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Estrone-3-sulfate acts at a postsynaptic site on rat CA1 pyramidal cells

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ESTRONE-3-SULFATE ACTS AT A POSTSYNAPTIC SITE ON RAT CA1 PYRAMIDAL CELLS

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ABSTRACT

Neurosteroids exert their effects in ways that are not clearly understood. This study investigated the mechanism of action of the neurosteroid, estrone-3-sulfate (E-3-S), an estradiol metabolite that rapidly induces epileptogenic activity *in* vivo and *in* vitro. Previous studies demonstrated that E-3-S decreases the amplitude of GABA-mediated inhibitory postsynaptic currents (IPSCs) in rat CA1 pyramidal cells. The blind whole-cell patch-clamp technique and direct GABA application were utilized to determine whether E-3-S acts at a presynaptic or postsynaptic site.

Bath application of 450 μM E-3-S led to a mean 83% reduction in GABA-evoked IPSC amplitude. Lesser (but not significant at $p<0.01$) effects were noted at lower doses, indicating that E-3-S acts dose-dependently at a postsynaptic site.

Application of E-3-S (4.5, 45, 450 μM) produced no significant change in resting conductance; however, 450 μM E-3-S decreased GABA-activated conductance and produced a negative shift in the reversal potential. This suggests that E-3-S reduces current flow through open GABA-activated Cl- channels.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

A. Steroid Hormones

Steroid hormones emerged early in evolution, acting as primitive regulators of growth. They diversified later into the gonadal steroids, glucocorticoids and mineralocorticoids [127,150,173]. In eukaryotes, steroid hormones play crucial roles in cellular division and growth, regulation of membrane permeability and in the maintenance of cell integrity. Steroid hormones are able to access all cells and organs of the body. Their lipophilic nature enables them to easily penetrate cell membranes and to cross the blood-brain barrier [201].

The brain is considered to be a target site for steroid hormones. Peripherally produced steroids have dramatic effects on neuroendocrine and behavioral functions and, during vertebrate development, these compounds are responsible for the regulation of several important brain neuronal functions [133]. During the late fetal and early postnatal periods, gonadal steroids influence the survival, differentiation and connectivity of neuronal populations in the brain and spinal cord [15,94,95,201]. Neuronal function continues to be influenced by steroid hormones in the adult brain [8,12,13,14,17,42,56, 93,94,116,124,127,134,154,158,174,175,176,182,200,201,215,219]. In addition to direct steroidal actions, biotransformation of steroids in brain tissues results in the formation of biologically active metabolites [2,3,12,13,14,33,35,42,45,53,60,80,84,86,95,102,103,109, 117,136,200,201,210,226].

In mammals, steroid hormones are derived from cholesterol and are primarily synthesized in the steroidogenic tissues of the testes, ovaries and adrenal gland. The placenta, during pregnancy, is a source of estrogens and progestins. Under certain developmental conditions (puberty and menopause), the adrenal gland serves as an added source of androgens and estrogens. Additionally, certain other peripheral tissues (e.g. adipose tissue) can produce significant quantities of estrogens from steroid precursors [62]. Interestingly, it has been found that the brain itself forms steroid hormones *de novo* [2,3,13,14,15,33,34,35,36,42,53,60,80,84,86,91,95,103,106,109,113,117,136,154,168, 169,175,195,199,200,201,206,207,226].

B. Brain Neurosteroidogenesis

The first hint that the brain might be capable of synthesizing steroid hormones came in 1976. At this time, it was found that the mammalian brain contains large amounts of the steroid precursor cholesterol, as well as its sulfate and lipid derivatives and those enzymes that are necessary for steroid biosynthesis [85]. Since that time, considerable further evidence has been obtained by various researchers for the *de novo* synthesis of steroid hormones by the brain. These researchers have demonstrated the presence, independent of peripheral steroidogenic sources, of several different steroid hormones in the brain and have confirmed the presence of the enzymes necessary for the synthesis of these hormones. To date, the *de novo* synthesis of multiple steroid hormones has been demonstrated in the mammalian brain (Figure 1) and in the brain of non-mammalian vertebrates, including birds and amphibians [195,199,200,201]. Steroid

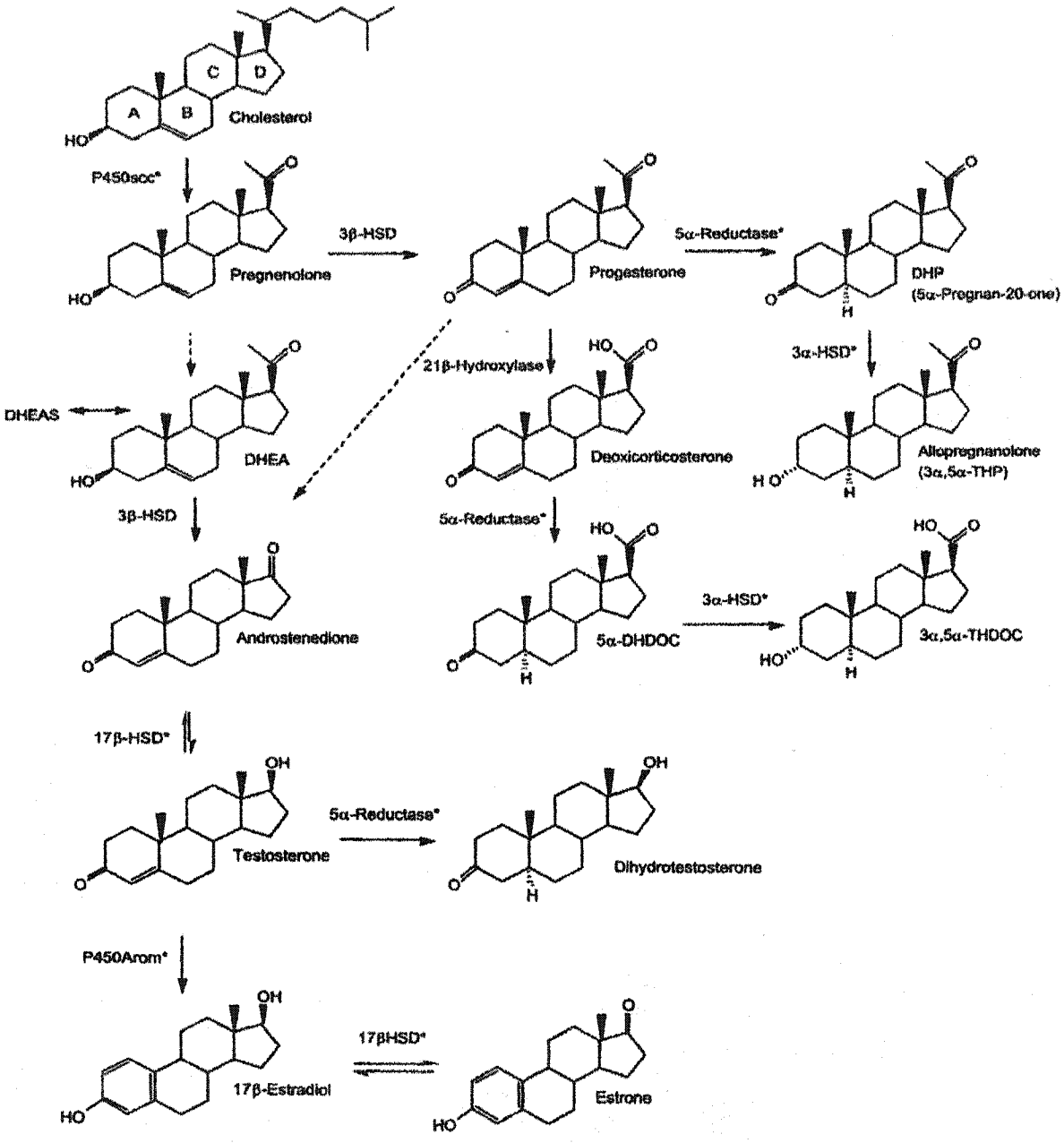

Figure 1: Neurosteroid biosynthesis in the brain. *Enzymes which are expressed in the human brain. The broken line indicates that several steroids have been omitted from the diagram at this step. Abbreviations: DHP, dihydroprogesterone; DHEA, dehydroepiandrosterone; DHEAS, dihydroepiandrosterone sulfate; THP, tetrahydroprogesterone; DHDOC, dihydro-11-deoxycorticosterone; THDOC, tetrahydro-11-deoxycorticosterone; P450scc, P450 side chain clevage; P450Arom, P450 Aromatase; HSD, hydroxisteroid oxidoreductase. Reprinted with permission from Ref. 16.

hormones that are synthesized in, or act upon, the CNS are referred to as "neurosteroids", a term which was introduced by Baulieu in 1981 [132,154].

The main precursor of various steroid hormones produced in peripheral steroidogenic glands is pregnenolone (PREG) [16]. Researchers have demonstrated the presence and accumulation of PREG, pregnenolone sulfate (PS), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S) and the fatty acid esters of these steroid hormones throughout the brains of several mammalian species [2,12,13,14,15,34, 35,42,80,86,91,95,113,130,136,154,169,200,201,226]. These hormones persist in brain tissues after removal of peripheral steroidogenic sources by adrenalectomy, castration and hypophysectomy [12,13,14,34,35,86,136,168,169, 200,201]. Some regional differences in the concentrations of these neurosteroids in the brain have been observed. For example, there is a higher concentration of PREG in the olfactory bulb than in other brain areas and, in the hypothalamus, a higher concentration of DHEA than in the remainder of the brain [14].

Studies were also conducted to verify that the persistent nature of neurosteroids in the brain after removal of peripheral steroidogenic sources was not due to retention of these hormones in the circulation. In these studies, radioactive DHEA and PREG were peripherally administered and a rapid release of these compounds from the brain was observed. Interestingly, PREG, DHEA and certain of their metabolites were found to occur, in the brain, at much higher concentrations than are normally found in the plasma (Table 1) [14,34,35,36].

Table 1: Brain vs Plasma Concentration of Neurosteroids.

	PREG	PREGS	PREGL	DHEA	DHEAS	DHEAL	PROG
Brain (ng/g)							
Intact	8.9 (±2.4)	14.2 (±2.5)	9.4 (±2.9)	0.24 (±0.33)	1.70 (±0.32)	0.45 (±0.13)	2.2 (±1.1)
Orx/adx	2.6 (±0.8)	16.9 (±4.6)	4.9 (±1.3)	0.14 (±0.13)	1.64 (±0.43)	0.29 (±0.12)	3.2 (±1.6)
Plasma (ng/ml)							
Intact	1.2 (±0.6)	2.1 (±0.9)	2.4 (±0.9)	0.06 (±0.06)	0.20 (±0.08)	0.18 (±0.05)	1.9 (±0.7)
Orx/adx	0.3 (±0.1)	nd*	1.3 (±0.3)	nm*	nm*	nm*	0.1 (±0.1)

Neurosteroid concentrations found in the brain of male rats (intact vs orx/adx) are compared with the concentrations of those same neurosteroids in the plasma of male rats (intact and orx/adx) Abbreviations: adx, adrenalectomized; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHEAL, dehydroepiandrosterone-lipoidal; orx, orchectomized; PREG, pregnenolone; PREGS, pregnenolone sulfate; PREGL, pregnenolone-lipoidal; PROG, progesterone. *nd, not detected; nm, not measured. Reprinted with permission from Ref. 14.

As further evidence for *de novo* brain neurosteroidogenesis, researchers conducted studies to confirm the presence, in brain, of those enzymes necessary for steroid biosynthesis. There are three key enzymes necessary for steroid biosynthesis: cytochrome P450 side-chain cleavage enzyme (P450scc), cytochrome $P450_{17\alpha,lysate}$ ($P450_{17\alpha,lysate)}$ and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD). These enzymes, and other enzymes involved in neurosteroidogenesis, have been found in various regions of the mammalian brain [14,33,45,53,60,84,95,103,117,136,154,200, 201,206,207,226].

Steroid biosynthesis begins with the formation of PREG. This process is initiated by cholesterol side-chain cleavage by P450scc, which has been demonstrated in several areas of the mammalian brain. This enzyme seems to occur in the myelinated region of the white matter in neonatal and adult animals [33,84,103,117,200,201,205]. It is synthesized mainly in glia by type I astrocytes, but can also be found in oligodendrocytes [136]. Expression of mRNAs for P450scc is constant from the neonatal period through adulthood [205].

$P450_{17\alpha,lysate}$ converts PREG to DHEA, which is present in abundant quantities throughout the brain. To date, DHEA formation has been demonstrated in purified glial cells, astrocytes and neurons that have been isolated from neonatal rat brains [2,3,111, 200]. It has been shown, however, that mRNAs for $P450_{17\alpha,lysate}$ are highly expressed, regardless of age, in the rat mesencephalon. Conversely, expression of $P450_{17\alpha,lysate}$ mRNA increases in the brain stem of the postnatal rat [103,200,201,226].

The dehydrogenation and isomerization of PREG and DHEA into progesterone (PROG) and androstenedione (AD) is catalyzed by 3β-HSD, which has been reported in the mammalian brain and in cultured neurons [3,45,60,103,200,201,206,210]. The conversion of PREG into PROG has been demonstrated in homogenates of discrete brain areas, including the hypothalamus, hippocampus, amygdala, septum and cortex [169,210]. Furthermore, i*n situ* hybridization has demonstrated the presence of 3β-HSD in several brain regions, including the olfactory bulb, striatum, cortex, thalamus, hypothalamus, habenula, septum, hippocampus and cerebellum and has shown that the expression of this enzyme varies by region [60]. 3β-HSD expression also seems to be age dependent. In the neonatal period, mRNAs encoding for 3β-HSD are expressed in the cerebellum and cerebrum at higher levels than in other brain areas [103,201,206]. This increased expression of 3β-HSD mRNAs suggests that there is a functional role for PROG and its metabolites during the neonatal period; it has been reported that these neurosteroids seem to promote axonal growth, myelination and synaptic contact of neurons [95,201].

Of interest is a recent study which found that, in the hippocampus (well known for its property of neuronal plasticity), 3β-HSD mRNA levels, as well as concentrations of PREG and PROG are highest on the day of birth and then decrease steadily with age. In this study, plasma levels of PREG and PROG were measured and found to be lower than hippocampal concentrations, suggesting that these neurosteroids were synthesized in the brain [82].

The major cell type involved in neurosteroidogenesis in the mammalian brain is the glial cell [14,200,201]. Immunohistochemical analysis with anti-P450scc antibodies has shown intense antibody binding in several vertebrate species, throughout the white-matter of the brain, where glial cells (astrocytes and oligodendrocytes) may be found. Immunohistochemical staining in glial cell cultures from newborn rats shows more intense staining by oligodendrocytes (myelinating glial cells) than by astrocytes [91,117,200,201]. However, both oligodendrocytes and astrocytes have been shown to produce PREG, the synthesis of which is the first step in steroidogenesis. These cells have been identified as the primary cell types, in the brain, involved in the synthesis of PREG [2,12,13,80,91,152,200,201]. Additionally, it is interesting to note that oligodendrocytes and astrocytes have been shown to express progesterone, glucocorticoid, estrogen and androgen receptors [92,93].

In addition to glial cells, other cell types in the brain are also thought to play roles in neurosteroidogenesis. Purkinje cells have been found to possess both P450scc and 3β-HSD. Furthermore, in several vertebrate species, Purkinje cells have been demonstrated to produce PREG, PS and PROG [195,199,200,201,205,206,207]. Steroidogenic acute regulatory protein, which is involved in the transport of cholesterol to the inner mitochondrial membrane (the location of P450scc) has been demonstrated in Purkinje cells [46]. Interestingly, 3β-HSD expression increases in Purkinje cells during the neonatal period [200,201,226]. In the rat hippocampus, P450scc, $P450_{17\alpha,lysate}$ and P450arom (the enzyme which converts androgens to estradiol) have been demonstrated in the CA1-CA3 pyramidal cell regions and in the granule cells of the dentate gyrus [97].

C. Effects of Neurosteroids on the Brain

To date, there are numerous documented examples of neurosteroid effects on the nervous system. Researchers are currently in the process of studying these effects at the cellular and molecular levels with the expectation that this research will lead to a greater understanding of the physiological, behavioral and clinical effects of neurosteroids.

The actions of neurosteroids on neurons are varied. In the fetal and early postnatal periods, neurosteroids act via genomic mechanisms to influence differentiation, survival, axonal growth and synaptic contact of neurons in both the brain and spinal cord [15,94,95,201]. In the adult, neurosteroids influence changes in the morphology and connections of neuronal cells. For example, it has been found that estrogens act to increase dendritic spine density in hypothalamic and hippocampal neurons [1,42,116, 125,134]. Additionally, in primary cell cultures, PROG has been found to stimulate myelin-specific protein synthesis in oligodendrocytes [15,94,95]. In fact, it has been shown that, 3-7 days following brain or spinal cord injury, synthesis of PROG, allopregnenolone (3α,5α-THP) and 5α-dehydroprogesterone (5α-DHP) from PREG-S increases dramatically in the area surrounding a lesion [42]. Interestingly, *in vivo* studies have demonstrated that PROG acts to regenerate sciatic nerves after cryolesions [14,15,102]. Research also suggests that, after brain injury, DHEA, PREG and other neurosteroids may act to limit brain damage and act to affect brain repair by down-regulating reactive astroglia, thus preventing the formation of gliotic scar tissue which, when present, acts as a physical barrier that prevents axonal growth [42,56].

Neurosteroids also act to influence behaviors, especially those related to reproduction [14,17,52,66,110,132,157,222]. Concentrations of brain steroids are not constant, but vary in relation to animal age and physiological change [8,14,134]. There are a number of physiological, behavioral and pathological phenomena that seem to be related to cyclical and physiological variation in brain neurosteroid concentration. These include: stress, depression and anxiety, aggression, personality traits, sexual function, post-partum depression, cognitive performance and seizure susceptibility [5,8,12,14, 16,17,25,43,49,50,51,52,66,67,68,73,87,95,110,127,132,136,141,155,157,164,175,176, 208,211,222,225].

It has long been known that certain steroid hormones influence the probability of seizure occurrence, causing an increase or decrease in the frequency and severity of seizures. As early as the1950s and 1960s, it was observed that estrogens had a proconvulsant effect in women with epilepsy [118,119,194]. To date, considerable further evidence has accumulated which indicates that alterations in neurosteroid concentrations (endogenous or administered) influence seizure susceptibility [5,6,10, 11,16,19,59,67,69,70,71,72,124,144].

Certain epileptic syndromes such as absence epilepsy and juvenile myoclonic epilepsy occur during puberty, corresponding to the changes in hormonal concentrations that occur during this time. In women, alternations in brain neurosteroid concentrations that occur with pregnancy, menopause, the use of oral contraceptives and specific stages in the menstrual cycle have been linked to seizures or to changes in seizure frequency [5,11,16,67,69,70,71,76,144]. This is very pronounced in women with epilepsy.

Many epileptic women experience increased seizure frequency immediately before and during menstruation. This is known as catamenial epilepsy. Although reports differ, menstrual exacerbation of epilepsy has been reported in up to 78% of patients [5,11,16,44,67,71,107,144,155,172]. In 1956, Laidlaw became the first to note the relationship between catamenial epilepsy and hormonal fluctuation in women. Laidlaw hypothesized that PROG could exert a slight, but significant, anticonvulsant effect [107]. This hypothesis was supported when it was found that there was a negative correlation between plasma PROG concentration and seizure frequency and that, in epileptic women, seizure frequency increases significantly with the rapid decline in PROG concentration that occurs at menstruation. It was also found, in this same study, that there was an increase in seizure frequency which occurred before ovulation; this led to the hypothesis that estrogen has proconvulsant activity [5].

Additional support for the hypothesis that a lack of PROG and its metabolites underlies catamenial seizures is the observation that there is an increased frequency of seizures in epileptic women with luteal insufficiency. In these women, who fail to produce adequate PROG during the luteal phase of the menstrual cycle, administration of PROG has been found to decrease seizure frequency [16,71,172].

Current research has focused on the relative concentration of estrogen relative to PROG. Researchers have found that seizures are exacerbated when the concentration of estrogen is high relative to the concentration of PROG. When PROG levels are elevated, the frequency of seizures decreases. During the luteal phase of the menstrual cycle, when PROG secretion is increased and estrogen secretion is decreased, many epileptic women

experience a reduced incidence of seizures. In clinical studies with women suffering from catamenial epilepsy, it has been found that administration of PROG or synthetic PROG leads to a reduction in seizure frequency. [5,107,118,164,172].

In women with focal epilepsy, intravenously administered PROG has also been found to significantly decrease interictal ("between seizures") spike frequency [6,16]. In women suffering from medically intractable focal or catamenial epilepsy, add-on therapy with cyclic natural PROG significantly reduces seizure frequency [16,69,70,72].

One method to decrease the production of ovarian hormones, such as estrogen, is via exogenously administered gonadotrophin releasing hormone (GnRH). After an initial stimulatory effect, GnRH acts, via negative feedback on the pituitary, to suppress the release of ovarian hormones. Research was conducted to study the effect of GnRH and GnRH analogs on seizure susceptibility. Add on-therapy with synthetic analogues of GnRH has been found to decrease the frequency or severity of catamenial seizures. Unfortunately, GnRH administration over long time periods is not feasible because severe bone demineralization results from its use [10,16,63,167,209].

It is hoped that current epilepsy research will lead to improved treatments for seizure disorders, as well as new insights into brain structure and function. There has been a resurgence of interest in the neurosteroids as possible therapeutic agents in the treatment of anxiety, posttraumatic stress disorder, depression, schizophrenia and seizure disorders [16,69,70,72,109,121,127, 165,166,172,171,131].

D. Steroid Hormones—Classic vs Nongenomic Mechanisms of Action

Many studies have been undertaken to determine the mechanism by which neurosteroids modulate seizure occurrence and neuronal activity in general. Nevertheless, these mechanisms are not yet fully understood. Research has demonstrated that neurosteroids act, not only through genomic mechanisms but also by direct modulation of membrane-bound receptors, such as those for γ-aminobutyric acid (GABA) [9,12,13,14,15,16,28,38,47,57,64,65,87,89,105,109,112,121,122,123,124, 125,126,127,132,135,136,137,139,148,153,154,160,162,175,176,184,185,192,196, 201,203,204,215,217].

According to the "classic" mechanism of action for steroid hormones (Figure 2), they exert their effects by entering a target cell via simple diffusion and then binding, with high affinity and specificity, to intracellular receptors. Binding of the steroid results in the activation of the receptor into a form which can interact with DNA. The activated steroid-receptor complex is then translocated to the genome, where it binds to the DNA-hormone-responsive elements on the chromatin. This results in an increase in mRNA synthesis. Thus, steroid hormones exert their effects through the regulation of transcriptionally directed changes in protein synthesis. These classic genomic effects tend to occur over time periods of hours to days; however, some genomically mediated effects have been demonstrated to occur over a time course of minutes—but not less than 10 or 20 minutes [16,62,87,109,127,136,175,176].

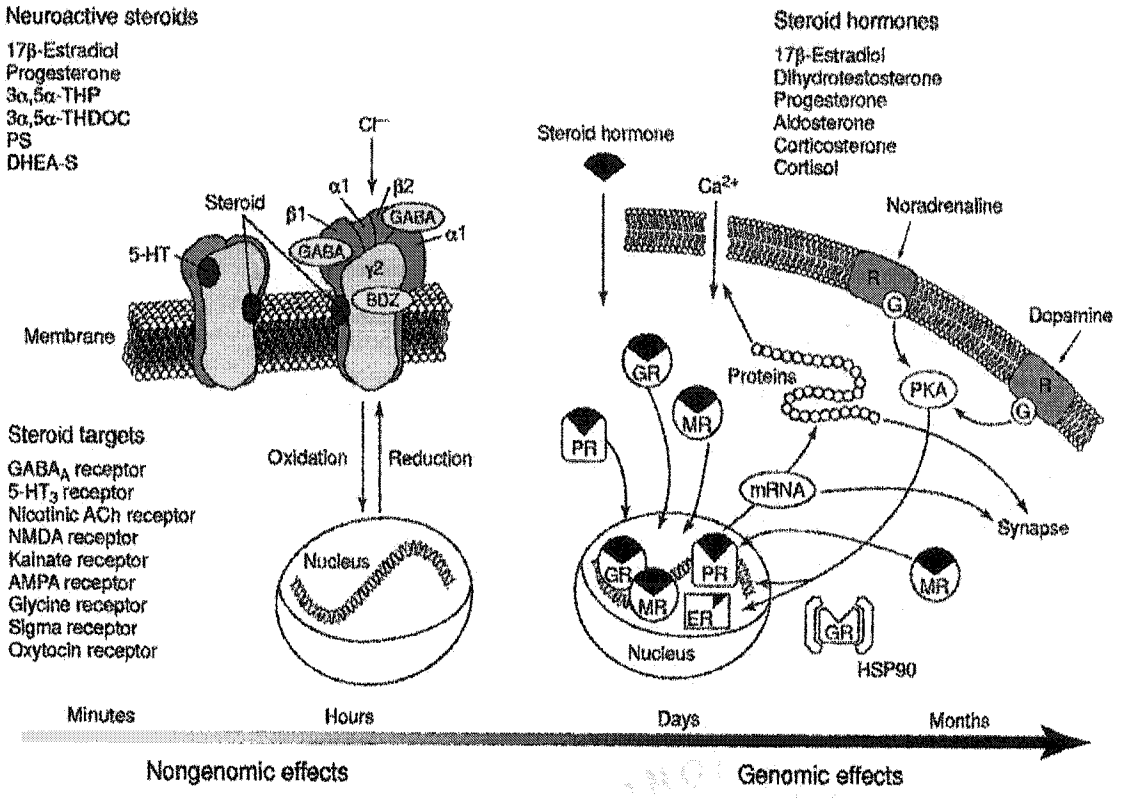

Figure 2: Neurosteroids exert both nongenomic and genomic effects. "Neuroactive steroids" is a term which is frequently used to refer to steroids that interact with neurotransmitter receptors. In the upper left-hand corner is a list of neurosteroids that are known to interact with neurotransmitter receptors. In the lower left is a list of neurotransmitter receptors that are modulated by neuroactive steroids. In the upper right corner is a list of typical steroid hormones. Some steroid hormones may also be classified as neuroactive steroids. The left side of this figure illustrates the nongenomic effects of steroid hormones, which can occur over milliseconds to hours; the right side illustrates the classical genomic effects of steroid hormones, which tend to occur over hours to days. Abbreviations: BDZ, benzodiazepines; DHEA-S, dehydroepiandrosterone sulfate; ER, estrogen receptor; G, G-protein; GR, glucorticoid receptor; HSP90, heat-shock protein 90; MR, mineralocorticoid receptor; PKA, protein kinase A; PR, progesterone receptor; PS, pregnenolone sulfate; R, receptor; THDOC, tetrahydrodeoxycorticosterone; THP, tetrahydroprogesterone. Reprinted with permission from Ref. 175.

In contrast to genomically mediated effects, which are slow in onset, long in duration and which persist after the steroid is removed from the tissue, many neurosteroids have been demonstrated to exert rapid neurotropic effects (Figure 2, Table 2). These rapid effects, which generally occur over milliseconds to seconds, result when certain neurosteroids bind to membrane bound receptors (such as those for inhibitory and/or excitatory neurotransmitters), thus, rapidly altering the excitability of neurons. These non-genomic effects are rapid in onset and are generally reversible after the steroid is removed [16,87,109,127,136,175,176].

There have been many studies concerning the non-genomic effects of neurosteroids. As a result of these studies, neurosteroids have been found to exert their non-genomic effects via modulation of a variety of neurotransmitter receptors (Figure 2). The most extensively studied example of the non-genomic effects of neurosteroids, and the one on which this paper will focus, is modulation of the GABA receptor.

E. GABA Receptors

In order for the CNS to function normally, a balance between excitation and inhibition must be maintained. In the CNS, GABA functions as the principal inhibitory neurotransmitter, and extreme changes in neuronal excitability can result even from small changes in GABAergic neurotransmission. GABA receptors are found throughout the brain and spinal cord; however, receptor subtypes are not evenly distributed [23,24,31,88, 99,151,159,212].

Table 2: Neurosteroids modulate a variety of neurotransmitter receptors.

Receptor	Steroid	Modulation	Effective Concentration
$GABA_A$	3α,5α-THP	positive	10^{-8}-10^{-5}M
	3α,5β-THP	positive	10^{-8}-10^{-5}M
	3α,5α-THDOC	positive	10^{-8}-10^{-5}M
	PS	negative	10^{-5}-10^{-3}M
	DHEA-S	negative	10^{-5}-10^{-3}M
Nicotinic Acetylcholine	progesterone	negative	10^{-6}-10^{-4}M
			10^{-5}-10^{-4}M
ACH	3α,5α-THP	negative	10^{-5}-10^{-4}M
Glycine	progesterone	negative	10^{-5}-10^{-3}M
	PS	negative	10^{-4}M*
$5\text{-}HT_3$	estradiol (17α and β)	negative	10^{-6}-10^{-4}M
	progesterone	negative	10^{-6}-10^{-4}M
	testosterone	negative	10^{-5}M*
	3α,5α-THP	negative	10^{-5}M*
	PS	none	10^{-5}M*
NMDA	17β-estradiol	negative	$5X10^{-5}$M
	PREG-S	negative	10^{-6}-10^{-3}M
	PREG-HS	negative	10^{-4}M*
	PS	positive	10^{-6}-10^{-3}M
AMPA	PS	negative	10^{-4}M*
Kainate	PS	negative	10^{-4}M*
	17β- estradiol	positive	10^{-8}-10^{-5}M
	progesterone	positive	10^{-5}-10^{-3}M
Oxytocin	progesterone	negative	10^{-9}-10^{-6}M
Sigma	DHEA-S	positive	10^{-7}-10^{-5}M
type 1	PS	negative	10^{-7}-10^{-5}M
(σ1)	progesterone	antagonist	10^{-8}-10^{-6}M

Abbreviations: ACH, acetylcholine; DHEA-S, dehydroepiandrosterone sulfate; PREG-S, pregnanolone sulfate; PREG-HS, pregnanolone hemisuccinate; PS, pregnenolone sulfate; THDOC, tetrahydrodeoxycorticosterone; THP, tetrahydroprogesterone.

*Only one concentration tested. With permission, from Ref. 175.

Gamma-aminobutyric acid was first found in the mammalian brain in 1950; however, its role as an inhibitory neurotransmitter in the CNS was not accepted for several years, until the discovery of bicuculline as an antagonist. Still, it was clear that GABA could have both pre- and postsynaptic inhibitory actions, which were bicuculline-insensitive, in many regions of the brain. These actions could be mimicked by the GABA analog, baclofen [41,89,99,100,170]. To distinguish these receptors from each other, Hill and Bowery (1981) introduced the $GABA_{A/B}$ receptor classification, in which the bicuculline-sensitive, baclofen-insensitive receptors are termed $GABA_A$ and the bicuculline-insensitive, baclofen-sensitive receptors are termed $GABA_B$. In recent years, a new class of GABA receptors, insensitive to both bicuculline and baclofen, has been discovered--these are known as $GABA_C$ receptors [20,21,90,96,99,100,135].

The $GABA_A$ receptor, sometimes known as the GABA-benzodiazepine-ionophore receptor complex, is a heteropentameric complex that has specific allosteric modulatory sites for benzodiazepines (however, not all $GABA_A$ receptors are influenced by benzodiazepines), barbiturates, neurosteroids and ethanol [21,90,99,100]. The $GABA_A$ receptor is found throughout the CNS (except the interpeduncular nucleus) and is a target for many psychoactive drugs (both CNS depressants and excitants) [20,23,89,99,135, 138,221]. It appears to be the most complex member of the same ligand-gated ion channel superfamily as the nicotinic-cholinergic, glutamate, 5-hydroxytryptamine and strychnine-sensitive glycine receptors [89,135,142,150,179]. Bicuculline and picrotoxin

are $GABA_A$ antagonists. Muscimol is a $GABA_A$ agonist. Many drugs that are used for the treatment of nervous system disorders are direct modulators of the $GABA_A$ receptor [89,98,135].

The $GABA_A$ receptor is made up of various membrane-spanning subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, and ρ_{1-3}), which form an integral chloride channel. The various subunits of the $GABA_A$ receptor are expressed in different combinations in various regions of the CNS; thus, there are multiple subtypes of the $GABA_A$ receptor. In other words, the heterogeneity of $GABA_A$ receptors is a result of the association of five subunits in a range of combinations. These subunits combine together to form a single ligand-gated ion channel complex [89,120,135,142,147,186,213]. In some brain regions, including the hippocampus, the coexistence of multiple $GABA_A$ receptor subtypes has been demonstrated [18,89,213,223]. The $GABA_A$ receptor is ionotrophic--it is functionally linked to its Cl^- channel. No activation of second messenger pathways (metabotrophic effects) have yet been described [21,89,135,187].

The electrophysiological responses as well as the physiological and pharmacological roles of $GABA_A$ receptor-channel complexes are dependent upon the subunit composition of the Cl^- channel [23,26,104,112,121,135,138,147,160,161, 162,185]. For example, research suggests that the α_6-subunit containing $GABA_A$ receptor is related to motor control. Ethanol-nontolerant rats, which are known to have a point mutation in the α_6-subunit of the $GABA_A$ receptor, exhibit a dramatically increased susceptibility to postural reflex impairment as a result of ethanol and benzodiazepine administration [104,135]. Several other physiological and pharmacological roles have

been suggested for various subunits of the $GABA_A$ receptor. For example, α_2 subunits have been linked to behavioral cocaine sensitivity, α_4 subunits are linked to seizures, pre-menstrual syndromes and stress, β_3 subunits may be involved in tremor, jerky gait, cleft palate, righting reflex and behavioral sensitivity to cocaine and γ_2 subunits are implicated in seizures, anxiety, righting reflex and abnormal gait [135].

Interestingly, it has been suggested that the complex $GABA_A$ receptor may have evolved from a relatively simple ligand-gated ion channel, composed solely of ρ-subunits. [89,150]. These holo-oligomeric receptors were first described in interneurons of the spinal cord. In recent years, a new type of GABA receptor has also been found in the vertebrate retina, cerebellum, hippocampus and optic tectum as well as in insects and bacteria [48,89]. There is evidence, however, that these receptors are functional only in the retina [90]. These new GABA receptors have been known by a variety of names: $GABA_C$, $GABA_{NANB}$, ("non-A, non-B"), $GABA_\rho$ and also as bicuculline-insensitive GABA receptors [20,21,89,90,96,99,100,135]. Although it has been recommended that the term $GABA_C$ be avoided, new pharmacological, structural, functional and genetic evidence, as well as evidence regarding cellular localization, has been presented which strengthens the case for classifying these receptors as distinct from their $GABA_A$ counterparts [21].

The $GABA_C$ receptor is composed entirely of ρ-subunits. It is not affected by a variety of substances that affect the $GABA_A$ receptor, including neurosteroids and benzodiazepines. It is insensitive to both bicuculline and baclofen [21,48,90]. The $GABA_C$ receptor is selectively activated by cis-4-aminocrotonic acid. Like the $GABA_A$

receptor, the $GABA_C$ receptor is ionotrophic and is directly linked to a Cl- channel [20,48,89]. Interestingly, the $GABA_C$ receptor has a higher sensitivity for GABA than the $GABA_A$ receptor and, unlike the $GABA_A$ receptor, does not desensitize. Furthermore, the $GABA_C$ channel remains open for a longer time and its current is smaller than that of the $GABA_A$ channel [21,48,90].

The $GABA_B$ receptor is insensitive to bicuculline, but is agonized by baclofen. Additionally, the $GABA_B$ receptor mediated effect is insensitive to picrotoxin and is antagonized by phaclofen. Like $GABA_A$ receptors, $GABA_B$ receptors are found in most sites throughout the CNS and peripheral nervous systems. $GABA_B$ receptors are not found in the lamina molecularis of the olfactory bulb or in the granule cell layer of the cerebellum [23,24,31,99,159,202,212,221]. The $GABA_B$ receptor is thought to be involved in the regulation of such functions as appetite, learning, mood, pain and neuroprotection; it has also been implicated in a variety of neurological disorders such as depression, anxiety, epilepsy and spasticity [99,100].

There are two main $GABA_B$ receptor subtypes and their classification is based upon anatomical location and physiological function. There are also presynaptic $GABA_B$ receptors and postsynaptic $GABA_B$ receptors. In several types of neurons, including hippocampal neurons, activation of postsynaptic $GABA_B$ receptors results in an outward K^+ current. This current is responsible for a prolonged, delayed inhibitory postsynaptic potential (late IPSC). Presynaptic $GABA_B$ receptors are responsible for modulation of a variety of neurotransmitters. This subtype also includes autoreceptors that, when activated, decrease Ca^{2+} influx into the cell. This decrease in Ca^{2+} influx leads to

decreased GABA release at the presynaptic terminal, which leads to excitatory disinhibition. A physiological role for $GABA_B$ receptors on excitatory nerve endings has not been established [46,83,99,100,143,180].

In addition to their ionotrophic effects, mediated by via ligand-gated ion channels, $GABA_B$ receptors also produce metabotropic (second messenger linked) effects. The $GABA_B$ receptor is coupled to K^+ or Ca^{2+} channels via guanine nucleotide-dependent proteins (G-proteins). These G-proteins activate second messengers that, in turn, modulate Ca^{2+} and K^+ channels, thereby controlling synaptic transmission and neuronal excitability [7,54,75,99,100,224].

Metabotrophically, activation of $GABA_B$ receptors can have two effects on the second messenger, cyclic AMP (cAMP). Activation of one type of $GABA_B$ receptor increases production of cAMP, via increasing adenylate cyclase activity. Another type of $GABA_B$ receptor is negatively coupled to adenylate cyclase activity--activation of this receptor decreases cAMP formation. Cyclic AMP then exerts its effects by activating protein kinase A (cAMP dependent protein kinase) which, in turn, is then free to phosphorylate its target proteins.

Additionally, $GABA_B$ receptors are also able to activate phospholipase A_2. This leads to release of arachadonic acid and its eicosanoid metabolites (fatty acid derivative signaling molecules). These eicosanoid metabolites have the ability to either act as second messengers within the cell or to diffuse to, and affect, adjacent cells. Furthermore, phospholipase C is activated by $GABA_B$ receptors. Phospholipase C hydrolyses phosphoinositides, leading to inositol trisphosphate (IP_3) formation. This

second messenger causes a release of intracellular Ca^{2+}. This leads to an activation of protein kinase C that, in turn, phosphorylates target proteins [99,100].

In the hippocampus, the question of whether $GABA_B$ receptor-mediated presynaptic inhibition is through modulation of K^+ and/or Ca^{2+} conductances is controversial. Some researchers have proposed that this inhibition is mediated through G-protein activation of presynaptic K^+ channels [220]. Others have proposed a mechanism involving direct modulation of Ca^{2+} channels [111].

Both $GABA_A$ and $GABA_B$ receptor types mediate inhibitory postsynaptic transmission in the brain. In the hippocampus, synaptically released GABA generates both fast ($GABA_A$ receptor-mediated) and slow ($GABA_B$ receptor-mediated) postsynaptic inhibition [145]. Early studies of the GABA-mediated inhibitory postsynaptic current (IPSC) described their spontaneous occurrence in chloride-loaded neurons; these spontaneous currents were called "synaptic noise." As a result of these studies, it was concluded that most, if not all, spontaneous inhibitory activity is a result of neuronal action potential firing. These studies also found that removal of action potentials (by the sodium channel blocker tetrodotoxin; TTX) led to a dramatic reduction in both the frequency and amplitude of spontaneous inhibitory activity [32,81]. Spontaneous activity was not, however, completely abolished by TTX. The small currents that remained were simply not detectable through the use of early sharp electrode methods.

The use of sharp microelectrodes presented researchers with a serious limitation—neuronal perforation significantly decreased resolution. By perforating the neuronal membrane with a sharp electrode, a relatively large electrical shunt was

introduced. This leak conductance rendered small amplitude changes in membrane current or voltage undetectable. Fortunately, this limitation of sharp electrode recording was overcome through the adaptation of a whole-cell patch recording method. The resolution of this technique enabled researchers to investigate previously undetectable small amplitude miniature spontaneous IPSCs. As a result, it was discovered that the firing of action potentials by principal neurons or by GABAergic interneurons is not necessary for the generation of miniature spontaneous IPSCs. It was subsequently concluded that continual spontaneous GABAergic activity is likely to be an important factor in the regulation of neuronal excitability. Furthermore, modulation of this activity has been found to be the mechanism by which several neurosteroids exert their effects on the CNS [32,151].

F. Inhibitory Neurosteroids and the GABA Receptor

Cashin and Moravek (1927) were the first to observe the rapid effects of steroids on neuronal excitability. They reported that intravenously injected cholesterol had an anesthetic effect [29]. Subsequent to that research, in 1941, Selye tested 75 steroids in order to determine if they had an anaesthetic effect. He reported the reversible sedative-anesthetic activity of PROG and deoxydorticosterone (DOC) and reported on the potency of their A-ring reduced metabolites as sedative-hypnotic agents [183]. Subsequently, in 1965, Atkinson et al. screened 168 pregnane steroids by injecting them intravenously into mice. They found that the vast majority of these steroids had sedative-hypnotic effects; however, 20 were observed to produce seizures [4].

Selye's initial observations of the rapid and reversible anesthetic effects of PROG and DOC and their ring-A reduced metabolites led to the development of a class of steroid anesthetics which were later introduced into clinical practice. These anesthetics included 21-hydroxy-5α-pregnane-3,20-dione (hydroxydione) and 3α-hydroxy-5α-pregnan-11,20-dione (alphaxalone). Steroid anesthetics were soon withdrawn from the market due to side-effects such as allergic reactions, involuntary muscle movement, occasional seizure onset and toxicity for newborns. These anesthetics also exhibited a lack of analgesic effects. As a result, interest in these compounds decreased [9,55,61,77,114,124,127,140]. Over the past several years, however, there has been a renewed interest in the mechanisms of action of steroid anesthetics [154,182].

Since anesthesia was rapidly induced by the steroid anesthetics, this seemed to preclude the assumption that all steroidal effects are genomic in nature. Further studies were conducted and it was soon demonstrated that the effect of the steroid anesthetics was not related to a slow-onset genomic mechanism of action [27,154]. It was initially assumed that the rapid effects of the steroid-anesthetics were "nonspecific" and that these agents acted, like cholesterol, by partitioning into membrane lipids and altering neuronal function. This hypothesis was supported by the results of early studies, which used liposomes to examine the effects of the anesthetic steroids, barbiturates, and volatile anesthetics on molecular mobility and local polarity [27,154,198,203]. Interestingly, however, it was observed that several of the more polar metabolites of the

steroids PROG and DOC had effects that were more potent than that of their more hydrophobic parent steroids [115,154]. This led researchers to investigate mechanisms of action other than lipid partitioning.

Schofield (1980) conducted one of the first studies linking steroids to the $GABA_A$ receptor complex. This study demonstrated that pentobarbital (a barbiturate) and alphaxalone both act to prolong GABA-mediated synaptic inhibition in the guinea pig olfactory cortex slice [178]. Further evidence supporting binding to the $GABA_A$ receptor complex was presented in the 1980s and 1990s. In one radioligand binding experiment, it was demonstrated that neurosteroids enhance [^{3}H] muscimol (a $GABA_A$ receptor agonist) binding to solubilized and purified $GABA_A$ receptor proteins in a preparation associated with a minimal amount of lipid. The results of this study supported binding to a specific site on the $GABA_A$ receptor complex and were contrary to the lipid-partitioning hypothesis [26,109]. Furthermore, the observation that intracellularly applied neurosteroids are inactive makes the possibility that neurosteroid effects on the $GABA_A$ receptor occur secondarily to lipid-partitioning unlikely. Intracellularly applied neurosteroids retain the ability to lipid-partition, but are not able to interact with the $GABA_A$ receptor through the usual mechanisms [109].

Various other radioligand-binding experiments were conducted. In one such experiment, it was found that alphaxalone (a steroid anesthetic), like barbiturates, increases binding of diazepam to the $GABA_A$ benzodiazepam binding site [64,65]. Further studies demonstrated more barbiturate-like actions of alphaxalone. This

compound was also found to: 1) displace the binding of [^{35}S]- t-butylbicyclophosphorothionate (TPBS), a non-competitive GABA antagonist and 2) enhance binding of muscimol (a $GABA_A$ receptor agonist) [65,123]. Furthermore, the presence of GABA was found to significantly increase steroid displacement of TBPS, suggesting that GABA facilitates steroid binding to the $GABA_A$ receptor complex [108].

Although study results suggested a barbiturate-like mechanism of action, researchers continued to question exactly where, on the $GABA_A$ receptor complex, steroid binding occurred. Initial studies with the steroid anesthetic alphaxalone showed that the benzodiazepine antagonist Ro15-1788 did not block its actions; thus, binding at the benzodiazepine recognition site was deemed unlikely [38,64]. Studies using pregnenolone and pregnanedione were also conducted. In these studies Ro15-1788 also failed to block steroid potentiation of the $GABA_A$ Cl^- current [28,37,64,188].

After initial studies, which mostly involved alphaxalone, researchers began studying the effects of other steroids on the nervous system. Due to their structural similarity to alphaxalone, the naturally occurring 3α-hydroxy ring-A reduced pregnane steroids were a logical first choice. The 3α-hydroxysteroids, which include the major metabolites of PROG and DOC, allopregnenolone and allotetrahydroDOC, have become the most extensively studied of the neurosteroids; they are also among the most active. To date, considerable evidence has accumulated which demonstrates that the rapid effects of many neurosteroids is due to their selective, high affinity interaction with the $GABA_A$ receptor complex [2,14,15,16,28,47,57,58,65,89,105,109,112,121,122,123,124,125,126, 127,135,137,139,148,153,154,160,162,175,184,185,201,203,204].

In radioligand studies, the 3α-hydroxysteroids allopregnenolone, allotetrahydroDOC, PREG and pregnanedione were demonstrated to enhance benzodiazepine binding to the $GABA_A$ receptor complex, as do the anesthetic barbiturates and alphaxalone. Also like the barbiturates and alphaxalone, allopregnenolone and allotetrahydroDOC were also found, in high concentrations, to directly stimulate $^{36}Cl^-$ uptake in a manner which is sensitive to picrotoxin [4,38,39,154]. Furthermore, tetrahydroprogesterone (THP) and tetrahydrodeoxycorticosterone (THDOC) have been demonstrated to have the ability to displace t-butylbicyclophosphorothionate (TBPS) from the Cl^- channel with greater affinity than barbiturates [57,123,176].

Research from electrophysiological studies has revealed, among other things, that the 3α-hydroxysteroids PREG, pregnanedione, THP, THDOC, allopregnenolone and allotetrahydroDOC act in a similar manner to the anesthetic barbiturates; however, their effects are not completely the same. Unlike barbiturates that, in addition to their effects on the $GABA_A$ receptor complex, also antagonize glutamate-mediated currents, the 3α-hydroxysteroids are specific for the $GABA_A$ receptor. Allopregnenolone, allotetrahydroDOC, and other neurosteroids, have been demonstrated to potentiate GABA-induced Cl^- currents in a reversible, dose dependent, manner [57,154].

Electrophysiological studies were conducted in an attempt to elucidate the mechanism of action by which neurosteroids modulate the $GABA_A$ receptor complex. The voltage clamp technique was utilized to study the effect of PREG and androsterone on the kinetics of IPSCs, evoked by the synaptic release of GABA, in rat hippocampal

neurons. It was found that the decay phase of the IPSC was significantly increased. This suggested a mechanism of action involving an increase in the mean time that the $GABA_A$ Cl^- channel was open [109].

In order to determine the effect of neurosteroids on the kinetics of the $GABA_A$ Cl^- channel, further studies with PREG, androsterone, allopregnenolone and allotetrahydroDOC, were conducted. These studies contradicted the hypothesis that steroid binding to the $GABA_A$ receptor complex acted to prolong the opening of single Cl^- channels. Instead these studies demonstrated that, like the barbiturates, steroid binding to the $GABA_A$ receptor complex acts to: 1) increase both the frequency of Cl^- channel opening and 2) change the distribution of single-channel events in a manner which favors channel openings of longer duration. In other words, steroid binding to the $GABA_A$ receptor complex increases the probability that a Cl^- channel will enter a long-duration, naturally occurring, open state [38,109,123,127,136,154,176,204].

In relation to the heterogeneity of the $GABA_A$ receptor complex, structure-activity studies have revealed that the presence of a 3α-hydroxy group within the A-ring of neurosteroids is necessary in order for these compounds to have an allosteric effect on the $GABA_A$ receptor complex [58,136]. It has also been found that different $GABA_A$ receptor isoforms exhibit different sensitivities for neurosteroids [112,121,136,185]. In general, it has been found that neurosteroids interact primarily with $GABA_A$ receptors that are composed of assemblies of α_1-β_1 and γ_2 subunits or those composed solely of β_2 subunits. A β subunit is required in order for a neurosteroid to interact with the $GABA_A$ receptor [135,160]. In one study, it was found that the efficacy of allosteric coupling was

greatly reduced by removal of the α subunit. Furthermore, this study concluded that the removal of the γ subunit decreased the affinity of the $GABA_A$ receptor complex for allopregnenolone; affinity of the $GABA_A$ receptor complex for alphaxalone was not only dependent upon the presence of the γ subunit, but also upon the subtype of the γ subunit [121]. Other studies have demonstrated that neurosteroid modulation of the $GABA_A$ receptor complex is inhibited by δ and ε subunits [136,161,162].

Despite intensive research, the exact location of neurosteroid binding on the $GABA_A$ receptor complex has not been determined. To date, there has been no convincing evidence that neurosteroid binding occurs at the barbiturate binding site; however, there has been significant evidence that binding occurs at a site separate from the barbiturate or benzodiazepine binding sites. Many researchers have suggested a novel "neurosteroid binding" site; however, this has yet to be convincingly demonstrated [38,74,105,204]. Further research in this area is needed.

G. Excitatory Neurosteroids and the GABA Receptor

Not all neurosteroids exert inhibitory effects on the CNS. It was first noticed in the late 1950s and early 1960s that certain steroid hormones have convulsant or proconvulsant properties. It was noticed that these effects, like those of the steroid anesthetics, occurred quite rapidly. Again, it was thought that these rapid effects were unlikely to involve a genomic mechanism of action. Studies were undertaken to determine if the convulsant neurosteroids have a mechanism of action that was similar to that of the steroid anesthetics [4,74,118,119,154,194].

One of the most interesting effects of a convulsant steroid on the GABA receptor is that of PS. At nanomolar concentrations, PS exerts a small potentiating effect on GABA evoked currents. At micromolar concentrations, however, PS exerts an excitatory effect by acting: 1) at the postsynaptic site to inhibit the $GABA_A$ receptor-mediated Cl^- current via a reduction of channel opening frequency and 2) at the presynaptic site to directly reduce the frequency of spontaneous GABA release [14,38,126,137,148,196]. In a recent study, it was demonstrated that PS reduces Cl- channel opening frequency by enhancing $GABA_A$ receptor desensitization [184]. Further studies have confirmed that PS can act to agonize or inhibit the $GABA_A$ receptor. In one such study, it was demonstrated that PS acts both to slightly increase benzodiazepine binding and to inhibit barbiturate-induced enhancement of benzodiazepine binding [127]. Pregnenolone sulfate also acts on other neurotransmitter receptors [14,124,127,154,201].

Radioligand studies were conducted, using PS, in an attempt to determine the mechanism by which this compound modulates the $GABA_A$ receptor complex. It has been found that PS increases binding to [^{3}H]- muscimol, a $GABA_A$ receptor agonist, at nanomolar concentrations; [^{3}H]- muscimol binding is decreased at micromolar concentrations. Pregnenolone sulfate also acts, at micromolar concentrations, to inhibit [^{35}S]TBPS (a $GABA_A$ antagonist) binding. Further research has demonstrated that PS exerts its antagonist effect on the $GABA_A$ receptor complex primarily via binding at the picrotoxin-TBPS recognition site [105,124,125,127].

Glucocorticoids, such as cortisol, cortisone and their reduced derivatives, have a complex mechanism by which they exert excitatory effects via interaction with the

$GABA_A$ receptor complex. Majewska et al. (1985) prepared synaptosomes from various brain regions of adrenalectomized rats and found that glucocorticoids, like PS, exert a mixed effect on GABA binding to the $GABA_A$ receptor complex. At nanomolar concentrations, PS and glucocorticoids increase GABA binding, whereas, at micromolar concentrations, GABA binding was inhibited [122].

In radioligand binding studies, glucocorticoids, like PS, have also been found to exert a mixed agonistic/antagonistic effect on the binding of [^{35}S]-TBPS. Like the $GABA_A$ receptor antagonist bicuculline, glucocorticoids increase binding of [^{35}S]-TBPS at nanomolar concentrations; at micromolar concentrations, [^{35}S]-TBPS binding was inhibited [127]. Additional research is needed in order to elucidate the mechanism of action by which glucocorticoids exert their modulatory effects on $GABA_A$ receptors.

One of the best-studied convulsant steroids is 3α-hydroxy-16-imino-5-beta-17-aza-androstan-11-one (RU5135), a synthetic steroid derivative. This compound, despite the fact that it is a 3α-hydroxylated derivative, has been found to cause sedation in rats. In contrast, convulsions occur when sublethal doses are administered. As one of the most potent $GABA_A$ receptor antagonists yet detected, RU5135 has an affinity for the $GABA_A$ receptor that is several hundred times greater than bicuculline (K_A value in the low nanomolar range). It has been suggested that RU5135 and bicuculline interact with a similar, if not identical, binding site. Additionally, RU5135 has also been demonstrated to modulate other neurosteroid receptors, such as strychnine-sensitive glycine receptors [37,89,123,146,154,188].

Studies with excitatory neurosteroids have furthered the search for a specific neurosteroid binding site on the $GABA_A$ receptor. Studies with these compounds have led some researchers to suggest that there may be multiple neurosteroid binding sites [127,129,149,153,192]. In fact, recent research strongly suggests that sulfated and unsulfated neurosteroids may act at separate sites [47,153,192]. In one study, steroid sulfates were tested to determine their effect on muscimol-induced chloride uptake by GABA agonists. It was noted that the $GABA_A$ modulatory effect (agonist or antagonist) is dependent upon the orientation of the A-ring sulfate moiety; 3α-hydroxysteroid sulfates were found to be excitatory, whereas 3β-hydroxysteroid sulfates were found to be inhibitory [47]. In another study, sulfated and unsulfated neurosteroids such as DHEA and DHEA-S were co-applied to neurons. In this study, the $GABA_A$ receptor was modulated simultaneously by both steroids, suggesting separate sites of action [149].

H. Estrogens

High-dose estrogens are well known convulsants [16,191,197,214,215,216,218]. The rapid effects of estrogens on the CNS have been described in various electrophysiological studies. In the hippocampus, estrogen priming has been found to lower seizure threshold [215]. Other studies have demonstrated that 17β-estradiol increases field potentials evoked via stimulation of the Schaffer collaterals. Wong and Moss (1991) found that, in some hippocampal CA1 pyramidal neurons, 17β-estradiol exerts a depolarizing effect [214].

It has been suggested that estrogens exert their convulsant effects, at least in part, via an agonistic effect on a subset of glutamate receptors known as α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) receptors [190,191,214,215]. In one study, it was demonstrated that 17β-estradiol, but not 17α-estradiol, causes an increase in the Schaffer collateral-activated excitatory postsynaptic potential (EPSP). This effect occurred in the presence of 2-amino-5-phosphovalerate (APV), an N-methyl-D-aspartate (NMDA) antagonist, and was found to be reversible. The effect was also blocked by 6-cyano-7-nitroquinoxaline-2,3-dione, an AMPA antagonist. In this study, ionophoresis was also used to apply glutamate and the EPSPs that were evoked through this method were also potentiated by 17β-estradiol. These results suggested a postsynaptic site of action [215].

One important effect of estrogens on the CNS is their role in synaptic plasticity. Estrogens have been demonstrated to induce the formation of dendritic spines. It is proposed that these dendritic spines, occupied by excitatory synapses, are induced to form after NMDA receptors are activated by estrogens [79,158,174,217].

I. Estrone-3-Sulfate--A Novel Excitatory Neurosteroid

Estrone is one of the three main estrogens derived from androstenedione. It is converted, in a reversible fashion, to estradiol. Estrone is secreted into the urine in several forms, including estrone-3-sulfate (E-3-S). Estrone is found in both the male and female rat; however, estrogen sulfotransferase, which converts estrone into E-3-S , is not present in the female rat brain. To date, no physiological role has been demonstrated for

E-3-S; however, this steroid hormone has been used to treat Alzheimer's disease and has been shown to improve the memory, orientation and calculating ability of these patients [22,78].

Estrone-3-sulfate is an epileptogenic metabolite of estradiol that has been demonstrated to exert rapid CNS effects. It has been shown to rapidly produce epileptiform activity both *in vivo* and in the *in vitro* hippocampal slice [128]. The rapid effects of E-3-S suggest a non-genomic mechanism of action.

Previous findings in this lab indicate that E-3-S (in micromolar concentrations) exerts rapid effects in the hippocampus. It was noted that bath application of E-3-S produced multiple population spikes in the Schaffer collateral-stimulated field potential within 15 minutes. This effect was found to be dose-dependent and reversible. Furthermore, the effect was diminished, but not blocked completely, by APV [40,156].

Additional studies, in this laboratory, demonstrated that E-3-S potentiates the orthodromically-evoked inward synaptic current in rat hippocampal CA1 cells. Following this observation, kynurenic acid was utilized to block glutamate-mediated excitatory postsynaptic currents (EPSCs) in order that IPSCs could be isolated and studied. Electrical stimulation, under these conditions, evoked a current which had a reversal potential of -60 mV and which was blocked by E-3-S. When the neurons were chloride-loaded via patch electrodes filled with KCl, the IPSC increased in amplitude and became inward, thus supporting the hypothesis that this was a chloride current. This current was eliminated both by E-3-S and by bicuculline (a $GABA_A$ antagonist) [30,156].

The above observations led to the current set of studies, which seek to further elucidate the mechanism of action for this novel convulsant neurosteroid. Specifically, these studies were designed to test the hypothesis that E-3-S acts to reduce the GABA-evoked IPSC at the postsynaptic site, in a dose-dependent manner [189].

II. MATERIALS AND METHODS

A. Hippocampal Slice Preparation

Hippocampal slices (400 μM) were prepared from 18-31 day old Sprague Dawley rats (n=62, Simonsen Labs, Gilroy, CA) [181]. Rats were housed under controlled temperature (73 ± 3 °F) and lighting (12 hours on/off, 325 lux, fluorescent light source) conditions. They were provided with food (Harlan Teklad Rodent Diet #2018) and water *ad libidum*.

For each experiment, a rat was placed into a chamber and anesthetized with methoxyflurane. The methoxyflurane was located in a separate (glass) container, through which air was pumped. The flow of air emerging from this container was directed into the chamber in which the rat was located. After the rat was anesthetized (stage III, plane II), it was decapitated and its brain was removed.

In order to remove the brain, a cut was made along the midline of the skull, the bone was peeled away with forceps, and a plastic spatula was used to gently sever any connections. The brain was immediately placed in icy (~0C), oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) [181]. ACSF contained (mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 10 dextrose. All chemicals used in these experiments were obtained from Sigma Chemical Company (6050 Spruce St., St. Louis, MO).

While submerged in icy (~0C), oxygenated ACSF, the brain was bisected and the cerebellum and lower brain stem were removed. The dorsal surface of the cerebral hemisphere was removed by making a cut dorsal to the corpus callosum, perpendicular to

the midsagittal plane and parallel to the long axis of the midbrain. The brain was glued, cut dorsal surface down, to the pedestal of a vibratome (Campden Vibroslice, model NVSL, World Precision Instruments, Inc., 175 Sarasota Center Boulevard, Sarosota, FL) using cyanoacrylic glue (Crazy Glue®, Elmer's Products, Inc., 180 E. Broad St., Columbus, OH) [181]. The pedestal of the vibratome was immersed in icy (~0C), oxygenated ACSF and 400 μM transverse slices of the hippocampus were prepared. A thickness of 400 μM was deemed to be optimal. With thinner slices, there is a decrease in the number of surviving neurons--presumably due to the fact that neuronal death occurs rapidly following dendritic damage. A thicker slice would have decreased visibility of the cell layers. Excess tissue surrounding the hippocampus was removed manually and slices were placed in an oxygenated holding container containing room temperature (~21-23C) ACSF for incubation. The time from decapitation until slices were placed in the incubation container was less than 10 minutes [181].

Hippocampal slices were incubated for a minimum of one hour prior to use. The incubation period allows activation of enzymes that help to remove surface debris, which is present due to tissue damage that occurs during slicing [181]. During experiments, hippocampal slices were transferred, as needed, to an air/fluid interface chamber (Tap Plastics, San Jose, CA, custom made, see Figure 3). The air/fluid interface chamber allows for excellent visibility while placing electrodes. Also, recording electrode contact with the bathing solution (evident due to electrical fluctuations seen with the patch recorder) coincides with contact with the surface of the brain slice--this allows determination of electrode depth in the tissue during experimentation. Finally, the

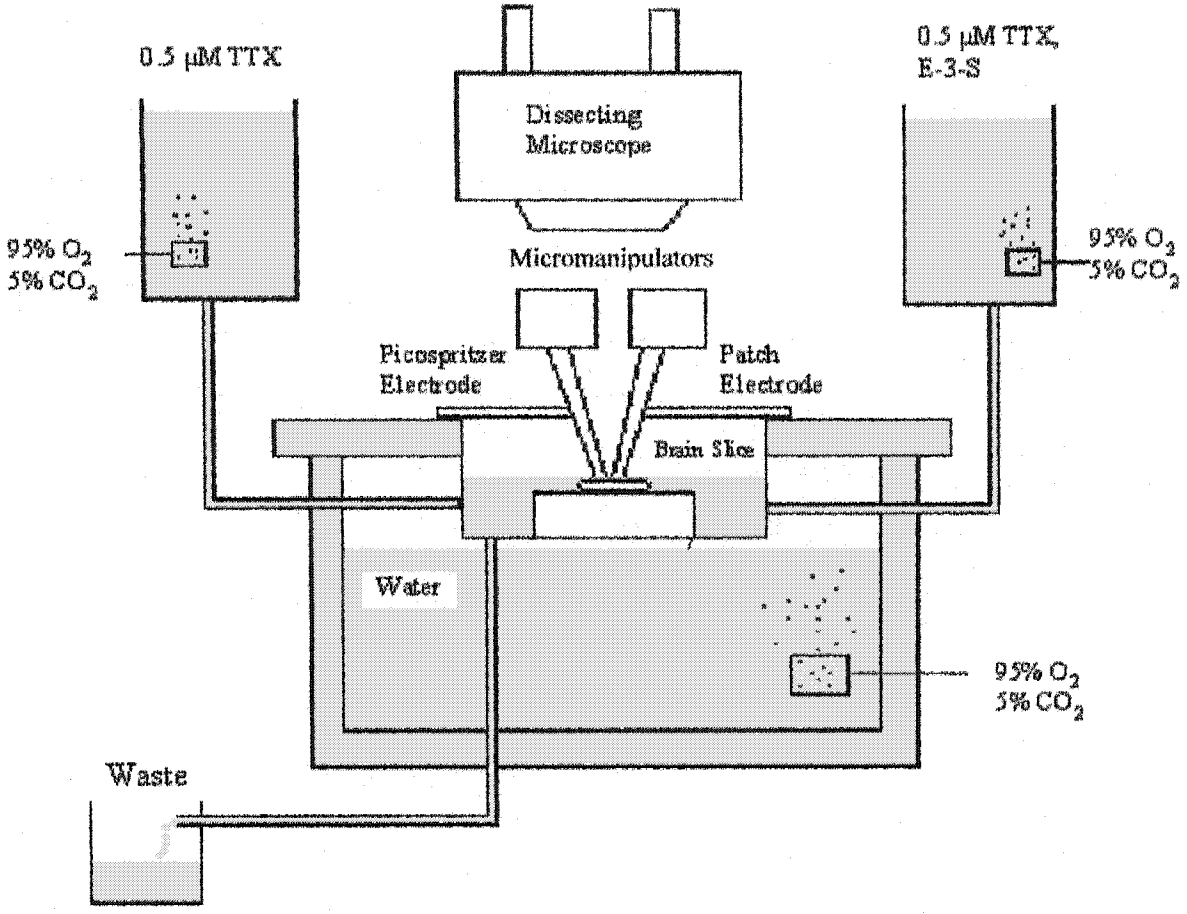

Figure 3: Air/Fluid Interface Chamber. During experiments, hippocampal slices were placed in an air/fluid interface chamber. Slices were bathed with 0.5 μM Tetrodotoxin (TTX; upper left-hand container) and control measurements were taken. The bath solution was then changed to a secondary source (upper right-hand container) which contained 0.5 μM TTX and 4.5, 45 or 450 μM E-3-S. ACSF was the diluent used to make bathing solutions. The level of the bath solution was maintained at an even level with the top of the hippocampal slices. ACSF was used as a diluent and bath solutions were oxygenated with a mixture of 95% O_2, 5% CO_2. As an additional measure to oxygenate the hippocampal slices and prevent drying, humidified 95% O_2, 5% CO_2 was bubbled through a chamber containing water (located below the hippocampal slice); this generated a humidified air source, which was directed over the slices. Hippocampal slices were visualized with a dissecting microscope and two borosillicate electrodes were placed into the CA1 pyramidal cell layer. One electrode contained 5mM GABA (diluent was ACSF) and was attached to a picospritzer; upon pressure ejection of 5mM GABA, an inhibitory post synaptic current was induced. The other electrode (the recording electrode) was attached to the patch clamp amplifier and contained a K+ gluconate/KCl filling solution. Electrodes were placed with the aid of micromanipulators.

air/fluid interface chamber minimizes capacitative coupling of the recording electrode to the bath solution, optimizing recording conditions [101]. During experiments, slices were perfused with a control bathing solution consisting of 0.5 μM tetrodotoxin (TTX; ACSF used as a diluent) or with a bathing solution consisting of 0.5 μM TTX and 4.5, 45 or 450 μM E-3-S (ACSF used as a diluent). Slices were perfused at a flow rate of 0.3 to 0.5 ml/min.

B. Whole Cell Recordings

Whole cell recordings were accomplished in a Faraday cage which contained only those objects required in close proximity to the brain slices: the air/fluid interface chamber, micromanipulators, bathing solutions and dissecting microscope [101]. Monoppolar recording was utilized--the recording electrode was referenced to a ground that was in contact with the fluid present in the air/fluid interface chamber. The Faraday cage surrounding the air/fluid interface chamber was also grounded in order to block electrical noise.

The hippocampus is arranged with a lamellar and laminar organization and circuitry and cell layers of this preparation have been well established. A dissecting microscope was utilized to visualize the CA1 pyramidal cell layer of the hippocampal slices--pyramidal and granule cell bodies are densely packed in cell layers that can be seen with low magnification [101]. While visualizing the CA1 pyramidal cell layer with the dissecting microscope, micromanipulators were used to aid initial electrode placement.

CA1 pyramidal cells were obtained (Axopatch-1D Patch Clamp and CV-4 1/100 headstage, Axon Instruments, Foster City, CA) using the blind, whole-cell patch clamp technique [18]. Data were collected, at room temperature, from cells that exhibited spontaneous synaptic activity and that had a resting membrane potential of at least –50 mV, a holding current below 0.20 nA and an initial access resistance below 80 MΩ.

Borosilicate recording electrodes (FHC, 9 Main St., Baudoinham, ME) were pulled (Flaming Brown Micropipette Puller P-80 PC, Puller-Sutter Instrument Co., San Rafael, CA) to obtain a resistance between 2-8 MΩ. These electrodes were filled with a solution containing (mM): 15 NaCl, 62.5 K^+ Gluconate, 62.5 KCl, 10 HEPES, 2 $MgCl_2$, 11 EGTA and 1 $CaCl_2$. The pH of the electrode filling solution was adjusted to 7.3 using 50% KOH. Tetrodotoxin (0.5 μM) was applied in bath form to prevent Na^+-dependent action potentials and to block any indirect effects of E-3-S.

Cells were voltage clamped at –70 mV. Whole cell conductance was calculated, in voltage clamp mode, by measuring the current response to a –5.0 mV, 10 Hz square wave pulse applied via the patch electrode (R=E/I, 1/R=G). A single IPSC was evoked every 3 minutes via pressure ejection (2-20 psi, 10-100 ms) of 5 mM GABA (prepared using ACSF as a diluent) using a Picospritzer II (General Valve Corp, Fairfield, NJ). The GABA was delivered through a borosilicate electrode (FHC) pulled to have a tip diameter between 1 and 5 μM. The GABA electrode was placed as close as possible to the recording electrode. Pressure and duration of the GABA pulse were adjusted in order to obtain an IPSC that had an amplitude of between 50-300 pA as well as the fastest rise time and shortest duration possible.

After a stable IPSC (amplitude change of less than 20% over 9 min.) was obtained, the bath solution was changed to a secondary source containing 4.5 45 or 450 μM E-3-S (ACSF was used as the diluent in making these solutions). In control experiments, no E-3-S was present in the secondary bath source. In some instances, after application of E-3-S for 30 min., the bath solution was changed back to the primary source (0.5 μM TTX) in order to determine if the effect of E-3-S could be reversed.

The input of a -5.0 mV square wave pulse, applied via the patch electrode, allowed for the measurement of resting conductance and GABA-activated conductance. This square wave pulse was introduced approximately 5 seconds prior to GABA-application. GABA was then applied and an IPSC ensued. Approximately 5 seconds after the end of the IPSC (after return to baseline), input of the -5.0 mV square wave pulse was stopped. The resting conductance, the current response to the square wave pulse prior to GABA application, is indicative of the number of open channels while the cell is at rest. The GABA-activated conductance, the current response to the square wave pulse at the peak of the outward current flow during an IPSC, is indicative of the number of channels open at that time.

C. Data Analysis

During experiments, data were collected in analog form on video tape using a modified video casette recorder (Panasonic Omnivision PV-4760 adapted by AR Vetter Co, Rebersburg, PA). Data were later digitized (1K @ 800μsec) using Computerscope

software (RC Electronics, Inc., 1988). Digitized data were analyzed using DATA-PAC II software (RUN Technologies, Inc., 1992).

The amplitude of GABA-evoked IPSCs was measured at the peak of the outward current. Resting conductance and GABA-activated conductance were calculated by measuring the current response to the -5.0 mV square wave pulse prior to GABA application and at the peak of the outward current following GABA application, respectively (V=IR, C=1/R). Figure 4 illustrates the points at which these measurements were made.

Data from multiple experiments were averaged within the four treatment groups (controls, 4.5, 45 and 450 μM E-3-S). Averaged data were statistically analyzed prior to E-3-S exposure and at 6 min. and 24 min. after E-3-S exposure, using a one-way analysis of variance and paired t-tests. Due to the potential therapeutic significance of these experiments, the level of significance was set at $p<0.01$.

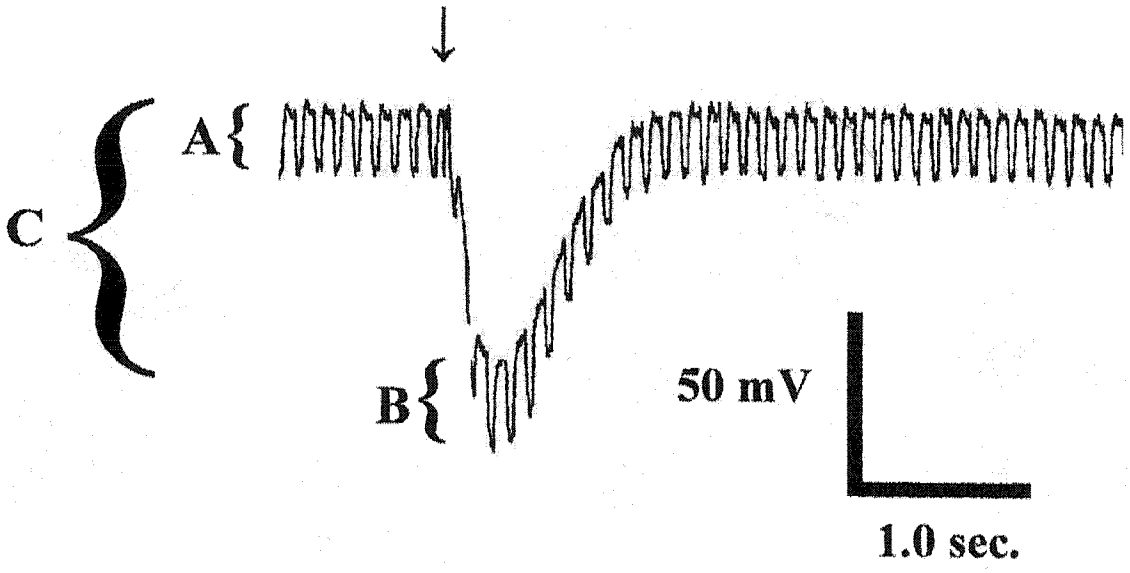

Figure 4: An IPSC with a -5.0 mV square wave pulse. Inhibitory post-synaptic currents (IPSCs) were evoked via pressure ejection of 5 mM GABA (↓). Prior to pressure ejection of GABA, a -5.0 mV square wave pulse was introduced via the patch electrode. This square wave pulse allowed for the measurement of resting conductance and GABA-activated conductance. To calculate resting conductance, the current response to the square wave pulse (A) was measured after the input of the -5.0 square wave pulse, but prior to GABA application. To calculate the GABA-activated conductance, the current response to the square wave pulse was measured at the peak of the inward current (B). Following these measurements, resting and GABA-activated conductances (V=IR; C=1/R) were calculated. GABA-activated IPSC amplitude was also measured (C). All measurements were conducted on digitized data, using DATA-PAC II software.

III. RESULTS

Data concerning the effects of E-3-S on the amplitude of the GABA-activated IPSC are summarized in Table 3 and Figures 5, 6, and 7. Bath application of 450 μM E-3-S resulted in a rapid and significant decrease in GABA-activated IPSC amplitude. The first significant effect occurred after 6 min. (paired t-test, p=0.0001), with a 45.5% mean reduction in IPSC amplitude. The IPSC continued to decrease in amplitude for approximately 24 min., when the response began to stabilize at approximately 17% of the pre-E-3-S value (n=14; paired t-test, p=0.0001).

Bath application of 45 μM E-3-S for 24 min. resulted in a mean 63.7% reduction in IPSC; however, this decrease was not statistically significant at the 0.01 level (n=14; paired t-test, p=0.013). No significant change in IPSC amplitude was seen after 24 min. of exposure to 4.5 μM E-3-S (n=8; paired t-test, p=0.246). No significant change was observed in control IPSCs (n=10; paired t-test, p=0.140).

Bath application of E-3-S made the reversal potential of the GABA-activated IPSC more negative. Using the Nernst equation, it was calculated that the Cl^- current, under these experimental conditions, should have a reversal potential of approximately -12 mV. The K^+ reversal potential was calculated to be -85 mV. During the course of these experiments, the reversal potential of the GABA-activated IPSC was measured and it was found to reverse at a mean of -17 mV (n=7, SD=10). The reversal potential of the GABA-activated IPSC after bath application of 4.5 μM E-3-S for 30 min. was similar to the reversal potential of control cells; the remaining current also reversed at a mean of

–17 mV (n=2, SD=5). Application of 45 μM E-3-S for 30 min. resulted in an IPSC with a reversal potential which averaged -36 mV (n=4, SD=5.8). After application of 450 μM E-3-S for 30 min., the reversal potential of the remaining current averaged –38mV (n=7, SD=4.7). In summary, with increasing concentrations of E-3-S, the reversal potential became more negative, moving away from the calculated Cl^- reversal potential (-12 mV) and towards the calculated K^+ reversal potential (-85 mV). These data are summarized in Figure 8.

Statistical analysis revealed no significant change in resting conductance after 24 min. of exposure to E-3-S at 4.5 (n=7, paired t-test, p=0.755) 45 (n=5, paired t-test, p=0.030) or 450 μM E-3-S (n=7, paired t-test, p=0.192) or in controls (n=7, paired t-test, p=0.425). Data concerning the effect of E-3-S exposure on resting conductance are summarized in Table 4 and Figures 9 and 10. All conductance data are expressed as nanosiemens (nS), the MKS (meter, kilogram, second) unit of conductance. A nanosiemen is equal to an inverse ohm and is sometimes called a "mho."

Exposure to E-3-S caused a significant change in GABA-activated conductance following 24 minutes of exposure to 450 μM E-3-S (n=7, paired t-test, p=0.001). No significant change in GABA-activated conductance occurred in cells exposed to 4.5 μM (n=8, paired t-test, p=0.405) or 45 μM E-3-S (n=5, paired t-test, p=0.209). Furthermore, there was no decrease in GABA-activated conductance in control cells (n=7, paired t-test, p=0.050). Data concerning the effect of E-3-S exposure on GABA-activated conductance are summarized in Table 5 and Figures 11 and 12.

Table 3: E-3-S Reduces GABA-Activated IPSC Amplitude.

	IPSC amplitude (before E-3-S)	IPSC amplitude (6 min. after E-3-S)	IPSC amplitude (12 min. after E-3-S)	IPSC amplitude (24 min. after E-3-S)
Control	100.8 ±37.7 pA (n=11)	84.2±45.3 pA (n=11)	79.7±35.4 pA (n=10)	81.1±43.7 pA (n=10)
4.5μM E-3-S	80.3±44.6 pA (n=9)	69.3±49.8 pA (n=9)	58.5±43.6 pA (n=8)	70.7± 51.8 pA (n=8)
45 μM E-3-S	69.2±34.3 pA (n=6)	46.7±27.7 pA (n=6)	31.0±17.7 pA (n=6)	25.1±9.6 pA (n=5)
450 μM E-3-S	101.4±37.8 pA (n=16)	55.3±27.2 pA* (n=16)	35.3±26.2 pA* (n=14)	17.6*±13.7pA* (n=14)

These data show how mean IPSC amplitude was affected by bath application of E-3-S (4.5, 45 and 450 μM) for 6, 12 and 24 minutes. The mean IPSC amplitude was measured at the peak of the outward current. Mean current amplitude (± one standard deviation) is shown. In the case of controls, the secondary bath solution did not contain E-3-S.
* Significantly different from control at $p<0.01$.

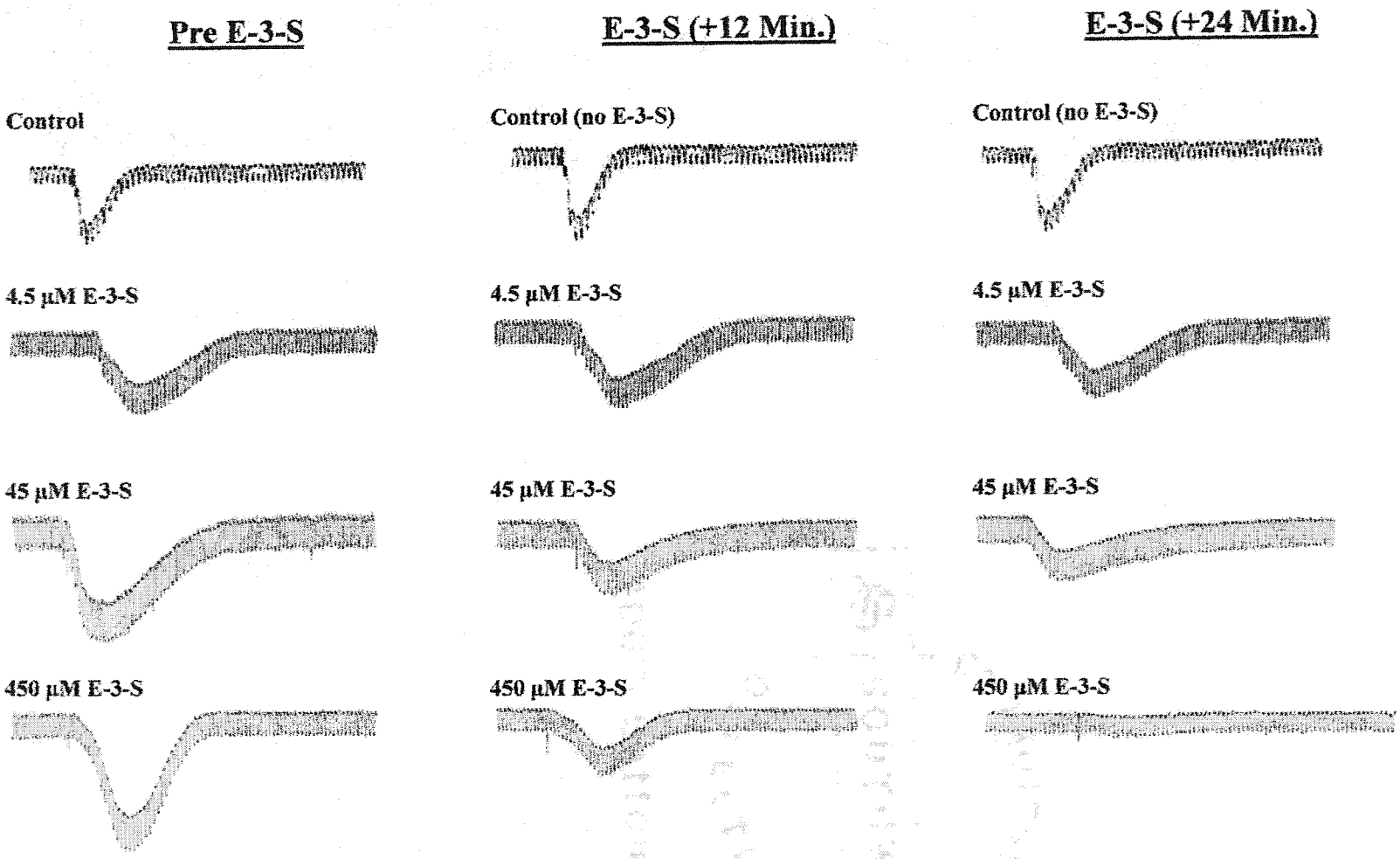

Figure 5: IPSC tracings. Application of 450 µM E-3-S caused a statistically significant ($p<0.01$) reduction in GABA-activated IPSC amplitude (beginning at 6 min.). Additionally, 450 µM E-3-S caused a reduction in GABA-activated conductance at 24 min. This appears as a decrease, over time, in the amplitude of the current response to a -5 mV, 10 Hz hyperpolarizing pulse. GABA-activated conductance was measured at the peak of the inward current (the IPSC) that followed GABA application. The pulse was applied beginning approximately 5 sec. prior to GABA application and appears, in the above tracings (obtained from 3 separate cells), as downward spikes. Stimulus artifacts indicate GABA application.

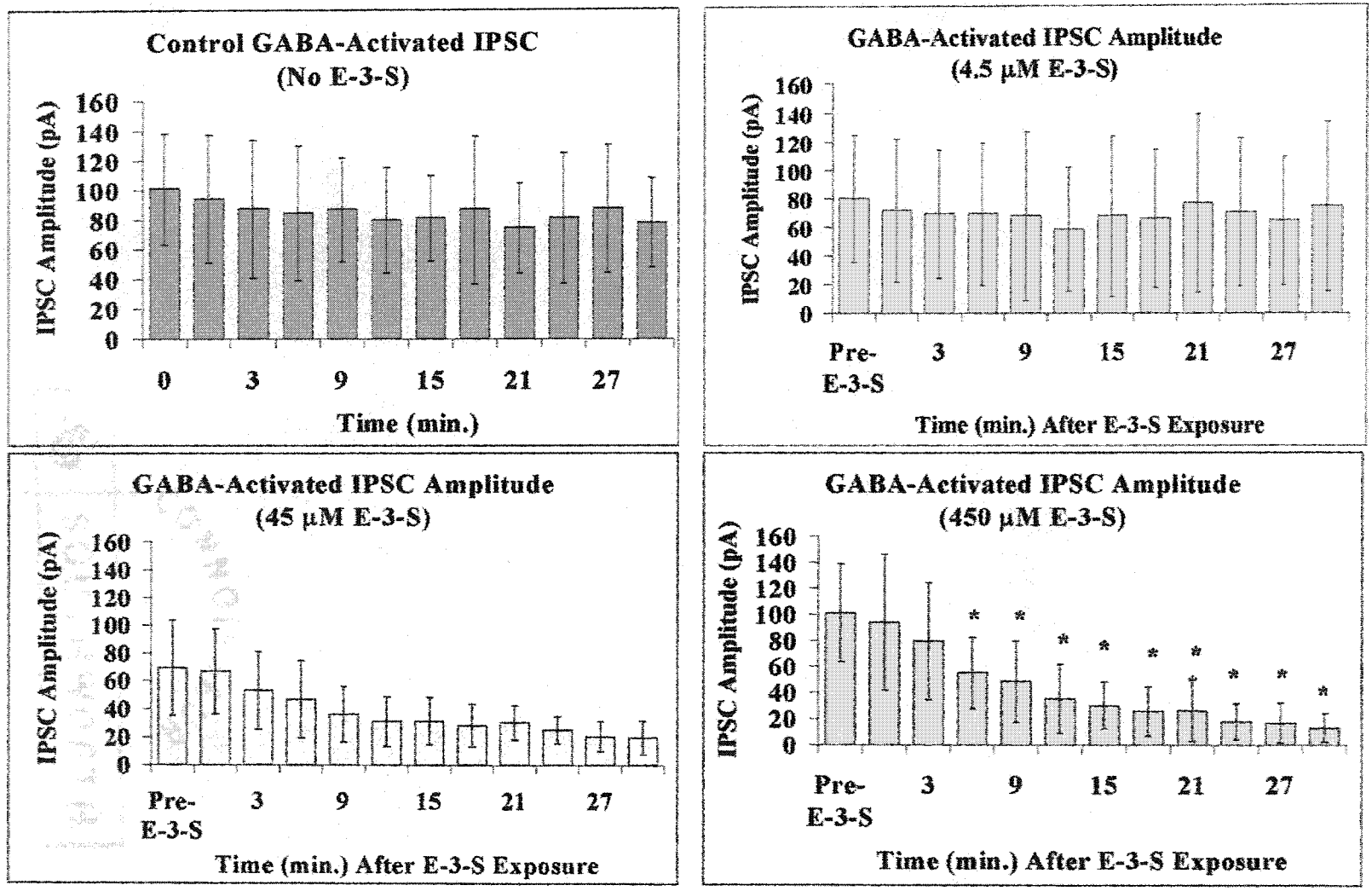

Figure 6: E-3-S causes a dose-dependent reduction in GABA-activated IPSC. Bath application of 450 μM E-3-S (n=14) resulted in a statistically significant decrease in IPSC amplitude, first seen after 6 min. No significant effect was seen in controls (n=10) or with 4.5 (n=8) or 45 μM (n=5) E-3-S. Error bars represent ± one standard deviation. * Statistically significant ($p<0.01$)

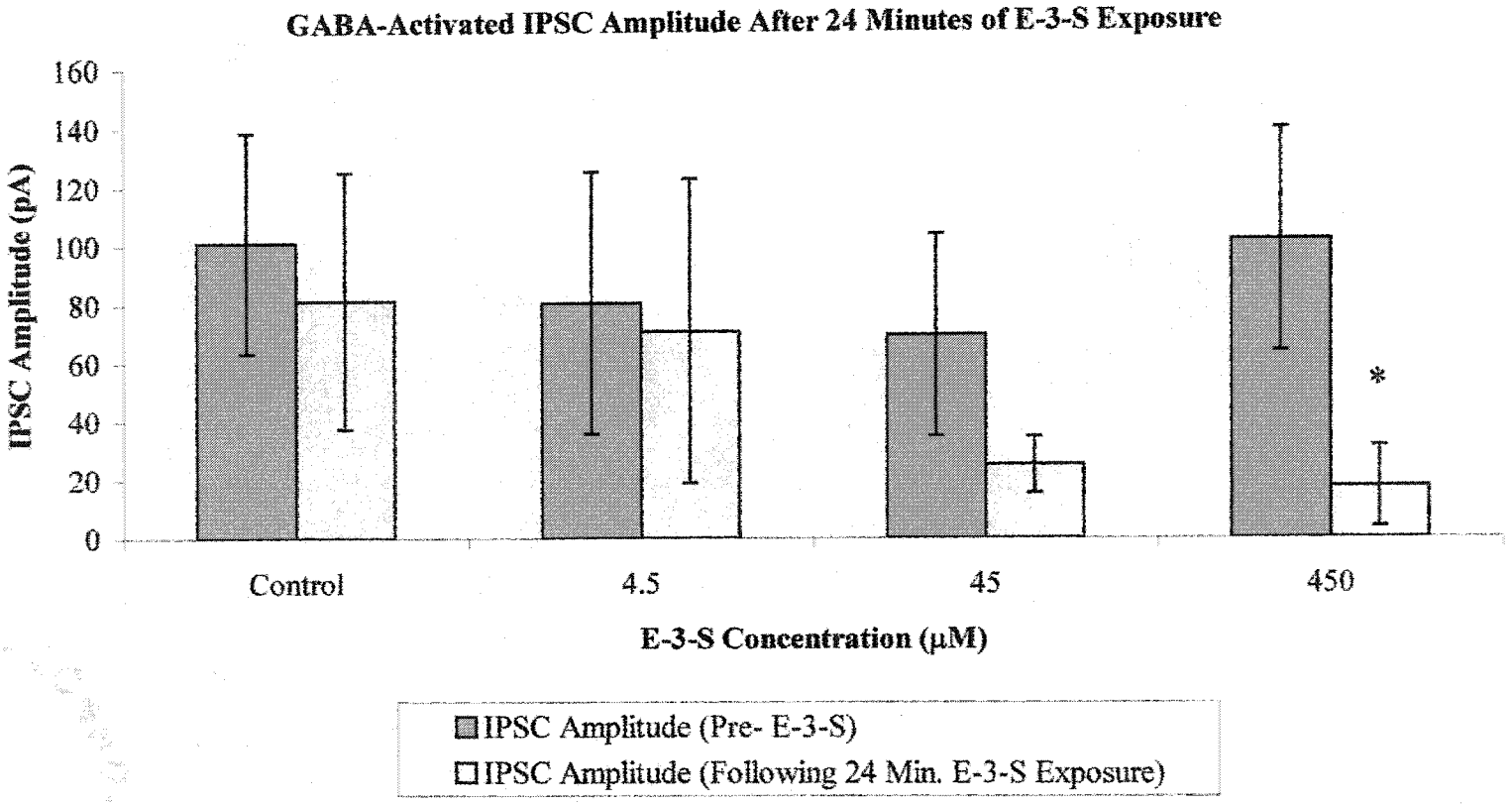

Figure 7: E-3-S causes a dose-dependent reduction in IPSC amplitude. Following 24 minutes of exposure to 450 μM E-3-S (n=14), a significant reduction in GABA-activated IPSC was observed. No significant effect was seen using 4.5 (n=8)or 45 μM (n=5) E-3-S or in the controls (n=10, no E-3-S present in secondary bath source). Error bars represent ± one standard deviation around the mean. * Statistically significant ($p<0.01$)

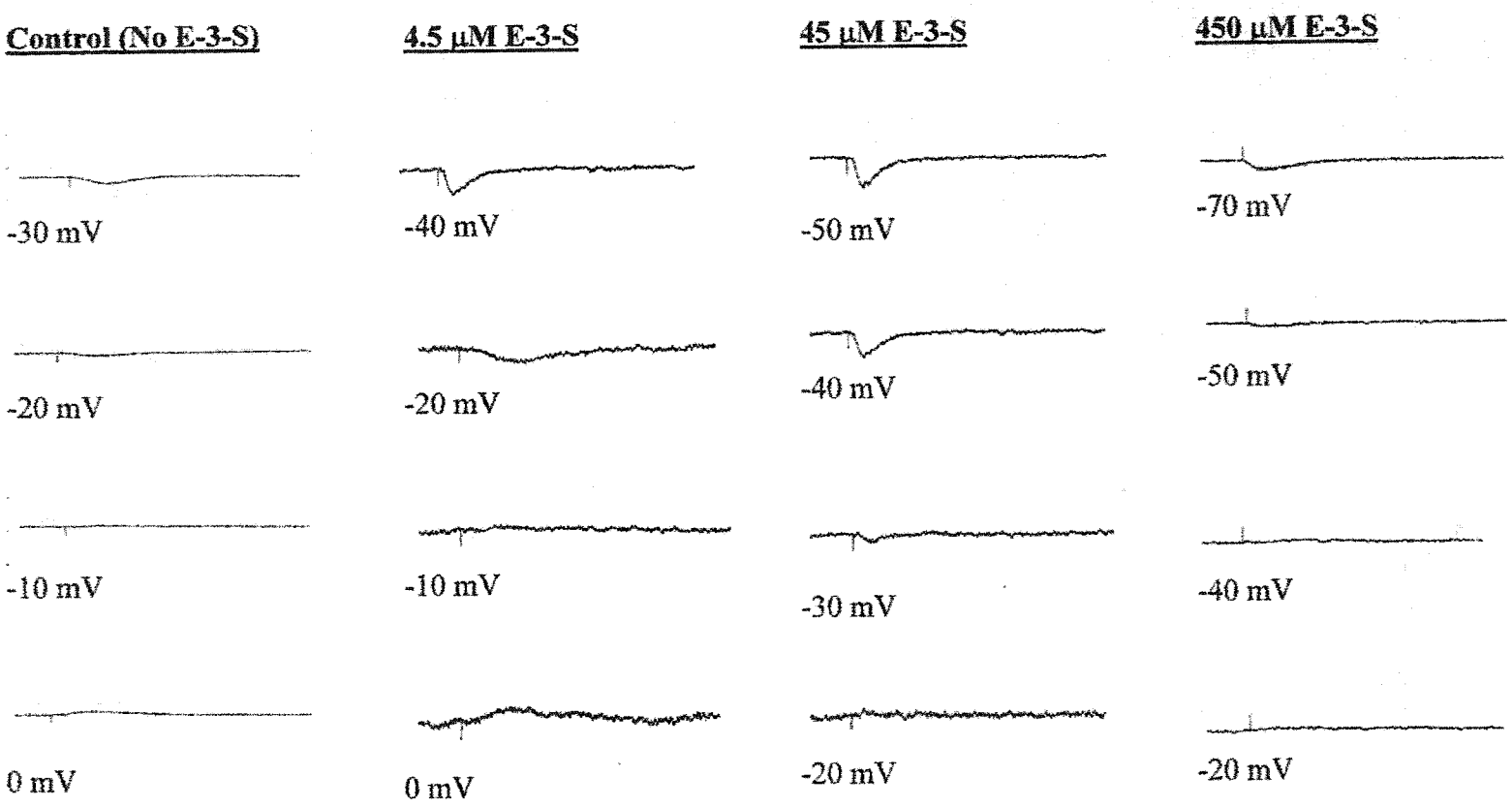

Figure 8: The reversal potential changes after E-3-S exposure. In most instances, treatment of hippocampal slices with E-3-S for 30 minutes did not completely block all GABA-induced currents. The reversal potential of the residual current became more negative with increasing concentrations of E-3-S. The reversal potential of the control (no E-3-S added) averaged -17 mV. After 30 minutes of exposure to 4.5 μM E-3-S, the mean reversal potential was also -17 mV. Exposure to 45 μM E-3-S resulted in a mean reversal potential of -36 mV; after exposure to 450 μM E-3-S, the mean reversal potential was -38mV. The stimulus artifact indicates application of 5.0 mM GABA. Above tracings are from 4 separate cells.

Table 4: E-3-S does not change resting conductance.

	Resting Conductance (Before E-3-S)	(6 min. after E-3-S)	(12 min. after E-3-S)	(24 min. after E-3-S)
Control	55.1 ±8.67 nS (n=11)	55.8±11.7 nS (n=10)	53.9±10.2 nS (n=9)	57.0±10.5 nS (n=7)
4.5μM E-3-S	56.1±6.15 nS (n=8)	56.4±3.34 nS (n=7)	53.4±5.57 nS (n=7)	55.2±5.71 nS (n=7)
45 μM E-3-S	55.0±7.05 nS (n=6)	48.9±11.1 nS (n=6)	49.3±10.2 nS (n=6)	49.2±10.2 nS (n=5)
450 μM E-3-S	46.7±8.31 nS (n=10)	45.8±9.12 nS (n=10)	46.3±7.70 nS (n=8)	46.3±7.66 nS (n=7)

These data demonstrate that bath application of E-3-S (4.5, 45 and 450 μM) did not have a significant effect ($p<0.01$) after 6,12 or 24 minutes. The mean resting conductance (± one standard deviation) was measured, at each time point, after the input of a -5mV, 10 Hz square wave pulse, but before GABA was applied to the cell. Measurements are in nanosiemens (nS). In the case of controls, the secondary bath solution did not contain E-3-S.

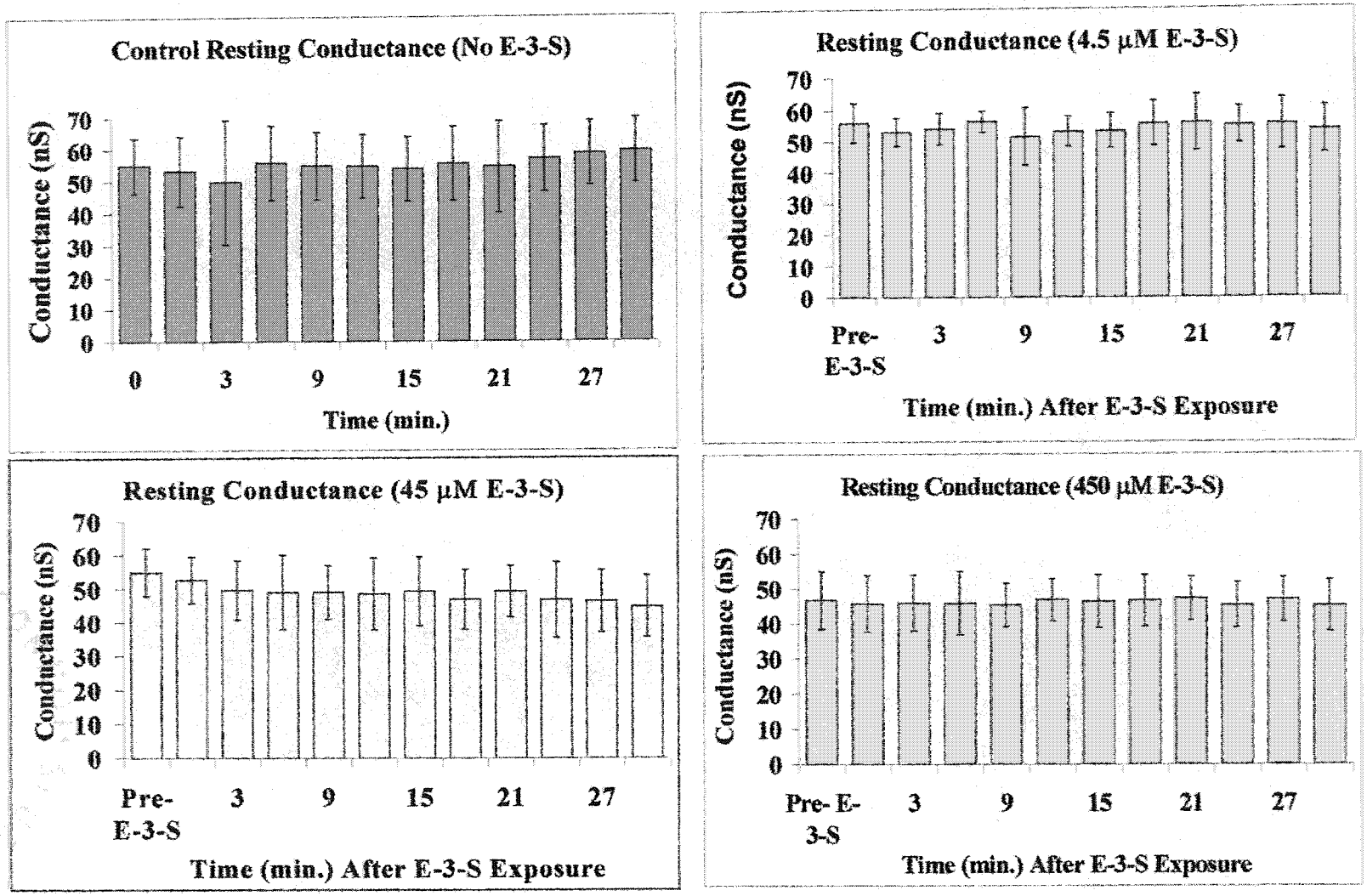

Figure 9: E-3-S does not alter resting conductance. Over time, following bath application of E-3-S (4.5, 45 and 450 µM; n=7, 5, and 7, respectively) and in controls (n=7), no statistically significant change in resting conductance occurred. Conductance was measured in nanosiemens (nS). Error bars represent ± one standard deviation around the mean.

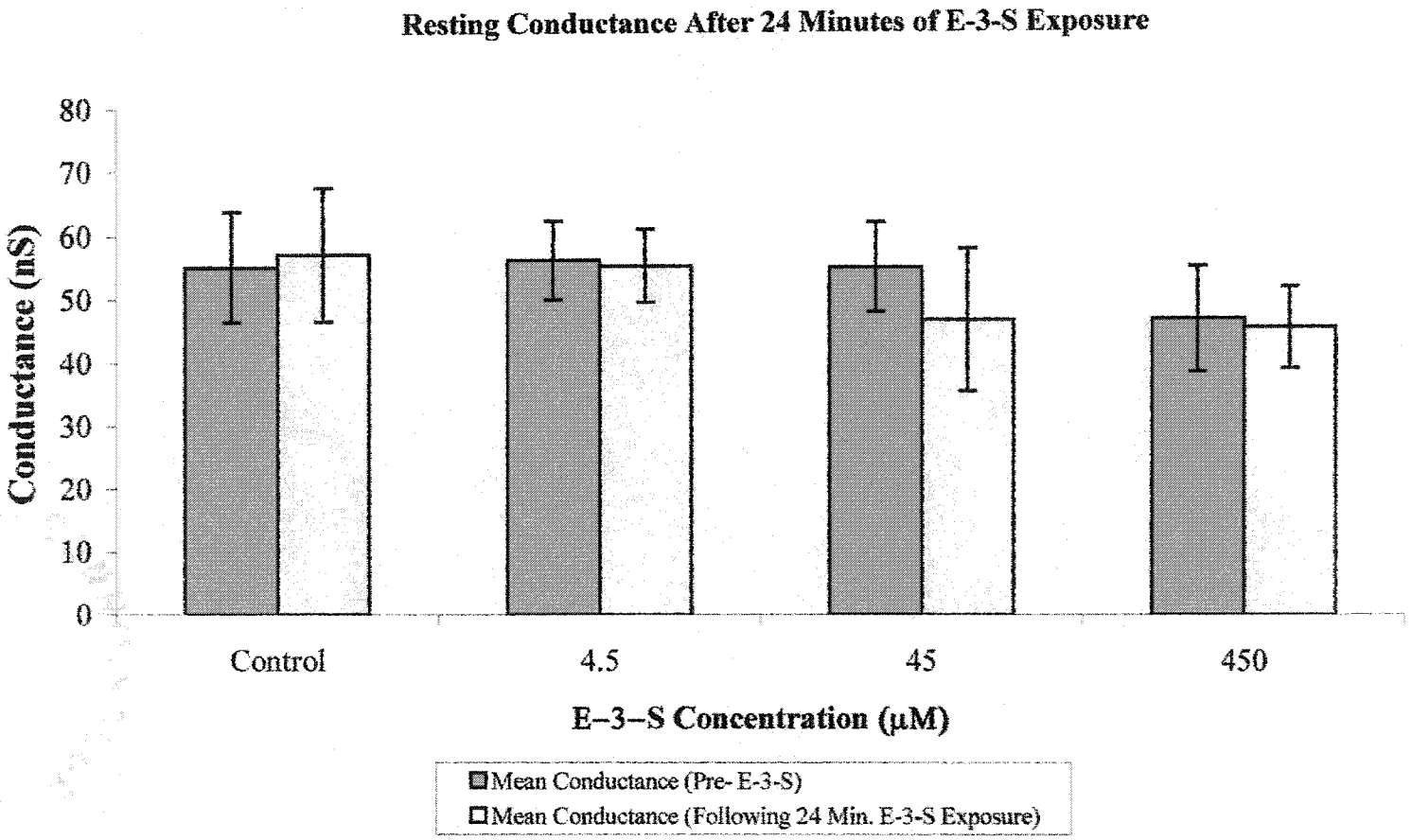

Figure 10: E-3-S does not significantly alter resting conductance. Following bath application of E-3-S (450, 45, 4.5 μM; n=7, 5 and 7, respectively) and in controls (n=7; no E-3-S added), no statistically significant ($p<0.01$) effect on resting conductance was noted. Measurements were taken following the input of a -5 mV, 10 Hz, square wave pulse and conductance was calculated in nanosiemens (nS). Error bars represent ± one standard deviation around the mean.

Table 5: E-3-S reduces GABA-activated conductance.

	GABA-activated conductance: (Before E-3-S)	(6 min. after E-3-S)	(12 min. after E-3-S)	(24 min. after E-3-S)
Control	76.9 ±11.5 nS (n=11)	72.3±13.7 nS (n=10)	72.5±12.3 nS (n=9)	69.5±16.1 nS (n=8)
4.5μM E-3-S	75.8±15.5 nS (n=8)	72.3±15.7 nS (n=7)	73.4±13.2 nS (n=8)	75.6±10.5 nS (n=8)
45 μM E-3-S	68.4±19.2 nS (n=6)	61.0±16.4 nS (n=6)	56.9±14.5 nS (n=6)	52.2±11.9 nS (n=5)
450 μM E-3-S	70.4±9.69 nS (n=10)	57.1±16.5 nS (n=10)	54.4±9.85 nS (n=8)	51.5±10.4 nS* (n=7)

These data illustrate how bath application of E-3-S (4.5, 45 and 450 μM) for 6, 12 and 24 minutes affects GABA activated conductance. The mean resting conductance was measured (± one standard deviation), after input of a -5mV, 10 Hz square wave pulse and after GABA pressure ejection, at the peak of the outward current. Measurements are in nanosiemens (nS). In the case of controls, the secondary bath solution did not contain E-3-S.

* Significantly different from control ($p<0.01$).

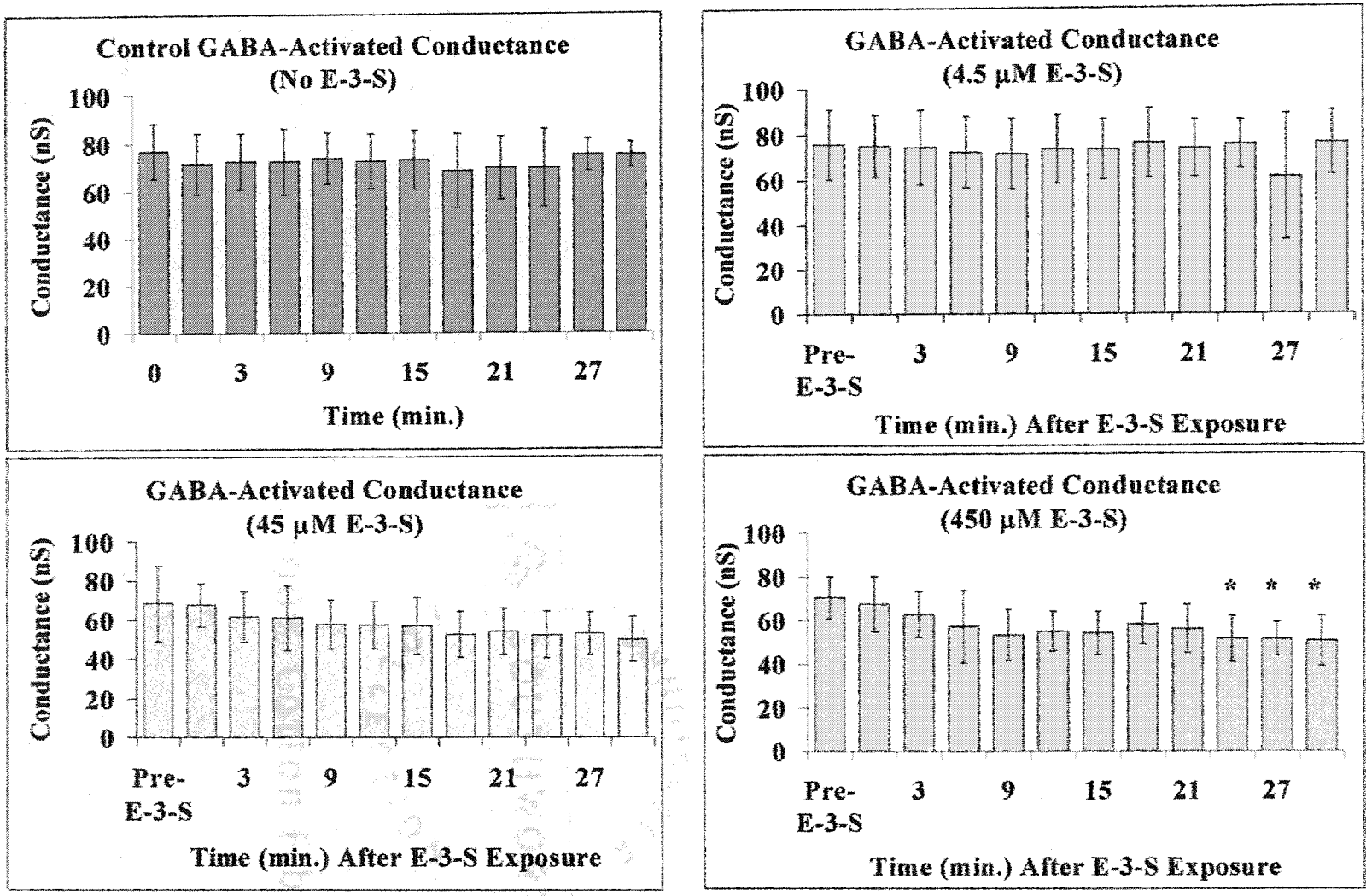

Figure 11: E-3-S reduces GABA-activated conductance. Over time, GABA-activated conductance was significantly reduced with bath application of 450 µM E-3-S (n=7). Conductance was measured in nanosiemens (nS). Error bars represent ± one standard deviation around the mean. * Statistically significant ($p<0.01$).

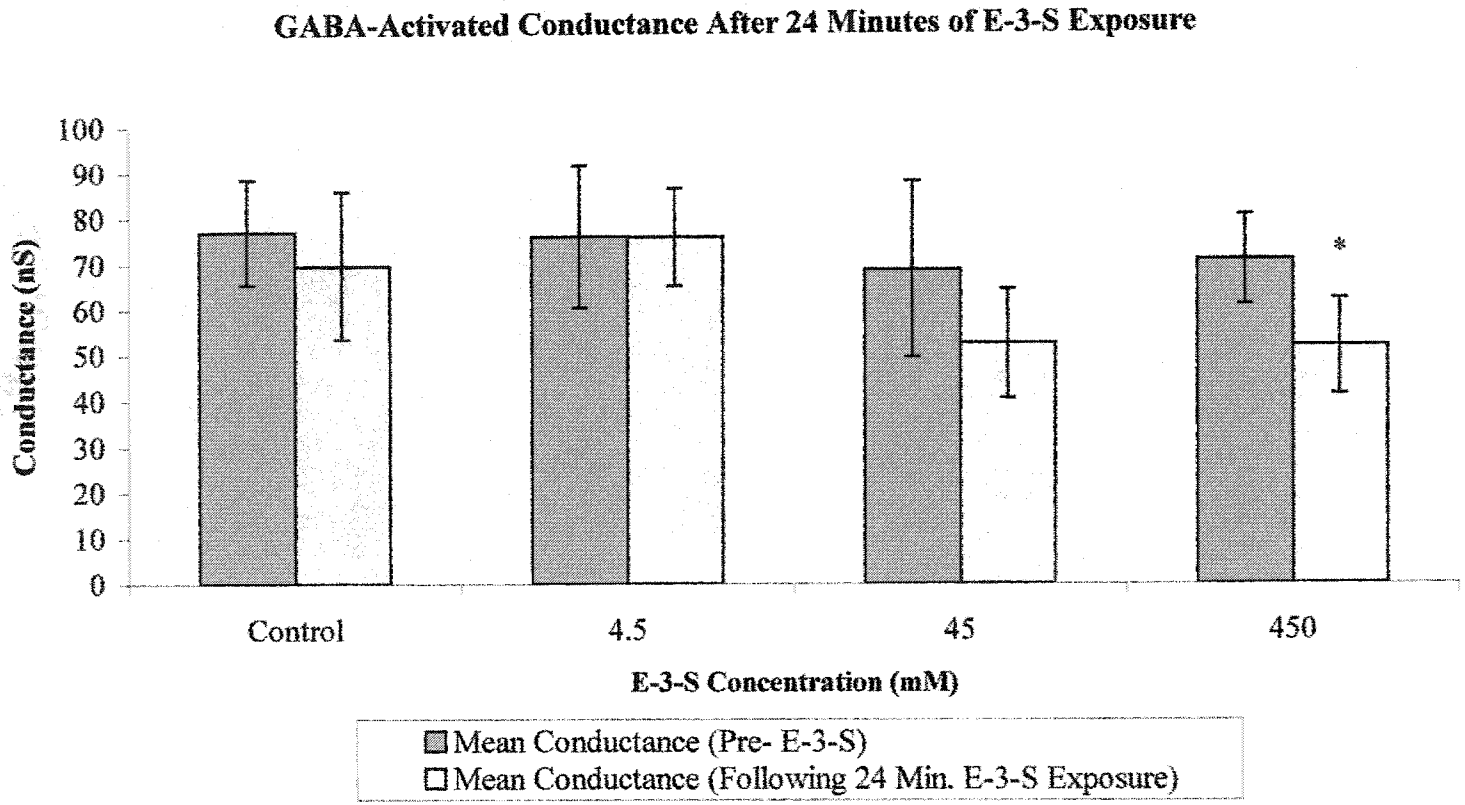

Figure 12: E-3-S causes a dose-dependent reduction in GABA-activated conductance. Following bath application of 450 μM (n=7) E-3-S for 24 min., GABA-activated conductance was significantly reduced. Conductance was calculated in nanosiemens, (nS). No statistically significant effect was seen with 4.5 (n=8) or 45 μM (n=5) E-3-S, or in the controls (n=8; no E-3-S added). Measurements were taken at the peak of the outward current following application of GABA and a -5 mV, 10 Hz, square wave pulse. Error bars represent ± one standard deviation around the mean. * Statistically significant ($p<0.01$).

IV. DISCUSSION

The significant reduction in GABA-evoked IPSC amplitude that was observed following bath application of 450 μM E-3-S supports a postsynaptic site of action for this steroid hormone metabolite. In this preparation, any significant change in amplitude points to a mechanism of action for E-3-S which involves interaction with the postsynaptic site rather than a presynaptic modulation in the amount of neurotransmitter release because: 1) a constant amount of GABA was applied to the cell through the use of pressure ejection, and 2) the TTX that was added to the bath solution, in addition to preventing action potentials, would also have acted to prevent any indirect presynaptic effects. If the reduction in GABA-evoked IPSC were due to a presynaptic modulation in the amount of neurotransmitter release, no effect would have been seen in this preparation.

Although the data in this study demonstrate a statistically significant (at the 0.01 level) reduction in GABA-activated IPSC amplitude only at an E-3-S concentration of 450 μM, previous experiments in this laboratory have demonstrated that 45 μM E-3-S significantly reduces synaptic currents in CA1 pyramidal cells [163]. One factor that may have contributed to the statistical insignificance of GABA-activated IPSC reduction with 45 μM E-3-S is the relatively low number of experiments which were conducted at this concentration level (n= 5). More experiments would most likely have the effect of placing 45 μM E-3-S in the statistically significant category. It should be noted that, with

p=0.013, the probability that 45 μM E-3-S causes a significant decrease in GABA-activated IPSC amplitude falls barely outside of the p=0.01 level.

An examination of control data for GABA-activated IPSC amplitude shows a marked, but statistically insignificant, trend in which the GABA-activated IPSC amplitude decreases over the course of the experiments. This trend is most likely due to cell resealing, a commonly encountered difficulty in the whole-cell patch clamp technique. In any case, the effect of cell resealing was not statistically significant. Furthermore, the low level of significance selected for this set of experiments (p=0.01) and the use of paired t-tests for data analysis help to ensure that any statistically significant reduction in GABA-activated IPSC amplitude is due to the effect of E-3-S and not cell resealing.

Because most previously studied neurosteroids have been found to exert their effects at the $GABA_A$ Cl^- channel, the experimental conditions of this study were designed such that the GABA-activated IPSC appeared to be primarily a $GABA_A$ Cl^- current (there was also minor K^+ component). This was achieved through the use of a recording electrode filling solution that contained an elevated concentration of Cl^- ions. Through dialysis, the Cl^- ions entered the cell and elevated the concentration of Cl^- in the cell interior. Upon GABA-activation, Cl^- ions flowed out of the cell, instead of into the cell, resulting in an inward, depolarizing, IPSC.

Using the Nernst equation, the reversal potential for K^+ in this system was calculated to be –85 mV; the Cl^- reversal potential was calculated to be –12 mV. In order to obtain the largest possible Cl^- current, cells were voltage clamped at –70 mV, far away

from the Cl^- reversal potential. Additionally, the holding potential of –70 mV was near the K^+ reversal potential; thus, the K^+ current was minimized. In summary, under these conditions, the GABA-activated IPSC was primarily a $GABA_A$ Cl^- current. This current was diminished significantly by 450 μM E-3-S. These two pieces of evidence strongly suggest that E-3-S acts as an antagonist at the $GABA_A$ receptor.

Further evidence suggesting that E-3-S acts to modulate the $GABA_A$ receptor complex is the observation that, over time, the reversal potential of the GABA-activated IPSC moved towards the K^+ reversal potential and away from the reversal potential for Cl^-. After 30 minutes of exposure to 450 μM E-3-S, the reversal potential of the GABA-activated IPSC changed from a pre-E-3-S value with a mean of approximately –17 mV (near the calculated the chloride reversal potential of -12 mV) to an IPSC with a mean reversal potential of –38 mV. This change in the reversal potential is reflective of an actual change in the currents underlying the IPSC and suggests antagonism of a certain ion channel rather than a generalized inhibition of GABA binding. For example, if the GABA-activated IPSC was composed solely of a Cl^- current, application of E-3-S would have resulted in a smaller IPSC, but the reversal potential of the current would have remained unchanged.

In most cases, bath application of 450 μM E-3-S for 30 minutes did not completely block the GABA-activated IPSC. The fact that the reversal potential of the remaining current (-38mV) is not at the calculated K^+ reversal potential (-85 mV) but, rather, nearer the calculated Cl^- reversal potential (-12 mV) suggests that the remaining current has a chloride component. This is not surprising, due to the fact that GABA has a

rather high affinity for its receptor ($K_A = 4.7 \times 10^6\ M^{-1}\ sec^{-1}$) [193]. The fact that the effective concentration of E-3-S is in the micromolar range strongly suggests that the affinity of E-3-S for the GABA receptor is much lower. Thus, in order to completely overcome binding of GABA it would be necessary to apply a higher concentration of E-3-S than was utilized in this study. Unfortunately, E-3-S is not soluble in ACSF at concentrations much higher than 450μM. As a result, the dose response curve was not able to be expanded to include another ten-fold increase in concentration. Furthermore, it should be noted that one drawback of the pressure ejection technique is that it is impossible to quantify the amount of GABA that actually reaches the cell. It is impossible to determine exactly where, in the depths of the hippocampal slice, that the pressure pipette is located. It is also impossible to determine the volume of GABA which is released from the pressure pipette once it has been placed in the tissue because the act of inserting the pipette into the tissue can result in a partial blockage of the pipette tip [101].

The fact that the decrease in GABA-activated IPSC after application of 45 and 450 μM E-3-S was observed to be only partly reversible is interesting because a non-genomic effect should be reversible after removal of the E-3-S. It is likely that the irreversible nature of this effect, in this preparation, is due to a difficulty in completely removing all of the E-3-S in the slice chamber through application of the control bath solution (0.5 μM TTX). The lipophilic nature of this neurosteroid may also have resulted in an inability to completely remove the E-3-S from the hippocampal tissue. Furthermore, it is possible that application of the control bath solution for an additional

period of time may have resulted in the complete return of the pre-E-3-S IPSC. Unfortunately, the length of time over which a cell can be successfully and reliably patch-clamped, without cell death or statistically significant resealing, exceeds the time that would have been required to test this hypothesis.

In previous experiments in this laboratory, the convulsant effect of E-3-S on field potentials was studied [156]. An interesting observation is that it was possible, with bath application of control ACSF, to completely reverse epileptiform activity invoked following application of 45 μM E-3-S. The effect of 450 μM E-3-S was found to be partly reversible with a longer wash duration. It must be noted, however, that the field potential is a multicellular effect and that it may be easier to remove enough of the E-3-S to influence an entire population of cells than it is to reverse the effect on a single cell.

Application of 450 μM E-3-S for 30 minutes significantly decreased the GABA-activated conductance. The decrease in GABA-activated conductance signifies a decrease in the number of channels that were open at the peak of the GABA-activated inward current. This further supports the hypothesis that E-3-S acts to block ion channels.

The resting conductance (leak conductance) is primarily due to current flow through K^+ channels that are open at rest. These K^+ channels are delayed rectifiers and, although they typically open in response to a depolarizing current, a certain number of channels are also open when the cell is in rest; these open channels serve a minor part in helping to maintain the resting membrane potential. The fact that the resting conductance

is not changed significantly by application of E-3-S for 30 minutes suggests that E-3-S does not affect the delayed rectifiers.

Additionally, a small part of the resting conductance is due to GABA leak at the presynaptic site. This GABA leak is the cause of miniature IPSCS (mIPSCs). Because GABA leak accounts for only a small part of the resting conductance, a decrease in mIPSCs would most likely not cause a significant decrease in the resting conductance. Data concerning mIPSCs were not collected during the course of these experiments. However, it is interesting to note that previous studies in this laboratory have demonstrated that bath application of 450 μM E-3-S for 30 minutes significantly decreases the occurrence of mIPSCs [163]. This supports the hypothesis that E-3-S acts to block the GABA receptor.

In order to understand the mechanism of action of E-3-S fully, more research is needed. Experiments could be carried out to test the hypothesis that E-3-S is an antagonist of the $GABA_A$ receptor Cl^- channel. In these studies, $GABA_A$ and $GABA_B$ antagonists could be utilized and the two components of the GABA-activated IPSC could be studied separately to determine the effect of E-3-S on each. Furthermore, studies should be conducted in order to verify that the effect of E-3-S is non-genomic. In these studies, a protein synthesis inhibitor such as cycloheximide could be utilized. Effects that persisted or occurred in the presence of the protein synthesis inhibitor would be non-genomic in nature.

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Kind regards,

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