential in the negative direction, indicating a decrease in $[Cl^-]_i$. In whole-cell recordings from P18-P28 neurons with 1 mM pipette Cl^-, the mean reversal potential (-100.1 ± 1.3 mV; $n = 5$) and calculated $[Cl^-]_i$ (2.27 ± 0.11 mM) measured in 1 mM $[K^+]_o$ were not significantly different from the mean values obtained in outside-out patches (-102.8 ± 2.0 mV and 2.06 ± 0.16 mM, respectively; $p > 0.3$). This difference was significant ($p < 0.002$) in 3.5 and 10 mM $[K^+]_o$. The change in reversal potential occurred slowly and was reversible, as shown in Figure 3*B*.

In previous studies, raising extracellular potassium inhibited Cl^- extrusion (Thompson et al., 1988a,b; Thompson and Gähwiler, 1989b). Payne (1997) estimated the apparent affinity (K_m) of KCC2 for $[K^+]_o$ to be ~5 mM. This led to the hypothesis that KCC2 could operate in reverse in the presence of elevated $[K^+]_o$ and accumulate both K^+ and Cl^-. As shown in Figure 4, raising $[K^+]_o$ reversibly shifted the reversal potential in the depolarizing direction. In these experiments, the cell was initially maintained in 3.5 mM $[K^+]_o$. The bath concentration of K^+ was then raised to 10 mM, resulting in a >15 mV depolarization in the reversal potential. This indicates a corresponding 3.1 mM increase in $[Cl^-]_i$. With low pipette Cl^-, raising $[K^+]_o$ increased $[Cl^-]_i$ by increasing the net influx of Cl^- via K^+-Cl^- cotransport.

Furosemide is a loop diuretic that blocks chloride cotransport. Application of furosemide in the presence of 10 mM $[K^+]_o$ shifted the reversal potential back toward initial levels (Fig. 4). Lowering $[K^+]_o$ to 3.5 mM in the presence of furosemide made the reversal potential more negative (Fig. 4), approaching levels observed with excised patches. This effect was reversible. In furosemide and 1 mM pipette Cl^-, both the reversal potential (-93.5 ± 6.8 mV; $n = 4$, 4 animals) and the calculated $[Cl^-]_i$ (3.19 ± 0.72 mM)

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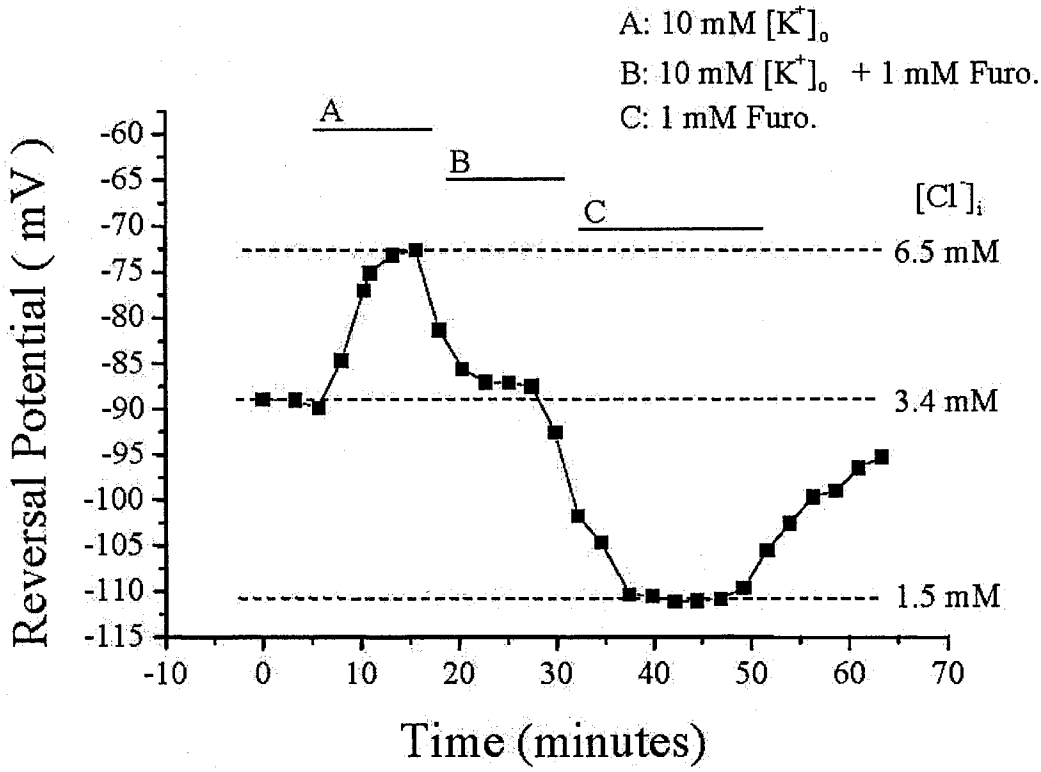

Figure 4. Potassium and furosemide alter intracellular chloride. A 10 min application of 10 mM $[K^+]_o$ reversibly depolarized the reversal potential and raised the $[Cl^-]_i$ by 3.1 mM. After washout, bath application of 1 mM furosemide hyperpolarized the reversal potential and lowered $[Cl^-]_i$ by 1.9 mM. In this figure, 0 min represents the first current-voltage measurement taken ~5 minutes after whole-cell mode was achieved. *Furo., Furosemide.*

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were not significantly different from the same parameters recorded in outside-out patches ($p > 0.2$). This effect is similar to the lowering of $[Cl^-]_i$ observed in 1 mM $[K^+]_o$. The combination of these results suggests that the Cl^- accumulation mechanism responsible for the deviations from low pipette Cl^- concentrations is a K^+-coupled Cl^- cotransporter.

To test the hypothesis that K^+-coupled Cl^- extrusion lowers $[Cl^-]_i$ in recordings with elevated pipette Cl^-, we examined the effects of manipulating $[K^+]_o$ and blocking transport with furosemide. Figure 5*A* summarizes the effects of manipulating $[K^+]_o$ and furosemide application on reversal potentials in PN 18-28 neurons. The changes in $[Cl^-]_i$ are shown in Figure 5*B*. When recordings were made with 1 mM pipette Cl^-, lowering $[K^+]_o$ to 1 mM produced a -12.7 ± 2.3 mV ($n = 5$) change in reversal potential, indicating a 1.54 ± 0.41 mM decrease in $[Cl^-]_i$. A similar change in both parameters was observed with 20 mM pipette Cl^-. Likewise, the mean changes in reversal potential and calculated $[Cl^-]_i$ when $[K^+]_o$ was raised to 10 mM were similar in both 1 and 20 mM pipette Cl^- groups. The effect of furosemide depended on the pipette Cl^- concentration. In 1 mM pipette Cl^-, furosemide shifted the reversal potential to more negative values and lowered calculated $[Cl^-]_i$. In the 20 mM pipette Cl^- group, furosemide produce a positive shift in the reversal and raised the calculated $[Cl^-]_i$.

The dependency of the action of furosemide on pipette Cl^- supports the hypothesis that Cl^- homeostatic mechanisms can operate either to accumulate or to extrude Cl^-. In 10 mM $[K^+]_o$ the reversal potential and calculated $[Cl^-]_i$ were still significantly lower than those obtained with outside-out patches ($p < 0.002$; $n = 5$; 20 mM $[Cl^-]_{pipette}$; $n = 3$; 40 mM ($[Cl^-]_{pipette}$). This suggests that although K^+-Cl^- cotransport plays an important role in maintaining $[Cl^-]_i$, other mechanisms may also contribute to Cl^- extrusion.

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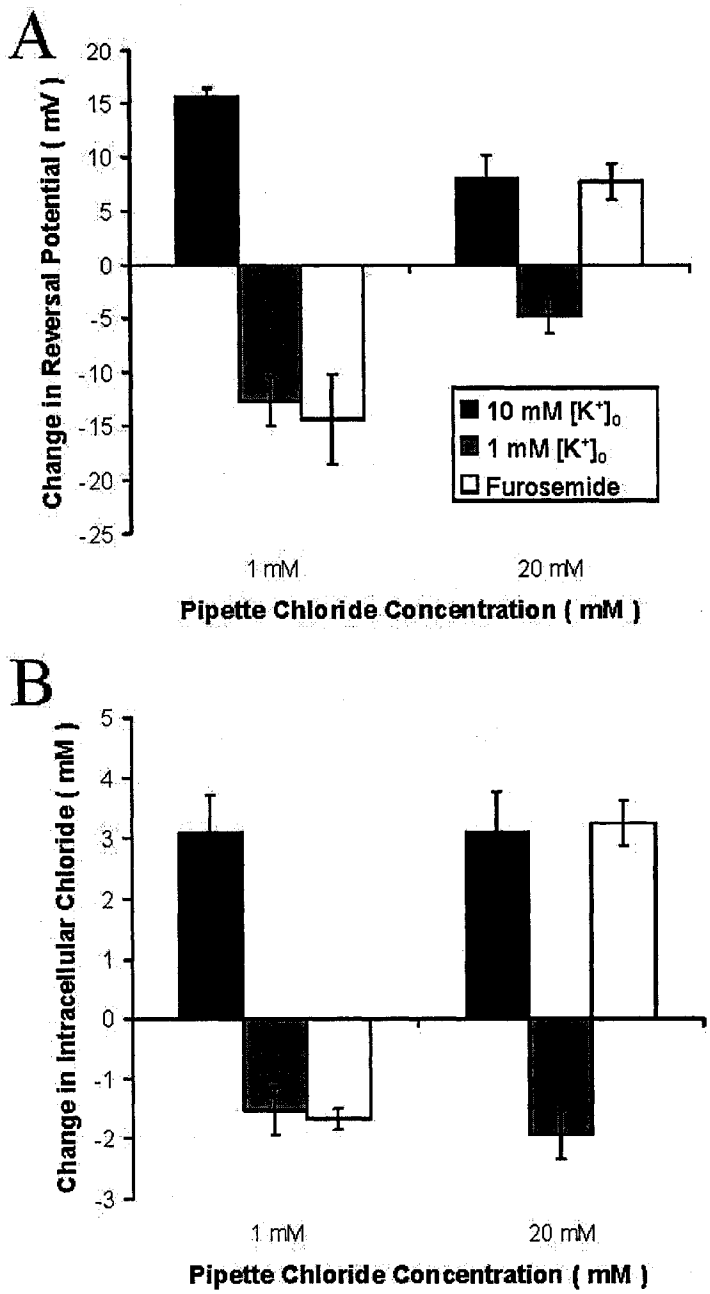

Figure 5. The action of furosemide depends on pipette chloride. *A*, Changes in reversal potential were smaller in 20 mM pipette Cl^- but resulted in similar changes in calculated $[Cl^-]_i$. *B*, Furosemide lowered the reversal potential and $[Cl^-]_i$ when pipette Cl^- was low and raised both the reversal potential and $[Cl^-]_i$ when pipette Cl^- was high. The magnitude of the effect of furosemide was not significantly different from that of the effect of lowering $[K^+]_o$ in 1 mM pipette Cl^- or raising $[K^+]_o$ in 20 mM pipette Cl^-.

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Developmental Regulation of KCC2 Function and Expression

Previous studies have indicated that KCC2 expression is developmentally regulated (Lu et al., 1999; Rivera et al., 1999). We tested the hypothesis that the reversal potential of Cl^- currents in neurons lacking KCC2 would be relatively insensitive to changes in $[K^+]_o$. Figure 6*A* shows the lack of effect of changes in $[K^+]_o$ on the Cl^- reversal potential in a P3 animal. The reversal potential immediately after establishment of whole-cell recording with 20 mM pipette Cl^- reached a value near -49 mV. Only minimal changes in the reversal potential were subsequently observed. In PN 3-6 neurons, neither raising $[K^+]_o$ to 10 mM (n=3) nor lowering $[K^+]_o$ to 1 mM (n=3) had any significant effect on the reversal potential for GABA responses (Fig. 6*B*), consistent with a lack of expression of KCC2. Comparison of the reversal potentials in 3.5 mM $[K^+]_o$ between P3-P6 and P18-P28 neurons provides further support for reduced function of KCC2 in neonates. As shown above in Figure 2*A*, the reversal potential and $[Cl^-]_i$ in P3-P6 neurons were significantly different from that in P18-P28 cells, although both groups were also significantly different from the measurements made in outside-out patches ($p<0.03$). Under conditions that support Cl^- accumulation by KCC2 (low pipette Cl^-), the reversal potential and $[Cl^-]_i$ in the PN 3-6 group were significantly lower than in PN 18-28 neurons ($p<0.05$). Conversely, with elevated pipette Cl^-, these values were significantly higher in PN 3-6 than in the older neurons ($p<0.002$). These results suggest that KCC2 does not substantially contribute to Cl^- homeostasis in PN 3-6 neocortical neurons.

The physiological data presented above are consistent with a developmental role of KCC2 in chloride homeostasis and suggest the hypothesis that KCC2 expression levels will be reduced in P3 neurons compared with P25 neurons. To test this hypothesis, we examined KCC2 mRNA levels in P3 and P25 neurons using single-cell rt-PCR

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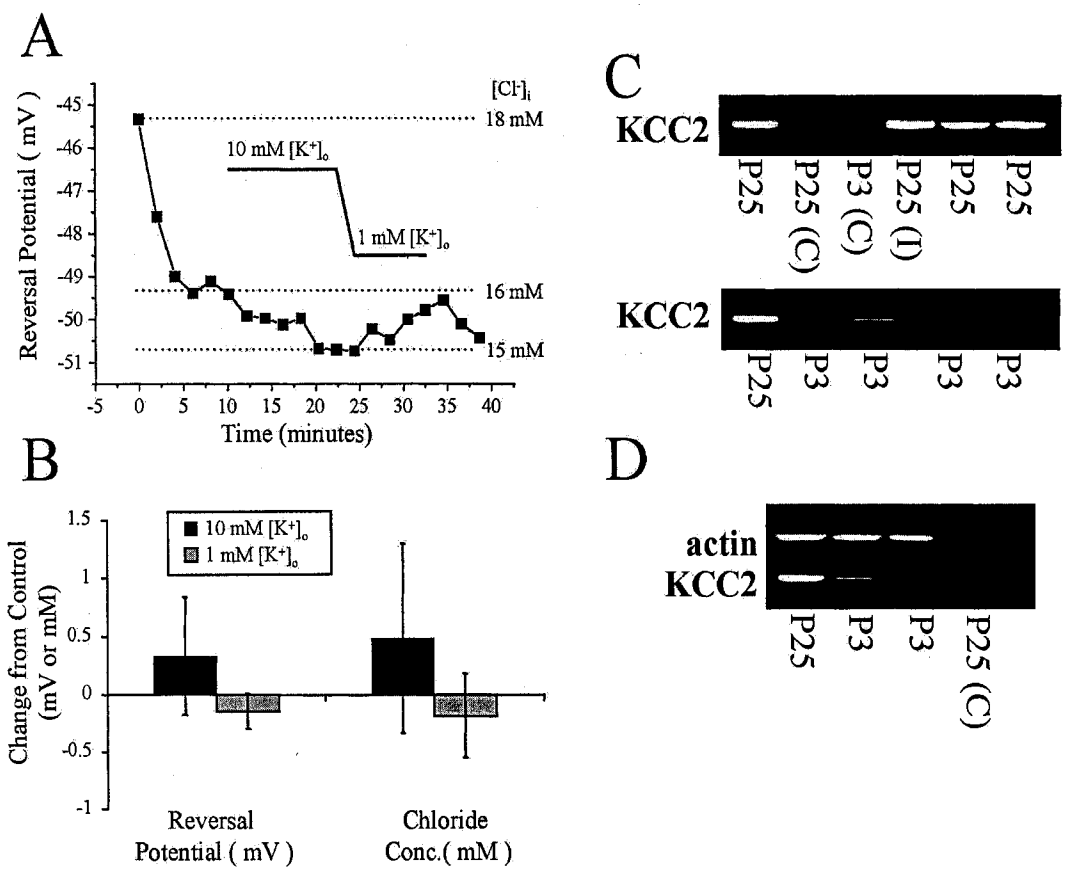

Figure 6. Single-cell rt-PCR of neurons from neocortical slices reveals developmentally regulated expression of KCC2 RNA. *A*, Manipulations of $[K^+]_o$ had no effect in this example recording from a P3 neuron with 20 mM pipette Cl^-. *B*, The mean changes in reversal potential and calculated $[Cl^-]_i$ were significantly less than those observed in P18-P28 neurons. Data from both 1 and 20 mM pipette Cl^- were pooled. *C*, Single neurons were analyzed from neocortical slices of P3 or P25 rats. rt-PCR, as described in Materials and Methods, was used to amplify a specific KCC2 fragment from pyramidal neurons or interneurons. *Control lanes* (C) refer to rt-PCR performed on samples taken by aspirating extracellularly within the slice. *D*, Cell contents from several of the cells in *C* were subjected to rt-PCR amplification of both KCC2 and actin.

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procedures (Fig. 6*C*). KCC2 mRNA was detected in all P25 neurons including pyramidal cells and interneurons. However, only one of four P3 neurons showed detectable levels of KCC2 mRNA. The presence of KCC2 in all P25 cells was unlikely to be caused by contamination during the rt-PCR procedure because control samples in which the cell cytoplasm was not harvested failed to reveal KCC2 mRNA expression. To ensure that the lack of KCC2 mRNA expression in P3 neurons was not caused by a failure to detect all mRNAs during the rt-PCR process, we examined, in the same sample, the expression of actin mRNA (Fig. 6*D*). Actin mRNA was detected in all P3 and P25 cells. The actin controls also allowed for the semiquantitative analysis of KCC2 levels in the one P3 neuron expressing KCC2 mRNA. The amount of KCC2 mRNA in the P3 neuron (relative to actin mRNA measured in the same cell) was 14% of that in P25 neurons. These data show that the developmental changes in K^+-coupled and furosemide-sensitive Cl^- accumulation and extrusion are correlated with the expression of KCC2 mRNA and reinforce the role of KCC2 in the developmental shift to lowered $[Cl^-]_i$.

Functional Consequences of Thermodynamic Driving Forces for Cotransporters

The results presented above support the role of KCC2 in both accumulation and extrusion of Cl^- under physiological conditions. To determine whether this action of KCC2 is consistent with the chemical forces acting on the transporter, we used a theoretical model to predict the thermodynamic driving forces as a function of $[Cl^-]_i$ and $[K^+]_o$. The direction of transport of an electroneutral cotransporter is governed by the chemical potentials of the ions transported [derived from Stein (1990) eq. 2.3]:

$$U = RT\sum_{j=1}^{n} m_j \ln\left(\frac{[X_j]_{in}}{[X_j]_{out}}\right)$$

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where n is the number of different ion species transported (KCC2, $n = 2$; NKCC, $n = 3$), m_j is the number of molecules transported for each species (for NKCC, $m_{Cl} = 2$; $m = 1$ otherwise), $[X_j]_{in}$ and $[X_j]_{out}$ is the concentration of ion species j inside and outside the cell, and RT is the product of the gas constant and absolute temperature. The thermodynamic driving force (U) is plotted in Figure 7 for two chloride cotransporter subtypes: NKCC and KCC2. The direction of transport in NKCC is relatively insensitive to changes in $[Cl^-]_i$ or $[K^+]_o$ over the range of physiological concentrations of the two ions. However, the direction of KCC2 transport is sensitive to small changes in $[Cl^-]_i$ and $[K^+]_o$ near physiological levels. Below the expected $[Cl^-]_i$ for mature pyramidal cells [~10 mM (R.A. DeFazio and S. Keros, unpublished observations)], KCC2 is predicted to accumulate intracellular chloride when $[K^+]_o$ is > 1 mM (Fig. 7, *gray shaded region*).

Discussion

Our results demonstrate the existence of a powerful homeostatic mechanism that maintains intracellular Cl^- concentration. Despite whole cell dialysis with the low Cl^- pipette solution, K^+-Cl^- cotransport activity raised the calculated intracellular Cl^- concentration by 0.7-1.6 mM. This is a net change in Cl^- concentration; in light of the continuous dialysis of the cell with the low Cl^- pipette solution, it is likely that the pumps were accumulating substantially more than ~1 mM chloride. Likewise despite elevated pipette Cl^- concentrations, reversal potentials indicated intracellular Cl^- concentrations well below those calculated from excised patches. This implies that the pumps were extruding at least several millimolar Cl^-. It is unlikely that NKCC, the pump traditionally associated with Cl^- accumulation, plays a role under these recording conditions because both lowering extracellular potassium (affecting only K^+-Cl^- cotransport) and cotransport antago-

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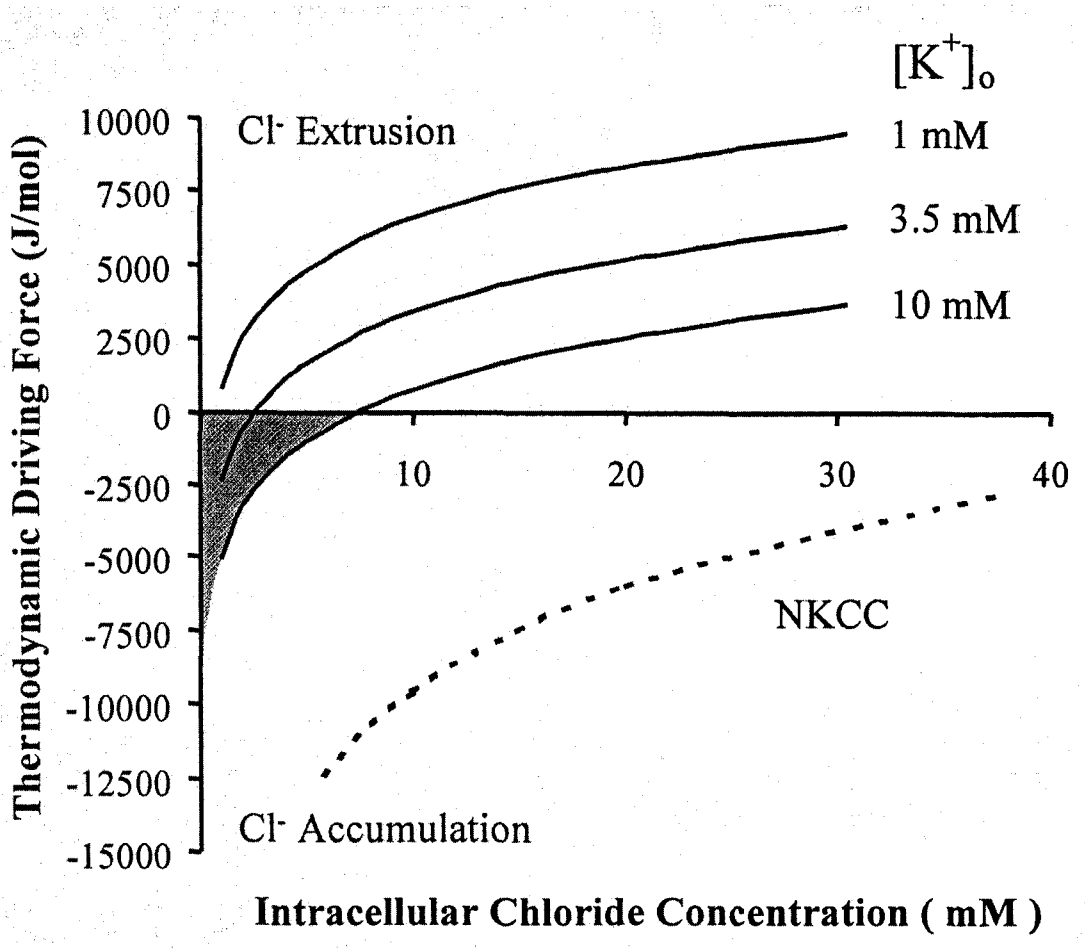

Figure 7. The thermodynamic driving force determines the direction of electroneutral Cl^- cotransport. The *x*-axis represents the intracellular Cl^- concentration. Along the *y*-axis, *positive values* of the driving force represent Cl^- extrusion, whereas *negative values* indicate Cl^- accumulation. The sum of the chemical potentials of Na^+, K^+, and 2 Cl^- inside and outside the cell as a function of intacellular Cl^- concentration is represented by the *dashed line* for 3.5 mM $[K^+]_o$. The *solid lines* represent the driving force for the cotransport of both K^+ and Cl^- (thus KCC2) at different $[K^+]_o$. The *gray shaded region* represents the range of ion concentrations that give rise to intracellular K-Cl accumulation; thus, at elevated $[K^+]_o$ and low $[Cl^-]_i$, thermodynamic calculations prediction the KCC2 activity will raise intracellular Cl^-.

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nism with furosemide (affecting both NKCC and K^+-Cl^- cotransport) lowered intracellular Cl^- to levels similar to those obtained in excised patches. This surprising result suggests that K^+-Cl^- cotransport function alone is sufficient for the homeostatic maintenance of intracellular Cl^-; i.e., under these recording conditions K^+-Cl^- cotransport is both the primary extrusion and accumulation mechanism in mature neocortical neurons.

Bicarbonate flux has been proposed to be a major contributor to $GABA_A$ receptor function (Kaila et al., 1993; Staley and Proctor, 1999). Our initial finding that the reversal potential with low pipette Cl^- was much more depolarized than those obtained in excised patches could be interpreted to reflect a strong bicarbonate flux (see Materials and Methods). However, when Cl^- accumulation via K^+-Cl^- cotransport was reduced by lowering extracellular potassium or by cotransport antagonism with furosemide, the reversal potentials were no longer significantly different from those of excised patches, which lacked a bicarbonate component. These manipulations of extracellular potassium and cotransport antagonism are not expected to modify intracellular or extracellular bicarbonate. These results support the contention that the depolarized reversal observed with low pipette Cl^- was caused by Cl^- accumulation via K^+-Cl^- cotransport and not by an unmasked bicarbonate efflux. Bicarbonate can be an important component of GABA responses (Kaila et al. 1993, 1997; Staley and Proctor, 1999); however, under the present recording conditions bicarbonate did not appear to play a major role in determining the response to GABA. A lack of a bicarbonate contribution to depolarizing GABA responses in hippocampal pyramidal cells has also been reported (Grover et al., 1993).

Differential expression of the transport proteins involved in Cl^- homeostasis has been proposed to explain differences in resting Cl^- concentrations between cell types (Rohrbough and Spitzer, 1996; Ulrich and Huguenard, 1997) and during development

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(Owens et al., 1996; Kakazu et al., 1999; Rivera et al., 1999). The absence of K^+-coupled Cl^- cotransport in our recordings from neonatal neurons correlated well with the reduced expression of KCC2 mRNA detected in single-cell harvests. Although other extrusion mechanisms must exist in neonatal neurons, the absence of a potassium-coupled mechanism is consistent with the elevated intracellular Cl^- concentration reported in neonatal neurons. In addition, because of a role for neuronal K^+-Cl^- cotransport in the maintenance of extracellular potassium (Payne, 1997), the lack of KCC2 expression in neonatal neurons may explain differences in extracellular potassium regulation observed during development (Hablitz and Heinemann, 1989).

The presence of a powerful potassium-coupled Cl^- cotransport mechanism has important functional implications. Our results suggest that K^+-Cl^- cotransport alone is sufficient for homeostatic maintenance of intracellular Cl^-. The Cl^- set point (the concentration at which the pump exhibits no net flux) is coupled to $[K^+]_o$. If intracellular Cl^- exceeds the set point, K^+-Cl^- cotransport can extrude Cl^-. If Cl^- goes below the set point, K^+-Cl^- cotransport can accumulate Cl^-. We have demonstrated the capacity of these pumps to maintain intracellular Cl^- concentrations despite whole-cell dialysis via a patch pipette. Such a Cl^- load or sink is substantially less than the physiological Cl^- load or sink because of $GABA_A$ receptor-mediated Cl^- flux, thus demonstrating reserve capacity, an important aspect of numerous physiological systems. It is clear that other Cl^- homeostatic mechanisms are present in the neuron. For example, the Cl^- reversal potential is still quite hyperpolarized in neurons from P3-P6 animals, suggesting that although immature cells have reduced KCC2 mRNA levels some other mechanism is lowering intracellular Cl^-.

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Payne (1997) proposed that KCC2 could contribute to extracellular potassium homeostasis. Although the relative contribution of neurons to the spatial buffering of extracellular potassium remains to be determined, one of the primary consequences of a rise in extracellular potassium would be elevation of the intracellular Cl^- concentration. In our experiments, raising extracellular potassium from 3.5 mM to 10 mM increased intracellular Cl^- ~3 mM (with either 1 or 20 mM pipette Cl^-). A 3 mM increase in intracellular Cl^- from a resting Cl^- concentration of 10 mM would raise the Cl^- reversal potential from –61 to –54 mV. Under these conditions, activation of $GABA_A$ receptors could depolarize the cell to –54 mV, much closer to the threshold for action potential generation in neocortical neurons. Large increases in extracellular potassium have been described during seizure-like activity both *in vivo* (Lux et al., 1974; Xiong and Stringer, 1999) and *in vitro* (Benninger et al., 1980; Swann et al., 1986; Hablitz and Heinemann, 1989). It is possible that elevations in neuronal Cl^- could contribute to the difficulty in controlling prolonged seizures.

The net effect on excitability of a K^+-dependent accumulation of intracellular Cl^- is not clear. A rise in extracellular potassium has multiple effects on neurons; in addition to increased intracellular Cl^-, input resistance decreases and the membrane potential depolarizes. In the present study at a holding potential of –70 mV, raising extracellular potassium from 3.5 to 10 mM decreased the input resistance by 41 ± 10 MΩ and induced a depolarizing current of 388 ± 122 pA. To a considerable degree the most prominent effect of an acute increase in extracellular potassium is membrane depolarization such as that described by Kaila et al. (1997). They demonstrated an ~20 mV depolarization because of a transient increase in extracellular potassium of up to 7.4 mM in response to by a high-frequency stimulus train. Such a depolarization is sufficient to place the mem-

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brane potential above the elevated Cl^- reversal potential and result in a hyperpolarizing response to synaptic GABA. Neuronal buffering of extracellular potassium probably does not have pathological consequences for neuronal function because the elevated intracellular Cl^- concentration is balanced by a decrease in input resistance and membrane depolarization.

Recent studies suggest a strong neuronal contribution to buffering of $[K^+]_o$ (Ransom et al., 2000; Xiong and Stringer, 2000). It is difficult to infer a direct role of KCC2 in these studies because the manipulations that would affect the cotransporter also altered the spontaneous activity that gave rise to the potassium transient. Lowering extracellular Cl^-, furosemide, and ouabain all raised the ceiling of the extracellular potassium transient (Xiong and Stringer, 2000). Although Xiong and Stringer conclude a role for the neuronal Na^+-K^+ ATPase, it is clear that breakdown of the potassium gradient because of inhibition of the Na^+-K^+ ATPase could also inhibit K^+-coupled Cl^- cotransport. KCC2-mediated Cl^- accumulation in elevated $[K^+]_o$ is consistent with an enhancement of the capacity of the homeostatic mechanisms that regulate extracellular K^+. In addition to its major role in neuronal Cl^- homeostasis, KCC2 expression may result in a more rapid return to normal levels of $[K^+]_o$ and/or a lower maximal level that $[K^+]_o$ can attain.

In summary, we have shown that developmental changes in the expression of KCC2 results in the coupling of $[Cl^-]_i$ and $[K^+]_o$ via the activity of the furosemide-sensitive K^+-coupled Cl^- cotransporter. $[K^+]_o$ manipulations in immature neurons had no effect on $[Cl^-]_i$, consistent with the low expression of KCC2 mRNA detected in cytoplasm harvested from these cells. Expression of KCC2 results in lowered $[Cl^-]_i$ and translates physiological changes in $[K^+]_o$ to marked changes in $[Cl^-]_i$. These data demonstrate

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a physiologically relevant and developmentally regulated role for KCC2 in Cl^- homeostasis and neuronal excitability.

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ECTOPIC ACTION POTENTIAL GENERATION IN CORTICAL INTERNEURONS DURING SYNCHRONIZED GABA RESPONSES

by

SOTIRIOS KEROS AND JOHN J. HABLITZ

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Abstract

In the presence of 4-aminopyridine and excitatory amino acid receptor antagonists, individual neurons in brain slice preparations exhibit large gamma-aminobutyric acid (GABA)-mediated responses as a consequence of synchronous GABA release from a network of interneurons. These synchronized GABA responses are frequently associated with ectopic action potentials (EAPs), which are thought to be action potentials initiated in distal axon terminals which subsequently travel antidromically toward the soma. Ectopic action potentials feature prominently in some models of epilepsy. Neocortical synchronized GABA responses propagate across the cortex, predominantly in superficial layers. The role that EAPs may play in contributing to laminar differences in the synchronized GABA response has not been addressed. Here we examined the occurrence of EAPs during synchronized GABA responses in neurons within layers I and II/III. EAPs occurred in 78% of layer I interneurons and in 25% of layer II/III interneurons (including chandelier cells). EAPs were not observed in layer II/III pyramidal neurons. The prevalence of EAPs in layer I interneurons provides a mechanism by which layer I can support both the initiation and propagation of synchronized GABA responses. Thus, layer I interneurons are a critical component of a network capable of synchronizing a propagating wave of GABA release across the neocortex.

Introduction

Cortical interneurons are known to form tightly coupled networks which can fire synchronously *in vivo* and *in vitro* (Michelson and Wong, 1994; Whittington et al., 1995; Bragin et al., 1995; Benardo, 1997; Swadlow et al., 1998). The mechanism of synchronization of interneurons may involve dendritic and axonal electrical coupling as well as

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recurrent axon collaterals (Benardo, 1997; Deans et al., 2001; Schmitz et al., 2001; Traub et al., 2001b). One widely studied model of interneuron synchronization involves application of 4-aminopyridine (4-AP) in the presence of excitatory amino acid (EAA) receptor antagonists (Aram et al., 1991; Michelson and Wong, 1991; Muller and Misgeld, 1991; Perrault and Avoli, 1991). In hippocampal and neocortical slices *in vitro*, this induces interneurons to burst synchronously, causing so-called synchronous or giant inhibitory post-synaptic potentials (IPSPs; Aram et al., 1991; Avoli et al., 1994). These synchronous events often have both depolarizing and hyperpolarizing components (Perrault and Avoli, 1992; Lamsa and Kaila, 1997) and are blocked by the $GABA_A$ receptor antagonist bicuculline (Aram et al., 1991; Avoli et al., 1994). Synchronous bursts can be evoked with extracellular stimulation and also occur spontaneously. 4-AP-induced events slowly propagate through the neocortex (Aram et al., 1991; Benardo, 1997; Hablitz and DeFazio, 1999).

Non-somatic action potentials, otherwise referred to as ectopic action potentials (EAPs), are generated distally in the dendrites and axons, and propagate toward the soma (Gutnick and Prince, 1974; Stasheff et al., 1993a; for review see Pinault, 1995). EAPs are prominent in several models of epilepsy and may be involved with the initiation or spread of seizure-like activity (Rosen and Vastola, 1971; Gutnick and Prince, 1972; Pinault and Pumain, 1985). EAPs can be recorded during spontaneous and evoked giant IPSPs from slices treated with 4-AP (Perreault and Avoli, 1989, 1992; Benardo, 1997). Studies in the hippocampus led to the conclusion that these EAPs originate in the axons and depend on synaptic activation of $GABA_A$ receptors (Perreault and Avoli, 1989; Avoli et al., 1998). Recent evidence in support of axo-axonic gap junctions (Schmitz et al., 2001; Traub et al., 2001a) suggests that EAPs in one axon could spread to axons of

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connected neurons, thus amplifying the network effects of EAPs. Studies of EAPs during giant IPSPs or synchronized GABA responses have been focused mainly on the hippocampus (Perreault and Avoli, 1992; Stasheff et al., 1993a; Lamsa and Kaila, 1997; Avoli et al., 1998; Traub et al., 2001b), where both pyramidal cells as well as interneurons display EAPs. Studies in the neocortex, however, indicate that interneurons are more likely to have EAPs (Benardo, 1997).

Synchronized GABA responses in upper cortical layers have been shown to differ from those in deeper layers. Laminar differences in the pattern of neocortical activity in both kindled and 4-AP-treated slices were seen by Barkai et al. (1994, 1995) who reported that paroxysmal neuronal events in superficial layers exhibited an inhibitory component which was absent in deeper layers. Yang and Benardo (2002) have shown that horizontal neocortical slices containing only superficial layers (layer I and II) can exhibit synchronized GABAergic activity when excitatory transmission is blocked. Voltage-sensitive dye-imaging studies have revealed that although all neocortical layers exhibit evoked synchronized GABA responses, such responses propagate preferentially in superficial layers of the cortex (Hablitz and DeFazio, 1999). The potential importance of layer I in maintaining propagating synchronized GABA responses is strongly supported by the fact that layer I is composed entirely of GABAergic neurons whose processes generally stay in the superficial layers (DeFelipe and Jones, 1988; Li and Schwark, 1994; Zhou and Hablitz, 1996). Furthermore, it is known that interneurons in general, and specifically layer I interneurons, are electrically coupled and capable of synchronized oscillatory firing (Whittington et al., 1995; Benardo, 1997; Galarreta and Hestrin, 1999; Fukada and Kosaka, 2000; Deans et al., 2001; Traub et al., 2001b; Chu et al., 2003). Biocytin labeling of layer I interneurons has revealed axonal arbors that can span 700 μm within

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layer I, and collaterals that descend as deep as layer IV (Zhou and Hablitz, 1996). Pyramidal cells in deeper layers as well as some deeper layer interneurons send both apical dendrites and axon collaterals into layer I (Martin, 1984; Kawaguchi, 1993; Marin-Padilla, 1990; Cowan and Wilson, 1994). This organization enables layer I interneurons to directly affect all layers of the neocortex. The fact that layer I interneurons can form tightly coupled networks and influence large areas of cortex suggests a potentially important role for these neurons in synchronized neocortical GABA responses. In the present study, we compared responses of neurons in layers I and II/III to determine the mechanism whereby layer I may be responsible for enabling the propagation of synchronized GABA responses. Portions of the work have appeared in abstract form (Keros and Hablitz, 2002).

Experimental Procedures

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using a protocol approved by the University of Alabama at Birmingham (Birmingham, AL, USA) Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and discomfort.

Postnatal days 17-24 Sprague Dawley rats were anesthetized with ketamine (100 mg/kg) and decapitated. The brains were rapidly removed and placed in an ice cold, oxygenated (95% O_2/5% CO_2) cutting solution containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 3 $MgCl_2$ and 1 $CaCl_2$. Coronal slices 300 μm thick were cut on a Vibratome (Ted Pella Inc., Riverside, CA, USA) from a block of brain containing

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the right sensorimotor cortex. Slices were stored for 45 min at 37 °C and then kept at room temperature until recording.

Slices were incubated for 30-60 minutes in the recording chamber before experiments in a solution containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 0.02 D-(-)-2-amino-5-phosphonopentanoic acid (APV), 0.01 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 0.05-0.01 4-AP. During recordings, slices were continually bathed in this solution at 32-35 °C via a peristaltic pump.

Whole-cell current clamp recordings were made from visually identified neurons in layer I and layer II/III using a Zeiss Axioskop FS (Carl Zeiss Inc., Thornwood, NY, USA) microscope with infrared differential interference contrast optics. Layer I interneurons were identified by their location in the cell-sparse zone, within 80 μm from the pial surface. Layer II/III pyramidal cells were selected based on their distance below the pia (200-300 μM), their triangular shape and prominent apical dendrites. Layer II/III interneurons were identified by their non-pyramidal morphology. Action-potential firing properties and biocytin labeling were used to confirm the location and identity of all cell types.

Electrodes had a resistance of 3-6 MΩ when filled with an intracellular solution containing (in mM): 134 K-gluconate, 0.5 KCl, 5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 NaGTP, 0.2 $CaCl_2$. The pH was adjusted to 7.3 with KOH and 0.5% biocytin was routinely added. The experimentally measured liquid junction potential was -14 mV. All reported voltages are corrected values. Series resistance was measured at the beginning and the end of experiments. Cells were rejected if series resistance initially exceeded 20 MΩ or changed by more than 25% during the course of the experiment.

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Synaptic activity was evoked in slices by electrical stimulation in layer I with a bipolar electrode placed 100-200 μm lateral to the recorded neuron unless otherwise noted. With stimulus duration set at 200 μs, stimulus intensities ranged from 100 to 400 μA. Stimulus intensity was adjusted in each slice as the smallest that would reliably trigger a GABA response. The shortest interstimulus interval used for evoking a GABA response was 45 s. Responses were recorded using an Axopatch 200A or MultiClamp 700A amplifier (Axon Instruments Inc., Union City, CA, USA), filtered at 2-5 kHz and digitized at 10 kHz using a Digidata 1200 acquisition system and Clampex Software (Axon Instruments). Data analysis and graphing was performed with Origin 7.0 (Microcal, Northampton, MA, USA). A paired Student's *t*-test was used for statistical analysis. Data is reported as mean±standard error of the mean.

Chemicals for these experiments were purchased from Fisher Chemicals (Fairlawn, NJ, USA) except for biocytin, D-glucose, potassium gluconate (Sigma-Aldrich, St. Louis, MO, USA), HEPES and EGTA (Calbiochem, San Diego, CA, USA). APV, CNQX and SCH 50911 were purchased from Tocris Cookson Inc (Ellisville, MO, USA).

Results

EAPs in Neocortical Neurons

Current clamp recordings of evoked responses in the presence of 4-AP and EAA receptor antagonists were obtained from visually identified neurons in layers I and II/III. These evoked responses were evaluated to determine the presence of EAPs using three established criteria (Perreault and Avoli, 1989, 1991, 1992): (1) EAPs occur at membrane potentials more negative than those required to elicit action potentials via current

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injection or EPSPs; (2) EAPs persist despite hyperpolarizing current injection; and (3) EAPs are of variable amplitude, and are almost always smaller than full action potentials. An example of EAPs in a layer I interneuron is shown in Figure 1. From rest, an evoked synchronized GABA response was observed as a slight hyperpolarization with several action potentials (Fig. 1*A*). GABA-mediated responses were hyperpolarizing at resting membrane potentials due to the low chloride internal solution used in the patch pipette. When the neuron was hyperpolarized with current injection to -100 mV, synchronized GABA responses were depolarizing and EAPs were observed rather than full-amplitude somatic action potentials (Fig. 1*B*). This suggests that the EAPs are responsible for triggering the full-amplitude somatic action potentials at rest, presumably through invasion of the initial segment. EAPs in layer I interneurons were typically seen in groups of 3 to 10 "spikelets" occurring near the peak of the synchronized GABA response. EAPs generally matched the timing and number of the full-amplitude action potentials seen at rest in the same neuron, implying that EAPs are sufficiently depolarizing at normal resting membrane potentials to bring the neuron to threshold. The *inset* is a close-up of three of the events in Fig. 1*B*. Note that the EAPs arise abruptly from the baseline and lack a hyperpolarizing afterpotential. EAP amplitudes ranged between 35 and 55 mV and varied from trial to trial as well as across neurons. EAP amplitudes varied with membrane potential but were always smaller than depolarization-evoked action potentials. EAPs were not suppressed upon hyperpolarization to -135 mV (data not shown). During current injection to threshold, the synchronized GABA response suppressed current-injection-induced firing (Fig. 1*C*), but EAP induced firing could still be observed during the evoked response. This observation emphasizes that the EAPs are responsible for triggering full-amplitude action potentials during the synchronized GABA response. A

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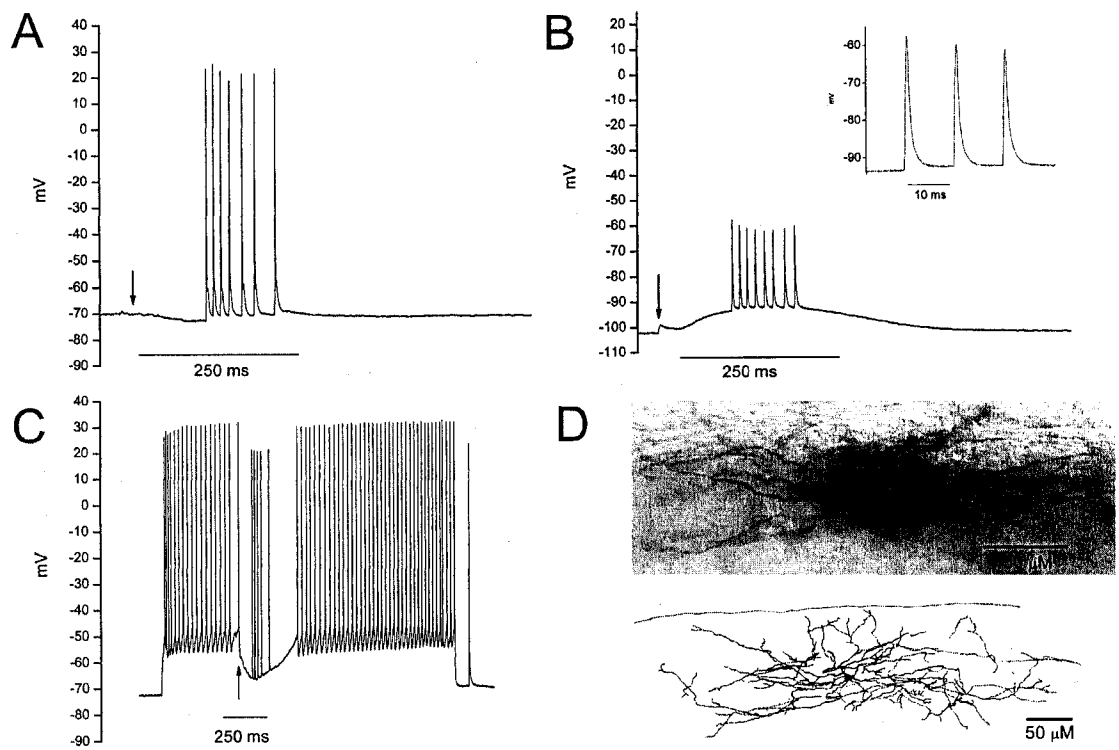

Figure 1. Properties of EAPs occurring during evoked synchronized GABA responses in layer I interneurons. *A*, At the resting membrane potential, EAPs trigger full-amplitude action potentials during an evoked synchronized GABA response. *Arrows* indicate the time of electrical stimulation. *B*, With hyperpolarizing current injection to keep the interneuron below threshold, individual EAPs are seen as small-amplitude events occurring during a depolarizing synchronized GABA response. The *inset* shows the first three EAPs from *B* on an expanded time scale. *C*, Depolarizing current injection was used to depolarize the neuron and evoke action potentials. The synchronized GABA response was hyperpolarizing, yet the EAPs triggered full-amplitude action potentials. Most layer I interneurons displayed fast-spiking characteristics upon current injection, as seen in *C*. *D*, Photomicrograph (*top*) and camera lucida drawing (*bottom*) of a typical biocytin labeled layer I interneuron. The pial surface is represented by the *continuous line* in the camera lucida drawing.

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photograph and camera lucida reconstruction of a typical layer I interneuron is shown in Figure 1*D*. Layer I interneurons typically had dendrites and axons which stayed primarily within layer I.

EAPs were present during the synchronized GABA response in 78% (25/32) of layer I interneurons. EAPs were not observed during synchronized GABA responses in layer II/III pyramidal cells (n=33) but were present in 25% (6 of 24) of layer II/III interneurons. Synchronized GABA response half-widths ranged from 120 to 500 ms, with an average of 275±25 ms (n=41). Response amplitudes and durations were not significantly different between cell types. The reversal potential of the synchronized GABA responses using an intracellular solution containing 1 mM chloride was -87±1 mV (n=30). There was no significant difference in the reversal potential measured in different cell types. In previous studies, GABA application under similar conditions resulted in reversal potentials near -90 mV (DeFazio et al., 2000; DeFazio and Hablitz, 2001). This agreement strongly suggests that responses observed here are GABA mediated.
Whereas most layer I interneurons exhibited EAPs, they were seen in only six of 24 layer II/III interneurons. Two of these six cells with EAPs were successfully labeled with biocytin. Both labeled cells had chandelier cell like morphologies. None of the 18 neurons without EAPs had chandelier-like morphologies. Figure 2*A* shows an evoked synchronized GABA response in a chandelier cell at rest. Synchronized GABA response and EAP properties in the chandelier cells were similar to those recorded in layer I interneurons (compare Fig. 2*A* with Fig. 1*A*). A photomicrograph and camera lucida reconstruction of a biocytin-labeled chandelier cell are shown in Figures. 2*B* and 2*C*. A limited dendritic spread and extensive axonal arborization with closely spaced axonal boutons arranged vertically, as observed here, are characteristic of chandelier cells in the

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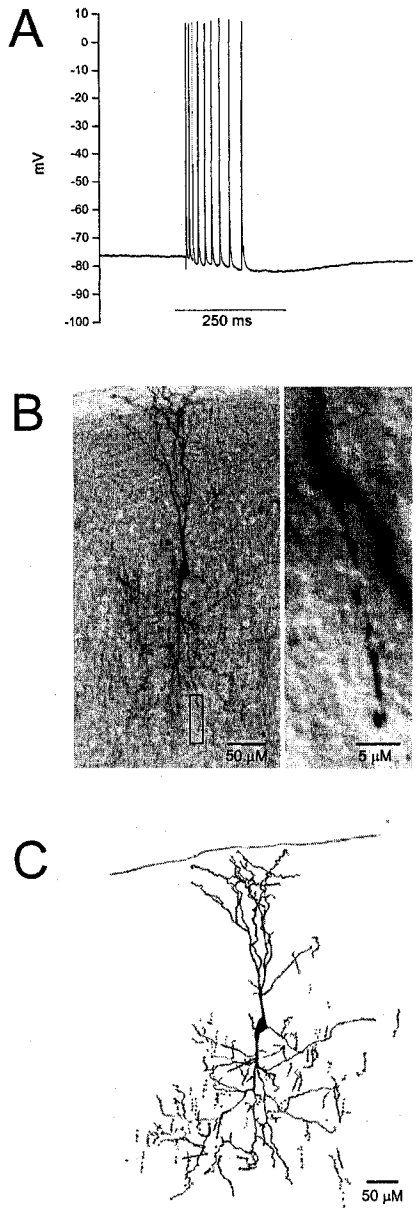

Figure 2. EAPs are observed during the synchronized GABA response in chandelier-type interneurons in layer II/III. *A*, An evoked synchronized GABA response with EAPs recorded from a chandelier cell. Chandelier cell responses were similar to those recorded in layer I interneurons. The *arrow* indicates the time of stimulation. *B*, Photomicrograph of a biocytin-filled layer II/III chandelier cell. The border between layer I and layer II/III is indicated by the *hash mark*. The photomicrograph on the *right* is a higher power picture of an axon segment taken from the area indicated by the *box*. *C*, Camera lucida reconstruction of the cell in *B*. The pial surface is represented by the *continuous line*. Note that the chandelier cell projects its dendrites into layer I.

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neocortex (DeFelipe, 1999). Chandelier cells make inhibitory synapses onto the initial segment of pyramidal cells (Freund et al., 1983; Douglas and Martin, 1990; DeFelipe, 1999). These neurons project their dendrites from the soma in layer II/III up through layer I toward the pial surface.

Comparison of Spontaneous and Evoked Events

Synchronized GABA responses occurred spontaneously, although the site and mechanism of initiation is unknown. Under our recording conditions, synchronized GABA responses occurred spontaneously in 25% (18 of 72) of the slices tested. Spontaneous synchronized GABA responses in these slices occurred at irregular intervals of 1-5 min. Figure 3*A* shows a spontaneous synchronized GABA response that occurred in a layer I interneuron. Electrical stimulation toward the end of this spontaneous synchronized GABA response evoked an IPSP (indicated by an *arrow*) but did not result in further depolarization or additional EAPs. In general, 10-40 s intervals were necessary before a synchronized GABA response could be evoked following either a spontaneous or evoked response. This indicates that synchronized GABA responses have a refractory period. An evoked synchronized GABA response from the same neuron as Figure. 3*A* is shown in Figure 3*B*, but from a membrane potential of -102 mV. From this potential, the amplitude of the evoked synchronized GABA response is greater than the spontaneous one in Figure 3*A*, yet the duration was the same and the number and timing of the EAPs were similar. In all slices tested, spontaneous synchronized GABA responses were of similar amplitude and duration when compared with evoked responses in the same neurons. EAPs were observed in all layer I interneurons recorded from slices with spontaneous synchronized GABA responses (n=15). The number of EAPs observed was similar in

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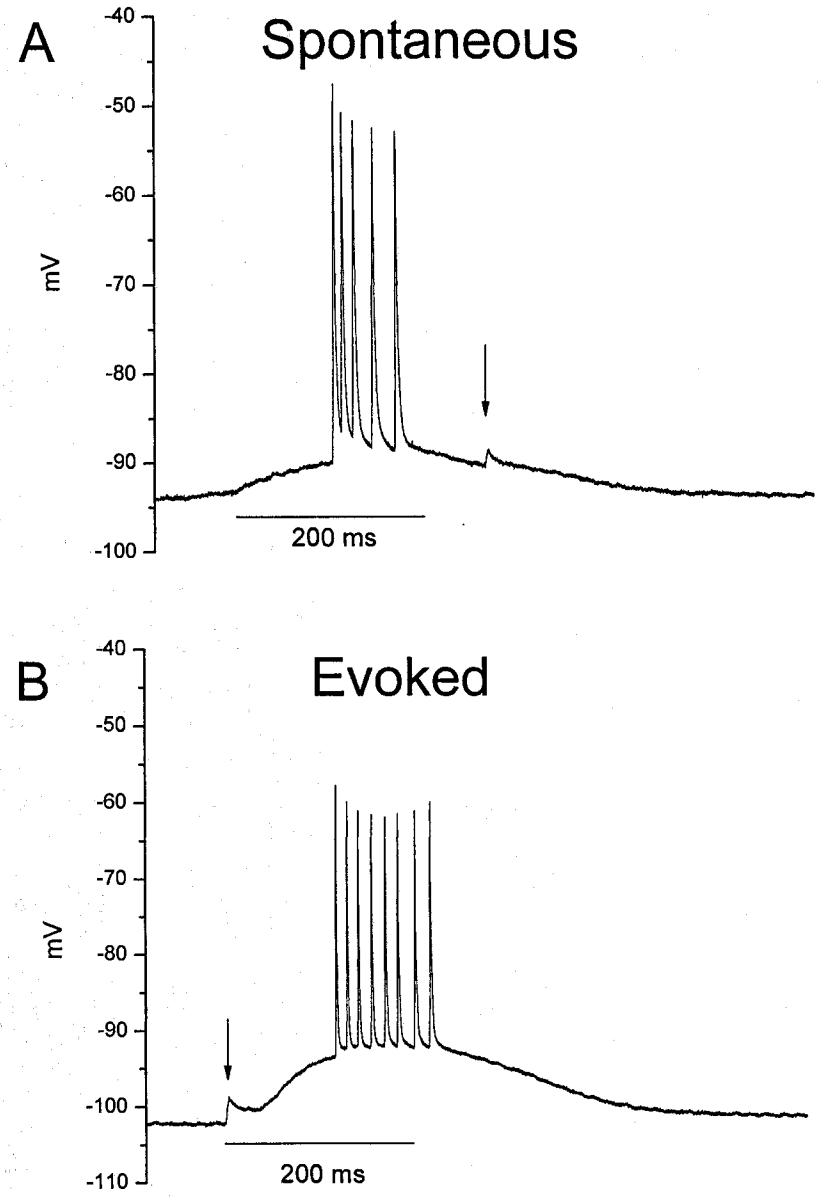

Figure 3. Spontaneous and evoked synchronized GABA responses have similar properties. *A*, Example of a spontaneous synchronized GABA response in a layer I interneuron. EAPs were observed in each interneuron which had spontaneous events. Electrical stimulation (*arrow*) during the decay of the synchronized GABA response failed to evoke another synchronized GABA response or additional EAPs. *B*, An evoked synchronized GABA response from the same interneuron as in *A* but from a more hyperpolarized membrane potential is similar to the spontaneous synchronized GABA response in *A*.

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both evoked and spontaneous responses from the same neurons. Thus, evoked and spontaneous synchronized GABA responses appear to be analogous events despite different methods or locations of initiation.

Prolonged GABA Responses

The majority of neurons tested were from slices which exhibited synchronized GABA responses and EAPs as described above. However, four slices (out of 72 tested) displayed prolonged GABA responses consisting of a burst of action potentials longer than 750 ms (Fig. 4*A*). Prolonged responses were defined as events having at least 35 EAPs. The prolonged responses were recorded from one layer I interneuron in each of four slices obtained from four different animals. Other slices from these animals had the non-prolonged GABA responses. Synchronized GABA responses occurred spontaneously in all four slices which had the prolonged responses, as opposed to only 20% (14 of 68) in slices without prolonged GABA responses, suggesting that GABA responses were more easily initiated in the former group. In a given neuron, prolonged evoked responses were similar in appearance to the spontaneous responses. In contrast to the non-prolonged GABA responses, which had refractory periods of up to 40 s during which another synchronized GABA response could not be evoked, prolonged responses could be evoked at intervals less than 5 s (data not shown). One of the prolonged responses from 4*A* is shown on an expanded scale in Figure 4*B*. The response began as a series of EAPs which eventually became full-amplitude action potentials as the membrane depolarized during the response. This observation emphasizes that the EAPs can trigger full-amplitude action potentials during GABA responses.

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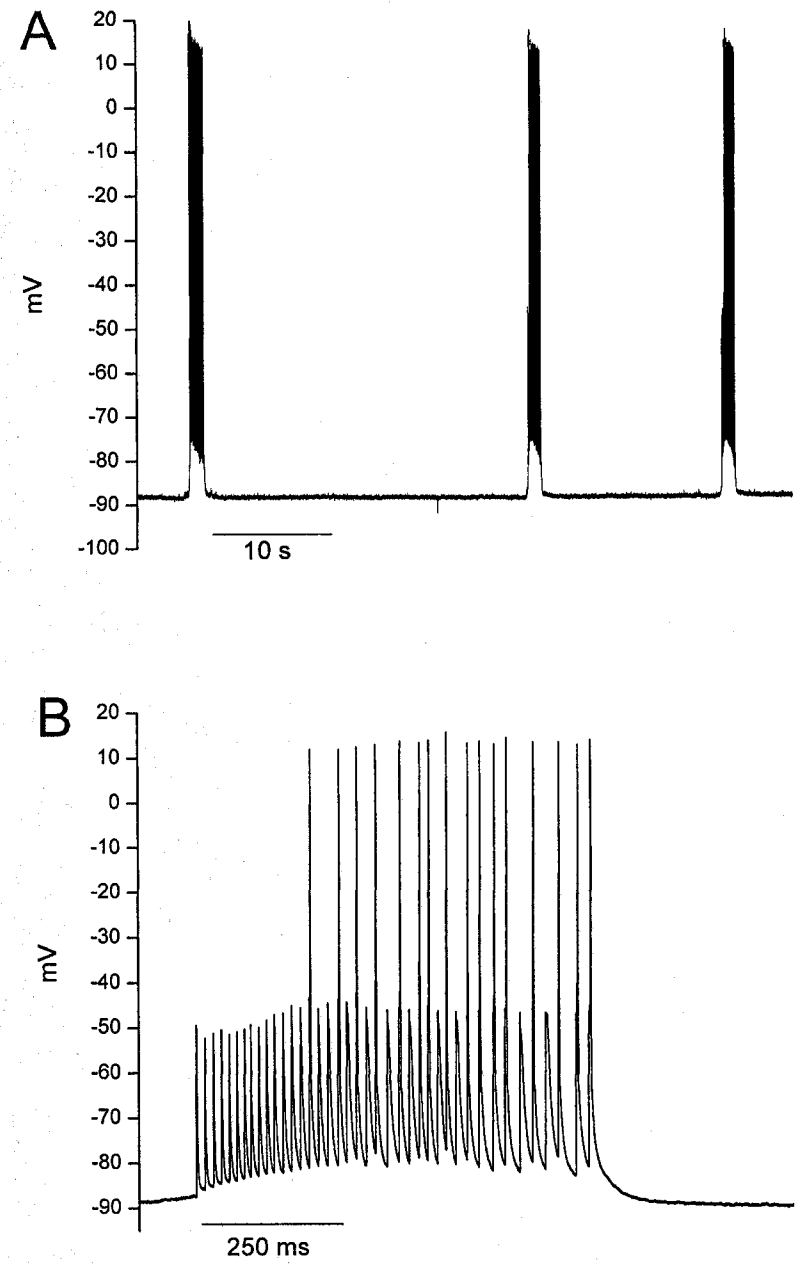

Figure 4. Layer I interneurons from a subpopulation of slices have prolonged synchronized GABA responses. Prolonged synchronized GABA responses, *A*, were spontaneous and occurred more frequently than spontaneous non-prolonged synchronized GABA responses. Full amplitude action-potentials were triggered during the prolonged response by the underlying EAPs despite hyperpolarizing current injection to -90 mV. *B,* A close-up of a prolonged synchronized GABA response from *A*. This example illustrates that individual EAPs are responsible for the full-amplitude action potentials which occur during the GABA response.

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Propagation of GABA Responses and Role of $GABA_B$ Receptors

In order to show that synchronized GABA responses were propagating events, interneurons were selected that were distal (1 mm) from the stimulation electrode. The experiment shown in Figure 5 indicates that under such conditions synchronized GABA responses occurred at a long latency. The average latency to the onset of the synchronized GABA response from stimulation that was 1 mm away from the soma was 766±233 ms (n=3). In contrast, all responses evoked from proximal (100-200 μm) stimulation began during the directly evoked IPSP, less than 40 ms after stimulation. The long latency from distal stimulation confirms that the observed GABA responses are slowly propagating events and are unlikely to be directly evoked synaptic events. In this particular example, two of the EAPs occur more than 200 ms after the stimulus (*arrow*), yet before the beginning of the synchronized GABA response. This observation suggests that an evoked synchronized GABA response might first initiate EAPs in the terminals of a given neuron as the response travels toward the soma. These EAPs then propagate more quickly along the axon and precede the synchronized GABA response at the somatic recording site.

Synchronized GABA responses are dependent on activation of post-synaptic $GABA_A$ receptors since the GABA response is blocked by bicuculline (Aram et al., 1991) and their reversal potential matches that of a chloride dependent response (Aram et al., 1991; Yang and Benardo, 2002). The $GABA_B$ antagonist SCH 50911 was used to determine if GABA acting on $GABA_B$ receptors influences synchronized GABA response properties.A synchronized GABA response from a layer I interneuron is shown in Figure 6*A* (*left*). After bath application of SCH 50911 (10 μM; Fig. 6*A*, *right*) the duration of the response increased as did the number of EAPs which occurred during the synchronized

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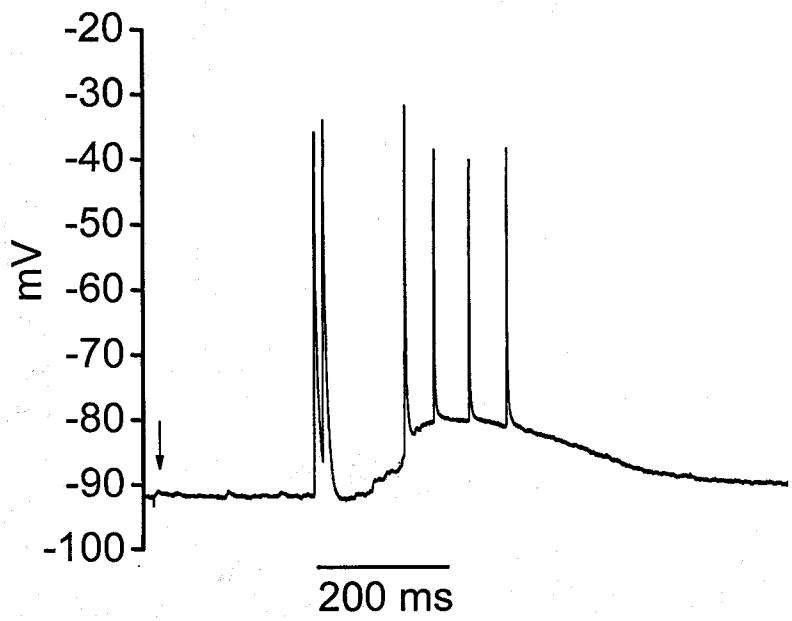

Figure 5. Evoked synchronized GABA responses are propagating events. In this example, the synchronized GABA response was evoked with electrical stimulation from a location distal to the interneuron. The synchronized GABA response begins 300 ms after the time of stimulation (*arrow*), indicating a slowly propagating event. Two EAPs are seen well before the onset of the synchronized GABA response.

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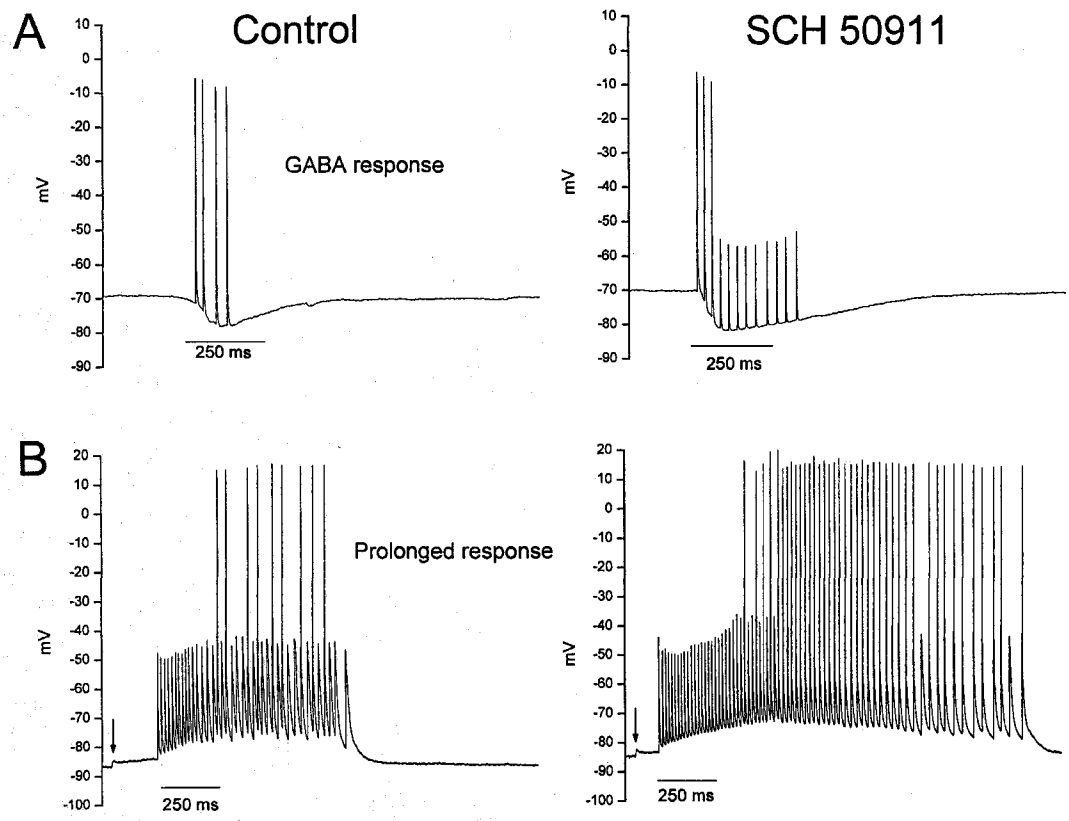

Figure 6. $GABA_B$ receptor antagonists enhance synchronized GABA responses and increase the number of EAPs. *A*, A spontaneous synchronized GABA response with EAPs in a layer I interneuron before (*left*) and after (*right*) bath application of the $GABA_B$ antagonist SCH 50911. SCH 50911 increases the amplitude and duration of the synchronized GABA response and increases the number of observed EAPs. *B*, Prolonged synchronized GABA responses before and after SCH 50911 (*left* and *right*, respectively). The *arrows* indicate the time of stimulation.

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GABA response. SCH 50911 increased the average half-widths of the synchronized GABA responses by 137±38 ms or 44±8% (P=0.002, n=11) and increased the average number of EAPs from 4.8 to 10.6 or 120 ± 33% (P=0.016, n=3). SCH 50911 also enhanced the response in a layer I interneuron with a prolonged synchronized GABA response (Fig. 6*B*). In this neuron, the evoked response began as a train of EAPs which triggered a few somatic action potentials. Application of the $GABA_B$ antagonist increased the duration of the response as well as the number of somatic action potentials. The latency of the response to stimulation as measured to the first EAP decreased from 150 ms to 75 ms. These experiments suggest that activation of $GABA_B$ receptors helps to limit the magnitude and duration of synchronized GABA response. In addition, initiation and propagation of the synchronized GABA response are not dependent on functioning $GABA_B$ receptors.

Discussion

We report three main findings in this study: (1) although synchronized neocortical GABA responses occurred in all cell types studied, EAPs were much more likely to be observed during these responses in layer I interneurons when compared to layer II/III pyramidal cells and layer II/III interneurons; (2) a subpopulation of slices had prolonged synchronized GABA responses, with large numbers of EAPs; and (3) $GABA_B$ receptors regulate the duration of the synchronized GABA response and limit the number of EAPs observed during a synchronized GABA response.

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Layer I Interneurons Exhibit Properties That Can Coordinate Activity in a Network of Interneurons

The specific site of origin of neocortical EAPs is unknown. EAPs were observed during synchronized GABA responses, despite hyperpolarization to very negative membrane potentials. Our results imply a distal site of initiation consistent with evidence from the hippocampus that EAPs are thought to be generated at axonal release sites (Stasheff et al., 1993a). We did not observe EAPs associated with the synchronized GABA response in pyramidal neurons. This is in contrast to the hippocampus where both pyramidal neurons and interneurons display EAPs during 4-AP-induced responses (Perreault and Avoli, 1989, 1992; Stasheff et al., 1993a; Avoli et al., 1998). The prevalence of EAPs in layer I indicates that these responses are a key element of these neurons' behavior and suggests a special role for layer I cells in contributing to the propagation of neocortical synchronized GABA responses.

In the presence of EAA antagonists, 4-AP-induced synchronized GABA responses have a reversal potential near that expected for chloride (Avoli et al., 1998; DeFazio and Hablitz, 2001), emphasizing the role of $GABA_A$ receptors. $GABA_A$ receptor activation can lead to a depolarizing current in cortical neurons (Gulledge and Stuart, 2003), and synchronized GABA responses imaged with voltage-sensitive dyes show depolarization in both the hippocampus (Sinha and Saggau, 2001) and neocortex (Hablitz and DeFazio, 1999). Thus we were able to distinguish EAPs that trigger somatic action potentials from somatic action potentials that may have occurred as a result of a depolarizing synchronized GABA response.

Layer I interneurons have long axons with multiple swellings representing presumed *en passant* synaptic contacts onto other layer I interneurons as well as deeper

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layer neurons (Zhou and Hablitz, 1996). A previous study has shown that 25% of paired recordings from late-spiking layer I interneurons demonstrated connections via chemical synapses (Chu et al., 2003). These observations suggest a powerful role for EAPs in interneurons. An EAP generated at a distal site on the axon and then traveling antidromically could be expected to depolarize more proximal release sites as the EAP travels along the axon. This depolarization could lead to GABA release onto postsynaptic neurons from all release sites that lie between the site of EAP initiation and the soma. Furthermore, back-propagation of action potentials into the dendrites (Stuart and Sakmann, 1994; Hausser et al., 1995; Stuart et al., 1997; Martina et al., 2000; Kaiser et al., 2001) would occur when an EAP arriving at the initial segment triggered a somatic action potential. Indeed, 4-AP enhances back-propagation in the distal dendrites of interneurons (Goldberg et al., 2003). In this way, an EAP generated in an axon would have excitatory effects throughout the interneuron's arborization.

There is evidence that layer I interneurons have dendrites that are electrically coupled (Gallereta and Hestrin, 1999; Chu et al., 2003), suggesting that back-propagating action potentials in one interneuron could cause a local depolarization in others. In addition, possible axo-axonic gap junctions between interneurons (Draguhn et al., 1998; Traub et al., 2001b) could allow the EAP to depolarize axons of connected interneurons to threshold, thus recruiting and synchronizing additional interneurons. While the role of gap junctions in GABA responses has not been specifically addressed in the neocortex, spontaneous synchronized GABA responses in the hippocampus are abolished by gap junction blockers (Yang and Michelson, 2001 ; Traub et al., 2001). Taken together, these data suggest that EAPs might be capable of synchronizing a population of interneurons

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and provide a mechanism for the coordinated activity necessary to sustain a propagating synchronized GABA response.

$GABA_B$ Receptors Limit the Number of EAPs

The cause and effect relationship between GABA release and EAPs has not been established because it has been difficult to isolate one phenomenon from the other. Both EAPs and the synchronized GABA response are abolished by either tetrodotoxin or $GABA_A$ antagonists (Aram et al., 1991; Stasheff et al., 1993b; Lamsa and Kaila, 1997; Avoli et al., 1998; Yang and Benardo, 2002). The GABA released during a propagating synchronized GABA response could be expected to activate both presynaptic and post-synaptic $GABA_B$ receptors. Our experiments with the $GABA_B$ antagonist SCH 50911 indicate that $GABA_B$ receptor activation normally shortens the duration of the synchronized GABA response, as seen from the increase in the half-widths of both regular and bursting-type synchronized GABA responses upon $GABA_B$ receptor inhibition. This is expected because $GABA_B$ autoreceptors located presynaptically are known to inhibit GABA release (Deisz and Prince, 1989; Davies and Collingridge, 1993; Deisz, 1999; Cobb et al., 1999). In addition to increasing the duration of synchronized GABA responses, the $GABA_B$ antagonist also increased the number of EAPs during the synchronized GABA response. If $GABA_B$ antagonism leads to extra GABA release, then our observations support a mechanism where EAP generation results from $GABA_A$ receptor activation, as has been suggested for the hippocampus (Avoli et al., 1998). This might result from GABA activating a depolarizing GABA current at hyperexcitable axon terminals.

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Role of EAPs in Chandelier Cells

Within layer II/III, the only neurons with EAPs that were positively identified were chandelier cells. Chandelier cells are a unique subtype of interneuron in the cortex and provide powerful inhibition through synapses on pyramidal cell initial segments (Freund et al., 1983; Douglas and Martin, 1990; DeFelipe, 1999). Epileptic foci show a loss of chandelier cell terminals and axons (Ribak, 1985; Freund and Buzsaki, 1988) suggesting that chandelier cells prevent initiation of epileptic activity in pyramidal cells. The existence of EAPs in chandelier cells might represent an important intrinsic feature of these cells. Although their specific inputs are not known, chandelier cells have dendrites which project up through layer I where they likely make synapses with layer I axons. Chandelier cells may work together with layer I cells to coordinate the synchronized GABA response. The presence of EAPs in chandelier cells, but not other layer II/III interneuron subtypes, indicates that neocortical EAPs may be limited to certain classes of interneurons.

Relationship Between EAPs, GABA Release and Excitation

The mechanism of EAP generation has been attributed to a 4-AP dependent increase in axon excitability (Kocsis et al., 1983). Extracellular potassium ion concentration ($[K^+]_o$) increases during the synchronized GABA response (Perreault and Avoli, 1992; Avoli et al, 1996; Louvel et al., 1994). This would excite axons and facilitate EAP generation through direct depolarization. Presynaptic $GABA_A$ receptors exist on hippocampal mossy fibers and regulate fiber excitability (Ruiz et al., 2003) and $GABA_A$ receptor activation in cortical neurons can be excitatory (Gulledge and Stuart, 2003). If $GABA_A$ receptors are present on layer I terminals or fibers, it presents a possible mecha-

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nism by which GABA can directly trigger EAPs. In the hippocampus, exogenous GABA can directly activate interneurons and lead to synchronous GABA release (Perkins, 2002). $[K^+]_o$ elevations can lead to intracellular chloride ($[Cl^-]_i$) accumulation through the KCC2 transporter (Payne, 1997; Rivera et al., 1999; DeFazio et al., 2000). Such a chloride accumulation would strengthen $GABA_A$ receptor induced depolarization and increase the likelihood of generating an EAP.

We propose that in the presence of 4-AP and EAA blockers a hyperexcitable, positive feedback-type state exists. 4-AP increases axon terminal excitability and also potentiates GABA release from presynaptic release sites. Stimulation or an intrinsic burst in an interneuron results in GABA release which activates $GABA_A$ receptors leading to a depolarization which contributes to further neuron excitability. Activity-dependent increases in $[K^+]_o$ depolarize the terminals directly and, together with possible presynaptic $GABA_A$ receptor activation, lead to the generation of EAPs. This leads to further GABA release as the EAP travels antidromically and stimulates GABA release from more proximal terminals. If EAPs propagate quickly along an axon, compared with the propagation of synchronized GABA response through the slice (Fig. 5), then EAPs may contribute to the advancing edge of the synchronized GABA response. This mechanism could set up a wave of activity that propagates to adjacent neurons through electrical connections as well as local $[K^+]_o$ increases which would affect other neurons in the vicinity. Imaging with voltage-sensitive dyes has shown that synchronized GABA responses evoked from lower layers are depolarizing and travel to the superficial layers before spreading laterally along the pial surface (Hablitz and DeFazio, 1999). The increased likelihood of EAPs in layer I interneurons might explain why the GABA response is more prominent in the upper cortical layers.

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Epileptic foci *in vivo* are associated with increases in $[K^+]_o$ (Moody et al., 1974). Seizure activity might be initiated when interneurons respond to this elevated $[K^+]_o$ by firing EAPs and leading to GABA release. On a background of elevated $[K+]_o$, $GABA_A$ currents are possibly excitatory. The interaction of EAPs and excitatory GABA release might then be responsible for synchronizing a network, and spreading abnormal activity far from the epileptic focus.

Possible Explanations for the Observed Distribution of EAPs

Pyramidal cells in the hippocampus display EAPs during synchronized GABA responses, suggesting that EAPs are not an interneuron specific phenomenon. It is not clear what accounts for the prevalence of EAPs in neocortical layer I interneurons. One explanation may lie in the existence of an activity-dependent potassium gradient. Holthoff and Witte (2000) have demonstrated that, upon intense electrical stimulation of layer VI, potassium levels in layer I are elevated as a result of a spatial potassium redistribution mechanism through a glial cell syncytium. The pattern of $[K^+]_o$ elevation seen matches the propagation of synchronized GABA responses measured using voltage-sensitive dye-imaging (Hablitz and DeFazio, 1999). During synchronized GABA responses in human tissue, $[K^+]_o$ elevations are positively correlated with field potential amplitudes and are greatest in the upper third (200-1200 μM) of the neocortex (Louvel et al., 2001), consistent with the idea that $[K^+]_o$ changes can preferentially occur in the upper layers. Redistribution of potassium to superficial layers would also contribute to $[Cl^-]_i$ accumulation via KCC2 and increase the probability that EAP-dependent GABA release would be excitatory.

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In summary, we have demonstrated that layer I interneurons are significantly more likely to have EAPs during synchronized GABA responses than layer II/III interneurons or pyramidal cells. The potential for EAPs to contribute to GABA release and interneuron excitability suggests that EAPs in layer I interneurons could play a crucial role in the synchronous activity necessary to mediate a propagating synchronized GABA response. These characteristics of layer I interneurons can account for previously observed laminar differences in 4-AP mediated synchronized GABA responses.

Acknowledgments

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SUBTYPE-SPEC GABA TRANSPORTER ANTAGONISTS SYNERGISTICALLY MODULATE PHASIC AND TONIC $GABA_A$ CONDUCTANCES IN RAT NEOCORTEX

by

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Abstract

GABAergic inhibition in the brain can be classified as either phasic or tonic. In many brain regions, GABA uptake via GABA transporters (GATs) can limit the time course of phasic currents arising from endogenous and exogenous GABA, as well as decrease a tonically active GABA current. GAT-1 and GAT-3 are the most heavily expressed of the four known GAT subtypes. The role of the various GAT subtypes in shaping GABA currents in the neocortex has not been explored. We obtained patch-clamp recordings from layer II/III pyramidal cells and layer I interneurons in rat sensorimotor cortex. Here we show that selective GAT-1 inhibition with NO711 decreased the amplitude of evoked inhibitory postsynaptic currents (IPSCs) and increased the decay time but had no effect on the tonic current. GAT-2/3 inhibition with SNAP-5114 had no effect on IPSCs or the tonic current. Coapplication of NO711 and SNAP-5114 markedly increased tonic currents and synergistically decreased IPSC amplitudes and increased IPSC decay times. The effects of the nonselective GAT antagonist nipecotic acid were similar to those of NO711 and SNAP-5114 together. We conclude that synaptic GABA levels in neocortical neurons are controlled primarily by GAT-1 but that GAT-1 and GAT-2/3 work together extrasynaptically to limit tonic currents. Inhibition of any one GAT subtype does not increase the tonic current, presumably as a result of increased activity of the remaining transporters. Thus, neocortical GAT-1 and GAT-2/3 have distinct but overlapping roles in modulating phasic and tonic GABA conductances.

Introduction

The inhibitory actions of GABA in the central nervous system can be divided into synaptic, phasic inhibition (i.e., inhibitory postsynaptic currents; IPSCs), and persistent,

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extrasynaptic tonic inhibition, seen as a tonically active conductance or current (Semyanov et al., 2004; Farrant and Nusser, 2005; Mody, 2005). Whereas phasic inhibition communicates information locally from a specific presynaptic neuron onto a postsynaptic neuron, tonic inhibition is involved in maintaining a general inhibitory tone (Brickley et al., 2001; Chadderton et al., 2004; Ulrich, 2003). GABA transporters (GATs) have been shown to modulate both tonic and phasic GABAergic signaling, particularly in the cerebellum and hippocampus (Nusser and Mody, 2002; Overstreet et al., 2000; Rossi et al., 2003; Semyanov et al., 2003). GATs can limit "spillover" of GABA from the synapse, promoting synapse independence (Isaacson et al., 1993; Overstreet and Westbrook, 2003; Rossi and Hamann, 1998). GAT antagonists are widely used as anticonvulsants (Adkins and Noble, 1998; Genton et al., 2001; Sills, 2003); brain GAT expression is altered in epilepsy, in brain malformations (Calcagnotto et al., 2002), after chemical injury (Zhu and Ong, 2004), and in patients with schizophrenia (Schleimer et al., 2004).

There are four identified GAT subtypes, classified in the rat as GAT-1, GAT-2, GAT-3, and BGT-1 (Borden, 1996). Initial studies indicated that GAT subtypes were differentially targeted to neurons and glial cells (Krogsgaard-Larsen et al., 1987), with GAT-1 restricted to neurons and GAT-3 restricted to glia. Recent studies show that the idea of the exclusive allocation of GAT-1 to neurons is simplistic, and GAT-1 has now been described on glial cells (Conti et al., 2004; Kinney and Spain, 2002; Ribak et al., 1996; Vitellaro-Zuccarello et al., 2003). In the mature neocortex, glial cell processes in all layers express (in decreasing abundance) GAT-3, GAT-1, and GAT-2 (Conti et al., 1998, 1999; Minelli et al., 1995, 1996, 2003) Neurons express (in decreasing amounts) GAT-1 and GAT-2 (Conti et al., 1999). However, neuronal GAT-1 is specifically and

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highly expressed at axon fibers and terminals (Chiu et al., 2002), whereas GAT-2 is not located at synapses (Conti et al, 1998; Minelli et al., 2003). In general, GAT-2 expression is low in the mature animals compared to the levels of GAT-1 and GAT-3 expression (Conti et al., 2004).

The first experiments investigating the role of GATs were conducted primarily with the GABA analog nipecotic acid, a nonselective GAT inhibitor (Schousboe et al., 1979). Nipecotic acid prolongs the decay of iontophoretically applied GABA and the late phase of evoked IPSCs (Dingledine and Korn, 1985; Hablitz and Lebeda, 1985). Inhibitors of GAT-1 such as tiagabine, SKF89976 and 1-(2-(((Diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO711) were the first widely available GAT subtype-specific antagonists. Differences were observed in the effects of the GAT-1-specific antagonists compared to those of nipecotic acid (Roepstorff and Lambert, 1992). The interpretation of these results was complicated because, as opposed to tiagabine and NO711, nipecotic acid is a GAT substrate. In addition, it is thought that nipecotic acid can result in heteroexchange for GABA via GATs (Solis and Nicoll, 1992), possibly confounding the conclusions of the role of GATs in regulating GABA concentrations.

GAT-1 antagonists increase the decay of evoked IPSCs (Engel et al., 1998; Rossi and Hamann, 1998; Overstreet and Westbrook, 2003; Roepstorff and Lambert, 1992; Thompson and Gähwiler, 1992) while generally having no effect on spontaneous IPSCs. GAT-1 inhibitors also increase a tonic conductance in both the cerebellum (Rossi et al., 2003) and the hippocampus (Nusser and Mody, 2002; Petrini et al., 2004; Semyanov et al., 2003). Evidence is emerging that non-GAT-1 transporters are also actively involved in GABA regulation. Tonic currents in cerebellar granule cells are increased by β-

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alanine, a transportable GAT-2/3 antagonist (Rossi et al., 2003), and β-alanine inhibits synaptically evoked transporter currents in neocortical glial cells (Kinney and Spain, 2002). (s)-(-)-1-[2-[tris-(4-methoxyphenyl)methoxy]ethyl]-3-piperidinecarboxylic acid (SNAP-5114) is a non-transportable antagonist with selectivity for GAT-2 and GAT-3 (Borden et al., 1994; Borden, 1996; Soudijn and van Wijngaarden, 2000). Few studies exist which have examined the effects of SNAP-5114 in the brain (Bolteus and Bordey, 2004; Dalby, 2000; Galvan et al., 2005).

In the present study, we used the whole-cell patch clamp technique to explore the role of specific and nonspecific GAT antagonists in modulating phasic and tonic currents in layer I interneurons and layer II/III pyramidal cells of the rat neocortex.

Experimental Procedures

Postnatal day 17 (P17) to P22 Sprague-Dawley rats were deeply anesthetized with ketamine (100 mg/kg) and decapitated. All experiments were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals by the use of a protocol approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. The brain was rapidly removed and submerged in ice-cold, oxygenated (95% O_2/5% CO_2) cutting solution containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 3 $MgCl_2$, and 1 $CaCl_2$. Coronal slices (300 μm) were cut from the right sensorimotor cortex with a Vibratome (Ted Pella, Inc., Riverside CA). Slices were stored in the cutting solution for 45-60 min at 37°C and then maintained at room temperature (22-24 C) until used for recordings.

Whole-cell voltage-clamp recordings were made from visually identified layer I interneurons and layer II/III pyramidal cells with a Zeiss Axioscop FS (Carl Zeiss Inc.,

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Thornwood, NY) microscope equipped with infrared differential contrast optics. Layer I interneurons were selected on the basis of their location in the cell-sparse area within 80 μm from the pia. Layer II/III pyramidal cells were identified by their pyramidal shape, the presence of a prominent apical dendrite, and their distance from the pia (200-300 μm). Roughly equal numbers of layer I and layer II/III pyramidal cells were used in this study. No significant differences were found between the two groups of cells; thus, the results were pooled.

Electrodes (KG-33 glass; Garner Glass, Claremont, CA) had resistances of 2-6 MΩ when filled with intracellular solution. Series resistance (Rs) and input resistance (R_{in}) was carefully monitored during each experiment with a 2-5 mV hyperpolarizing voltage step. Rs was estimated by measuring the peak of the transient current according to the formula $R_s = V_{step}/I_{transient}$. Experiments were excluded from analysis if Rs exceeded 20 MΩ or changed by > 25% during the experiment. Rs was not compensated.

The extracellular recording solution contained (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 0.01 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 0.02 D-aminophosphonovalerate (APV), and 0.01 SCH50911. Slices were placed in a 1 mL recording chamber, submerged in the recoding solution, and perfused via a peristaltic pump at a flow rate of 4 ml/min. The solution was heated to 32-35°C via an in-line heater (Warner Instruments, Hamden, CT), and the temperature was monitored by a thermistor placed in the recording chamber. All drugs were bath applied.

The intracellular pipette solution for most evoked IPSC and GABA pressure application ("puff") experiments contained (in mM): 125 KCl, 10 HEPES, 0.5 EGTA, 2 MgATP, and 0.2 NaGTP. The liquid junction potential was experimentally measured to

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be 5 mV. Intracellular solution for tonic current and reversal potential measurements contained (in mM): 120 Cs-$MeSO_4$, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and 5 lidocaine N-ethyl bromide (QX314). The liquid junction potential for this solution was 15 mV. The pH of the intracellular solutions was adjusted to 7.3 with either KOH or CsOH, and the osmolarity was adjusted to 295-300 mOsm with sucrose. Reported potentials have been corrected for the liquid junction potential. Some evoked IPSC and pressure application experiments were also performed by the use of the Cs-$MeSO_4$ internal solution. Our observations were not affected by the choice of internal solution used; thus, all data were pooled. The estimated chloride reversal potential for the KCl-based solution was 0 mV, and the experimentally measured reversal potential for the Cs-$MeSO_4$ solution was -30 mV. Neurons were voltage clamped at -65 mV (KCl solution) or +45 mV (Cs-$MeSO_4$ solution); thus, the chloride-mediated currents were inward (negative) with KCl and outward (positive) with Cs-$MeSO_4$. In the figures all currents are shown as outward currents, with the axis labeled "-pA" or "-nA" to designate the experiments recorded with KCl.

Voltage-clamp recordings were made with a Multiclamp 700A amplifier (Axon Instruments, Riverside, CA). Cells were held for at least 10 min after breakthrough before beginning data recording was begun. IPSCs were evoked by the use of a bipolar tungsten electrode placed on the surface of the slice within layer I, and 100 μm lateral to recorded neuron. Stimulus durations and intensities ranged from 80 to50 μs and 40 to 120 μA, respectively, and were adjusted to reliably yield the smallest possible amplitude IPSCs. Except where noted, IPSCs were evoked every 15 s and alternated in each individual neuron between a single stimulus and a "train" stimulation (three stimuli at 166 Hz).

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$GABA_A$ agonist puffs were controlled with a picospritzer (General Valve, Fairfield, NJ) and delivered via a patch pipette at intervals of 15 s. The pipette was placed 10-20 μm from the cell soma and contained either GABA (10 μM) or muscimol (3 μM) dissolved in (in mM): 125 NaCl, 3.5 KCl, 20 HEPES, and 10 glucose; the pH was adjusted to 7.3 with NaOH. Delivery pressure was fixed at 6 psi and the duration (8-25 ms) was adjusted to yield initial currents of 500-3000 pA. In the absence of drugs, GABA-evoked currents had stable amplitudes and kinetics for > 45 m (n = 3). Pressure application of the puff solution without GABA yielded no detectable current (n = 2; see also DeFazio et al., 2000). Increasing the duration of pressure application in the same neuron predictably increased the recorded GABA currents but had negligible effects on response kinetics over a wide range of test current amplitudes (500-3000 pA).

Data was filtered at 2-5 kHz (IPSCs) or 500 Hz (pressure application) and digitized at 10 kHz with Clampex 8 software (Axon Instruments). Data were analyzed with Clampfit 9 software (Axon Instruments). IPSCs were analyzed to measure peak amplitudes relative to baseline holding current, and decay times are listed as the time between 80% and 20% of the peak amplitude. Unless otherwise mentioned, the traces shown in the figures and reported values were obtained by averaging the last 3 min of data for both IPSCs (six total trials) and pressure application experiments (12 total trials) during the control, drug, and wash periods. Spontaneous IPSCs were detected and analyzed with the template searching method in Clampfit 9. The baseline holding current for tonic current measurements was determined from the average holding current of three 100 msec epochs free of spontaneous IPSCs. Tonic currents were calculated as the change in holding current after 3 min of treatment with 20 μM bicuculline. Reversal potential calculations

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were mathematically corrected for series resistance error and analyzed as described previously (DeFazio et al., 2000).

Origin 7.5 (Microcal, Northampton, MA) was used for graphing and additional analysis. Error bars represent the standard error of the mean. A Wilcoxon signed rank test for matched pairs or a Wilcoxon rank-sum test was used to calculate *P*-values. A *P*-value less than 0.05 was considered statistically significant.

Ionic salts were obtained from Fisher Chemicals (Fairlawn, NJ), and all other compounds were purchased from Sigma-Aldrich (St. Louis, MO) except for HEPES (Calbiochem, San Diego, CA) and CNQX, APV, and SCH50911 (Tocris Cookson, Inc, Ellisville, MO).

Results

To evaluate the possible role of GATs in modulating upper-layer neocortical $GABA_A$ currents, we pharmacologically isolated $GABA_A$Rs by recording in the presence of the excitatory amino acid (EAA) antagonists APV and CNQX and the $GABA_B$ antagonist SCH50911.

Effect of Nipecotic Acid on IPSCs and GABA-Induced Currents

Extracellular stimulation in layer I resulted in an evoked IPSC in the recorded neuron (Fig. 1*A*, *thick line*). Bath application of the nonselective GAT antagonist nipecotic acid (1 mM, *thin line*) caused a significant decrease in the IPSC amplitude and an increase in the decay time. Similar results were obtained in this neuron using a train of three stimuli at 166 Hz, a protocol designed to increase presynaptic GABA release compared to that produced with single-stimulus protocol (Fig. 1*B*). The *inset* shows the rising phase of

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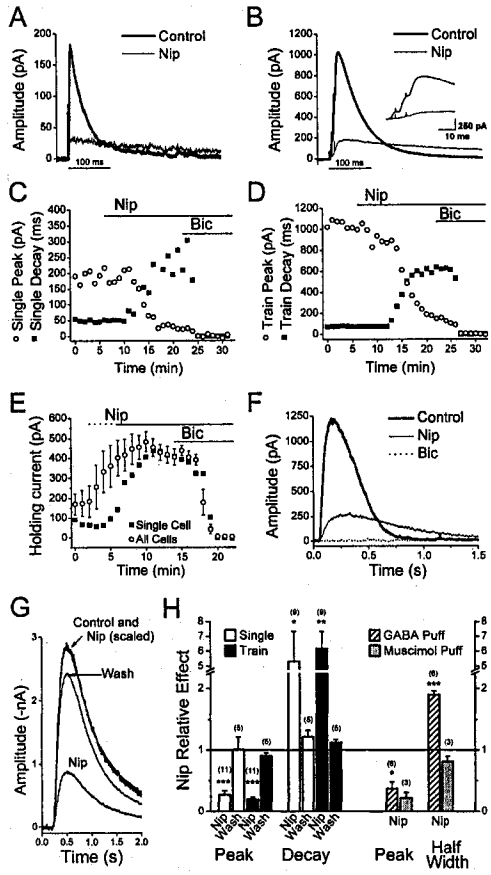

Figure 1. The effects of nipecotic acid on evoked IPSCs and GABA induced currents. *A*, Nipecotic acid (1 mM; *Nip*) dramatically reduced the amplitude and increased the 80% to 20% decay time of single-stimulus IPSCs. *B*, Train evoked IPSCs were similarly affected in the same neuron. IPSC *traces* in all figures represent the average of six trials. The *inset* in *B* shows the rising phase of the currents resulting from the three stimuli of the train protocol. *C*, Time course of the effects of nipecotic acid on the IPSC. Bicuculline (20 μM; *Bic*) abolished the responses. *D*, Train stimulation of the same cell from *C*, showing that nipecotic acid affects larger amplitude events similarly to the smaller single evoked IPSCs. *E*, Nipecotic acid treatment (8 min) activated a $GABA_A$ tonic conductance as seen by an increase in the holding current. Bicuculline completely reversed this effect. *Open circles* represent pooled results of cells treated with nipecotic acid for 8 to 12 min. The holding current was adjusted relative to bicuculline as the zero level. *F*, Nipecotic acid reduced the amplitude and increased the decay time of GABA puff (10 μM) currents. *G*, Muscimol (3 μM) evoked currents were reduced in amplitude by nipecotic acid whereas there was no change in the decay time. The control current and scaled nipecotic acid current are indistinguishable when superimposed. *H*, Summary of the effects of nipecotic acid. GABA- or Muscimol-evoked current *traces* represent the average of 12 trials. The *solid line* indicates no change from control. Numbers of experiments in parentheses and level of statistical significance are shown above individual *bars* ($*p < 0.05$; $**p < 0.01$, $***p < 0.001$ for this and all subsequent figures).

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the train IPSCs on an expanded time scale. Figsures 1*C* and 1*D* depict the time course of the effects of nipecotic acid on IPSC amplitude and decay time. It can be seen that the $GABA_A$ antagonist bicuculline (20 μM) completely abolished the IPSCs. In addition to its effects on IPSCs, nipecotic acid also induced large increases in the holding current (Fig. 1*E*). The change in the holding current upon application of bicuculline was used as the measure of the tonic current. The average tonic current in nipecotic acid-treated cells was 440 pA (SD 56; $n = 5$). A decrease in the Rin accompanied the increase in the holding current (data not shown). In addition, there was an increase in the standard deviation of baseline noise, as seen in the trial in the presence of nipecotic acid in Figure 1*A*. This is consistent with the activation of a tonic GABA current resulting from persistently elevated extracellular GABA levels caused by uptake inhibition.

One explanation for the reduction in IPSC amplitude caused by nipecotic acid is the possible inhibition of presynaptic GABA release. Although $GABA_B$ receptors were blocked with SCH50911, release may have been inhibited by hyperpolarization or shunting resulting from the activation of tonic inhibition. Thus, we examined the effects of nipecotic acid on currents elicited by GABA puffs, where the amount of GABA release can be kept constant (Fig. 1*F*). Bath application of nipecotic acid caused a reduction of GABA current amplitude and increased the decay time of the GABA-evoked currents. The magnitude of these effects was similar to those seen with the IPSCs, implying that the actions of nipecotic acid on IPSCs are primarily postsynaptic.

Both GABA and nipecotic acid are substrates for GATs and can compete for binding to GATs and subsequent transport. We used the $GABA_A$ agonist muscimol, which is not a substrate for GATs, to see how nipecotic acid affects an agonist which is not transported. Nipecotic acid reduced the amplitude of muscimol (3 μM)-evoked cur-

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rents (Fig. 1*G*). Nipecotic acid had no effect on the kinetics of the muscimol currents, as seen when the trace is scaled to the size of the control. The lack of an effect on the decay time of muscimol currents is the expected result for an agonist which has no transporter to remove it from the extracellular space. The decay time of the Muscimol-evoked current provides an approximation of the theoretical time course of GABA current activation in the absence of GABA uptake.

Figure 1*H* summarizes the results of our experiments with nipecotic acid on IPSCs and agonist puffs. Absolute measurements for this and other figures are listed in Table 1. It can be seen that single and train evoked IPSC amplitudes were significantly decreased by nipecotic acid, whereas decay times were increased. We found that these effects were reversible upon wash, which contrasts to the general observations of others (Dingledine and Korn, 1985; Roepstorff and Lambert, 1992). Both GABA and muscimol current amplitudes were decreased to a similar extent as the evoked IPSCs. This points to a postsynaptic mechanism of action to account for the decrease in amplitude.

Given that nipecotic acid induces a large tonic current, the uptake-dependent decrease in the amplitude of $GABA_A$ currents could be explained by a reduction in the chloride driving force as a result of persistent chloride flux through $GABA_A$Rs (DeFazio and Hablitz, 2001; Ling and Benardo, 1995), desensitization of $GABA_A$ receptors, or a reduced number of unbound $GABA_A$ receptors caused by elevated extracellular GABA levels. To test whether an elevation of the tonic current altered the driving force for chloride, we measured the reversal potential of GABA puffs by measuring the zero current level during a series of voltage steps before and after a 10 minute application of nipecotic acid. Using the Cs-$MeSO_4$ solution, the chloride equilibrium potential shifted from -30 mV (SD 12) to -5 mV (SD 10; $n = 4$; data not shown). On the basis of our holding poten-

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Table 1. Effects of GABA Uptake Inhibitors on Phasic GABA Currents

	Amplitude, pA		Decay Time†, ms		
Treatment	Control	Drug	Control	Drug	*n*
		Single IPSPs			
100 μM SNAP	140 (86)	141 (100)	29 (19)	28 (16)	17
20 μM NO711	349 (264)	284 (243)*	18 (13)	42 (72)*	23
10 μM NO711 + 40 μM SNAP	265 (197)	79 (85)**	30 (15)	187 (333)*	9
20 μM NO711 + 100 μM SNAP	140 (91)	28 (20)*	30 (12)	801 (643)*	7
1 mM Nipecotic Acid	159 (99)	38 (31)***	22 (12)	110 (136)*	11
		Train IPSPs			
100 μM SNAP	316 (144)	318 (252)	40 (11)	48(17)	17
20 μM NO711	573 (380)	414 (285)*	45 (24)	130 (82)*	23
10 μM NO711 + 40 μM SNAP	472 (290)	146 (115)**	46 (11)	550 (380)**	9
20 μM NO711 + 100 μM SNAP	263 (130)	101 (48)*	48 (9)	1200 (510)**	7
1 mM Nipecotic Acid	470 (290)	87 (52)***	43 (20)	286 (225)**	11
		GABA Puffs			
100 μM SNAP	638 (383)	715 (504)	241 (54)	257 (50)	5
20 μM NO711	838 (313)	1306 (605)**	285 (70)	465 (116)**	8
10 μM NO711 + 40 μM SNAP	1460 (970)	265 (238)*	288 (41)	797 (146)*	8
1 mM Nipecotic Acid	910 (600)	401 (440)*	380 (133)	714 (231)*	8
1 mM Nipecotic Acid (Muscimol)‡	1870 (1090)	434 (475)	677 (32)	554 (123)	3

Values represent the mean and (standard deviation). Statistical significance versus control is listed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; (Wilcoxon matched-pairs test). † 80%-20% decay time except for GABA puffs, which are reported as half-widths. ‡ Muscimol puffs instead of GABA puffs.

tial of +45 mV when using Cs-$MeSO_4$, this represents a decrease in the chloride driving force of 33% after treatment with nipecotic acid (i.e., $[V_m\text{-}E_{cl}]$ decreased from 75 mV to 50 mV). Although this decrease in driving force likely contributes to the decreased GABA currents, it cannot entirely account for the 60% to 80% reduction in our observed IPSC and GABA puff amplitudes. In addition, the effects of nipecotic acid on GABA current amplitudes were similar when recording with the KCl-based intracellular solution, where chloride flux would be less likely to cause elevations in intracellular chloride concentrations sufficient to significantly alter the chloride equilibrium potential. From these observations we conclude that $GABA_A$ receptor desensitization or receptor occu-

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pancy is a major cause of the nipecotic acid induced reduction of GABA-dependent current amplitudes.

Synergistic Effects of SNAP-5114 and NO711

The role that individual GAT subtypes play in modulating the effects of GABA in the neocortex has not been investigated. Using the GAT-1-specific antagonist NO711 and the GAT-2/3-selective antagonist SNAP-5114 we quantified the relative contribution of GAT-1 and GAT-2/3 to the effects seen with nipecotic acid. Figure 2*A* shows a plot of holding current as a function of time. The average holding current in the presence of bicuculline was set to zero for each condition. The change in the holding current after the application of bicuculline represents the tonically active $GABA_A$ conductance. The tonic currents were larger in the presence of nipecotic acid or a combination of SNAP-5114 and NO711 than those currents found in the control case or with either SNAP-5114 or NO711 alone. The tonic currents (as measured by the difference in holding currents between the *arrows*) for each condition are shown in Figure 2*B*. Neither 20-100 μM NO711 nor 100 μM SNAP-5114 increased the tonic current from control levels. However, lower doses of SNAP-5114 and NO711 (40 μM and 10 μM, respectively) significantly increased the tonic current when used together. At higher concentrations, the effect of coapplication of NO711 and SNAP-5114 matched the effect of nipecotic acid. These results show that both GAT-1 and GAT-2/3 are involved in regulating tonic GABA currents. The synergistic effects of SNAP-5114 and NO711 argue against non-specificity of either antagonist and suggest that GAT-1 and GAT-2/3 each function at less than maximal capacity under control conditions.

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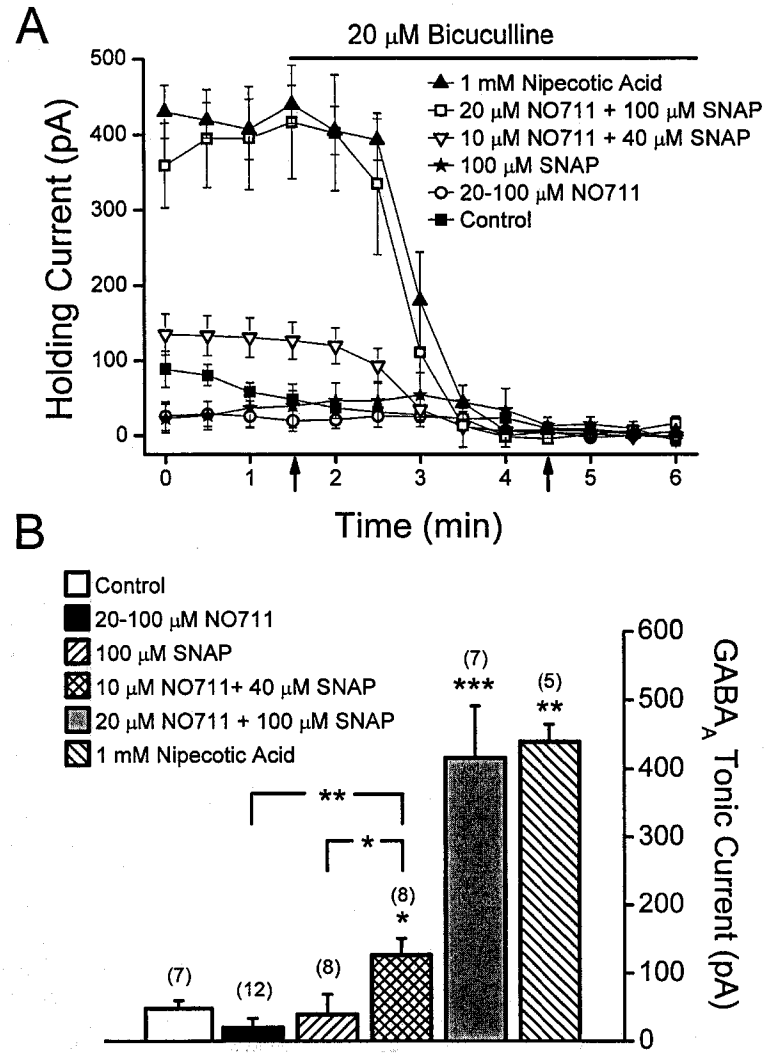

Figure 2. Inhibition of both GAT-1 and GAT-2/3 is necessary to induce a tonic current. *A*, Neurons were held for 10 minutes and then treated with the antagonists indicated for 8-10 minutes before the application of 20 μM bicuculline. The zero current level was set as the average holding current after complete wash-in of bicuculline. In the presence of SNAP or NO711, the change in the holding current upon the addition of bicuculline was similar to control. Treatment with NO711 plus SNAP or treatment with nipecotic acid alone led to bicuculline-induced changes that were greater than control. *B*, Summary of the effects with various GAT inhibitors. Neither NO711 nor SNAP changed the tonic current relative to control. Coapplication of SNAP and NO711 resulted in a statistically significant increase in the tonic current, when applied at a total concentration below that of either antagonist alone. Increasing the concentration of SNAP and NO711 increased the tonic current to the level seen with nipecotic acid. Tonic currents were calculated as the difference in the current at the time points indicated by the arrows in *A*.

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NO711 (20 μM) reduced the amplitude and increased the decay time of both single and train evoked IPSCs (Fig. 3*A*, *left* and *right* respectively). In contrast, SNAP-5114 (100 μM) had little effect on evoked IPSCs (Fig. 3*B*). Coapplication of NO711 and SNAP-5114 (10 μM and 40 μM) reduced the amplitude of evoked IPSCs and markedly increased the decay time (Fig. 3*C*). The scaled responses highlight that the initial decay closely matches that of the control IPSC but is followed by a long, slow second decay component. Higher concentrations of NO711 and SNAP-5114 (20 μM and 100 μM, respectively; see Fig. 4) increased the decay time of both single and train evoked IPSCs to as much as 4000 msec.

Roughly half of the experiments (43%, 7/16) involving coapplication of NO711 and SNAP-5114 resulted in IPSCs with two distinct components to the decay, as seen in Figure 3*C*. However, NO711 plus SNAP-5114 also resulted in IPSCs which lacked the initial fast decay (Fig. 3*D*). In this example, both nipecotic acid and the coapplication of NO711 and SNAP-5114 resulted in an IPSC with similar kinetics. Nipecotic acid treatment resulted in cells with one decay component (as seen in Figs. 1*A*, 1*B* and 2*D*) in 64% of experiments (7/11), and IPSCs with two clear decay components were observed in the other 36% (4/11) of experiments. Whether the fast component is observed in a given neuron may depend on the extent of desensitization at synaptic GABA receptors (see Discussion).

Despite its effects on evoked IPSCs, NO711 (20 μM) had no effect on the decays [9.4 msec (SD 1.6) versus 9.7 msec (SD 1.6)], amplitudes [55 pA (SD 18) versus 54 pA (SD 20)] or frequency [3.4 Hz (SD 1.7) versus 3.1 Hz (SD 1.9)] of spontaneous IPSCs (*n*

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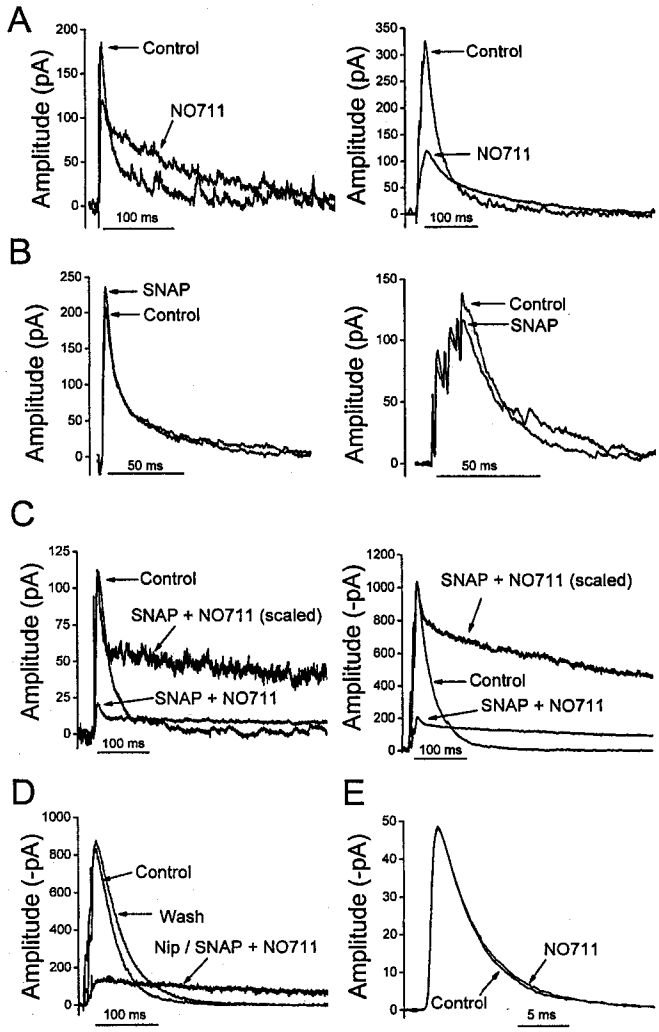

Figure 3. Effects of subtype-specific nontransportable GAT antagonists on IPSCs. *A*, The GAT-1 antagonist NO711 (20 μM) reduced the amplitude and prolonged the decay of single (left) and train (right) evoked IPSCs (same neuron). *B*, The GAT-2/3 antagonist SNAP (100 μM) had no effect on either single (left) or train (right) evoked IPSCs. The two examples in *B* are from different neurons. *C*, Coapplication of NO711 (10 μM) and SNAP (40 μM) profoundly reduced the amplitude and prolonged the decay of evoked IPSCs. In these examples taken from different neurons, scaled traces reveal two clear components to the decay. *D*, The effects of nipecotic acid were reversible, and SNAP and NO711 together mimicked the effects of nipecotic acid. In this example, a train IPSC was first treated with nipecotic acid, which decreased the amplitude and increased the decay time of the IPSC. This effect was reversed upon washout of nipecotic acid. Subsequent application of SNAP and NO711 produced results nearly identical to those produced by application of nipecotic acid. *E*, Despite its effects on evoked IPSCs, NO711 had no effect on spontaneous IPSCs. Each trace is the average of roughly 400 events from the same neuron.

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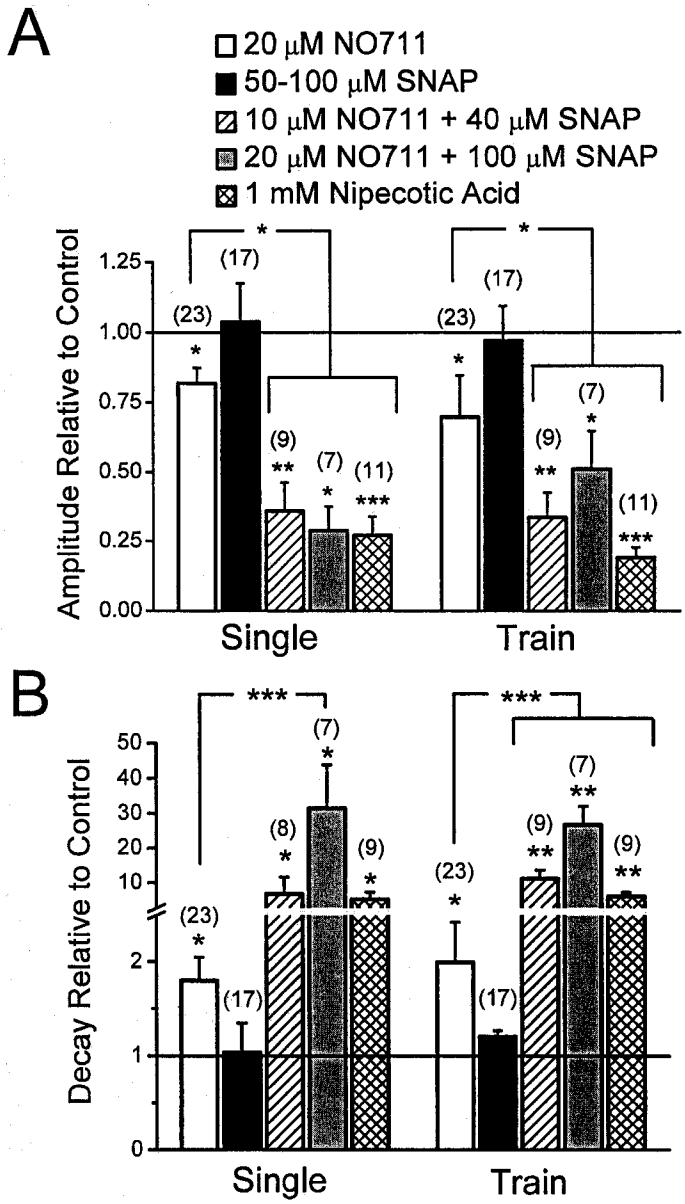

Figure 4. Summary of effects of GABA transporter antagonists on evoked IPSCs. *A*, NO711 decreased the amplitude of both single and evoked IPSCs, whereas SNAP had no effect. The decrease in IPSC amplitude upon cotreatment with SNAP and NO711 was statistically significant compared to both control and NO711 alone, and similar to results obtained with nipecotic acid. *B*, NO711 nearly doubled the decay time of single and evoked IPSCs. Cotreatment with high concentrations of N0711 and SNAP significantly prolonged the decay of single IPSCs compared to results of treatment with NO711 alone. Both cotreatment concentrations of NO711 and SNAP, and treatment concentrations of nipecotic acid significantly increased the decay of train IPSCs relative to the results produced by treatment with NO711 alone. These data demonstrate the synergistic effect on IPSC amplitudes and decay times when GAT-2/3 is inhibited in addition to GAT-1.

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= 7). Averaged spontaneous IPSCs before and after NO711 treatment are shown in Figure 3*E*. We did not observe any spontaneous events after treatment with nipecotic acid or coapplication of NO711 and SNAP-5114. This may have been caused by an inability to resolve spontaneous IPSCs, possibly because of a reduction in spontaneous IPSC amplitudes together with an increase in baseline noise resulting from an increase in the tonic current.

Our results with the effects of various combinations of GAT antagonists on IPSCs are summarized in Figure 4. SNAP-5114 had no effects on its own but worked synergistically with NO711 to mimic the effects of nipecotic acid. Unlike nipecotic acid, neither NO711 nor SNAP-5114 is a substrate for GATs, emphasizing that our observed effects on IPSCs are independent of the mechanism of transporter inhibition.

GAT Inhibition and GABA-Induced Currents

We next tested the effects of GAT subtype-specific antagonists on responses to GABA puffs. NO711 increased the amplitude and increased the half-width of GABA-induced currents (Fig. 5*A*). The effect on the amplitude was in contrast to the decrease seen with NO711 treatment on IPSCs. SNAP-5114 had little effect on GABA responses (Fig. 5*B*). These results show that the effects of the uptake antagonists on GABA-evoked currents generally mimic those observed with evoked IPSCs, with the exception of NO711 by itself. This observation implies that the $GABA_A$ receptors activated by GABA puffs are not the same as those activated by synaptic GABA release (see Discussion).

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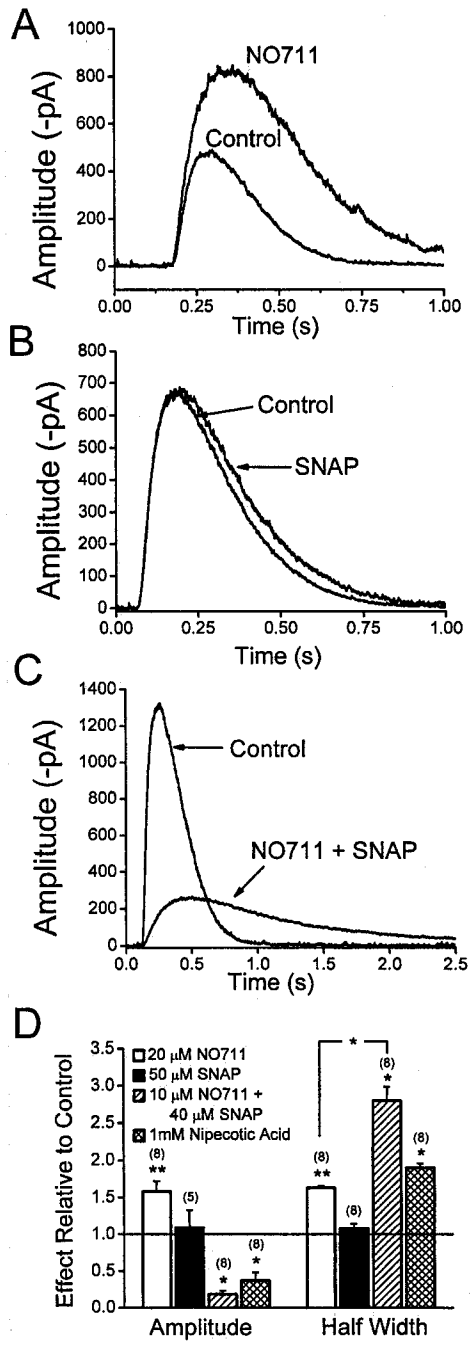

Figure 5. Effects of GAT antagonists on GABA puff currents. *A*, NO711 (20 μM) increased both the amplitude and decay time of 10 μM GABA puff currents. *B*, SNAP (50 μM) had little effect. *C*, Coapplication of NO711 (10 μM) and SNAP (40 μM) reduced the amplitude and prolonged the decay of GABA puff currents. *D*, Summary of the effects of GABA transporter antagonists on GABA puffs. The effects on GABA puff decays were consistent with the IPSC results. In contrast, the effects of NO711 on GABA puffs were opposite to those seen in IPSCs, where NO711 decreased peak amplitudes. Coapplication of NO711 (10 μM) and SNAP-5114 (40 μM) reduced the amplitude and increased the decay time (*C*). A summary of these results is shown in *D*.

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Discussion

Our findings indicate that neocortical interneurons and pyramidal cells exhibit a tonic $GABA_A$ conductance and that GAT-1 and GAT-2/3 act together in neocortex to modulate both tonic and phasic $GABA_A$ currents. We also found that the effects of the combination of GAT-1 and GAT-2/3 antagonists closely mimic the effects of nipecotic acid. This suggests that some previously reported effects of nipecotic acid which were attributed to nonspecific actions may instead be evidence of GAT-2/3 activity.

To investigate the possible GAT subtypes involved in modulating neocortical GABA currents, we used two selective GABA uptake inhibitors, NO711 and SNAP-5114. NO711 is a potent inhibitor of GAT-1, which has an IC50 of .38 μM and is three orders of magnitude more selective for GAT-1 than for other GAT subtypes (Borden et al., 1995). SNAP-5114 is moderately selective for GAT-3 over GAT-2, with an IC_{50} of 5 and 21 μM, respectively, and has an IC50 of 388 μM at GAT-1. The concentrations of SNAP-5114 used in our experiments (40-100 μM) would likely inhibit both GAT-3 and GAT-2. Despite evidence that GAT-2 is mainly localized to the leptomeninges and blood vessels (Durkin et al., 1995), there is evidence for some expression on neurons and glia (Conti et al., 1999). Our results suggest that functional GAT-2 and GAT-3 transporters are present on neurons and glia and regulate tonic and phasic $GABA_A$ conductances.

Both GAT-1 and GAT-2/3 Must be Inhibited to Increase Tonic Currents

$GABA_A$ antagonists block a tonically active current in hippocampus (Bai et al., 2001; Nusser and Mody, 2002; Semyanov et al., 2003), cerebellum (Brickley et al., 1996; Kaneda et al., 1995), and neocortex (Salin and Prince, 1996). In hippocampus and cerebellum, the tonic current is increased when GABA uptake via GAT-1 is inhibited

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(Nusser and Mody, 2002; Rossi et al., 2003; Semyanov et al., 2003). In contrast to the observations in other brain regions, our results indicate that GAT-1 antagonists alone do not increase the tonic current in neocortical pyramidal cells and interneurons. The tonic current was dramatically increased, however, when uptake was broadly inhibited with the non-selective GAT antagonist nipecotic acid or with coapplication of NO711 and SNAP-5114. This observation implies that the tonic current is limited by both GAT-1 and GAT-2/3. The effect on the tonic current of inhibiting one subtype alone is masked by uptake by the remaining subtypes. This suggests that under control conditions neither GAT-1 nor GAT-2/3 are working at full capacity. A similar conclusion was reached by Dalby (2000) on the basis of in vivo observations with selective and nonselective GAT antagonists in the thalamus. It was hypothesized that GAT-3 activity limits the effect of GAT-1 inhibition and that, thus, colocalized GAT-1 and GAT-3 act as "connected sinks" to limit the effect of selective blockade (Dalby, 2003).

The tonic current activated by nipecotic acid and by the coapplication of SNAP-5114 and NO711 persisted for > 10 min despite evidence that $GABA_A$ receptors can undergo both slow (Bianchi and Macdonald, 2002; Overstreet et al., 2000) and fast (Galarreta and Hestrin, 1997; Jones and Westbrook, 1995, 1996) desensitization. It may be that increases in GABA levels were insufficient to significantly desensitize $GABA_A$ receptors. Our tonic currents reached a peak at roughly 5 min with a slight decline over the next 2 min before stabilizing (Fig. 1*E*). This decline may represent a slow accumulation of intracellular chloride, as suggested by our measurements of a change in $GABA_A$ reversal potential during this time. A gradual onset of desensitization may also contribute to the observed decline in the tonic current from its peak.

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It is believed that the $GABA_A$ receptors mediating the tonic current are primarily located outside the synapse (Mody, 2001). Synaptic and extrasynaptic $GABA_A$ receptors vary in their subunit composition (Nusser et al., 1995; Brunig et al., 2002; Crestani et al., 2002; Wei et al., 2003; Farrant and Nusser, 2005). The general consensus indicates that synaptic receptors are composed predominantly of a γ2 subunit in association with α1, α2, or α3, whereas extrasynaptic receptors contain α4, α5, or α6, together with either δ or γ2 (Farrant and Nusser, 2005). Deletion of the α5 subunit reduces the tonic current in the dentate gyrus (Caraiscos et al., 2004), as does loss of δ and α4 subunits (Peng et al., 2004). The $GABA_A$ receptor subunits found extrasynaptically tend to desensitize less rapidly and have a higher affinity for GABA (Saxena and MacDonald, 1996; Tia et al., 1996; Bianchi and MacDonald, 2002; Yeung et al., 2003). Although there is yet no specific evidence in neocortex linking the tonic current to extrasynaptic receptors, it is reasonable on the basis of data from hippocampus and cerebellum to postulate that extrasynaptic $GABA_A$ receptors with distinct biophysical properties are primarily responsible for sensing the ambient GABA levels which give rise to the tonic current.

Decreased Uptake Can Desensitize Synaptic $GABA_A$ Receptors

Nonselective inhibition of GATs resulted in a decrease in the amplitude of evoked IPSCs. This decrease could be caused by either presynaptic or postsynaptic effects. Presynaptic $GABA_B$ receptors, which are known to decrease GABA release (Cobb et al., 1999; Davies and Collingridge, 1993; Deisz, 1999; Deisz and Prince, 1989), were blocked in our experiments. A decrease in synaptic release could still be expected because the increased tonic current can suppress excitability (Brickley et al., 2001; Chad-

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derton et al., 2004; Ulrich, 2003) or could activate putative presynaptic $GABA_A$ receptors (Ruiz et al., 2003; Kullmann et al., 2005). However, the amplitudes of GABA puffs were reduced to a similar extent as the evoked IPSCs. Because the output of GABA from the puffer pipette was kept constant, this reduction in amplitude likely represents postsynaptic effects. The reduction in both IPSC amplitudes and GABA puffs resulting from nipecotic acid or SNAP-5114/NO711 treatment was measured concomitantly with an increased tonic current. In this environment, postsynaptic receptors are possibly open or desensitized because of bound GABA as a result of elevated background GABA levels. There would thus be fewer unbound $GABA_A$ receptors available to be activated by synaptically released GABA, resulting in smaller evoked IPSCs.

Evoked IPSC Kinetics in the Presence of Uptake Antagonists Suggest Two Distinct Populations of $GABA_A$ Receptors

Both nipecotic acid and coapplication of SNAP-5114 and NO711 increased the decay time of evoked IPSCs. In some cases, IPSCs required several seconds to decay to baseline. This is ample time for GABA to diffuse many microns away from synaptic GABA receptors (Destexhe and Sejnowski, 1995; Clements, 1996; Wahl et al., 1996). The $GABA_A$ current observed during these long decays is therefore likely caused primarily by "spillover" activation of extrasynaptic receptors or activation of synaptic receptors at nearby synapses (Rossi and Hamann, 1998; Overstreet and Westbrook, 2003).

Half of our experiments with nonselective inhibition of GATs resulted in IPSCs with one decay component (Fig. 1*A* and 1*B,* and Fig. 3*D*) while the rest had two distinct decay components (Fig. 3*C*). Scaled responses show that, in IPSCs with two decay components, the initial decay was fast and closely matched the decay of the control IPSC, but

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that the second component was extremely slow. The slow components were not well represented by an exponential function (data not shown), and baseline noise prevented accurate measurement near the tails of the IPSCs. We thus used the time to fall from 80% to 20% of the peak as our decay measurement, which is largely a measure of the slow component.

The presence of two decay components would correlate well with the existence of two distinct populations of $GABA_A$ receptors distinguished by location, biophysical properties, or both. We propose that the fast component represents activation of unbound synaptic $GABA_A$ receptors, while the slow component reflects spillover out of the synapse induced by reduced uptake. If synaptic receptor availability is sufficiently reduced, perhaps by desensitization, then the fast component may disappear and give rise to the events with only a slow component, as seen in Figure 1*A* and 1*B,* and Fig. 3*D*. This also implies that extrasynaptic receptors are less sensitive to desensitization.

GAT-1 Limits Desensitization at Synapses

In neocortical neurons, GAT-1 is highly localized to axon terminals (Chiu et al., 2002; Conti et al., 2004; Pow et al., 2005). Selective inhibition of GAT-1 with NO711 resulted in an increase in the decay time and a decrease in the amplitude of evoked IPSCs. NO711 did not increase the tonic current, suggesting that the overall increase in extracellular GABA was small or otherwise limited largely to synapses. Evidence from the hippocampus also suggests that NO711-induced GABA elevations are primarily restricted to the synapse (Overstreet et al., 2000).

NO711 had no effect on either the amplitude or the decay of spontaneous IPSCs. It may be that the effects of GAT-1 inhibition manifest only when multiple fibers are

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stimulated, as is likely with evoked IPSCs, or when fibers have multiple closely spaced release sites, which may result in transmitter pooling (Scanziani, 2000; Arnth-Jensen et al., 2002). It has also been shown that unitary IPSCs are preferentially prolonged when synaptic density is high (Destexhe and Sejnowski, 1995; Overstreet and Westbrook, 2003). An alternate explanation for the lack of effect on spontaneous IPSCs may be that desensitization of postsynaptic receptors is promoted by activity-dependent release of GABA. If we assume that there are roughly 600 inhibitory synapses per neocortical neuron (Beaulieu et al., 1992) and that they are functionally equivalent, then a spontaneous IPSC frequency of 3 Hz would mean that release from any given synapse would occur only every several minutes. Even with synaptic uptake inhibited, this would likely be sufficient time for GABA to completely diffuse out of the synapse, thus limiting desensitization before the next spontaneous event. Evoking IPSCs every 15 s, as in our experiments, may have been sufficiently frequent to allow synaptic GABA levels to slowly build and desensitize $GABA_A$ receptors at those synapses, possibly via a slow desensitization (Overstreet et al., 2000). The effects of uptake inhibition were not dependent on the initial amplitude of evoked IPSCs, as GAT antagonists had similar effects on both single evoked IPSCs and the larger train evoked IPSCs.

In contrast to evoked IPSCs, NO711 increased the amplitude and half-width of responses to GABA puffs. It is unclear which populations of $GABA_A$ receptors are activated by the GABA puffs; thus, comparisons with evoked IPSCs can be difficult. It may be that puff application of GABA preferentially targets extrasynaptic receptors. In addition, the relatively slow kinetics of puff currents would likely mask the effects of desensitization as the reduced uptake allows GABA to spread over a larger area and activate larger numbers of receptors. When SNAP-5114 was applied together with NO711, the

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amplitude of GABA puffs was instead decreased, while the half-width was significantly increased (Fig. 5*D*), despite the reduction in amplitude. Because SNAP-5114 plus NO711 also induces a large tonic current, the reduction in GABA puff amplitude could be caused either by receptor desensitization or by the reduced availability of unbound receptors. The half-widths of GABA puffs in the presence of SNAP-5114 and NO711, as well as nipecotic acid, were similar to those seen with muscimol puffs under control conditions (Table 1). The facts that the binding affinity of GABA and muscimol is similar for many $GABA_A$Rs (Ebert et al., 1997), and that muscimol is not subject to any transport mechanisms suggest that GABA uptake was completely inhibited in the presence of those drugs.

The Effects of GAT-2/3 Are Primarily Extrasynaptic

The GAT-2/3 antagonist SNAP-5114 had no effect on evoked IPSCs. However, when coapplied with NO711, it acted synergistically to increase decays and reduce amplitudes. Our data are consistent with the picture derived from immunohistochemical evidence in the rat neocortex, where GAT-1 on neurons and glia are primarily concentrated at synapses and where GAT-2 and GAT-3 are expressed diffusely at extrasynaptic locations on glia, with possible extrasynaptic expression of GAT-2 on neurons (Fig. 6). Glial cells also express nonsynaptic GAT-1. Thus, GAT-1 inhibition elevates synaptic GABA levels, and GABA can then spill out of the synapse. GABA spread and extracellular $GABA_A$ activation, however, is limited by GAT-2/3 located nonsynaptically on neurons and glia. Inhibition of GAT-1 allows GABA to leave the synapse, but it is not until up-take via GAT-2/3 is also blocked that GABA levels rise enough to result in a measurable tonic current. Because GAT-2/3 inhibition has no effects on its own, GAT-1

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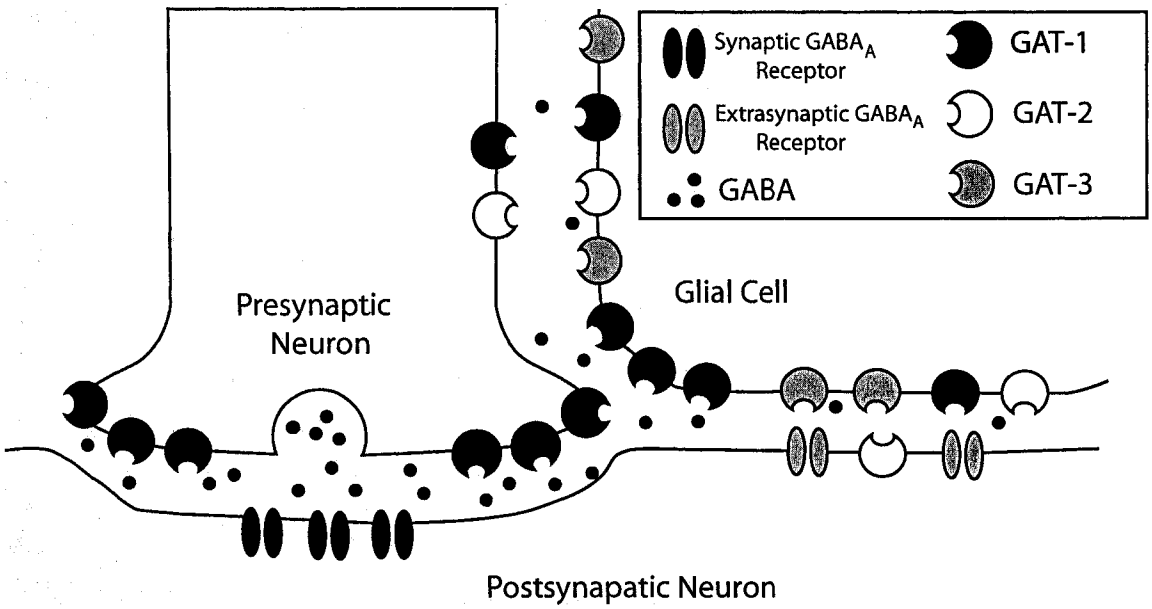

Figure 6. Summary illustration of possible location of GAT transporter subtypes and $GABA_A$ receptors. GAT-1 is localized to axon terminals on both neurons and glia, whereas glia express GAT-3 and some GAT-2 at nonsynaptic locations. Neurons also express extrasynaptic GAT-2. GABA released from presynaptic neurons is largely kept from leaving the synapse by GAT-1. GAT-1 inhibition allows synaptic GABA levels to increase, which leads to desensitization of synaptic $GABA_A$ receptors. GAT-1 inhibition also allows some GABA to escape the synapse, but activation of a tonic current is prevented by GAT-2/3. Inhibition of both GAT-1 and GAT-2/3 leads to prolonged synaptic currents and a large tonic current.

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activity seems sufficient to constrain GABA mostly within the synapse, while GAT-2/3 works together with GAT-1 to maintain low levels of extracellular GABA. Evoked GABA release results in both GAT-1 and GAT-2/3 currents in neocortical glial cells (Kinney and Spain, 2002), confirming immunohistochemical data and supporting our theory that GAT-2/3 is involved in extrasynaptic GABA regulation.

There is recent evidence that GAT4 (the mouse analog of the rat GAT-3) is potently inhibited by zinc (Cohen-Kfir et al., 2005), which is released together with glutamate at some glutamatergic terminals. Zinc has also been shown to increase extracellular GABA levels in the hippocampus (Takeda et al., 2004). This points to a role for GAT-3 in increasing GABA-mediated inhibition in response to increased excitatory activity. The extrasynaptic location of GAT-3 makes this transporter well suited for this task, but it remains to be seen whether this mechanism exists in the neocortex.

Nipecotic Acid as a Nonselective GAT Antagonist

GATs are highly influenced by substrate concentrations on both sides of the membrane (Wu et al., 2003) and are easily reversible (Richerson and Wu, 2003). Nipecotic acid is a substrate for GATs (Krogsgaard-Larsen et al., 1987) and can result in heteroexchange of GABA (Honmou et al., 1995; Szerb, 1982; Solis and Nicoll, 1992), in which one molecule of GABA is transported out of the cell as nipecotic acid is transported into the cell. In addition, nipecotic acid has been reported to act as a $GABA_A$ receptor agonist (Barrett-Jolley, 2001; Draguhn and Heinemann, 1996). Activation of a tonic current by nipecotic acid as seen by us and others (Dingledine and Korn, 1985; Draguhn and Heinemann, 1996; Hablitz and Lebeda, 1985; Wu et al., 2001) could be explained by both GABA heteroexchange and the $GABA_A$ agonist properties of nipe-

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cotic acid. Because GAT-1 was considered the predominant neuronal GABA transporter, differences in the results obtained with nipecotic acid and GAT-1-specific transporters have been attributed to these nonspecific effects of nipecotic acid (Roepstorff and Lambert, 1992). Nonspecific effects of SNAP-5114 and NO711 have not been reported, and their synergistic action in increasing the tonic current suggests a direct effect on GAT-mediated uptake. A contribution of heteroexchange or agonist properties of nipecotic acid to our results cannot be eliminated. However, the fact that the tonic current was equally enhanced by nipecotic acid and the combination of SNAP-5114 and NO711 indicates that it is primarily inhibition of GAT-2/3 by nipecotic acid which accounts for the differences in effect between nipecotic acid and selective GAT-1 antagonists. It is likely that GAT-2 or GAT-3 plays a larger role in previously studied systems than was originally thought.

GAT antagonists are known anticonvulsants. Seizure disorders such as epilepsy can originate and spread in specific areas of the brain (see Timofeev and Steriade, 2004). Because the GAT subtypes are differentially distributed in various brain regions (Chiu et al., 2002; Engel et al., 1998; Vitellaro-Zuccarello et al., 2003), the ability to inhibit specific GAT subtypes could become a powerful tool in treating epilepsy and other seizure disorders. Specifically, the best ratio of selective GAT antagonist dosages could be chosen for a given disorder phenotype and possibly maximize beneficial effects while minimizing unwanted side effects.

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SUMMARY AND CONCLUSIONS

GABA is responsible for two types of intracellular inhibitory signaling in the brain (Mody, 2001). Phasic inhibition is the result of direct activation of GABA receptors after vesicular release. This results in an IPSP which is generally brief, decaying to zero within 10s of milliseconds. Phasic inhibition is local and primarily involved in communicating information between two neurons. In contrast, the second type of signaling, tonic inhibition, is more likely to act over a distributed area when GABA acts on extracellular GABA receptors.

The experiments in this dissertation have described two mechanisms by which tonic and phasic GABA-dependent currents are controlled in the neocortex. First, the transporter KCC2 contributes to maintaining low intracellular chloride levels, which directly determines the potential strength of currents through $GABA_A$ receptors resulting from both phasic and tonic inhibition. Second, GATs are responsible for controlling GABA concentrations after release. On the basis of their location, the various GAT subtypes can modulate the time course and concentration of GABA differentially between the synapse and extracellular spaces.

We also described how GABA releasing interneurons are prone to firing EAPs in a 4-AP-induced model of hyperexcitability and postulated how this may result from GABA-mediated excitation. Although this hypothesis was not tested directly, our experiments with KCC2 imply that the known potassium elevations which occur in this model system should result in elevated intracellular chloride levels. These elevated chlo-

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ride levels would in turn directly lead to depolarizing $GABA_A$ currents. This depolarization may be sufficiently strong to trigger EAPs at axon terminals or other initiation sites.

The Clinical Relevance of the Developmental Switch of KCC2

Intracellular chloride concentrations are high in immature rat cortical neurons, which results in depolarizing $GABA_A$ currents (Rivera et al., 1999). The onset of expression of KCC2 between postnatal day 5 (P5) and P14 lowers intracellular chloride, resulting in the depolarizing to hyperpolarizing shift in $GABA_A$ activity (DeFazio et al., 2000; Khirug et al., 2005; Rivera et al., 1999). The importance of KCC2 expression is emphasized by the fact that mice die immediately after birth when KCC2 is genetically knocked out (Hubner et al., 2001). This result was surprising because of the postnatal onset of KCC2 expression in the cortex. Hubner et al. determined that spinal cord motoneurons undergo an embryonic rather than a postnatal KCC2-dependent developmental shift in chloride. This observation suggests that depolarizing $GABA_A$ currents is an important part of neuronal development.

Decreased KCC2 function is associated with hyperexcitability and can result from neuronal injury. Tornberg et al. (2005) developed a hypomorphic KCC2-deficient mouse which exhibited increased anxiety-like behavior and had increased susceptibility to pentylenetetrazole-induced seizures. Decreased KCC2 function is reported to result from anoxic and ischemic white matter injury (Malek et al., 2003), after axonal injury in dorsal motor neurons of the vagus (Nabekura et al., 2002), and in an "undercut" neocortical model of chronic injury (Jin et al., 2005). Decreased KCC2 expression or function as a result of injury would reduce the strength of GABA-mediated inhibition and contribute to hyperexcitability and epileptogenesis.

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Drugs which potentiate $GABA_A$ currents, such as phenobarbital, have been successfully used to treat seizures for nearly 100 years. Primarily because of its use in adults, phenobarbital use was begun for the treatment of neonatal seizures. Seizures in neonates are associated with significant mortality (Scher et al., 1993) and infant seizures are generally treated with phenobarbital or phenytoin as first-line agents despite the lack of firm evidence that the seizures themselves result in increased mortality. There are no studies which compare the outcomes of treatment with phenobarbital with those of treatment with placebo (Booth and Evans, 2004) because of the fact that such treatment has been the standard of care for decades. Over half of infants with seizures do not respond to phenobarbital or phenytoin therapy (Painter et al., 1999), and many drugs must be additionally employed as second-line agents. The relative ineffectiveness of these widely administered drugs, coupled with the fact that antiseizure medications can have significant side effects, clearly indicates that adequate seizure control in infants is lacking with currently available therapies.

The fact that developmental KCC2 expression is responsible for mediating a shift from depolarizing GABA currents to hyperpolarizing GABA currents in rodents leads to two obvious questions. Is this mechanism conserved in humans? If so, when does this switch occur? There are as yet no published studies addressing this issue. However, if we assume that this developmental switch occurs postnatally in humans, then it is understandable that GABA potentiating agents such as phenobarbital may actually not have their intended effect of increasing inhibition and suppressing seizures. There is little evidence that the seizures may themselves be harmful (Booth and Evans, 2004; Scher, 2003). In addition, many infant seizures spontaneously stop without intervention. Because treating infant seizures is currently the standard of care, it is considered unethical

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to conduct studies with a nontreatment group. Any evidence that drugs such as phenobarbital may not be effective or safe in infants might be sufficient to allow the initiation of clinical studies designed to test the effectiveness of GABA potentiators and alternate therapies.

Neocortical GABAergic Networks Can Support Propagating Depolarizing Responses

4-AP is a proconvulsant potassium channel antagonist that induces hyperexcitability in brain slices (Perreault and Avoli, 1989). The addition of excitatory amino acid receptor antagonists to 4-AP in the neocortex reveals GABA-mediated depolarizing responses (Aram et al., 1991; Benardo, 1997). These responses are concentrated in the upper layers of the neocortex (Yang and Benardo, 2002) and can propagate over a wide area (DeFazio and Hablitz, 2005). Upper layer interneurons have been shown to be synaptically and electrically connected (Chu et al., 2003; Galarreta and Hestrin, 1999). This demonstrates that neocortical interneurons are interconnected in a synchronizable network which is capable of supporting abnormal excitatory activity. GABAergic networks may therefore be responsible for contributing to seizure initiation or propagation.

EAPs are observed in many neurons during depolarizing GABA responses and are a key feature of the 4-AP slice model of hyperexcitability. In the hippocampus, both interneurons and pyramidal cells have EAPs (Perreault and Avoli, 1992; Stasheff et al., 1993; Traub et al., 2001a). In contrast, published studies of the neocortex illustrate EAPs only in interneurons, whereas pyramidal cells are not shown to have EAPs (Aram et al., 1991; Benardo, 1997); however, this lack of observation was not explicitly stated by the authors. EAPs are initiated in axons and propagate antidromically toward the

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soma, where they can back propagate into the dendrites and thus cause depolarization throughout a neuron's arborization. Thus, EAPs are candidates for contributing to the depolarization seen in this model as well as possibly encouraging synchronization and GABA release.

We had three main objectives in our study of depolarizing GABA responses. First, we wished to determine whether EAPs were more likely to be observed in certain classes of neurons. Second, we wanted to determine whether there were specific properties of upper layer neurons which would explain the observation that GABA responses are more prominent in the upper layers of the neocortex. Finally, we wished to elucidate the role of EAPs during GABA responses and establish a mechanism which may explain their generation.

Unequal Incidence of EAPs in Neocortical Cell Types

We recorded GABA responses from layer I interneurons and layer II/III interneurons and pyramidal cells and quantified the presence of EAPs in the various cell types. None of the pyramidal cells had evidence of EAPs, whereas EAPs were observed in the majority of layer I interneurons. This observation was in striking contrast to data from the hippocampus, where pyramidal cells usually fire EAPs during GABA responses. The presence of EAPs in layer I interneurons was consistent with previously published reports. EAPs were observed in only 25% of layer II/III interneurons. This suggests that, whereas only neocortical interneurons fire EAPs in this model, EAPs are not ubiquitous in interneurons. Two of the six layer II/III interneurons with EAPs were positively identified by biocytin labeling to be chandelier cells. Wheras identity of the other four cells was inconclusive, the presence of EAPs in layer I interneurons and chandelier cells

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implies that certain cell types are predisposed to firing EAPs. The propensity of layer I interneurons to fire EAPs, together with evidence that depolarizing GABA responses are concentrated in upper layers, strongly implicates layer I interneurons in supporting synchronous excitability.

The Possible Role of KCC2 in Depolarizing GABA Responses and the Distribution of EAPs

Enhancing GABA release with $GABA_B$ antagonists prolonged the GABA responses and increased the number of observed EAPs. This observation implicates GABA as a trigger of EAPs. While the precise mechanism of EAP generation is unknown, it is believed to result from depolarization of axon fibers or terminals (Kocsis et al., 1983). 4-AP blocks potassium channels, leading to terminal depolarization and hyperexcitability, and additional depolarization as a response to GABA via $GABA_A$ receptors may be the initiating event in the generation of EAPs. The link between 4-AP and depolarizing GABA responses is likely to be KCC2. Potassium is elevated during synchronized GABA responses (Perreault and Avoli, 1992; Louvel et al., 1994), and this elevation would be translated into increased intracellular chloride concentrations as potassium and chloride are cotransported via KCC2 into neurons.

Our data from the second article fail to answer the cause and effect question between EAPs and synchronized GABA responses but instead solidify their association. However, why are interneurons in layer I more likely to exhibit EAPs than interneurons in layer II/III are? One answer may be that there is some property of layer I interneuron terminals that predisposes them to firing EAPs. However, another answer may lie in their anatomical location. There is evidence which suggests that a glial cell syncytium

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redistributes elevations in potassium to layer I (Holthoff and Witte, 2000). It is possible that 4-AP-mediated increases in extracellular potassium are unequally distributed throughout the cortical layers and are instead concentrated in the upper layers. Thus, layer I interneurons may experience higher potassium concentrations than lower layers do. This would contribute to increased excitability of axon terminals in layer I and to increased intracellular chloride concentrations, which would then contribute to more strongly depolarizing chloride currents.

The hippocampus has a relatively low seizure threshold when compared to the neocortex (Burnham, 2002; Luciano, 1993), especially in immature animals (Adbelmalik et al., 2005). In contrast, seizure onset in aged animals often begins in the neocortex (Weissinger et al, 2005). Therefore, although temporal lobe epilepsies are most common, the neocortex may be important for the spread and maintenance of seizure activity that is initiated at a distant focus (Koch et al., 2005), especially since it is well established that potassium elevations accompany such activity. If indeed EAPs are the result of increased potassium levels and if EAPs contribute to interneuron synchronization, then layer I interneurons, together with layer II/III chandelier cells, might be the primary neurons responsible for neocortical seizure activity.

GAT Regulation of GABA

The regulation of GABA concentrations in both the synapse and extracellular space by GATs is well established (Farrant and Nusser, 2005; Mody, 2005; Semyanov et al., 2004). While all four GATs have been cloned for more than 10 years (Borden, 1996), only specific antagonists to GAT-1 have been widely available. Insight into the function of the various GAT subtypes has been based on the assumption that GATs are

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differentially distributed to neurons and glia. GAT-1 was thought to be located only on neurons, so GAT-1 antagonists such as tiagabine and NO711 were employed as the drugs of choice to examine the role of uptake on neuronal currents. However, evidence now indicates that the three main GAT subtypes involved in GABA regulation, GAT-1, GAT-2, and GAT-3, are all found on both neurons and glia (Conti et al., 2004). The commercial availability of a new GAT-2 and GAT-3 semiselective antagonist, SNAP-5114, provides a useful tool in helping to segregate the actions of GAT-1 from those of GAT-2 and GAT-3 (Bolteus and Bordey, 2004; Dalby 2000; Galvan et al., 2005).

While the role of GATs has been widely studied in areas such as the hippocampus, few studies exist which examine the function of GATs in the neocortex. The experiments reported in the third article explored the role of GATs in modulating tonic and phasic currents in the neocortex. We used a combination of selective and nonselective GAT antagonists to demonstrated that GAT-1 and GAT-2/3 have distinct yet overlapping roles in modulating neocortical GABA currents and that the effects of selective antagonists are synergistic rather than additive.

Effects of Selective GAT Inhibition

We utilized the whole-cell patch-clamp technique to record evoked IPSCs in layer I interneurons and layer II/III pyramidal cells. We found that the GAT-1-selective antagonist NO711 increased the decay time of evoked IPSCs and decreased their amplitude. This is consistent with electrophysiological data obtained from the hippocampus and with data which show extensive GAT-1 expression at neocortical synapses (Chiu et al., 2002; Conti et al., 1998, 1999, 2004). The effects on the decay suggest a prolongation of synaptic or perisynaptic GABA levels, possibly resulting in spillover (Overstreet

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and Westbrook, 2003), whereas the decrease in amplitude has been attributed to post-synaptic receptor desensitization (Overstreet et al., 2000). We did not observe an increase in the tonic current upon application of NO711, and this was in contrast to results in both the hippocampus and cerebellum.

There is only one published study which has looked at non-GAT-1 activity in the neocortex. With the use of β-alanine, a competitive antagonist and substrate for both GAT-2 and GAT-3, Kinney and Spain (2002) found that evoked transmitter release resulted in roughly equal GAT-1 and GAT-2/3 currents in neocortical glial cells. Because evoked release leads to measurable GAT-2/3 currents, it would follow that inhibition of GAT-2/3 would result in changes to extracellular GABA concentrations and thus changes in IPSC kinetics. Despite the results of Kinney and Spain, we did not observe changes in IPSCs upon application of the GAT-2/3 antagonist SNAP-5114; neither did we see any increase in the tonic current.

Inhibition of Both GAT-1 and GAT-2/3 Result in Synergistic Effects

We studied the effects of nonselective GAT inhibition with the use of two approaches. We used the classical nonselective competitive GAT inhibitor nipecotic acid, as well as a combination of NO711 and SNAP-5114. The effects on the tonic current and on IPSCs with each approach were nearly identical. First, we observed a large increase in the tonic current. Second, IPSC amplitudes were markedly decreased, and their decays were dramatically prolonged.

On the basis of the results with the tonic current, we concluded that both GAT-1 and GAT-2/3 actively transport GABA out of the extracellular space to limit the tonic current. However, each has extra capacity, such that inhibition of any one subtype does

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not result in a measurable increase in the tonic current. When both GAT-1 and GAT-2/3 are blocked, there no longer exists a mechanism to transport GABA back into neurons and glia, and this results in a large tonic current. If extrasynaptic GABA levels are elevated, one would expect the rate of diffusion out of the synapse to be limited. GABA may even diffuse into the synapse, depending on the relative synaptic and extrasynaptic concentrations of GABA. The increase in the tonic current was observed together with the decrease in IPSC amplitude. Nonselective GAT inhibition reduced the amplitude of IPSCs significantly more than inhibition of GAT-1 alone did. We believe that the GAT antagonist-mediated decrease in IPSC amplitude is the result of GABA receptor desensitization. The fact that IPSC amplitudes were further decreased when GAT-2/3 was inhibited in addition to GAT-1 suggests that the GABA elevations which give rise to the tonic current are not limited to extrasynaptic spaces.

GAT-2/3 Antagonists as Possible Therapeutic Drugs

Our results from the study involving the use of selective antagonists and reported in the third article are consistent with the current understanding that GAT-1 is the primary neuronal GAT subtype. If one were to look solely at the results with SNAP-5114, one might conclude that GAT-2/3 plays a negligible role in the neocortex. However, SNAP-5114 significantly potentiated the effects of GAT-1 inhibition. This result is possible only if GAT-2/3 is continually working to transport GABA. The lack of effect of SNAP-5114 alone may imply that GAT-2/3 works mainly as a backup system for GAT-1 or that the primary role of GAT-2/3 is to shuttle GABA into cells for metabolic purposes rather than to be a regulator of neuronal signaling. On the other hand, it may be that GAT-2/3 does indeed control inhibitory signaling in a manner not brought out by our

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experimental protocol or conditions. What is clear, however, is that there is functioning GAT-2/3 in the neocortex and that these transporters deserve further study. Of particular interest is the possible development of therapeutic drugs based on the idea that GAT-2/3 can be inhibited to increase a tonic current, at least in conjunction with GAT-1 antagonists. GAT-1 inhibitors such as tiagabine are currently used as drugs in the treatment of seizure disorders (Sills, 2003). Our data show that GAT-1 inhibition has a primarily synaptic mechanism of action. Combining GAT-2/3 and GAT-1 inhibitors may make it possible to more selectively augment tonic inhibition in the brain, thus increasing the threshold of seizure generation by overexcitation.

The expression, location, and function of the various GAT subtypes vary among brain regions (Conti et al., 2004). This is partly evidenced by the fact that, contrary to our results in the neocortex, selective GAT-1 antagonists increase the tonic current in the hippocampus (Nusser and Mody, 2002). This emphasizes the need for a variety of approaches to treat seizure disorders with methods that may exploit the regional differences and diverse etiologies of seizure disorders (Timofeev and Steriade, 2004). It may be possible to titrate the levels and ratio of GAT-1 and GAT-2/3 antagonists to best treat a specific disease phenotype in a particular individual.

A Reevaluation of Nipecotic Acid

Nipecotic acid has been used as a tool to study the role of GABA uptake for > 30 years (Schousboe et al., 1979). After the discovery of GAT-1 and GAT-1 selective inhibitors (Borden, 1996), a widely cited study (Roepstorff and Lambert, 1992) comparing nipecotic acid and tiagabine reported significant differences in the effects of the two drugs. It was concluded that nipecotic acid must have nonspecific effects and it was advised that nipecotic acid no longer be used to study GABA uptake. One such

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vised that nipecotic acid no longer be used to study GABA uptake. One such nonspecific effect is heteroexchange of GABA (Honmou et al., 1995; Szerb, 1982; Solis and Nicoll, 1992). Nipecotic acid is a substrate for GATs, and it is thought that as one molecule of nipecotic acid is transported into the cell, GABA is transported out of the cell. In addition to blocking reuptake of GABA, the possible transporter-mediated release of GABA via heteroexchange could lead to the large tonic currents caused by nipecotic acid (Dingledine and Korn, 1985; Draguhn and Heinemann, 1996; Hablitz and Lebeda, 1985; Wu et al. 2001). In our experiments, however, the effects of nipecotic acid were identical to those obtained when highly potent and specific GAT-1 and GAT-2/3 antagonists were used in tandem. This argues against nonspecific actions of nipecotic acid accounting for our observation of a large increase in the tonic current and the drastic changes on IPSCs. Instead, the previously reported results obtained with nipecotic acid are best explained by the nonselectivity of nipecotic acid. GAT-2 and GAT-3 are now known to be expressed in the hippocampus where these effects were observed. Thus, differences between nipecotic acid and GAT-1 antagonists can be accounted for by the presence of GAT-2/3 in these systems. The most likely explanation for the origination of the concept of heteroexchange is simply that GATs are reversible (Richerson and Wu, 2003). Nipecotic acid is a substrate for the transporter, and application of nipecotic acid would be expected to result in significant levels of nipecotic acid within neurons and glia. Eventually, the inward rate of the transporter would slow as the outward rate increased, and intracellular GABA could be transported out of the cell and into the extracellular space. This interpretation is supported by a recent study which compared the inward and outward flux of GABA through GAT-1 in the presence of nipecotic acid, NO711, and other GAT antagonists (Sitte et al., 2002).

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Tight regulation of the effects of GABA is critical for information processing and for the prevention of overexcitation which might lead to seizure. The studies contained in this dissertation have explored the control of neocortical GABAergic signaling via KCC2, GATs, and the ability of certain neurons to fire EAPs in a model of hyperexcitability. It is hoped that this information will contribute to a body of knowledge which leads to continued progress in the care and treatment of individuals who are affected with seizure disorders.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE APPROVAL FORMS

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: February 2, 2005

TO: John J. Hablitz, Ph.D.
CIRC-510 0021
FAX: 975-5097

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Neocortical Epilepsy During Development
Sponsor: NIH
Animal Project Number: 050104241

On January 27, 2005, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	B	48

Animal use is scheduled for review one year from January 2005. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 050104241 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: February 3, 2003

TO: John J. Hablitz, Ph.D.
CIRC-510 0021
FAX: 975-5097

FROM: Clinton J. Grubbs, Ph.D., Chairman
Institutional Animal Care and Use Committee

SUBJECT: Neocortical Epilepsy During Development (NIH) 030104241

On January 29, 2003, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	B	48

Animal use is scheduled for review one year from January 29, 2003. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Sotirios T. Keros

Graduate Program Neurobiology

Title of Dissertation Inhibitory Mechanisms in Rat Neocortex

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
John J. Hablitz	**, Chair**	
Michael J. Friedlander		
Lori L. McMahon-Wakefield		
David S. Weiss		
Michael J. Wyss		

Director of Graduate Program

Dean, UAB Graduate School

Date

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