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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

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ABSTRACT

EXPRESSION OF AXONAL $GABA_A$ RECEPTORS IN RAT DORSAL ROOT DURING DEVELOPMENT. Sean P. Roddy, Kaoru Sakatani, and Jeffery D. Kocsis. Department of Neurology and Section of Neurobiology, Yale University, School of Medicine, New Haven, CT, and VA Medical Center, West Haven, CT.

During the course of development, rat optic nerve fibers are depolarized by γ-aminobutyric acid (GABA); the depolarizing actions of GABA are very pronounced in the neonate, but are virtually absent in the adult. In contrast to these central nervous system myelinated axons, rat dorsal root axons respond with a large depolarization upon application of GABA even in the adult. We examined the GABA-induced depolarization in developing rat lumbar dorsal roots using a modified sucrose-gap technique. The dorsal roots displayed distinct GABA-induced depolarizations from birth through adulthood. Specifically, the depolarization was 2.51 ± 0.14 mV at one week, 1.16 ± 0.09 mV at two weeks, and 5.60 ± 0.40 mV at four weeks of age. However, the potential change observed in the four week-old was no different from that of a ten week-old adult rat. Comparing the depolarizations in the three time periods, the time-to-peak value in the one week-old was shorter than the others and was accompanied by a plateau level less than that at peak. Application of GABA to ventral spinal roots had no effect confirming that it is the primary afferent sensory fibers and not the motor fibers which are GABA-sensitive. Stimulation for release of endogenous GABA and blockade of GABA uptake by nipecotic acid (NPA) produced no depolarization in dorsal root. This

is in contrast to the optic nerve in which NPA elicits a distinct depolarization. These results demonstrate that unlike the optic nerve whose responses to GABA attenuate during the first few weeks and which displays endogenous release of GABA, peripheral dorsal roots exhibit GABA-sensitivity that peaks in the mature adult and dorsal root excitability during development is not influenced by GABA released from Schwann cells. The transient decrease in the amplitude of GABA-induced depolarization between one and two weeks of age correlates with myelination and with growth of fibrous connective tissue. The increase in magnitude of depolarization between two and four weeks of age may be due to increase in $GABA_A$ receptor density, a change in the electrochemical gradient, or a change in the subunit structure of the $GABA_A$ receptor.

ACKNOWLEDGEMENT

I would like to thank my advisor, Dr. Jeffery D. Kocsis. If it weren't for his criticism, enthusiasm, encouragement and support, the completion of this project would not have been possible. I would also like to acknowledge Robert Bhisitkul who, while working toward his PhD, taught me the fundamental techniques of dissection and the sucrose gap. Additionally, I would like to thank Barry Birch, a fellow medical student, who brought both humor and music to the lab on those long research days. Finally, I would like to indicate that funding for this project was supported in part by the Medical Research Service of the Department of Veteran Affairs, the National Multiple Sclerosis Society, and the National Institute of Health.

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INTRODUCTION

Neurotransmitter release and subsequent receptor activation have been traditionally localized to the synapse, and receptors have been observed solely on dendrites and cell bodies. However, there has been evidence for the presence of asynaptic $GABA_A$ receptors on both myelinated and unmyelinated axons (Brown & Marsh, 1978, Morris *et al.*, 1983, Bhisitkul *et al.*, 1987). In fact, $GABA_A$ receptor density along the length of the axon is often high when compared with endogenous stores of the neurotransmitter.

In the central nervous system, $GABA_A$ receptors have been shown to be transiently present on rat optic nerve axons during development and postulated to play a role in the terminal arborization patterning along the optic tract (Sakatani *et al.*, 1992). These receptors appear to affect axonal excitability through a shunting of excitatory current and thus can exert a pre-synaptic inhibitory effect. The specific physiologic role for axonal $GABA_A$ receptors is currently unclear.

GABA Pharmacology and Metabolism

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian central nervous system (Krnjević, 1974, Snyder, 1984). GABA exerts this inhibition by interacting with the two known GABA receptor subtypes, $GABA_A$ and $GABA_B$ (Simmonds, 1983, Bowery *et al.*, 1984, Bormann, 1988, Stephenson, 1988). The $GABA_A$ receptor is an integral membrane protein intimately associated with a chloride ionophore; whereas the pharmacologically distinct $GABA_B$ receptor regulates

calcium and/or potassium channel activity by modulation of adenylate cyclase through interaction with a GTP binding protein (Bormann, 1988). Unlike the monoamines which are present in nanomole/gram levels in the central nervous system, GABA is present in μmole/gram concentration levels (Tallman & Gallager, 1985). GABA does not cross the blood brain barrier in any significant amount.

GABA is synthesized in the neuron by the α-decarboxylation of L-glutamic acid, a major excitatory neurotransmitter (Cooper *et al.*, 1986). This reaction requires the enzyme glutamic acid decarboxylase (GAD) and the cofactor pyridoxal-5'-phosphate. Optimum reaction rates occur at pH 6.5. The reverse reaction has not been demonstrable either *in vivo* or *in vitro* and GAD is present only in retinal tissue and the central nervous system. GABA-transaminase (GABA-T) is the enzyme responsible for catalyzing the breakdown of exogenous GABA to succinyl semialdehyde; it requires pyridoxal-5'-phosphate as a cofactor and functions optimally at pH 8.2. Cell fractionization techniques and electron microscopy have shown that GABA-T is found in association with mitochondria and GAD is found in association with the synapse. GAD has a lower affinity for pyridoxal-5'-phosphate than does GABA-T so a reduction in the levels of this cofactor leads to epileptiform seizure activity in animal models.

GABA Receptor Subtypes

The $GABA_A$ receptor is a hetero-oligomeric protein believed to be a member of a gene superfamily (along with the nicotinic acetylcholine and the strychnine-sensitive glycine receptor) of ligand-gated ion channels who demonstrate significant DNA sequence

homology (Barnard *et al.*, 1987, Schofield *et al.*, 1987, Levitan *et al.*, 1988, Pritchett *et al.*, 1989). This receptor has been implicated in both pre-synaptic and post-synaptic inhibition. The $GABA_A$ channel mediates chloride transport across the membrane in response to agonist binding with receptor activation occurring on a millisecond time scale. $GABA_A$ receptors are widely distributed throughout the brain, but are most highly concentrated in the granule cell layer of the cerebellum and the frontal cortex of the cerebrum (Bowery *et al.*, 1987).

In addition to GABA, the $GABA_A$ agonist binding site recognizes muscimol (5-aminomethyl-3-hydroxyisoxazole), 3-aminopropanesulfonic acid (3-APS), δ-aminovaleric acid (DAVA), isoguvacine and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) (Morris *et al.*, 1983, Bormann, 1988) and is competitively antagonized by bicuculline, a plant alkaloid, (Davidoff, 1972, De Groat, 1972, Barolet *et al.*, 1985). The convulsant drugs picrotoxin and t-butyl-bicyclophosphorothionate (TBPS) are non-competitive antagonists that act at a site proposed to be on the chloride ionophore (Smart & Constanti, 1986). There are binding sites for chloride ions on the $GABA_A$ receptor near or within the pore which appear to regulate the flow of anions through the channel (Bormann *et al.*, 1987). Single channel recording studies indicate that two anion-binding sites are present on the $GABA_A$ receptor complex.

The $GABA_A$ response can be potentiated by the binding of several other classes of drugs to specific sites distinct from the agonist binding site. Binding at the potentiating sites does not in itself cause opening of the chloride ionophore and both the potentiating site and the agonist binding site must be occupied concurrently for receptor

activation. However, a significantly smaller concentration of agonist is required for a given response when the potentiating site is occupied. Whereas barbiturates increase the chloride conductance by an increase in the mean open time of the chloride channel, benzodiazepines, when bound to the receptor in conjunction with a $GABA_A$ agonist, increase the current through the $GABA_A$ receptor channels by increasing the frequency at which the ionophore opens (Study & Barker, 1981, Ong & Kerr, 1984, Stephenson, 1988). Two other classes of compounds have been shown to interact with the $GABA_A$ receptor to produce a potentiating effect. These are the antihelminthic avermectins (Pong & Wang, 1982), and steroids including progesterone derivatives (Lambert *et al.*, 1987) and the steroid anesthetic alfaxalone (Harrison & Simmonds, 1984).

Unlike the $GABA_A$ receptor, the $GABA_B$ receptor is distinct from the ion channel upon which it exerts an effect (Wojcik & Neff, 1984, Hill, 1985). It functions in inhibition both pre-synaptically and post-synaptically by the use of a second messenger system, specifically a pertussis toxin-sensitive GTP-binding protein, to cause an increase in outward potassium conductance and/or a decrease in inward calcium conductance (Inoue *et al.*, 1985, Karbon & Enna, 1985, Holz *et al.*, 1986). In the brain, $GABA_A$ receptors outnumber $GABA_B$ receptors except in areas such as the molecular layer of the cerebellum, superior colliculus, globus pallidus, lateral amygdaloid nucleus, and the pontine nucleus where $GABA_B$ receptors predominate (Wilkin *et al.*, 1981, Bowery *et al.*, 1983). In the spinal cord, $GABA_A$ receptors are located in the dorsal and ventral laminae I-X in contrast to $GABA_B$ receptors which are concentrated in laminae I-IV. $GABA_B$ receptors are bicuculline-insensitive, are activated stereospecifically by (-)-

baclofen (4-amino-3-[4-chlorophenyl]-butanoic acid), have no known specific antagonists, and are not potentiated by benzodiazepines or barbiturates (Bormann, 1988).

$GABA_A$-Mediated Inhibition

The direction of ion flow through a chloride-selective membrane pore depends on the electrochemical gradient for chloride which is determined by both the resting membrane potential and the chloride concentration gradient (Hille, 1984). There is an uneven distribution of chloride across the cell membrane with concentrations of extracellular chloride being 10-20 times higher than intracellular concentrations. The cytoplasm has a negative potential relative to the extracellular space. The concentration gradient drives the anion intracellularly while the negative charge drives the anion extracellularly. Activation of the $GABA_A$ channel in response to agonist binding causes an increase in chloride conductance and if membranes were dependent only on chloride, the resulting current would change the cell membrane potential, bringing it toward a new equilibrium state known as the equilibrium potential for chloride or E_{Cl}. The E_{Cl} can also be thought of as that membrane potential, in a membrane permeable only to chloride ions, at which there is no net movement of chloride ions. The E_{Cl} can be determined by using the Nernst equation:

$$E_{Cl} = \frac{RT}{F} \ln \frac{[Cl^-]_i}{[Cl^-]_o}$$

R is the Gas constant (8.314 $JK^{-1}mol^{-1}$)
T is the temperature in Kelvin
F is the Faraday constant (9.648 x 10^4 $Cmol^{-1}$)

$[Cl^-]_i$ is the intracellular chloride concentration
$[Cl^-]_o$ is the extracellular chloride concentration.

The real membrane potential (E_m), however, depends on all ion conductances (g_{ion}) in the cell. This can be illustrated by combining the Nernst and Goldman equations to give the following equation:

$$E_m = \frac{g_{Na}E_{Na}+g_KE_K+g_{Cl}E_{Cl}+g_{HCO_3}E_{HCO_3}+\cdots}{g_{Na}+g_K+g_{Cl}+g_{HCO_3}+\cdots}$$

When $GABA_A$ channels open, the relative conductance of chloride is so large in comparison to all other conductances that the membrane potential becomes that of E_{Cl}. If E_{Cl} alone was used to determine a new equilibrium state, this would assume that GABA induced no other ion transport. There is, however, evidence for HCO_3^- conductance through the $GABA_A$ channel (Sakatani *et al.*, 1992). It is therefore more appropriate to use E_{GABA} than E_{Cl}.

Generally speaking, there is a hyperpolarization in cortical neurons but a depolarization in sympathetic and dorsal root ganglia, primary afferent terminals (Simmonds, 1983) and optic nerve (Sakatani *et al.*, 1992) in response to GABA. For example, studies in primary afferent frog and rat cell bodies have determined E_{GABA} to be -35mV (Levy, 1977). In this case, since E_{GABA} is less negative than the resting membrane potential, there must be an outwardly directed chloride conductance and a depolarization of the primary afferent terminal in response to agonist binding.

Axonal $GABA_A$ receptors appear to exert their inhibitory effect pre-synaptically. That is, activation of these receptors decreases axonal excitability and can decrease the

probability of propagation of an excitatory stimulus across to the subsequent synapse. GABA can inhibit depolarization to threshold (a critical membrane potential less negative than that at rest at which a cell generates an action potential) by two mechanisms. The first occurs generally in cortical neurons where E_{GABA} is more negative than rest. Activation of the $GABA_A$ channels results in hyperpolarization and the membrane potential is brought further away from threshold reducing the probability of generating an action potential in the event of a depolarizing stimulus. The second occurs regardless of change in membrane potential. $GABA_A$ channels stabilize the cell membrane and shunt current in response to excitatory stimuli through an appropriate increase in chloride conductance. Shunting is an extremely effective means of inhibition even when E_{GABA} is between the baseline membrane potential and threshold for a given neuron. The cell becomes essentially clamped at the E_{GABA} while $GABA_A$ channels are activated (White, 1990). Whenever an excitatory potential is applied to the cell, the chloride conductance dissipates the depolarizing current and an action potential is not generated.

$GABA_A$ Receptor Desensitization

As stated above, agonist binding to the $GABA_A$ receptor results in an increased chloride conductance, but this conductance will fade in a biphasic manner even if the concentration of the agonist is maintained (White, 1990). This decrease is called desensitization and can be interpreted as a change in the receptor from an active to an inactive state. At GABA concentrations greater than 10 μM, currents in adrenal chromaffin cells through $GABA_A$ channels decreased following initial peak response

(Bormann *et al.*, 1985). The time course of desensitization was described by the sum of two exponentials indicating that $GABA_A$ receptors have both a fast and a slow desensitization as evidenced by the biphasic fading response. The first decrease is the "fast" desensitization and the decrease after the initial change in rate is the "slow" desensitization (White, 1990). This change in current is dependent upon agonist concentration, as shown by the fact that higher concentrations produce more rapid desensitizations. Recovery from the desensitized state was described approximately as a single exponential. In the presence of agonist, a $GABA_A$ channel in a dorsal root ganglion neuron will open and produce an inward current that immediately decreases at a given rate. After a given period of time, the rate then shifts abruptly to a more gradual rate. A concentration of at least 10 μM GABA is necessary for any desensitization at all. It has been proposed that rapid desensitization is due to a cAMP-dependent protein phosphorylation and slow desensitization is due to some other structural change upon dephosphorylation, but this remains speculative (Tehrani *et al.*, 1989).

Axonal $GABA_A$ Receptors

$GABA_A$ receptors have been identified on the axons of dorsal but not ventral roots indicating selective expression on sensory nerve fibers (Bhisitkul *et al.*, 1987). This raises questions as to why they are present and what their function is. Three possibilities have been proposed. The first assumes that receptors in the axonal membrane outside the spinal cord and at points distant from the axon terminal are not intended to be physiologically active. Instead, these proteins, which are integrated into the plasma

membrane in the cell body, are merely being transported to their intended site of function at a central synapse. The next theory addresses the possibility of synaptic drift. That is, axonal $GABA_A$ receptors are activated by distally diffused neurotransmitter released at the synapse. This would produce a further decrease in excitability along the axon. This theory seems unlikely due to the extreme distance between the site of release of GABA in the spinal cord and the known location of the peripheral axonal $GABA_A$ receptor adjacent to the dorsal root ganglion. The last theory assumes that non-neuronal cells such as the Schwann cell release GABA in an attempt to modulate transmission along the sensory fibers. This may take place either during normal adult life as a secondary modification of sensory input or during early postnatal development as a means of effecting the terminal arborization patterns of neurons by influencing neurite formation in growth cones.

$GABA_A$ Receptor Expression During Development

$GABA_A$ receptors have been shown to have an age-dependent pattern of expression in rat optic nerve. In a study by Sakatani *et al.* (1992), the depolarization in the membrane potential in a ten day postnatal (P-10) rat after application of 1 mM GABA averaged 5.7 mV, and this response was much attenuated at P-14. The absence of a response after two weeks of age indicates a progressive developmental decrease in $GABA_A$ receptor expression in the central optic nerve fibers.

Application of nipecotic acid (NPA) has been shown not only to block GABA uptake mechanisms (Johnston *et al.*, 1976), but also to stimulate the release of GABA

into the extracellular space (Brown *et al.*, 1980). Through the use of NPA, Sakatani *et al.* (1992), demonstrated that endogenous stores of GABA can depolarize optic nerve axons during the first two weeks of life. NPA, when applied in 1 mM concentrations to developing optic nerve, induced up to a 4.0 mV depolarization in the first week of life but this effect was not present after the second week.

Purpose

GABA has been shown to play a transient role in the developing central nervous system of rats, as demonstrated by its effects on the optic nerve. This $GABA_A$ receptor-induced effect can be reproduced through blockade of GABA uptake mechanisms by superfusion with NPA. This may be due to some role that GABA plays in neuronal calcium levels and neurite extension in the optic nerve during development. The adult rat optic nerve, on the other hand, does not appear to express the $GABA_A$ receptor whereas the adult dorsal root in the peripheral nervous system does. The question remains as to whether there is a such a change in the presence of the $GABA_A$ receptor in the early postnatal dorsal root. This study attempts to assess if there is any difference in $GABA_A$ receptor expression in dorsal root during development. Also, to assess the possibility of endogenous GABA release as a means of altering axonal excitability and thus effecting the growth cone, NPA was administered at various times during development.

METHODS

Dissection and Solutions

Wistar rats (seven days to ten weeks old) were deeply anesthetized with sodium pentobarbitol (60 mg/kg) by either intraperitoneal or subcutaneous injection and exsanguinated by carotid section. The spinal cord was exposed by laminectomy and the dura mater was incised. The fourth and fifth lumbar spinal roots were excised without ganglia. The roots were placed in a modified Krebs' solution at room temperature (20-24°C), saturated with a gas mixture of 95% O_2 and 5% CO_2 and allowed to equilibrate in the dissecting solution for 30-40 minutes.

The modified Krebs' solution was composed of (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.3 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, and pH 7.4. In addition, solutions containing γ-aminobutyric acid (GABA ; 1 mM), muscimol (1 mM), baclofen (100 μM), bicuculline (100 μM), and nipecotic acid (NPA; 1 mM) were made by adding the appropriate amount of the drug to the Krebs' solution. Isotonic sucrose solution was composed of 320 mM sucrose and isotonic-KCl solution was composed of (in mM): 3 NaCl, 124 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.3 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, and pH 7.4.

Sucrose Gap Recordings

A modified sucrose gap technique was used to measure both membrane potentials and compound action potentials (Kocsis & Waxman, 1983). Use of this technique requires placement of two extracellular electrodes in opposing compartments. Spinal

roots were placed across three superfusion compartments of a plexiglass chamber with the proximal end of the root relative to the spinal cord placed in the test compartment. The roots were sealed into place at the two junctions between compartments with petroleum jelly (Figure 1). All chambers were perfused individually at a constant flow rate of 1-2 milliliters per minute. In the test compartment, the Krebs' solution produces a physiologic medium in the test compartment to which drugs could be added. The isotonic sucrose solution in the middle compartment acts as a high resistance medium forcing all currents generated in the test chamber to flow through the cytoplasm of the axons in the spinal root toward the reference chamber. The reference chamber was perfused with isotonic-KCl solution for the purpose of depolarizing the neurons, thereby allowing an extracellular electrode placed in the bath to act as if intracellular. The reference chamber electrode and the test chamber electrode together measured the relative potential difference between bath and cytoplasm.

A variable current was delivered to the spinal root in the test compartment through a bipolar Teflon-coated stainless steel electrode. The current was generated in a stimulus isolator unit (Model 850A; W-P Instruments, Inc.) that was connected to an interval generator (Model 830; W-P Instruments, Inc.) and a pulse module (Model 831; W-P Instruments, Inc.). Compound action potentials and DC membrane potentials were recorded across the two outer compartments through Ag-AgCl electrodes embedded in agar (dissolved in 3 M KCl), and connected to a high input impedance DC-coupled differential amplifier (Axoprobe 1A; Axon Instruments). Output was sent to a VCR storage device (Neuro-Corder Model DR-886; Neuro Data Instruments Corp.), an

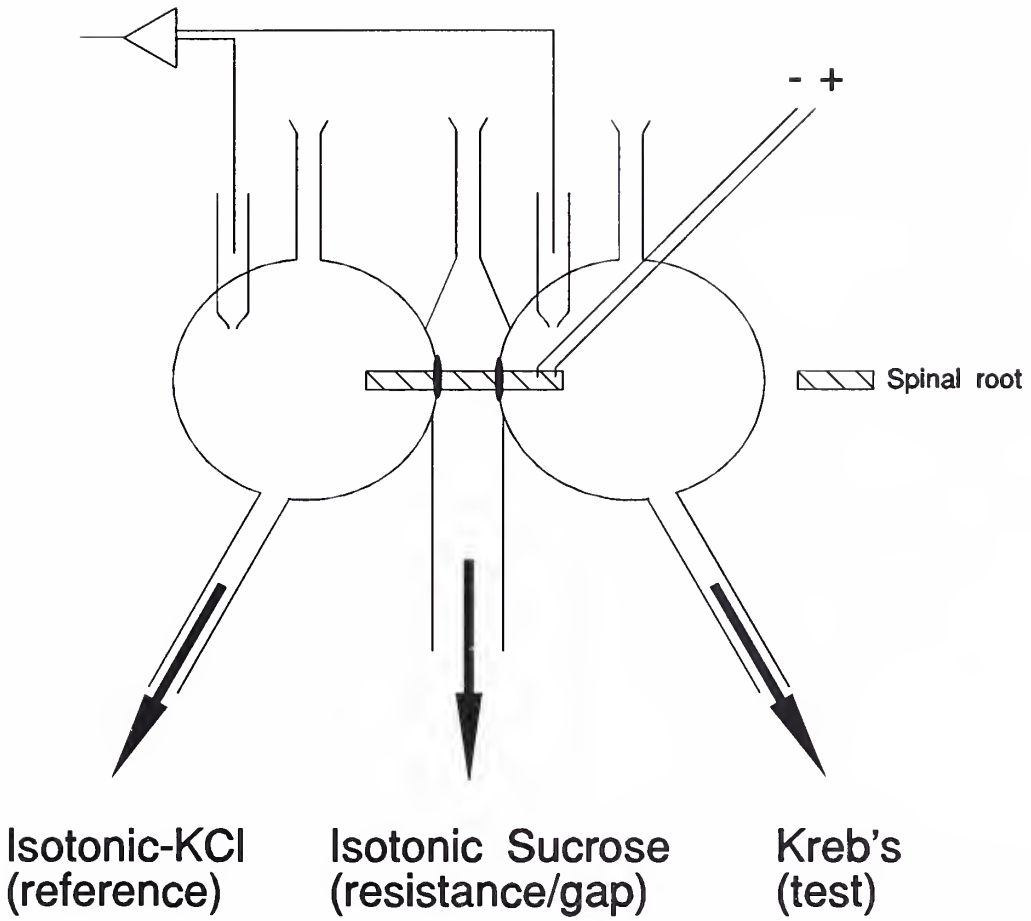

Figure 1: Schematic diagram depicting a dorsal root sealed across the three compartments of the modified sucrose gap chamber with the placement of a bipolar stimulating electrode in the test compartment.

oscilloscope (Model 5440; Tektronix, Inc.), and a chart recorder (Model R4-160VS; MFE Corp.) as real-time tracings with a millivolt scale as the ordinate. The magnitude of the initial compound action potentials was used as a screening method to ensure the use of healthy neurons. Roots whose compound action potentials were greater than twenty millivolts were used.

Statistical Analysis

Statistical significance of the difference in mean values (with standard errors) of the depolarizations was determined using analysis of variance (ANOVA). Significance was noted when the p value was less than 0.05. The designation of one week includes data from P-7 and P-8 rats, two weeks from P-13, P-14, and P-15 rats, and four weeks from P-27 and P-28 rats. Depolarizations were expressed as mean $\pm$ standard error.

Project Responsibility

The author (Sean P. Roddy) performed this project under the supervision of Jeffery D. Kocsis, Ph.D. (Professor of Neurology), at the Neuroscience Research Center of the West Haven VA Medical Center in a laboratory that was evaluating $GABA_A$ receptor plasticity in development and regeneration. Within this broad context, a smaller project was designed and carried out by the author. Dissections, solution preparation, and data analysis were performed by the author. Also, the sucrose gap apparatus including the gap chamber, electrodes, oscilloscope, amplifier, and chart recorder were assembled and maintained throughout the process of data collection by the author.

RESULTS

The effect of GABA and related drugs on reactivity of rat spinal roots was studied during the course of maturation. GABA both decreased the absolute amplitude of the compound action potential (CAP) and produced an age-dependent depolarization in lumbar dorsal roots. Compound action potentials were generated by stimulation of the spinal root axons in the test chamber of the sucrose gap with a bipolar electrode and recorded across the two outer compartments. Recordings in dorsal spinal roots demonstrated a characteristic rise, peak amplitude, and exponential decay as described by Bhisitkul *et al.*, (1987). The recordings had amplitudes of 20-30 mV in the first two weeks of life but increased to 40-50 mV at four weeks of age. Half-widths (width of an action potential at a point halfway between baseline and peak) of these CAP's averaged 1.0-1.2 milliseconds at one week of age and 0.8-1.0 milliseconds at two and four weeks of age.

The decrease in the magnitude of the dorsal root CAP occurred immediately after introduction of GABA to the test compartment. Notice the depolarizing shift in baseline of the GABA-applied tracing in Figure 2A. Since the peak of the CAP did not shift, the reduction in absolute amplitude must have resulted from the membrane depolarization. After application of GABA, half-widths were somewhat increased and decay was slightly prolonged. The difference in membrane potential between a control and GABA-perfused dorsal root was maintained after generation of a CAP.

The GABA-induced depolarizations of dorsal roots were statistically different in

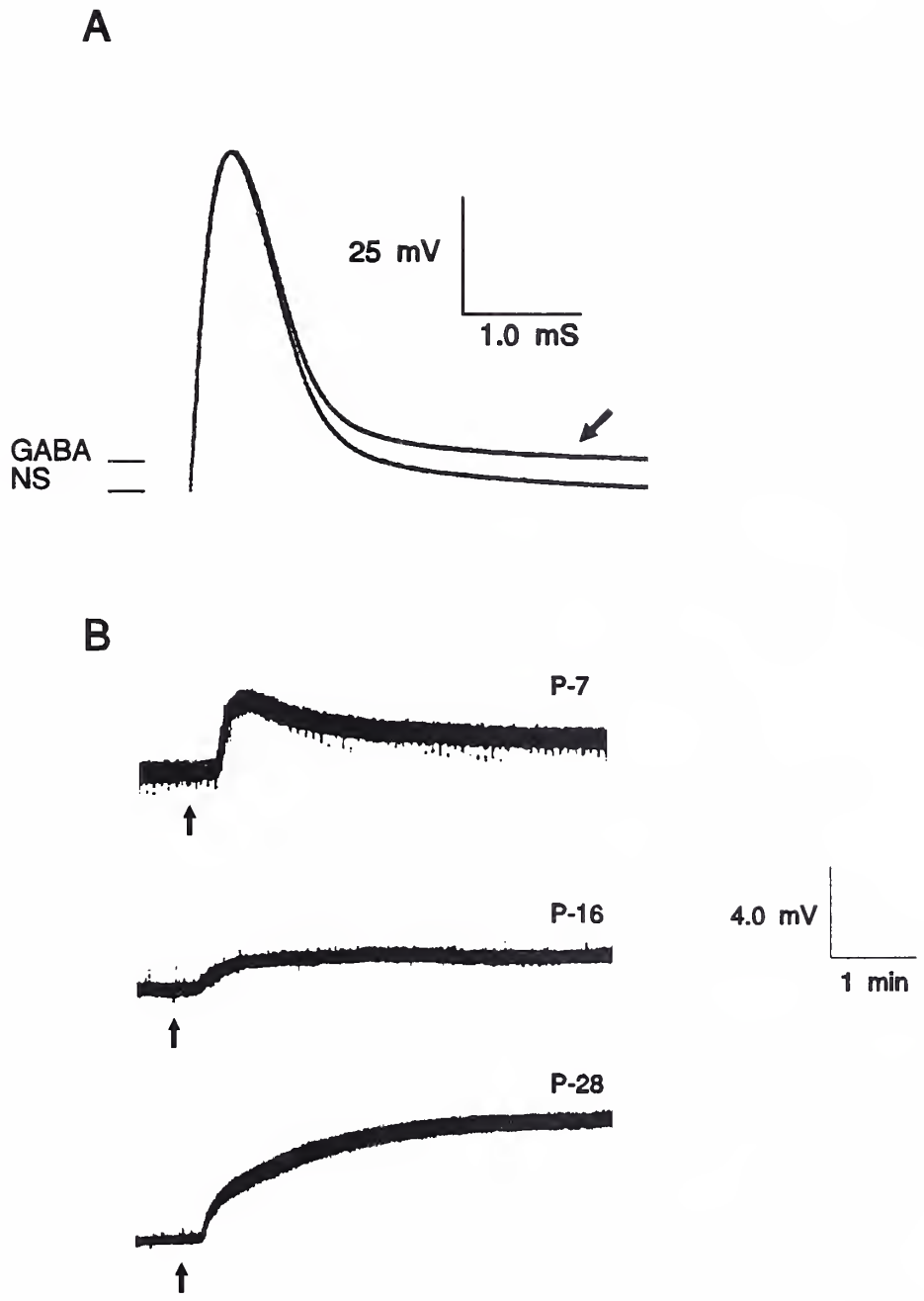

Figure 2: (A) The compound action potentials of lumbar dorsal root demonstrating the decrease in absolute amplitude after the administration of 1 mM GABA in a P-28 rat. The arrow indicates the action potential in the presence of GABA. NS denotes normal (Krebs') solution. (B) DC recordings of the membrane potential in P-7, P-16, and P-28 dorsal root in response to 1 mM GABA showing the three age-dependent categories of depolarization.

amplitude at the age of one week, two weeks, and four weeks ($p < 0.0001$) (Figure 2B). Specifically, these depolarizations seen after application of 1 mM GABA had values of 2.51 ± 0.14 ($n=11$) at one week, 1.16 ± 0.09 ($n=11$) at two weeks, and 5.60 ± 0.40 ($n=3$) at four weeks. However, the potential change observed at age four weeks was no different than that of a ten week-old adult rat indicating maturation of the GABA response by four weeks. There was a decrease in the magnitude of the depolarization between one and two weeks of age by 54% but an increase between two and four weeks such that the magnitude of the depolarization in the four week-old is 223% of that in the one week-old. A summary of dorsal root depolarization in response to perfusion with 1 mM GABA is shown in Figure 3.

In the one week-old rat, there was a rapid time to peak depolarization. However, the peak level of depolarization was not maintained and the membrane potential decayed quickly to a new plateau level that remained above the original baseline. The two week-old had a less rapid time to peak. Unlike the one week-old, the membrane potential at peak depolarization at two weeks was maintained throughout application of GABA. The four week-old had a similar pattern of GABA-induced depolarization as in the two week-old. However, the four week-old had the slowest time to peak and the largest depolarization in response to GABA. Dorsal roots in rats between two and four weeks of age produced progressively increasing amplitudes of depolarization to GABA with maturation. The maximum response to GABA was seen in the four week-old. The depolarization in the one week-old was 45% of that at four weeks and in the two week-old was 21% of that at four weeks.

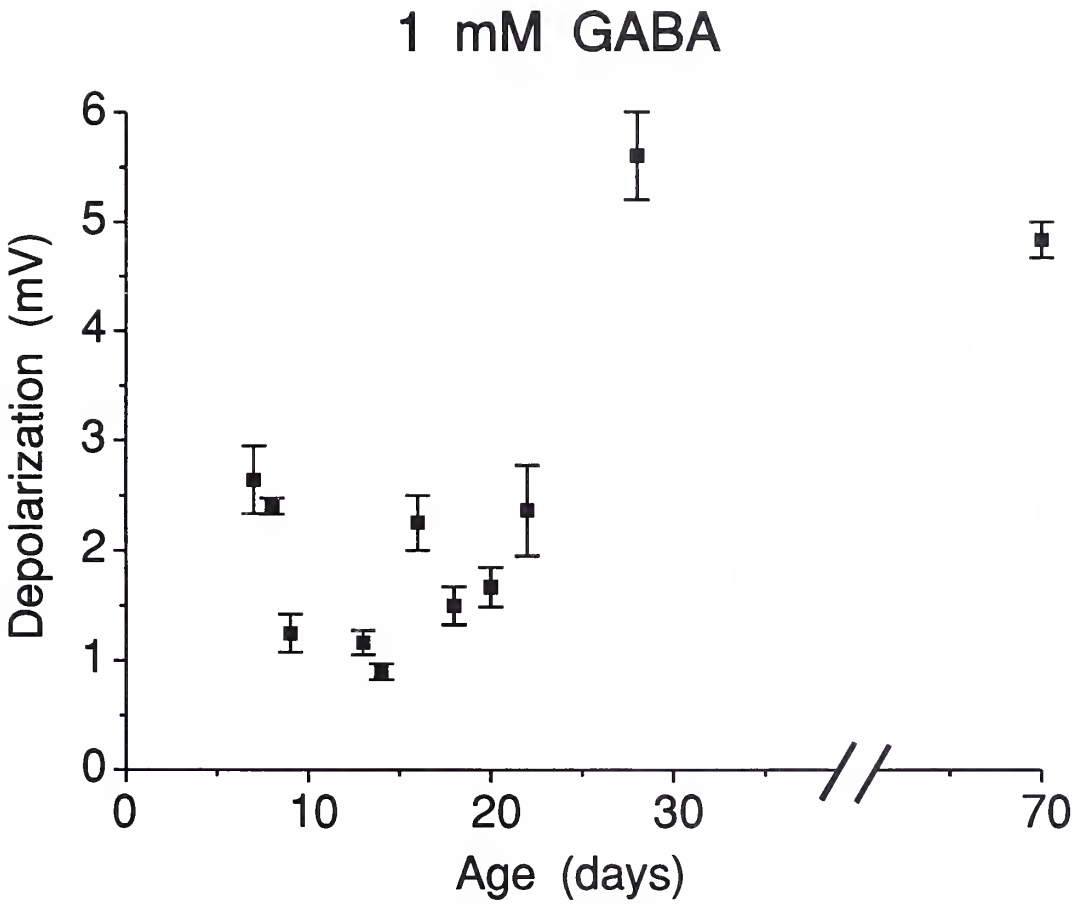

Figure 3: Mean peak depolarization in membrane potential (with bars denoting standard error) after application of 1 mM GABA to rat dorsal root.

It has been shown that the depolarizing action of GABA on adult rat dorsal root is due to the $GABA_A$ receptor. To determine GABA receptor subtypes in the neonate, experiments used specific $GABA_A$ and $GABA_B$ agonists and antagonists. The $GABA_A$ receptor agonist, muscimol, at a concentration of 1 mM produced a peak depolarization in the dorsal root at one week of 2.60 $\pm$ 0.31 mV (n=3), at two weeks of 1.45 $\pm$ 0.17 mV (n=4), and at four weeks of 4.60 $\pm$ 0.14 mV (n=2) (Figure 4A). These values are not statistically different from those produced by GABA at equivalent ages. Depolarizations by muscimol have similar times to peak as those produced by GABA and the amplitudes of depolarization and plateau levels were consistent. A summary of dorsal root depolarization in response to perfusion with 1 mM muscimol is shown in Figure 5.

Since a $GABA_A$ agonist produced a response, a competetive $GABA_A$ antagonist in the presence of GABA should decrease maximal response. Therefore the $GABA_A$ antagonist, bicuculline, was used in conjunction with GABA to examine competitive blockade of the $GABA_A$ receptor in the postnatal period. Dorsal roots in the sucrose gap chamber were perfused with 100 μM bicuculline for twenty minutes prior to application of 1 mM GABA. This produced peak depolarizations at one week of 1.35 $\pm$ 0.17 mV (n=4), at two weeks of 0.56 $\pm$ 0.17 mV (n=5), and at P-28 of 2.40 $\pm$ 0.28 mV (n=2) (Figure 4B). In all three age categories, a statistically significant decrease was demonstrated in the amplitude of peak depolarization when compared to GABA alone. The depolarizations produced by GABA in the presence of bicuculline were 43-54% of those produced by GABA alone at any given age. To determine $GABA_B$ receptor effects on the responses, baclofen was used in 100 μM concentration as a selective $GABA_B$

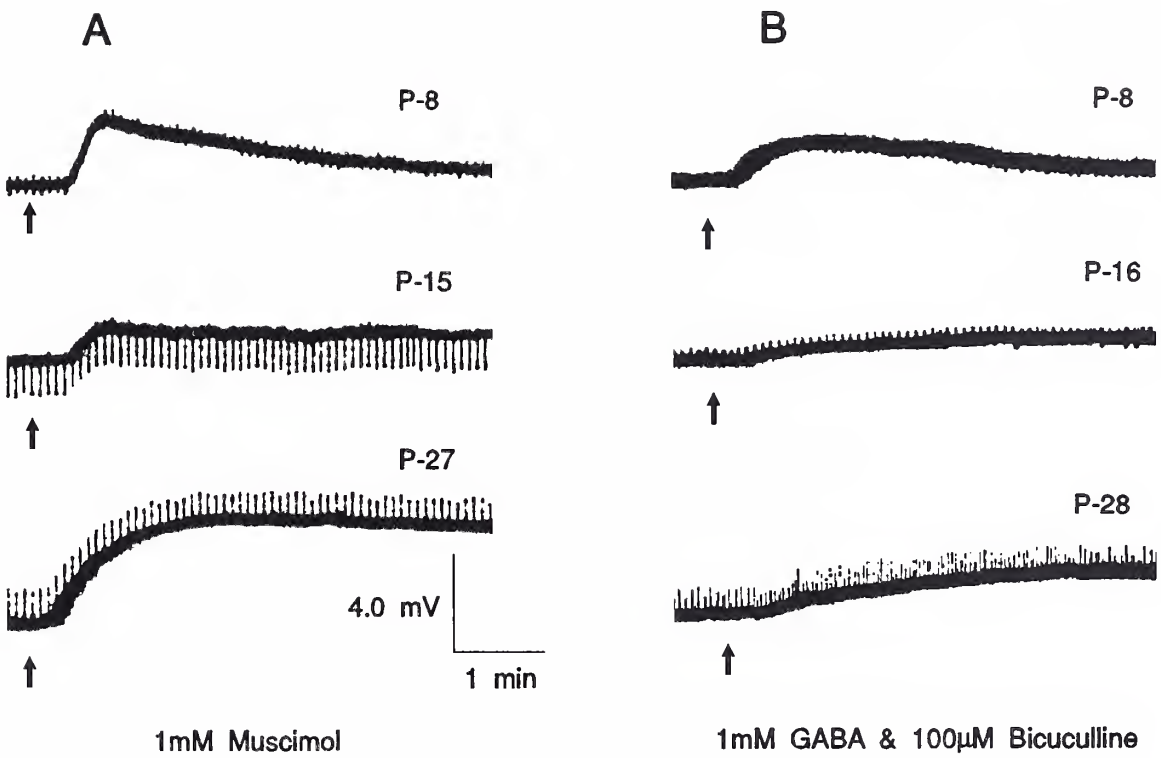

Figure 4: (A) Effect of muscimol (1 mM), a $GABA_A$ agonist. (B) Effect of GABA (1 mM) after a 20 minute infusion of bicuculline (100 μM), a $GABA_A$ antagonist. All figures are DC recordings of relative membrane potential. Infusion of each drug is begun at the arrow.

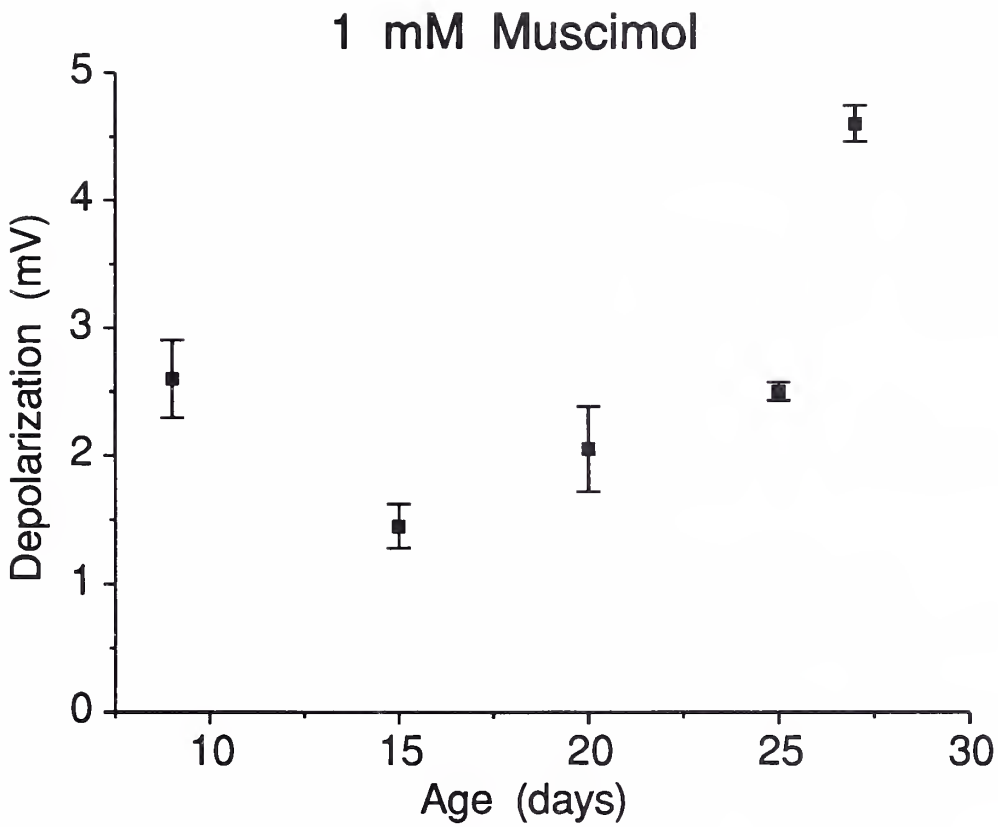

Figure 5: Mean peak depolarization in membrane potential (with bars denoting standard error) after application of 1 mM muscimol to rat dorsal root.

agonist. Application of baclofen to the dorsal root produced no change in resting membrane potential at both ages P-5 (n=4) and P-14 (n=4) (Figure 6A). This implies that $GABA_A$ and not $GABA_B$ receptors are involved in depolarization of dorsal root axons from birth to adulthood.

GABA uptake and release mechanisms that have been shown to exist centrally in the neonatal optic nerve were examined in the peripheral nervous system by application of 1 mM NPA to neonatal dorsal root. NPA stimulates release of endogenous GABA and blocks uptake mechanisms (Johnston *et al.*, 1976, Brown *et al.*, 1980). However, application of NPA on dorsal roots demonstrated no effect at both P-7 (n=4) and P-14 (n=4) (Figure 6B). This indicates that endogenous GABA does not play a role in determining axonal excitability. To determine if there is selective expression of $GABA_A$ receptors on sensory and not motor fibers during development of spinal roots, ventral roots were tested at both ages P-15 (n=4) and P-37 (n=4). Application of GABA did not produce a depolarization with ventral roots (Fig 6C). This indicates that there is selective expression of $GABA_A$ receptors on primary afferent sensory fibers and not on motor fibers during development as well as in adulthood.

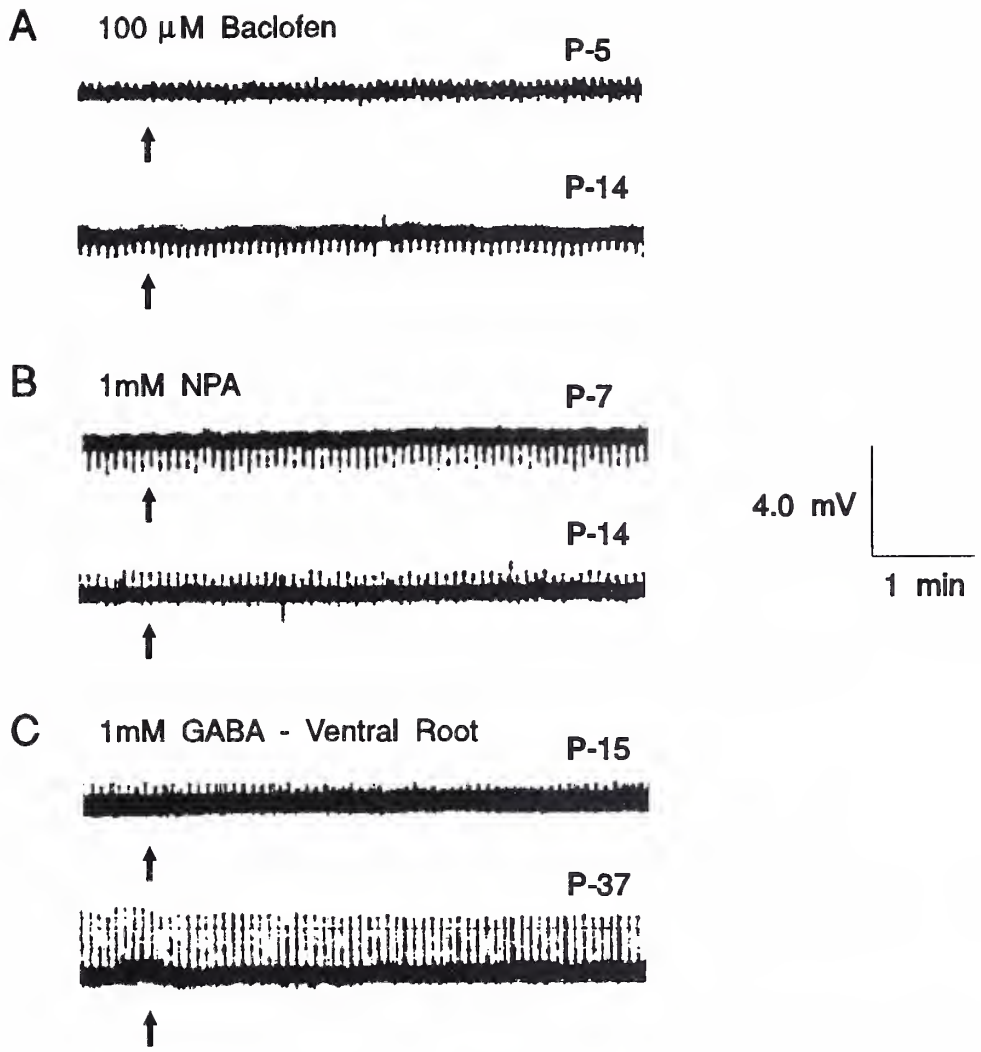

Figure 6: (A) Effect of baclofen (100 μM), a $GABA_B$ agonist. (B) Effect of NPA (1 mM). (C) Effect of GABA (1 mM) on ventral roots. All figures are DC recordings of relative membrane potential. Infusion of each drug is begun at the arrow.

DISCUSSION

GABA has been shown to alter axonal excitability in the developing central nervous system through axo-glial interaction in rat optic nerve. GABA also induces a $GABA_A$ receptor mediated depolarization in optic nerve axons which progressively decreases during the first two weeks of life. In contrast, the adult dorsal root axons in the peripheral nervous system express $GABA_A$ receptors during adulthood. This study demonstrated the effects that GABA exerts on membrane potential in dorsal root both vary significantly through the first four weeks of life and alter CAP amplitude. Additionally, there does not appear to be GABA-mediated modulation of dorsal root axonal excitability.

The results of this study indicate that GABA, through the $GABA_A$ receptor, can alter both the compound action potential and the membrane potential of rat lumbar dorsal spinal root differentially during development. After application of GABA, the absolute amplitude of the CAP was decreased but the peak voltage level of the action potential was nearly unchanged. Therefore, the GABA-mediated depolarization in baseline membrane potential was the cause for a decrease in action potential amplitude. GABA brought the membrane potential closer to threshold and, after an adequate excitatory current stimulus, the subsequent depolarization from the CAP was somewhat smaller in amplitude and larger in half-width.

The GABA-induced depolarizations observed in this study were mediated by $GABA_A$ and not $GABA_B$ receptors present on primary afferent sensory fibers; $GABA_A$

receptor mediated responses were not observed motor fibers. Responses were mimicked by the $GABA_A$ agonist, muscimol, blocked by the $GABA_A$ antagonist, bicuculline, and were absent after application of the $GABA_B$ agonist, baclofen. Additionally, superfusion of GABA to lumbar ventral spinal roots did not alter the membrane potential.

A significant change in the magnitude of the GABA-mediated depolarization in dorsal roots was noted during the first four weeks of life. Initially the amplitude of depolarization decreased from one week postnatal to two weeks postnatal but then steadily increased until maximum responses were achieved in the fourth week of life. Not only did the amplitude of the depolarization change during these first four weeks but the characteristics of these depolarizations changed as well. Looking at the one week-old, the peak depolarization after application of GABA was not sustained and the membrane potential decayed to a lower plateau level that remained above baseline. This decay phenomenon is characteristic of desensitization which is common in $GABA_A$ receptors (Tehrani *et al.*, 1989, White, 1990). Desensitization and a short time-to-peak value occurred only during the first week of life. The desensitization and reduction in the magnitude of depolarization can have two basic etiologies. First, there may be a reduction in expression of the $GABA_A$ receptor on the dorsal root axon. A decrease in the number of receptors would result in a decreased chloride conductance and a smaller depolarization. Alternatively, there may be shielding of axonal $GABA_A$ receptors in the two week-old by some developing tissue. It is well established that the first and second week of life is the time frame in which the process of myelination of the primary afferent sensory neurons occurs. Myelin has previously been shown to cover axon regions

containing other ion channels. For example, certain types of potassium channels are located under the mylein sheath in mammalian axons (Waxman & Ritchie, 1985). Using this barrier model, one can assume that GABA diffuses to spinal root axons more readily at one week than at two or four weeks of age. In contrast to the one week-old, the characteristic shape of the depolarization at two and four weeks of age appear to be similar. The responses differ only in the magnitude of the depolarization. This would suggest some type of developmental change in a two week time course secondary to an increase in $GABA_A$ receptor density, to a change in the electrochemical gradient, or to a change in the subunit structure of the $GABA_A$ receptor.

There is a transient presence of $GABA_A$ receptors in optic nerve during development. It has been postulated by Sakatani *et al.*, (1992), that these receptors play a role in altering neurite extension at the terminal axons in early development. The actions of the growth cone in formation of neuronal terminal arborization patterns and coding of information appear to be regulated by calcium levels in the terminal axon (Kater *et al.*, 1988). Large increases in the levels of intracellular calcium have been found to inhibit growth cone motility and large decreases inhibit neurite outgrowth. It appears that a given resting level of calcium is necessary for normal growth cone function. Since action potentials depolarize the cell, activate voltage-sensitive calcium channels in the terminal axon, and therefore increase calcium levels in the terminal axon, neurons in which there is active elongation of the growth cone are sensitive to changes induced by action potentials.

A train of such depolarizations causes cessation of extension of neurites in the

growth cone. This is where GABA appears to be important. GABA reduces threshold at low concentrations by both depolarizing the axons and increasing conduction velocity (Sakatani *et al.*, 1992). Although GABA depolarizes the membrane potential at higher concentrations, it also decreases input resistance and allows for shunting of excitatory current by an increased chloride conductance. This shunting mechanism clamps the axon at a point below threshold. Thus, the level of GABA in the extracellular space has an effect on the excitability of the nerve. Low levels would increase the probability of producing an action potential and therefore stop neurite extension whereas higher levels would inhibit the cell from reaching threshold and allow for continued arborization. Endogenous release of GABA from either pre-myelinated axons or glial cells can be stimulated by the addition of NPA (in addition to inhibiting GABA uptake) to a central nervous system white matter tract such as rat optic nerve in the first week of life. However, this is not the case in the peripheral nervous system. Application of NPA to rat dorsal root did not effect the resting membrane potential. The glia in the peripheral nervous system, namely Schwann cells, do not affect impulse conduction through a GABA-mediated increase/decrease in axonal excitability.

Endogenous sources of GABA which are present and activate $GABA_A$ receptors in the developing central nervous system optic nerve are absent in the peripheral nervous system dorsal spinal roots. $GABA_A$ receptors, though capable of modulating axonal excitability, do not appear to play a role in the developing dorsal root and its terminal arborization patterning. These reults emphasize the regionalization and specificity of axo-glial interactions in the developing nervous system.

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