Effects of Estrogen in the Basolateral

Amygdala of the Rat Brain

by

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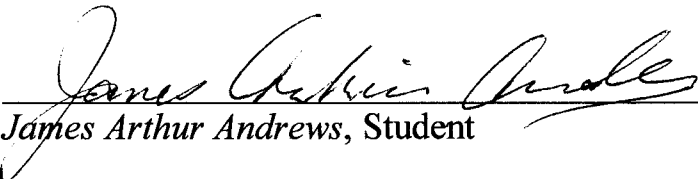
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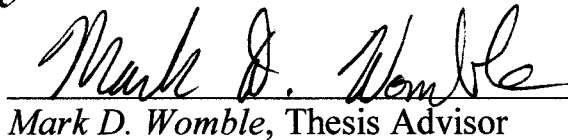
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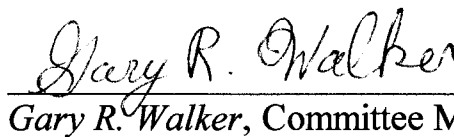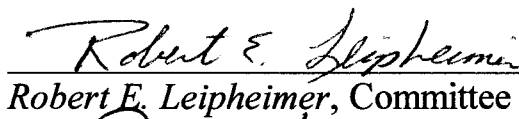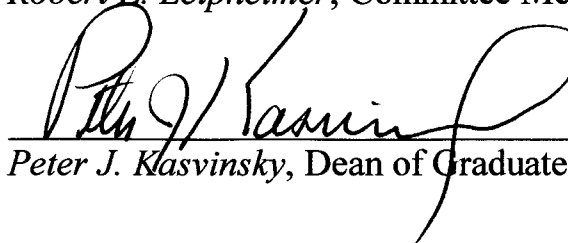
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James Arthur Andrews

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Abstract

Clinical evidence has shown estrogen may delay the onset of Alzheimer's disease and protect against neuronal damage associated with stroke. Intracellular recordings (current-clamp) were made to characterize the effects of estrogen in the basolateral amygdala (BLA) of the rat brain. Excitatory postsynaptic potentials (EPSPs) were elicited by stimulation of afferents in the external capsule. Estrogen was found to decrease EPSP amplitude in a rapid (20-30 min) fashion. Similarly, reduction of spontaneous synaptic activity occurred upon estrogen treatment. EPSP amplitudes returned to normal within 20 minutes of estrogen washout. 4-hydroxy tamoxifen (4-OHT), and estrogen receptor antagonist, prevented the estrogen-induced decrease in EPSP amplitude, suggesting dependence on an estrogen receptor. Estrogen treatment had no effect on neuronal input resistance, accommodation response, resting membrane potential, or action potential firing frequency. Preliminary data showed no change in inhibitory postsynaptic potential (IPSP) amplitude, suggesting estrogen might act on the presynaptic cell. These findings imply that estrogen may be protecting neurons from excitotoxic injury associated with stroke through the modulation of glutamate release.

Table of Contents

Table of Figures

Introduction

Estrogen is a female hormone that appeared to protect neurons from cell death (Behl *et al* 1995; Goodman, 1996; Green, 1996; Reibel *et al.*, 2000; Singer, 1996, 1998) and may play a protective role against several neurological diseases such as Alzheimer's disease (Birge *et al*, 1997; Henderson *et al*, 1994, 1997; Kawas *et al.*, 1997), Parkinson's disease (Bedard *et al.*, 1977), and cerebral ischemia (Hurn and Macrae, 2000; Sawada *et al.*, 2000; Rusa *et al.*, 1999; Fung *et al.*, 1999). Thus, postmenopausal women have the lowest levels of circulating estrogen and the highest probability of developing AD. It is currently not known how estrogen protects individuals from AD, nor is the actions of estrogen in the brain fully understood. The present study was undertaken to examine the actions of estrogen on neuronal function and synaptic transmission in the mammalian brain.

Alzheimer's disease

Alzheimer's disease (AD) is a disorder of the central nervous system that involves neuron and synapse loss, the presence of senile plaques, neurofibrillary tangles, and neocortical atrophy (Reviewed by Bondi and

Lange, 1998). In 1907, Alois Alzheimer, a German physician and neuropathologist, first described the morphological symptoms we now know today as AD. Dr. Alzheimer studied the brain of a patient who had died from dementia and found several distinct morphological changes in the AD brain (Reviewed by Bondi and Lange, 1998). These symptoms presently affect 50% of all people over the age of 85 and are predicted to affect 14 million people by the year 2050 (Reviewed by Birge *et al.*, 1997). Once diagnosed, average life expectancy is 7 to 10 years, of which most will be spent in a long-term care facility. In their final stages, AD patients are thoroughly debilitated by the neurological effects. AD patients experience a slow and painful decay of all senility and eventually die while in a permanent vegetative state (Reviewed by Bondi and Lange, 1998).

Neurofibrillary tangles and senile plaque formation significantly contribute to neuron loss, the subsequent decrease in synapses and neurotransmitter content, and the neocortical atrophy seen in AD patients. Unfortunately, the neuritic plaques and neurofibrillary tangles associated with AD can only be found through post-mortem staining. If these morphological changes are later found in brain tissue taken from a dementia patient, there is conclusive evidence that the patient suffered from AD (Reviewed by Bondi and Lange, 1998).

Neurofibrillary tangles are associated with the cytoskeleton of neurons affected by AD. Neurofibrils, also known as neurofilaments, are long strands of linked protein subunits. Neurofibrillary tangles are formed from neurofibril subunit deformation, and this directly impacts structure and function of the neurofibrils (Kandel *et al.*, , 1995). Transport proteins and structural support proteins of neurofibrils lose their delicate organization and become a chaotic filamentous intertwining structure (Reviewed by Bondi and Lange, 1998). Neurofibrillary tangles cause death in neurons by inhibiting the efficient transport of materials throughout the neuron. Neurons with neurofibrillary tangles eventually die from the lack of essential transportation of cellular components (Kandel *et al.*, , 1995).

Neuritic plaques are found in the areas between neurons of such areas as the amygdala, hippocampus, and other cortical regions (Reviewed by Bondi and Lange, 1998). The neuritic plaques are formed by deposits of insoluble β-amyloid protein. β-amyloid is cleaved from amyloid precursor protein (APP), a soluble protein normally found in the brain. The soluble form of ß-amyloid is a protease released in response to neuronal injury where it plays a neuroprotective role. It is believed that an alteration in the normal metabolism of this protein can contribute to the development of AD by making the cleaved β-amyloid protein insoluble and cytotoxic (Chao *et*

al., 1998). Soluble β-amyloid has been shown to be non-cytotoxic in some cases, and insoluble β-amyloid that forms neuritic plaques has been found to be neurotoxic to hippocampal neurons in culture (Cotman, *et al.*, 1992; Pike, *et al.*, 1991).

The toxicity of β-amyloid may result from its actions as a protease. After cleavage of their substrates, proteases release free radicals as by-products. Free radicals in solution can oxidize cellular components essential for a variety of cellular processes. Free radical production is believed to cause the toxicity of the β-amyloid plaques (Maury, 1995). Under normal conditions, the soluble form of ß-amyloid peptide is helpful to the central nervous system, but due to a change in the position of cleavage from its precursor protein, APP, the insoluble form of the peptide ironically becomes a detriment to neuronal functioning (Maury CPJ, 1995). The change in cleavage site is believed to be inherited, and this may underlie the genetic susceptibility that some families display for early onset of AD (Chao *et al.*, 1994).

Psychological signs of AD include unsteady development of dementia and a gradual impairment of memory, judgment, and concentration (Reviewed by Bondi and Lange, 1998). Since AD is the most common cause of dementia (Henderson., 1997), all cases of dementia especially in the

elderly have a very high chance of being a symptom of AD. Dementia in AD is marked by one or more of these disorders: aphasia (problems with language), apraxia (problems with carrying out motor activity despite having the physical ability), agnosia (problems with object recognition despite intact sensory function), and impaired planning, abstracting, and sequencing (Reviewed by Bondi and Lange, 1998).

AD patients may suffer from depression at early stages of the disease and they may exhibit abnormal fits of rage as the disease progresses (Reviewed by Bondi and Lange, 1998). These fits of rage are closely associated with other personality changes found in AD. Behavioral symptoms of AD probably result from the neurodegeneration occurring in the limbic structures, such as the amygdala, as well as in higher cortical areas (Reviewed by Bondi and Lange, 1998).

Treatment of Alzheimer's Disease

Currently, a suggested means of therapy and prophylaxis for AD patients is the administration of vitamins A, C, and E (Reviewed by Bondi and Lange, 1998). These vitamins are good antioxidants, which act to subdue the cytotoxicity of β-amyloid. Vitamin E (α-tocopherol) has shown

to play a protective role against the neurotoxic effects of β-amyloid in animal models (Jaffe *et al.*, 1994), , and it also shows the potential of delaying AD due to its strong antioxidant activities (Birge *et al.*, 1997). The antioxidants absorb the free radicals generated by β-amyloid, and other sources of free radicals such as cyclo-oxygenase, a chemical released upon an inflammatory response. Some functions of cyclo-oxygenase include involvement in the production of prostaglandins and mediation of excitotoxic cell death via the glutamatergic N-methyl-D-aspartate receptor. Anti-inflammatory drugs such as indomethicin can reduce the risk of developing AD by 50% (Birge, *et al.*, 1997).

Several currently prescribed AD medications stop the actions of enzymes at the axon terminal from cleaving used neurotransmitter. Two of the most commonly prescribed medications for AD at this time are donezapil (Aricept) (Birge *et al.*, 1997) and tacrin (Cognex) (Henderson *et al.*, 1997). These medications have been reported to slow the onset of AD symptoms by inactivating cholinesterases (Birge *et al.*, 1997). These enzymes are responsible for breaking down acetylcholine at cholinergic synapses. The patients receiving donezapil experience an increase in the amount of acetylcholine at synapses (Birge *et al.*, 1997) between intact cholinergic

basal forebrain neurons and efferent neurons such as those located in the basolateral amygdala, hippocampus, and higher cortical areas.

Estrogen has also been suggested as a treatment for AD (Birge *et al.*, (1997). Neuroprotective effects of estrogen have been implicated in studies of cytotoxicity (Behl *et al*, 1995: Goodman. 1996; Green *et al.*, 1996; Singer *et al.*, 1996, 1998), and it has been shown that estrogen increases blood flow to the brain during clinical trials with AD patients (Ohkura *et al.*, 1994). In addition, estrogen replacement therapy has been suggested, through multiple epidemiological studies, to possibly delay AD onset and slow cognitive decline in patients experiencing AD symptoms (Brenner *et al.*, 1994).

Heredity of Alzheimer's Disease

Genetic research has shown that heredity plays a large role in the likelihood of individuals to develop AD. The chance of developing AD increases four times if a first-degree relative has the disease, and it has been found that 80% of all AD occurrence is attributed to heredity (Birge *et al.*, 1997). The additional 20% may be caused by environmental factors such as head injuries and chemical insults that may have occurred at a time as far back as childhood (Birge *et al.*, 1997). The inherited trait that represents

individual susceptibility to AD development is believed to be the ApoE epsilon-4 allele (Holtzman *et al.*, 1995). ApoE protein is normally released in response to the neurodegeneration and other neuritic insults occurring in the brain (Mahley, 1988; Boyles *et al.*, 1989; Boyles *et al.*, 1990; Poirier *et al.*, 1991)). AD patients have been tested for the mutated ApoE protein. It has been found that people who have experienced head trauma with the "bad" ApoE epsilon-4 allele have been found to develop AD at a greater frequency than those with the wild-type ApoE gene (Mayeux *et al.*, 1995) . A malfunctioning ApoE protein is not able to help the neurons recover from injury. Normal functioning ApoE protein helps the injured neurons by making cholesterol available for cell membrane repair. Correspondingly, neurons located in areas of the brain affected by AD contain a low amount of cholesterol, and the delivery of cholesterol to these regions is markedly impaired (Mason *et al*, 1992).

Estrogen

Estrogens represent a class of several closely related steroid hormones, of which 17-β-estradiol (estrogen) is the major form synthesized

by the ovary (Johnson and Everitt, 1995). Synthetic pathways for estrogen are numerous (Fig. 1). Aromatase is an important enzyme in this pathway because it helps to control local estrogen blood levels (Johnson and Everitt, 1995). This enzyme has a wide distribution throughout different tissues in the body; including brain tissue (Balthazart and Ball, 1998). The mammalian limbic and hypothalamic regions contain high levels of aromatase (Balthazart and Ball, 1998). Activity of this enzyme is controlled in the ovarian cells by local blood levels of follicle stimulating hormone (Johnson and Everitt, 1995). Follicle stimulating hormone and luteinizing hormone are secreted from the anterior lobe of the pituitary gland, and control the blood levels of estrogen in a negative feedback loop with blood levels of estrogen (Johnson and Everitt, 1995). Aromatase is responsible for the aromatization of testosterone into estrogen and the aromatization of androstenedione into estrone (Balthazart and Ball, 1998). Estrone is an estrogen easily converted to 17β-estradiol (Johnson and Everitt, 1995). Thus, the presence of aromatase in other tissues gives those tissues the chance to capitalize on an extra dose of estrogen if blood levels of testosterone are at a concentration to permit aromatization. Luteinizing hormone participates in the regulation of estrogen synthesis earlier in the pathway. The levels of this hormone modulate the rate of pregnenolone,

formation from cholesterol (Johnson and Everitt, 1995). Pregnenolone is a

steroid precursor for testosterone/estrogen synthesis (Johnson and Everitt,

1995).

acetate

cholesterol

Pregnenolone

16α hydroxypregnenolone

Dehydroepiandrosterone

androstenediol

16α hydroxy dehydroepiandrosterone

16α hydroxy androstenediol

3β hydroxysteroid

dehydrogenase

+ isomerase

Progesterone

17α hydroprogesterone

androstenedione

testosterone

16α hydroxyandrostenedione

16α hydroxytesterone

Aromatase

Aromatase

5α reductase

Pregnanediol

20α dihydroprogesterone

Estrone

17β-estradiol

Estratriol

21α-hydroxylase

Cortisol

11 deoxycortisol

5α dihydrotesterone

The Genomic Pathway of Estrogen Action

Estrogen has traditionally been described as acting on tissues through a slowly developing and long-lasting mechanism commonly referred to as the genomic pathway. In this pathway, estrogen influences the cell by initially binding to an estrogen receptor (ER) , which is found at highest concentration in the cell nucleus (Li *et al.*, 1997). After becoming activated, the following sequence of events has been reported to occur (Reviewed by Tsai and O'Malley, 1994). The estrogen-receptor complex can interact with transcription factors . These transcription factors regulate specific sequences of DNA . In the absence of estrogen, the ER exists bound to heat shock proteins and remains inactive . After estrogen binding, the receptor undergoes a conformational change at its ligand-binding domain, and the receptor becomes activated at protein activating functions. This leads to dissociation of the receptor from the heat shock protein followed by ER dimerization, the coupling of two ERs. To activate the ER, the receptor must be in the presence of estrogen or a receptor agonist . Removal of the heat shock proteins through other means is not sufficient for activation of the ER . After the ER dimerizes, the ER is now able to bind to a specific DNA complex and interact with transcription factors to activate or inactivate the target gene . Gene activation leads to mRNA production and increased

synthesis of a specific protein that is then responsible for production of the estrogen-induced cellular response . The period between activation of ER mediated transcription and post-translational modification of the final protein product may involve hours or days .

The Estrogen Receptor (ER)

There are two well-described estrogen receptors, ERα and ERß, although some evidence points to the possibility of a third receptor type (Kuiper *et al.*, 1997). ERα and ERß belong to a superfamily of steroid/thyroid receptors . Within this nuclear receptor superfamily, estrogen receptors belong to the largest subgroup that also includes glucocorticoid receptors, androgen receptors, progesterone receptors, and mineralocorticoid receptors . There is a high degree of conservation amongst these receptors in their hormone-binding and DNA-binding domains (Kuiper *et al.*, 1997; Tsai and O'Malley, 1994; Wrenn, 1993).

In rat and human neurons, ER and other androgen receptor proteins have been detected in neurons (Couse *et al.*, 1997) with distributions from nuclei to far reaching cytoplasmic extensions such as dendrites and axon terminals (Greco *et al.*, 1998; Puy *et al.*, 1995). In the rat brain, neurons

containing ERß and ERα are distributed together in several cortical amygdaloid nuclei (Puy *et al.*, 1995) and some areas of the hypothalamus (Shughrue *et al.*, 1998; Weiland 1997). ERß, without the presence of ERα, is broadly expressed throughout the rat brain. Both isoforms of ER may have different actions in cells because of their different DNA binding domains (Shughrue, *et al.*, 1998). Complex cellular responses can arise because both ERα and ERß can dimerize with their homologue or heterodimerize with the other type of ER (Katzenellenbogen *et al.*, 1996). When ERs bind to DNA, both DNA recognition sites of the dimerized ERs recognize their own specific sequence. When heterodimerization occurs it is possible to have differences in function due to the differentiation in binding characteristics of ERα and ERß (Shughrue, *et al.*, 1998).

The ER protein is large protein with a number of functionally important motifs along its chain of amino acids. cDNA evidence indicates that ERß consists of 485 amino acids with a calculated molecular weight of 54,200 Daltons. Isomer homology at the ligand-binding domain of ERs is about 90%. Homology between DNA binding motifs is 50% (Kuiper, *et al.*, 1997). The differences between ERα and ERβ have developed to allow different receptors to activate different areas of the genome while being activated by a common hormone.

Antagonism of the ER

Estrogen replacement therapy increases the incidence of different types of cancer in female reproductive tissues. Extensive research with uterus and mammary tissue led to the discovery of antagonists to inhibit some of these side effects of estrogens. Drugs such as Tamoxifen, ICI, and Raloxifene competitively inhibit the estrogen receptor, and thus decrease the chances of cancer developing in some peripheral tissues (Gradishar and Jordan, 1997; Jordan, 1998). However, both Tamoxifen and Raloxifene are known to also have agonistic actions on the ER (Berry and Metzger. 1993; Ali *et al.*, 1993; Metzger *et al.*, 1995). In addition, the agonistic actions of these drugs differ from each other. Cellular responses to estrogen are very complex, and have yet to be completely defined. Understanding how the separate regions of the ER function while initiating cellular changes is integral to understanding the intricacies of ER antagonism/agonism duality.

Antagonism for the ER is an important part of modern medicine. To have an effective ER antagonist is to have the best of both worlds; drugs that can block the cancer associated with estrogen without reducing the beneficial actions of estrogen. It is not unusual for ER antagonists to have

15

more than one action in patients. The selective estrogen receptor modulator (SERM) tamoxifen is an ER antagonist, which is regularly prescribed for those considered to be at high risk for developing breast cancer. Chances of developing breast cancer drop by 45% after tamoxifen treatment (Smigel, 1998). At the same time, bone density increases so that fewer fractures occur, which is an important benefit of estrogen replacement therapy. However, there is an unfortunate increase in endometrial cancer incidence (Smigel, 1998). This phenomenon of inactivation/activation or antagonism/agonism is further exemplified by postmenopausal women experiencing increases in bone density when receiving the SERM raloxifene for treatment of osteoporosis (Black *et al.*, 1983). Understanding how SERMs work in different tissues and considering their actions on the individual estrogen receptor types (ERα and ERß) will help us to find clues that will eventually lead to the finding of a SERM with the ability to mimic the positive estrogenic influences but block the carcinogenic responses.

Defining the mechanism by which SERMs selectively activate and inactivate the ER is key in understanding how these chemicals can be both an asset and a liability. Activating functions (AF-1 and AF-2) are regions of the ER, which are activated following a specific conformational change by the ER (Jensen EV *et al.*, 1973). It is this conformational change in the ER

that is altered or inhibited by tamoxifen, raloxifene, and similar antagonists (Jordan, 1998). AF-1 is located in the N-terminus of the ER (Kumar *et al.*, 1987). AF-2 is located in the ligand-binding domain (Kumar *et al.*, 1987). The conformational change needed to activate AF-2 is a folding of helix 12 of the ER to uncover the AF-2 domain (Shiau *et al.*, 1998). This folding of helix 12 on the ER is inhibited by ER antagonists, and thus the AF-2 domain remains inactive (Shiau *et al.*, 1998). This does not mean that the other ER activating function is inactive. Helix 12 is sterically hindered by an alkyl-amino-ethoxy side chain of the antagonist molecule that is otherwise structurally similar to estrogen (Jordan, 1984).

Although the AF-2 domain of the ER is inhibited by ER antagonists, the AF-1 region may remain active through mitogen-activated protein kinase pathways (MAPK) (Kato *et al.*, 1995). When the AF-1 region is activated by SERMs, it initiates some of the same cellular changes as estrogen. It is ironic that treatment with antiestrogens invokes some estrogenic responses.

Non-Genomic Mechanism of Estrogen Action

Until recently, estrogen was thought to exert its action only via the genomic (nucleic acid/ protein synthesis) pathway, a process that requires

hours or longer to produce a cellular response. However, rapid effects of estrogens and other steroid hormones were found in frog and fish oocytes in the 1980's. (Zakon., 1998) Since these initial reports, additional studies have shown similar rapid cellular responses to estrogen and androgens in several tissues, including the brain (Balthazart *et al.*, 1998; Gorzynska and Handelsman., 1995; Woolley., 1999).

These recent findings of estrogenic and androgenic actions taking place in minutes or even seconds indicate that the traditional genomic pathway is not the only mechanism by which steroid hormones may exert their actions. The problem we now face is discovering a new pathway by which estrogen can function in a much shorter time frame.

An example of the rapid action of steroidal hormones is seen in Sertoli cells of the rat testis (Gorzynska and Handelsman., 1995). Testosterone treatment causes a rapid (20-40 second) rise in cytosolic calcium levels. An identical rapid cellular response was also seen during the application of testosterone that had been conjugated to a large protein. (Gorzynska and Handelsman., 1995). Since the known steroid receptors are found in the cytosol and the nucleus, their activation depends upon the steroid penetrating the cell membrane and reaching their ligand-binding domain. With a protein attached to the steroid hormone, diffusion across the cell membrane is

impaired. Actions such as those described in the Sertoli cells of male rats (Gorzynska and Handelsman., 1995) strongly suggest the presence of a cell surface steroid receptor. Since androgen receptors are very closely related to ER in structure and function, the existence of androgen membrane receptors in rat Sertoli cell membranes suggests that cell surface membrane receptors for estrogen may also exist.

Another possibility for the rapid action of estrogen is that the known receptors (ERα and/or ERβ) are working in a fashion other than the genomic pathway. Signal transduction pathways are a common mechanism for receptors and some evidence suggests that estrogen receptors may evoke their rapid actions via signal transduction mechanisms. Pyramidal neurons in the hippocampus are remarkably similar to those found in the BLA. In CA1 neurons of the hippocampus, estrogen activates the cAMP pathway to induce AMPA/kainate currents, which are normally activated by glutamate (Gu and Moss., 1996); similarly, in neuroblastoma cells estrogen initiates a mitogen-activated protein kinase (MAPK) cascade of events (Watters *et al.*, 1997). Growth factors such as epidermal growth factor and insulin-like growth factor also act through the MAPK pathway. These factors enhance the genomic response caused by estrogen, suggesting the possibility that the pathways of action for these chemicals may be intersecting.

In the MAPK pathway, there is a membrane receptor-associated tyrosine kinase initially activated by a growth factor, and the resulting cascade includes activation of the proteins ras, raf, and MAPK respectively (Hill CS and Treisman, 1995; Pelech SL and Sanghera, 1992: Sanghera, 1992). Introduction of the activated forms of any of these proteins into the cytosol of a cell had the effect of increasing ER activity at the AF-1 domain much like introduction of the growth factors (Kato *et al.*, 1995). Further observation showed that the Ser-118 residue of the ER was phosphorylated following activation of the MAPK cascade (Kato *et al*, 1995). Phosphorylation of this serine residue is important for the activation of the AF-1 domain of the ER. Introduction of 4-hydroxy-tamoxifen, an active form of tamoxifen, into the cytosol initiates phosphorylation identical to that of estrogen, whereas the stronger antagonist ICI 164,384 does not initiate a response (Kato *et al.*, 1990). Not only is this a possible pathway of SERM agonism, but this information also suggests that a possible method of blocking estrogen action is through the blocking of phosphorylation of the estrogen receptor.

The Basal Lateral Amygdala (BLA)

The basolateral nucleus of the amygdala (BLA) is one of several

nuclei that make up the amygdala, an almond-shaped region located deep

within the temporal lobe of the cortex (Martin, , 1996). As part of the limbic

system, the amygdala plays important roles in emotion and memory

formation (Martin, , 1996). The BLA consists primarily of pyramidal type

neurons that are very similar in structural and functional characteristics to

neurons of the hippocampus (Washburn and Moises, 1992b), another

important component of the limbic system (Martin, , 1996). The amygdala

and the hippocampus receive afferent innervation from the basal forebrain

(Emson *et al.*, 1979; Woolf and Butcher, 1982; Carlsen *et al,.* 1985). The

BLA is the amygdaloid region that has been shown to receive the highest

density of cholinergic inputs in the region (Ben-Ari *et al.*, 1977; Hellendall

et al., 1986). Functionally, this region is thought to be responsible for

giving emotional significance to memories (Martin, , 1996).

Cholinergic inputs from the basal forebrain begin to decrease in

number in AD. The cause of the decreased amount of cholinergic inputs in

AD is the death of afferent basal forebrain cholinergic neurons (Bartus *et al.*,

1982). The BLA is a region intimately related to the etiology of AD, in fact

it is one of the first regions of the brain to show the evidence of

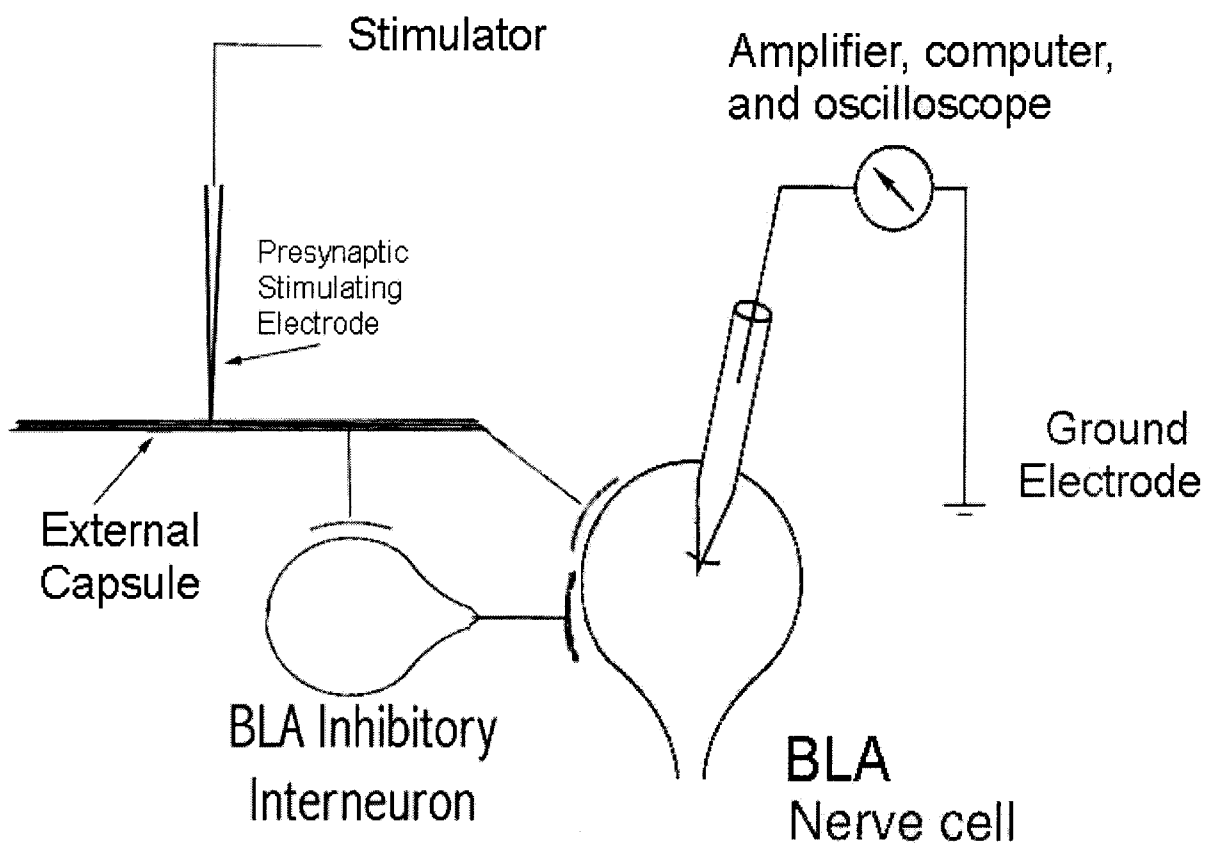neurodegeneration upon the onset of AD. The many psychological

dysfunctions associated with AD such as memory loss, emotional instability, and depression may originate from this region's incapacity to function properly following AD-inflicted neuronal cell death (Bartus *et al.*, 1982).

A major afferent pathway to the BLA is the external capsule (EC). This pathway carries both cholinergic and glutamatergic inputs to BLA neurons (Washburn and Moises, 1992). Brief electrical stimulation of the EC evokes in BLA neurons a rapid excitatory postsynaptic potential (EPSP) response, which is often followed by a delayed inhibitory postsynaptic potential (IPSP) response. Neurons initiate EPSPs from direct innervation via EC. However, in the case of an IPSP, neurons receive their signal via an interneuron. Interneurons receive excitation from a presynaptic source such as the EC, and generate an IPSP in postsynaptic cells (Rainnie *et al.*, 1991a, b). A long-lasting depolarization of BLA neurons is mediated by the synaptic release of acetylcholine, but this response is only seen following high frequency EC stimulations (Washburn and Moises., 1992c), which are not used in the present study.

Clinical evidence suggests that estrogen may play a protective role against the onset of AD (Birge *et al*, 1997; Henderson *et al*, 1994, 1997; Kawas *et al.*, 1997). Estrogen has also been reported to have effects on cognition (Birge *et al.*, 1997; Ripich *et al.*, 1995) and long-term potentiation

(Cordoba and Carrer, 1997). Since the amygdala is an early and severely affected target of the neurodegeneration associated with AD, it was of interest to investigate the actions of estrogen on neurons of this region. This project was thus undertaken to test whether estrogen altered the functional properties of BLA neurons or their synaptic inputs.

Stimulator

Amplifier, computer,
and oscilloscope

Presynaptic
Stimulating
Electrode

Ground
Electrode

External
Capsule

BLA Inhibitory
Interneuron

BLA
Nerve cell

Materials and Methods

Long-Evans rats of random sex and aged 3 weeks to 1 year were used in this study. Rats were housed on 12 hour light/dark schedule and received food and water *ad libitum*. All procedures involving live animals were approved by the Animal Care and Use Committee, Youngstown State University. Procedures were modified from Washburn and Moises (1992b). Animals were sacrificed by decapitation, their brains removed promptly, and placed in oxygenated and ice-cold artificial cerebrospinal fluid (ACSF). The ACSF was made in 1.5 liter batches daily including the following constituents with concentrations in millimolars 124 NaCl, 3.5 KCl, 1.5 MgSO$_4$, 1.0 anhydrous monobasic NaH$_2$PO$_4$, 26.2 NaHCO$_3$, 11.0 glucose, and 3.0 CaCl$_2$. Three to five slices of ventral forebrain containing the amygdala were cut on the horizontal plane at a thickness of 400 μm using a Vibroslicer (Campden Instruments). The slices were held at room temperature (23°C) in ACSF bubbled continuously with 95% O$_2$/ 5% CO$_2$ for one hour before any recordings were attempted. This assured time for the cells to recover from the vibrotome. Individual slices were placed the recording chamber as needed. The slice was held between two layers of nylon mesh submerged in continuously flowing ACSF at room temperature.

Intracellular recordings were obtained from neurons of the BLA using glass microelectrodes filled with 2.7M KCl/ 0.4M K acetate (pH 7.0) and having resistances of 70-120 MOhms. Recordings were amplified with the Axoclamp 2B in Bridge mode (Axon Instruments) and displayed on a Tektronix 2213A oscilloscope and Cole Parmer chart recorder. Signals were also fed to a computer interface (INDEC, Sunnyvale California) that digitized the analog signal for storage and analysis by a microcomputer-based program (pCLAMP, Axon Instruments).

Only cells with membrane potentials greater that -55 mV and over shooting action potentials were included in this study. Under our recording conditions, the vast majority of BLA cells were not spontaneously active but cells could be made to fire by passage of direct depolarizing circuit through the electrode. Neuronal input resistance was determined by passing an incremental series of current pulses (0.2 nA increments; range, -1.0 nA to +0.4 nA; 450 msec duration) through the recording electrode and measuring the resultant voltage deflections.

EPSPs were elicited in BLA neurons by delivering voltage pulses of 0.1 msec duration originating from an A310 Accupulser (World Precision Instruments), isolated via an A360 Stimulus Isolator (World Precision Instruments), and introduced through a bipolar stimulating electrode placed

on the surface of the slice over the external capsule (EC). While the EC could be easily identified visually, we relied on anatomical landmarks in the slice and diagrams of stereotaxically defined sections (Paxinos and Watson, Academic Press, 1998) for guiding placement of the recording electrode in the BLA. Typically, the stimulus intensity delivered was that which evoked an EPSP whose amplitude was just below threshold for generating an action potential when recordings were made at the resting membrane potential. Membrane potential was manually adjusted by intracellular injection of direct current through the recording electrode, and was held at -65 to -75 mV when characterizing EPSPs elicited by EC stimulation.

Estrogen (17- β-estradiol) (Research Biochemical International) and/or 4-OH-tamoxifen (Calbiochem) were applied to the slice by switching the bath superfusate from normal ACSF to ACSF containing known concentrations of the drugs. A 2 μM Estrogen concentration in ACSF was prepared by the addition of 1.2 ml of daily-made 100 μM stock solution to 60 ml saline. A 60 nM 4-OH-tamoxifen solution was prepared by the addition of 12 μL of 0.06μM stock solution stored at 0°C to 60 ml saline. All stock solutions were prepared with 95% ETOH as a solvent.

Tests were performed on neurons that passed criteria listed above for membrane health. Tests were conducted in a series, which included current

stimulation, single-pulse presynaptic stimulation, and train presynaptic stimulations. Voltage recordings were taken for all tests in the series, and some recordings were used in determining resting membrane potential. To decipher the early and late membrane resistances, the injected current and voltage responses were used in accordance with Ohm's Law. EPSP amplitudes were measured from resting membrane potential to the maximum point on the voltage traces resultant of single-pulse stimulation. The number of action potentials fired was counted during the 450 ms current stimulation at 800 pA of depolarizing current. Accommodation was analyzed by comparing experimental recordings to control. The results of the experiment were analyzed for statistical significance using a two-tailed t-test.

During the course of the experiments involving estrogen, the bath superfusate was changed from control saline to estrogen and back to control saline for washout. During the course of experiments involving 4 OH-tamoxifen, the bath superfusate was changed in the following manner: control, 4-OH-tamoxifen, control for washout, saline containing estrogen and 4-OHT (to test blocking), control saline for washout, and saline containing estrogen. The series of tests were conducted for 20 minutes after switching the bath to the next solution in sequence. New slices were placed in the recording chamber at the conclusion of an experiment.

Results

The experiments presented here were designed to investigate the actions of estrogen on synaptic transmission in the BLA. Perfusion of BLA neurons with ACSF containing estrogen (2 μM) resulted in a significant decrease in excitatory transmission and EPSP amplitude (Fig. 3). The mean EPSP amplitude in control ACSF was 9.9 ± 2.8 mV (± SD; n=7). Approximately 20 minutes after the bathing saline was changed to ACSF containing estrogen, mean EPSP amplitude was reduced to 0.8 ± 1.2 mV (n=7, p <0.001 from control). Switching the saline flow back to control ACSF was followed by a complete recovery of evoked EPSP amplitude, which returned to a mean amplitude of 9.6 ± 3.4 mV (n=7), a value not significantly different from the control amplitude.

When observing EPSP recordings (Fig. 4b), the change in EPSP amplitude upon addition of estrogen is seen as a gradual decrease of EPSP amplitude within 20 to 30 minutes. Frequently (4 out of 7 cells), estrogen completely eliminated an evoked EPSP. Upon wash, the evoked EPSP amplitude recovered and the cell once again showed normal synaptic responses within 30 minutes. Spontaneous synaptic activity was also reduced in the presence of estrogen (Fig. 5a-c). The level of spontaneous

activity in control cells (Fig. 5a) is higher than that observed for cells in the presence of estrogen (Fig. 5B). Typically, cells with lower EPSP amplitudes had correspondingly lower levels of spontaneous activity.

Effects of ethanol, the vehicle used to dissolve estrogen and tamoxifen, on EPSP amplitude were studied as a control against the possibility of ethanol playing a role in the effects of estrogen on EPSP amplitude (Fig. 8). Mean EPSP amplitude in the presence of ACSF containing 2% ethanol was 9.0 ± 1.4 mV compared to mean control EPSP amplitude of 9.9 ± 2.9 mV (n=2). These findings indicate that alcohol alone has no effect on EPSP amplitude.

Effects of estrogen on synaptic transmission were also evident during multiple (train) stimulations of the presynaptic pathway (n=9). When the recordings of train stimulation are superimposed, the effects of estrogen in comparison to control ACSF are visible as a lower amplitude response to the stimulation (Fig. 6a). The reversibility of the response to estrogen is seen as a return of the control train response to normal (Fig. 6b). Same cell traces of train stimulation responses in the presence of control ACSF (Fig. 7a), estrogen (Fig. 7b), and wash (Fig. 7c) confirm the effect of estrogen on responses to train stimulation.

Preliminary data suggested that estrogen did not alter IPSP amplitude. When comparing IPSP amplitudes in control ACSF and ACSF plus estrogen (Fig. 10A), there appeared to be no noticeable difference. Individual traces in figures 11a-c further emphasize the similarity of IPSPs in the presence of normal ACSF, estrogen, and wash.

Estrogen appeared to have no effect on early membrane resistance (Fig. 12), late membrane resistance (Fig. 13), resting membrane potential (Fig. 15), number of action potentials (Fig. 16), or accommodation response (Fig. 17).

Membrane resistance was calculated from the size of the voltage deflection produced during the intracellular injection of a 450 ms, 400 pA hyperpolarizing current pulse. BLA pyramidal neurons normally respond to a hyperpolarizing current with an initial peak negative voltage response, followed by depolarizing (upward) voltage sag (Fig. 12) This is consistent with studies of neurons characterized in this region (Washburn and Moises, 1992b) The depolarizing sag is due to the hyperpolarization-induced activation of outward current (Womble and Moises, 1992). The input resistance of BLA neurons was thus determined at 2 time points: at the initial peak of the voltage response (early resistance) and just prior to the end of the current pulse (late resistance). Estrogen had no significant effect on

either the early or late membrane resistances. Mean early membrane resistance for control neurons in the presence of control estrogen and after estrogen washout were 61.5 ± 15.6 MΩ, 59.0 ± 13.0 MΩ, and 70.2 ± 9.9 MΩ respectively. These values are consistent with previous reports (Washburn and Moises, 1992b; Womble and Moises, 1992; Gean and Shinnick-Gallagher, 1988, 1989) Mean late membrane resistances for neurons in the presence of control ACSF, estrogen, and wash were 53.5 ± 13.6 MΩ, 52.0 ± 14.4 MΩ, and 63.4 ± 11.7 MΩ respectively.

Estrogen appeared to have no effect on the resting membrane potential of BLA neurons. Mean values of resting membrane potentials recorded in control ACSF, estrogen, and wash were not significantly different. Mean resting membrane potentials recorded in the presence of ACSF, estrogen, and wash are -62.6 ± 6.3 mV, -64.8 ± 7.5 mV, and -62.5 ± 9.3 mV respectively.

Estrogen also appeared to have no effect on action potential frequency in BLA neurons. Action potentials are easily distinguished by a sharp depolarization followed by a sharp hyperpolarization. Action potential frequency was measured by the number of action potentials generated during 450 ms pulse of 800 pA depolarizing current. In control ACSF, the mean action potential number was 5.9 ± 2.5 APs. Cells in the presence of estrogen

and wash had mean AP numbers of 4.6 ± 3.0 APs and 7.8 ± 2.2 APs respectively. In addition, the recordings were examined for any change in accommodation response (fig. 17). Accommodation response is seen as a relative change in the frequency of action potential firing over time. Upon examination, no significant differences were found in the accommodation response of neurons studied under saline flow containing control ACSF and estrogen. Additionally, no changes were found when the bath was switched from ACSF containing estrogen back to normal ACSF.

To test the specificity of the estrogen response, an estrogen receptor antagonist was used to block estrogen receptors. The chosen antagonist, 4-OHT was used at 60 nM concentration. This concentration was used because 4-OHT's binding affinity for ERα is over 300 times that of estrogen (Kuiper *et al.*, 1997) The effects of this drug on BLA neurons (n=2) were examined to study the drug's blocking and direct effects on the cell. These preliminary results may point to possible roles estrogen has in the brain.

4-OHT has the effect of significantly increasing the EPSP amplitude ($p < 0.05$)(Fig. 16). Mean EPSP amplitude for recordings made in the presence of 4-OHT are 15 ± 0 mV. This is significantly greater than the mean of EPSP amplitudes recorded in control ACSF (9.9 ± 2.9 mV, $p < 0.05$). 4-OHT prevented the inhibitory effect of estrogen on EPSP

33

amplitude. Concurrent 4-OHT and estrogen perfusion gave no significant change in the EPSP amplitude (mean= 9.8 ± 4.6 mV).
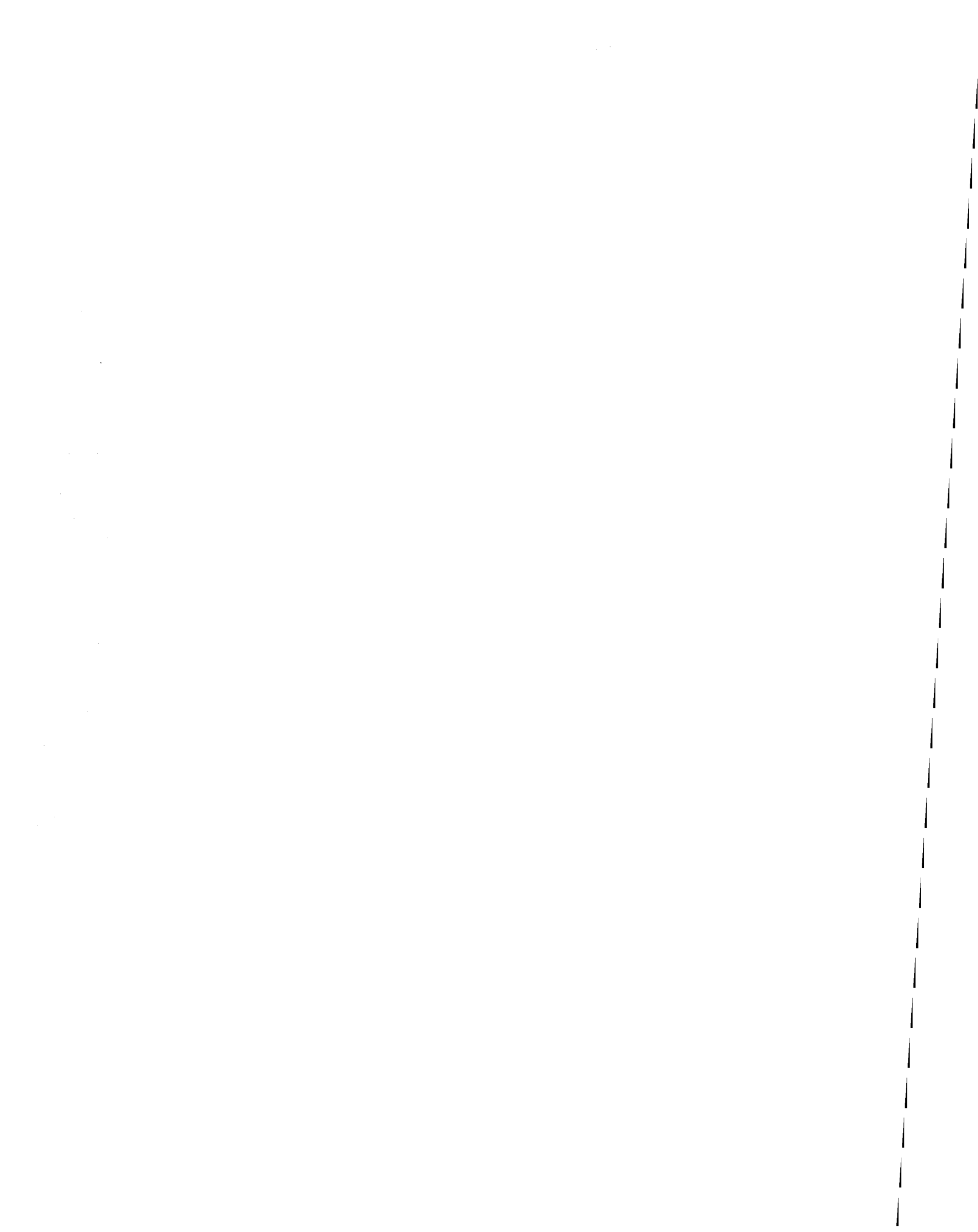
4-OHT alone or concurrent perfusion of 4-OHT and estrogen appeared to have no effect on early membrane resistance (Fig. 17), late membrane resistance (Fig. 18), resting membrane potential (Fig. 19), frequency of action potential number (Fig. 20), or the accommodation response (not shown).

4-OHT and concurrent perfusion of 4-OHT and estrogen had no significant effect on the early or late membrane resistances. In control ACSF, the mean value of early membrane resistances was 63.8 ± 14.8 MΩ. Cells in the presence of 4-OHT and concurrent perfusion of 4-OHT and estrogen had mean early membrane resistances of 54.0 ± 8.5 MΩ and 62.5 ± 16.3 MΩ respectively. The mean value of late membrane resistances in control ACSF was 62.6 ± 6.3 MΩ Cells in the presence of 4-OHT and concurrent perfusion of 4-OHT and estrogen had mean late membrane resistances of 62.5 ± 7.2 MΩ, and 72.5 ± 3.5 MΩ respectively.

4-OHT and concurrent perfusion of 4-OHT and estrogen also appear to have no effect on the resting membrane potential of BLA neurons. In control ACSF, the mean value of resting membrane potentials was -62.6 ± 6.3 mV. Cells in the presence of 4-OHT and concurrent perfusion of 4-OHT

and estrogen had mean resting membrane potentials of -62.5 ± 3.5 mV and - 72.5 ± 3.5 mV respectively. These values were not significantly different.

4-OHT and concurrent perfusion of 4-OHT and estrogen appeared to have no effect on action potential number. In control ACSF, the mean number of action potentials was 5.9 ± 2.8 APs. Recordings in the presence of 4-OHT and concurrent perfusion of 4-OHT and estrogen had mean action potential numbers of 5.0 ± 2.8 APs, and 3.0 ± 1.5 APs respectively. In addition to action potential frequency, the recordings were examined for any change in accommodation response. Upon examination, no noticeable differences were found in accommodation response of neurons studied in control ACSF, ACSF plus 4-OHT, or concurrent perfusion of 4-OHT and estrogen (Fig. 17a, b).
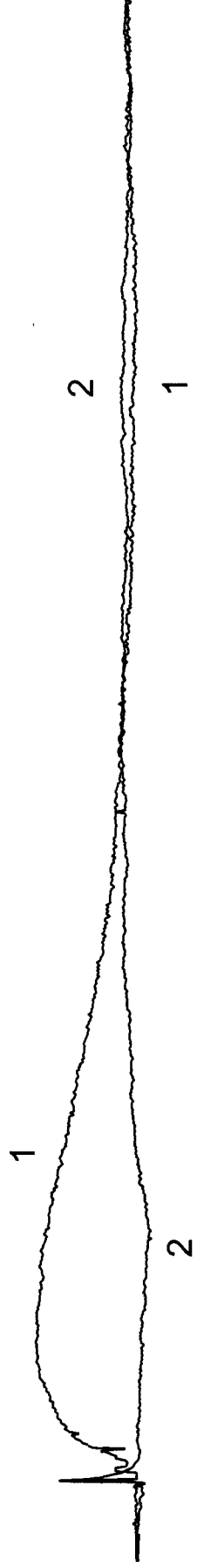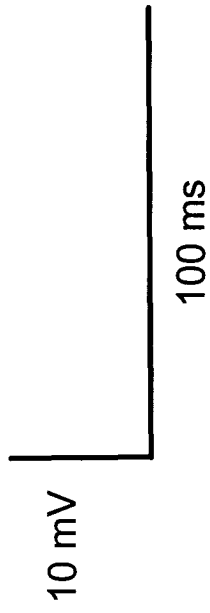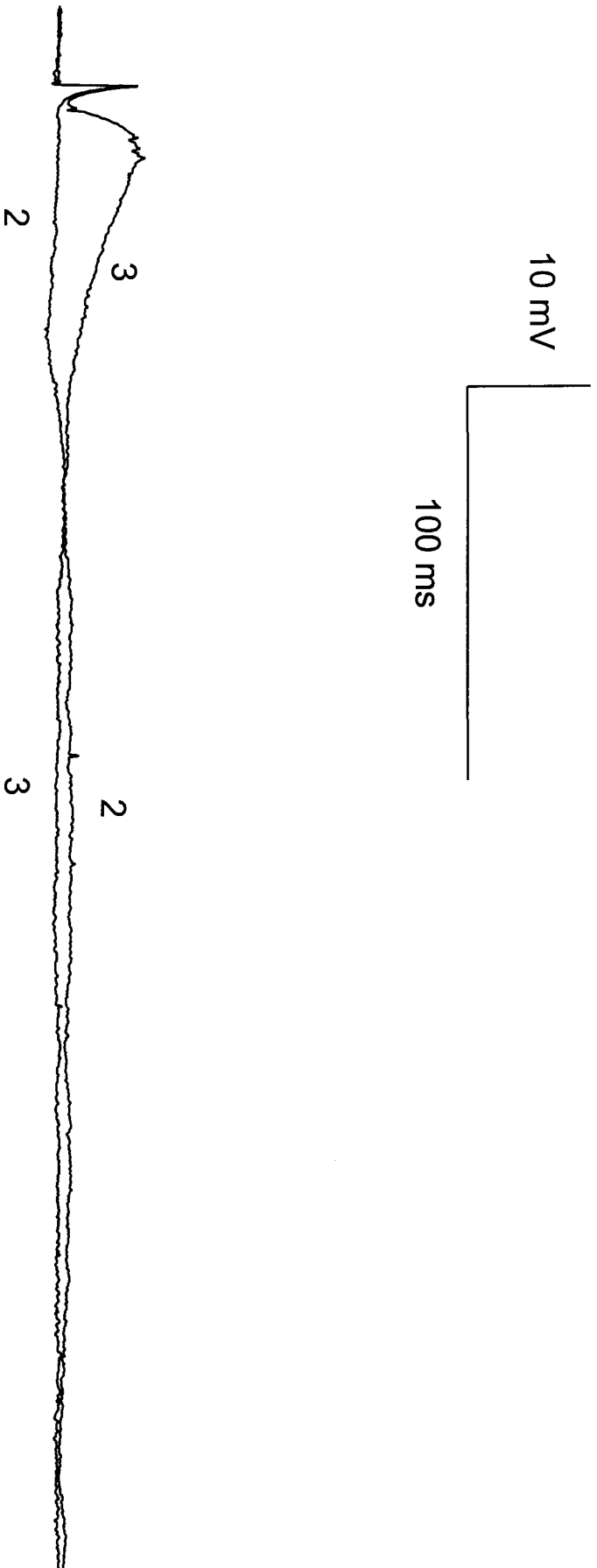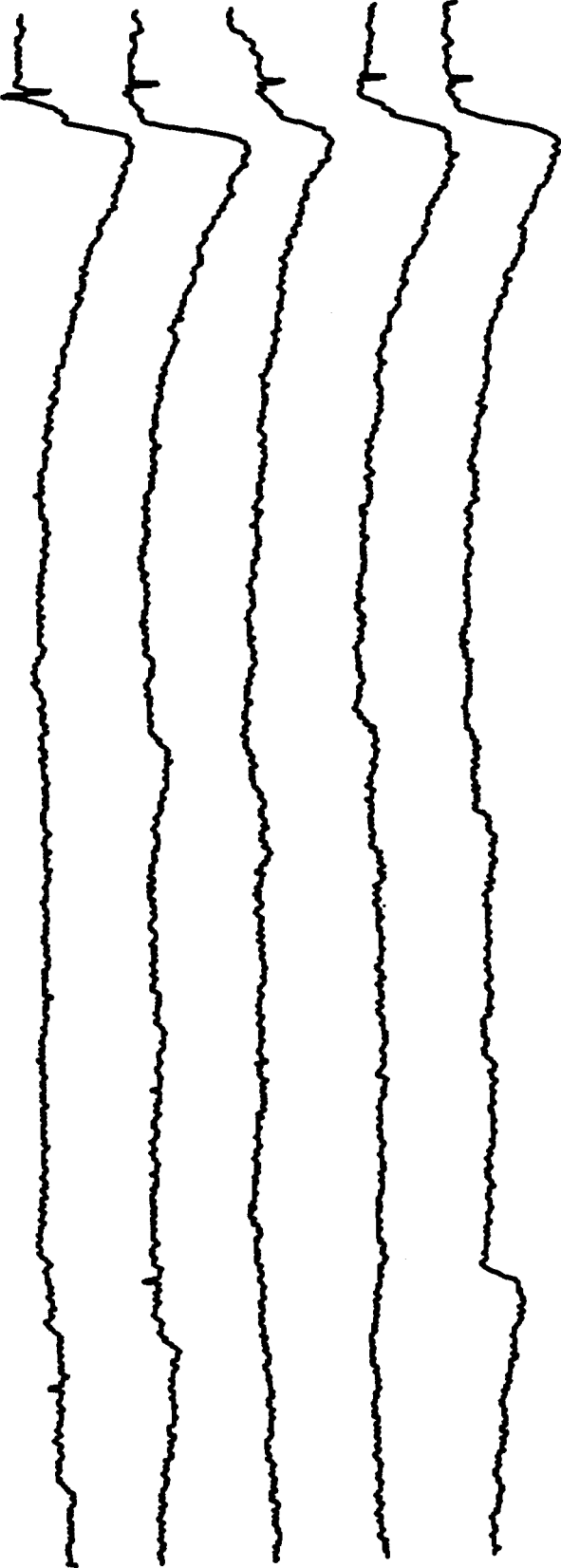
Effect of Estrogen on EPSP Amplitude

4a)

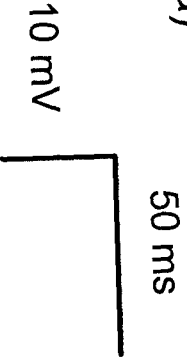10 mV
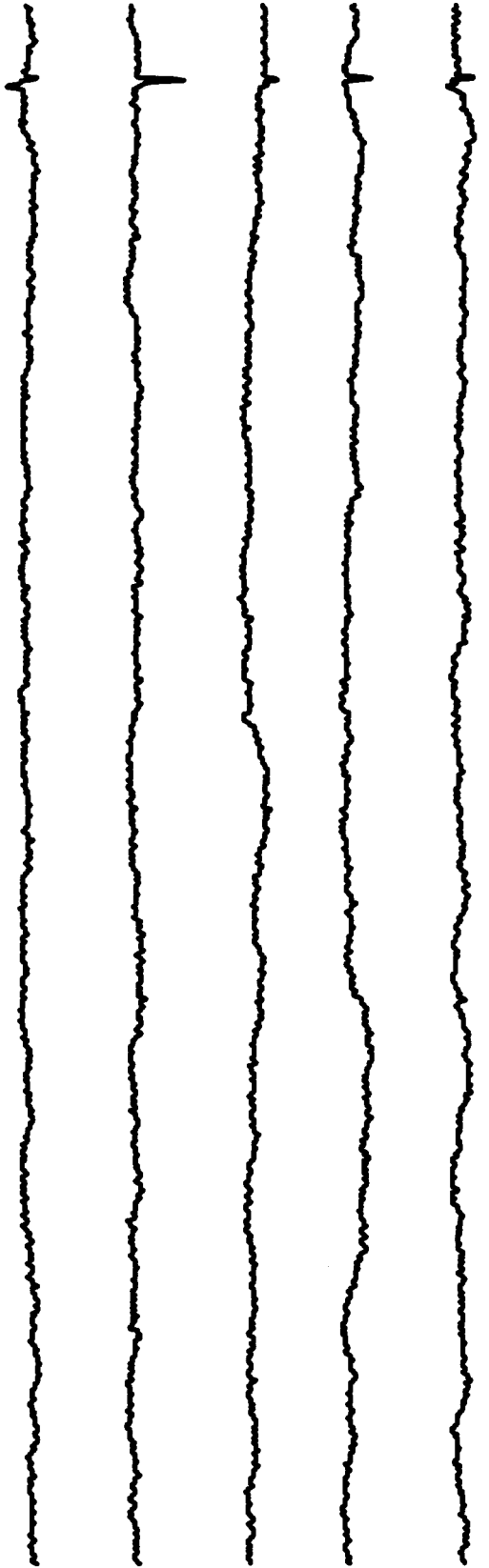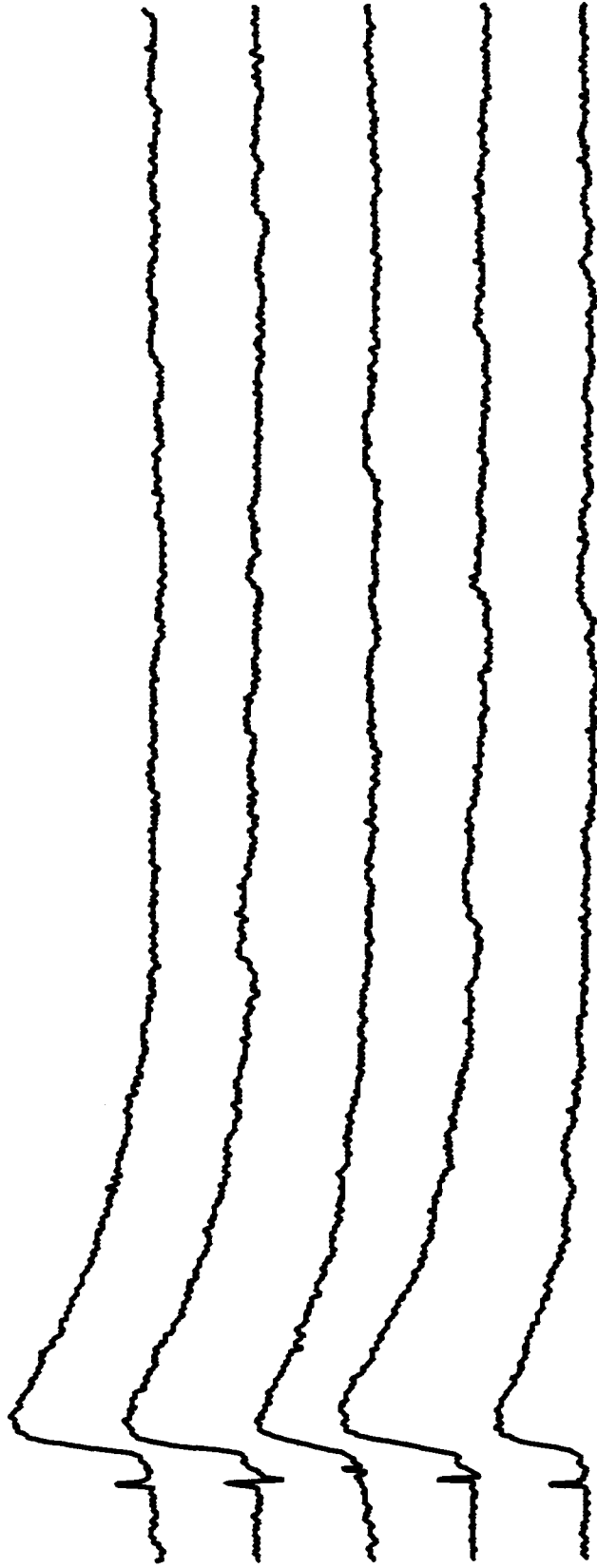
100 ms

1

2

1

2

4b)

10 mV

100 ms

2

3

3

2

5a)

10 mV

50 ms

5b)
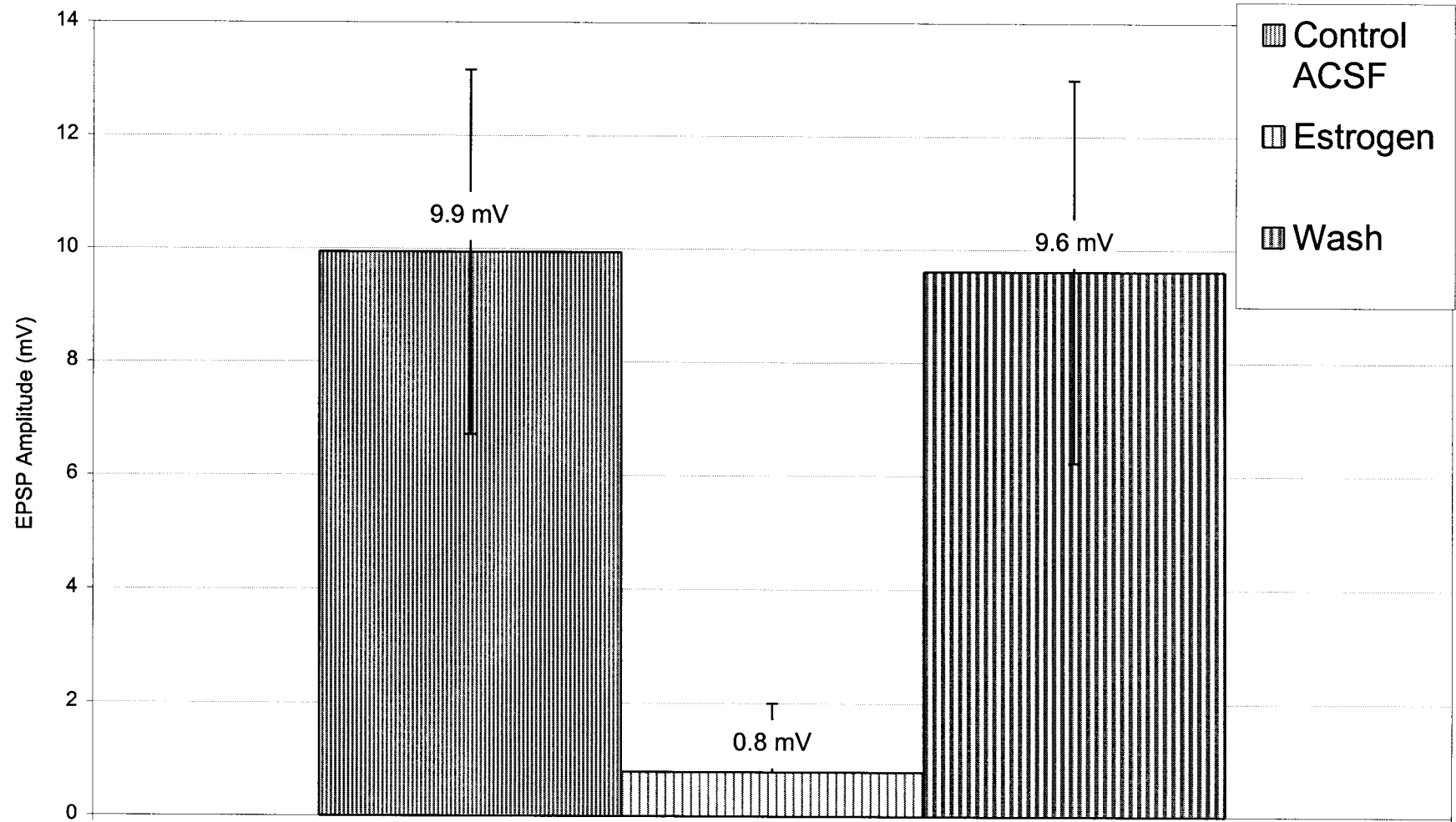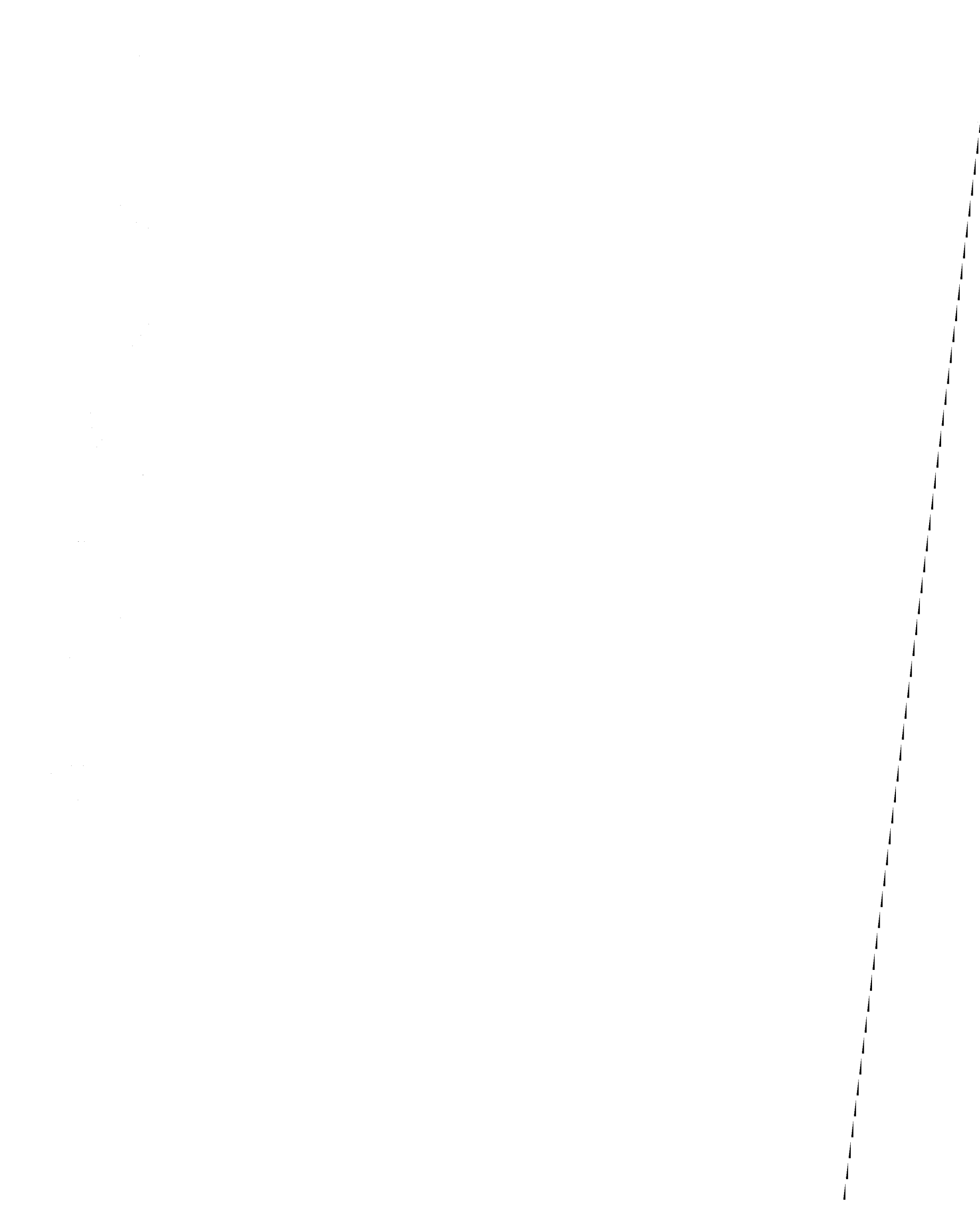
10 mV

50 ms

5c)

50 ms

10 mV

6a)

10 mV

500 ms

1

2

1

2

*
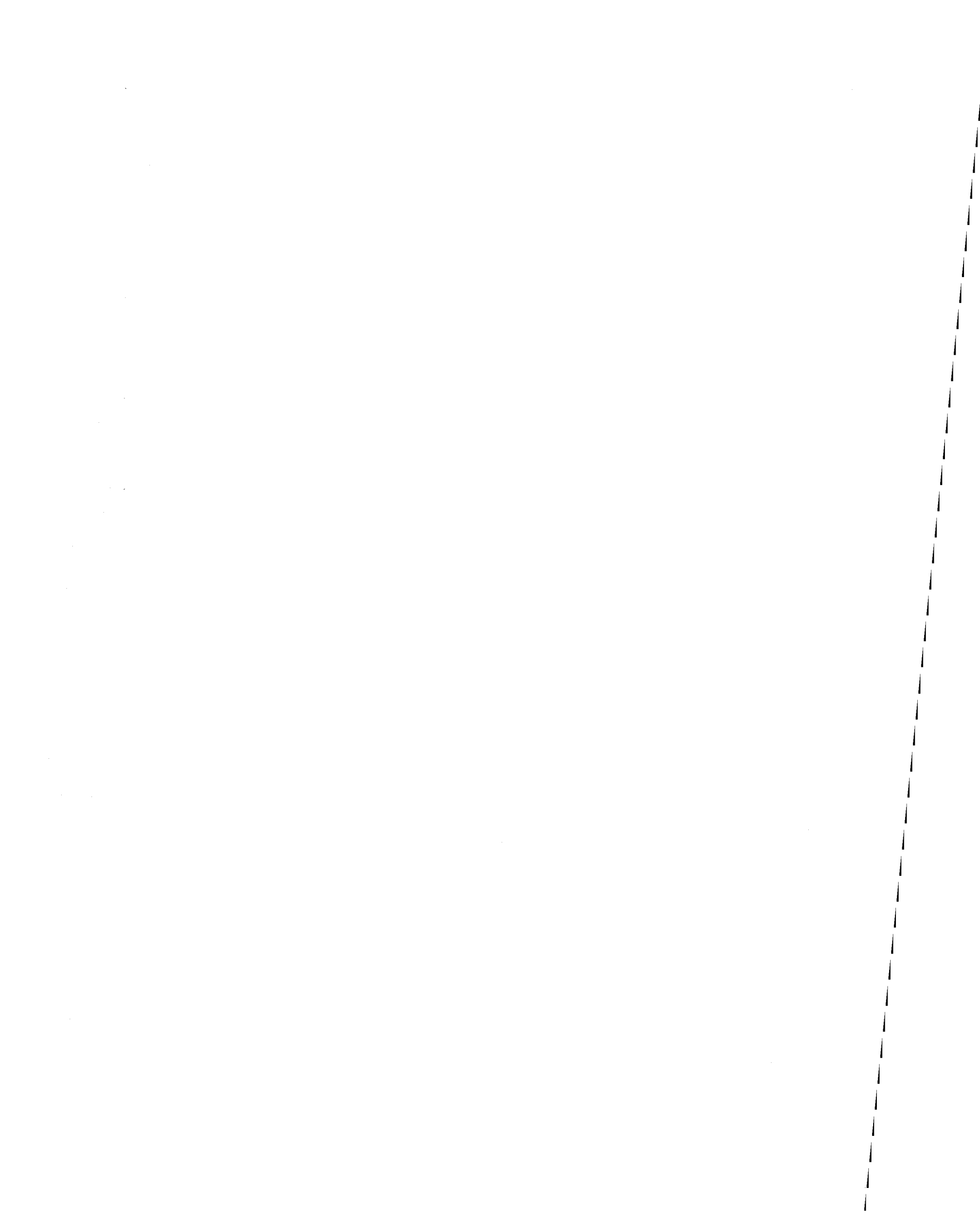
6b)
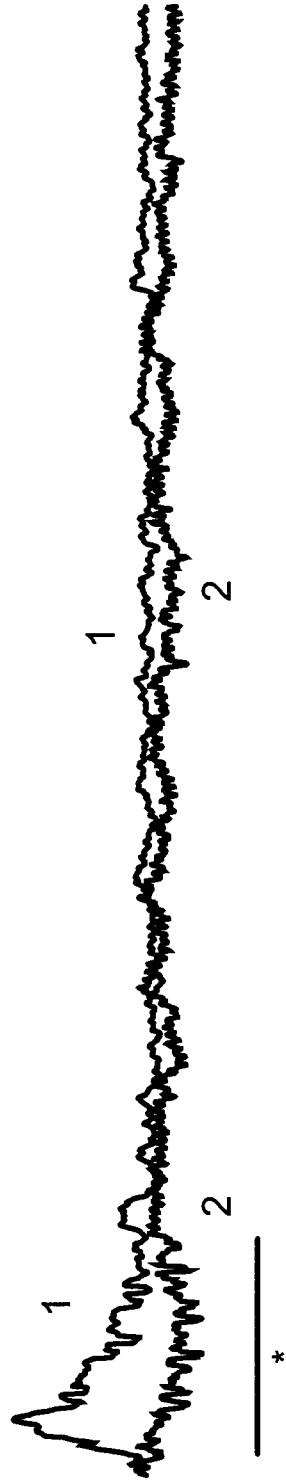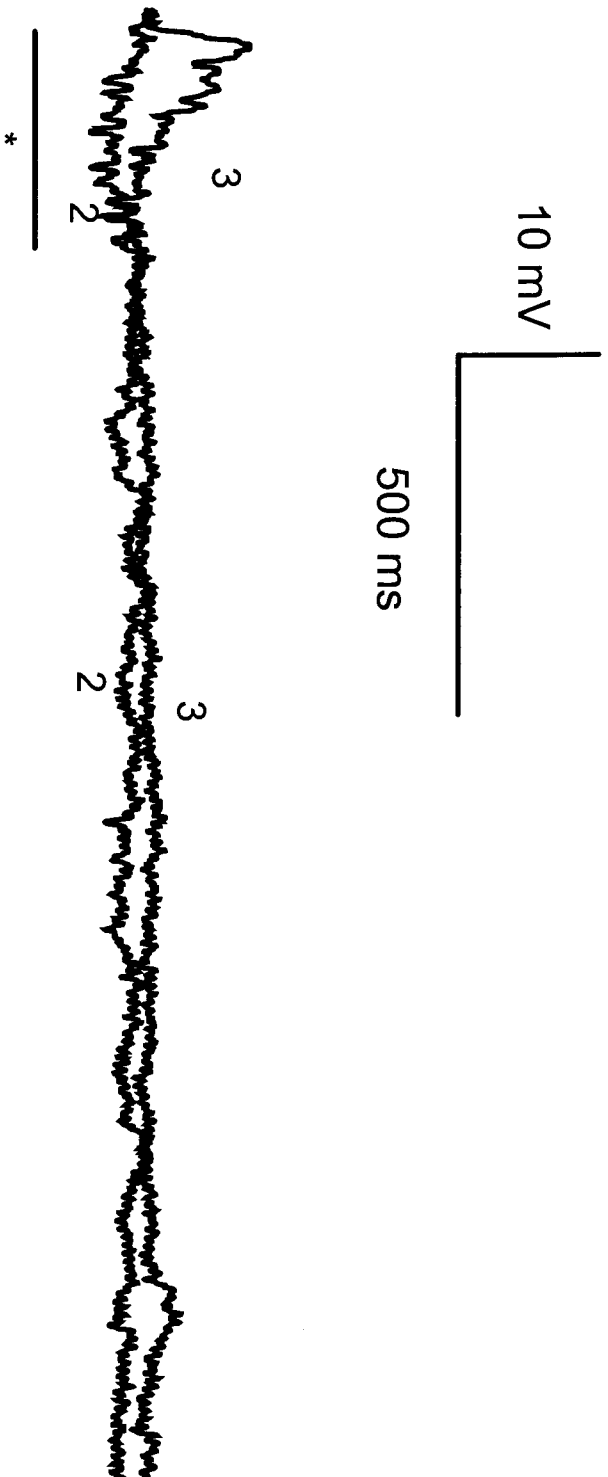
10 mV

500 ms

7a)

10 mV

250 ms

*
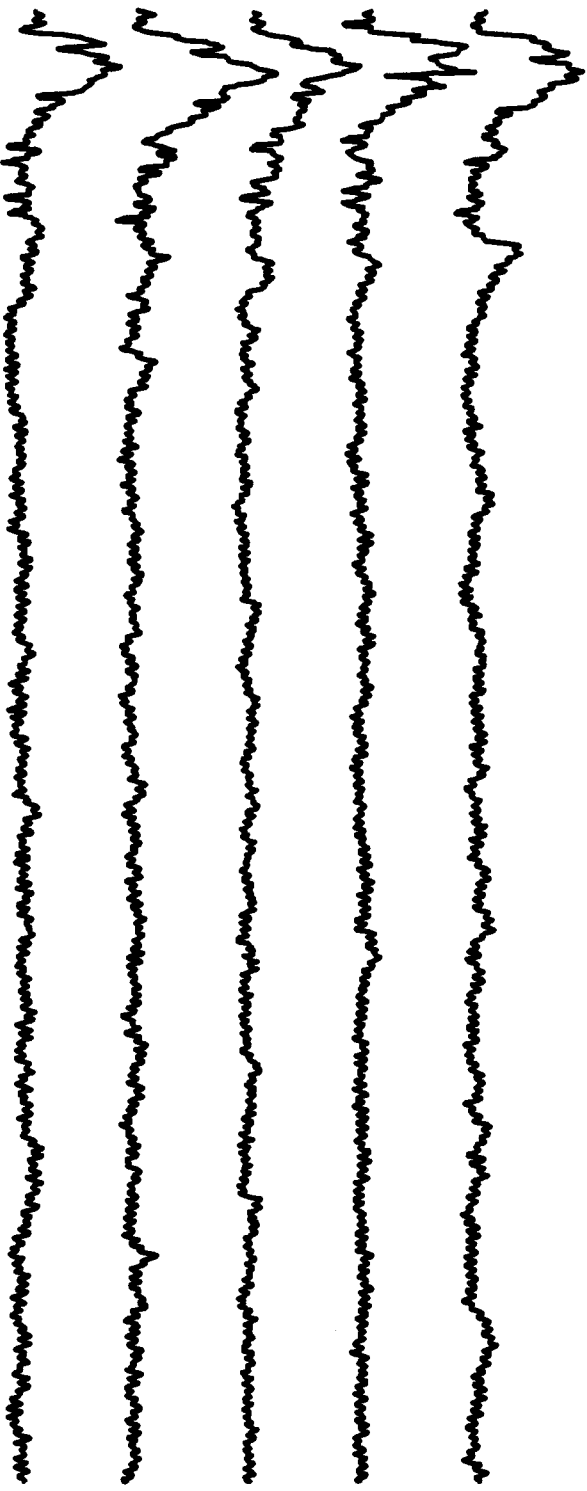
7b)

10 mV

250 ms

*
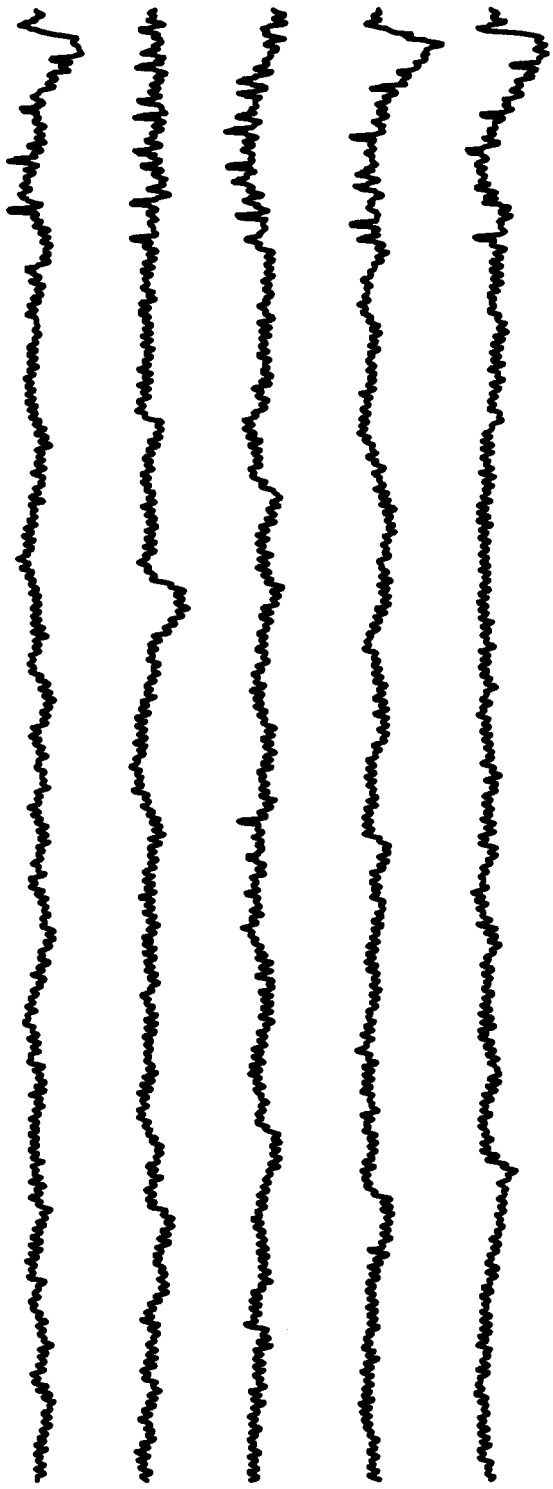
7c)

10 mV

250 ms

*
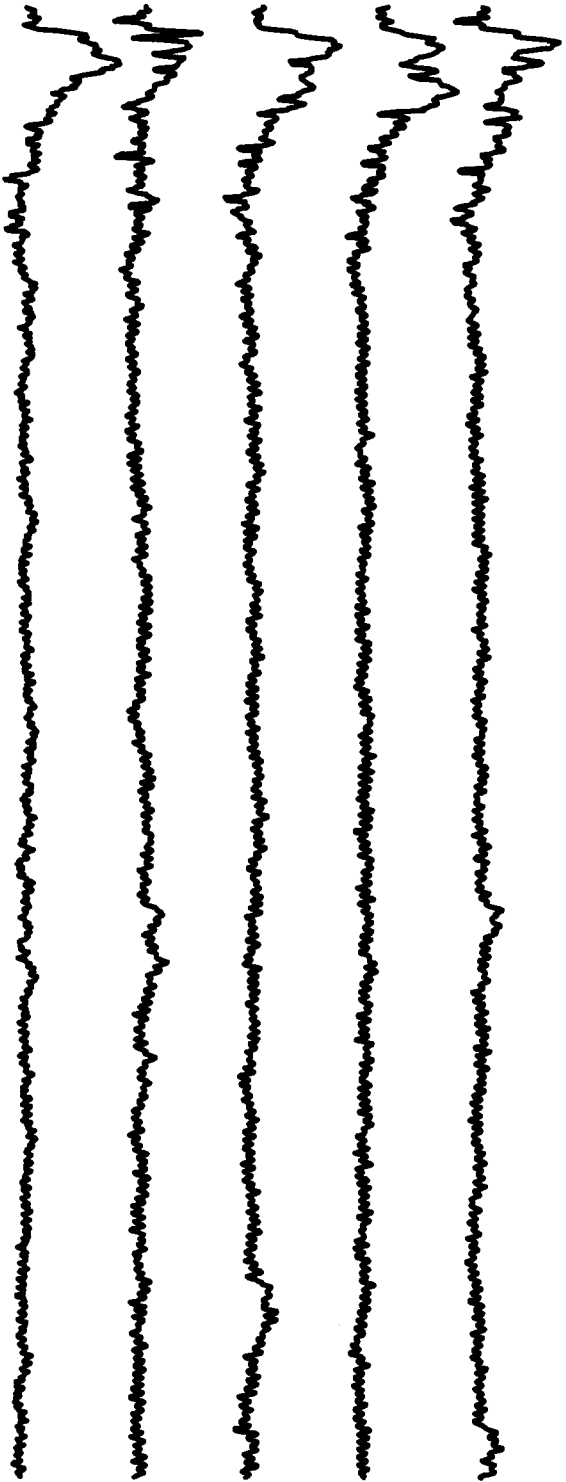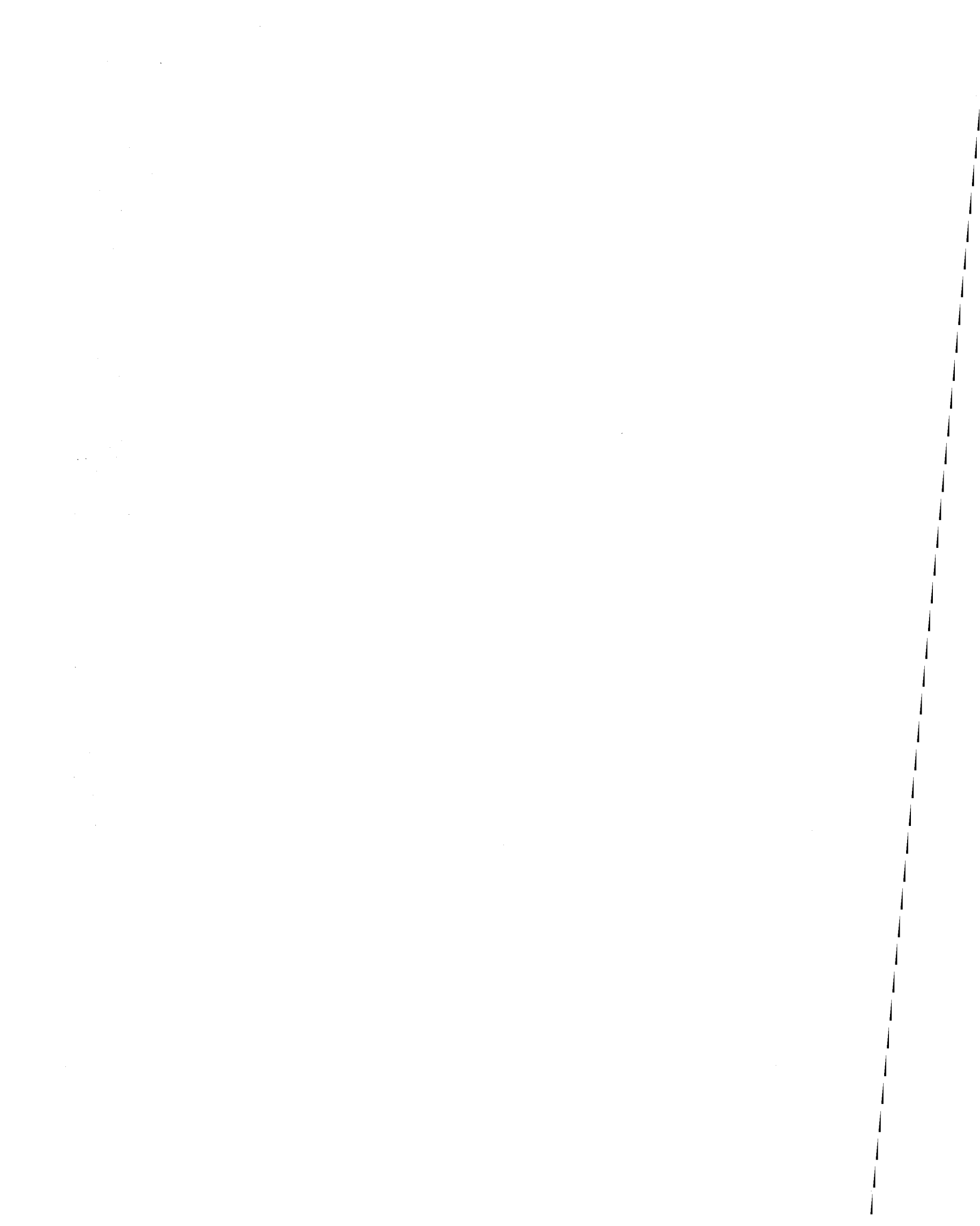
Effect of Ethanol Vehicle on EPSP Amplitude

9a)

10 mV

100 ms

(9b)

10 mV

100 ms

3

2

2

3

10a)

10 mV

500 ms

10b)

10 mV

500 ms

3

2

2

3

3

11a)

10 mV

500 ms

11b)
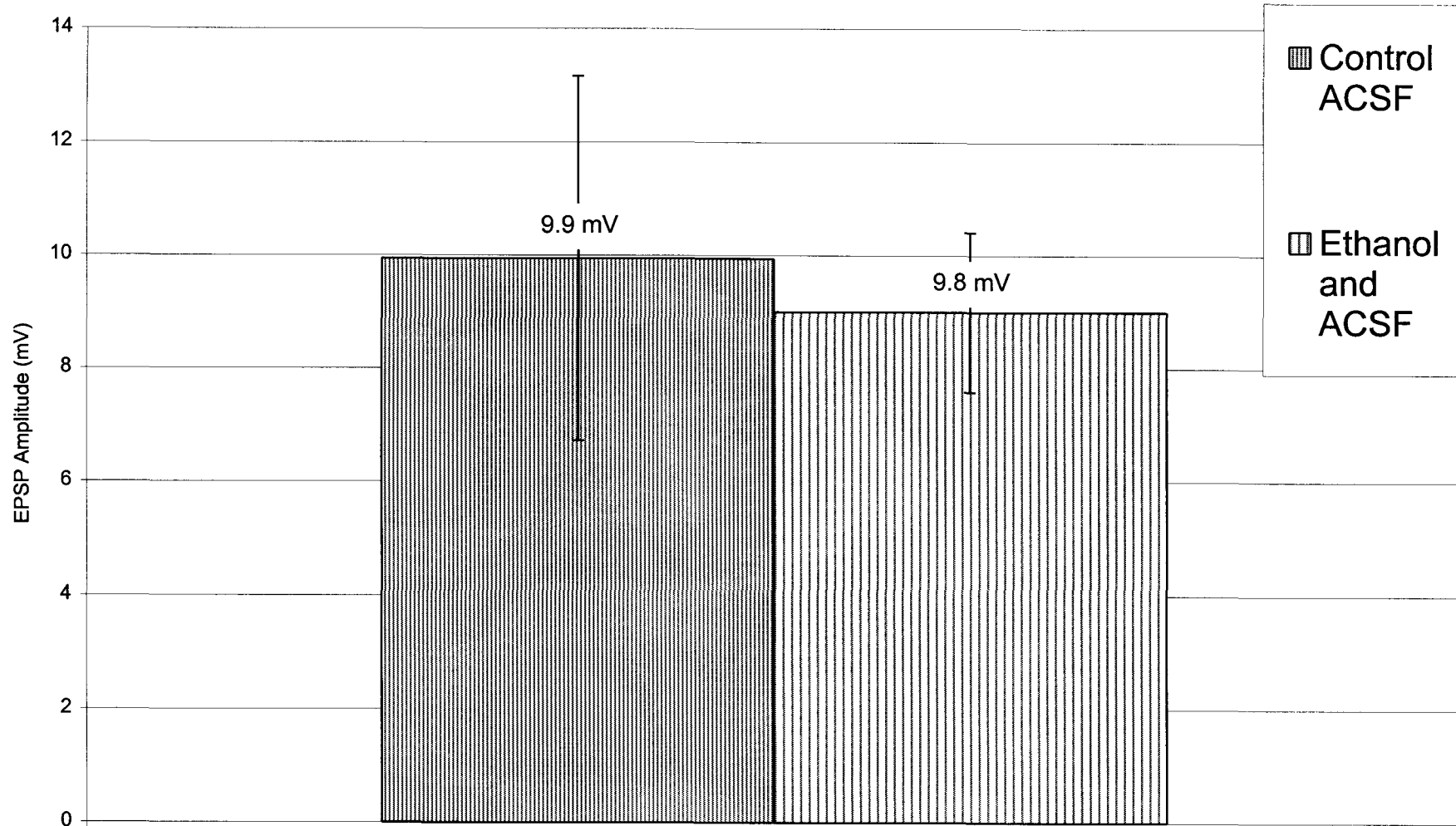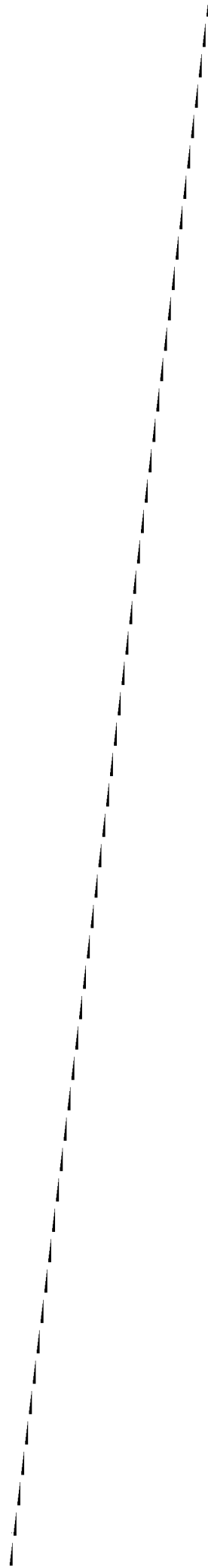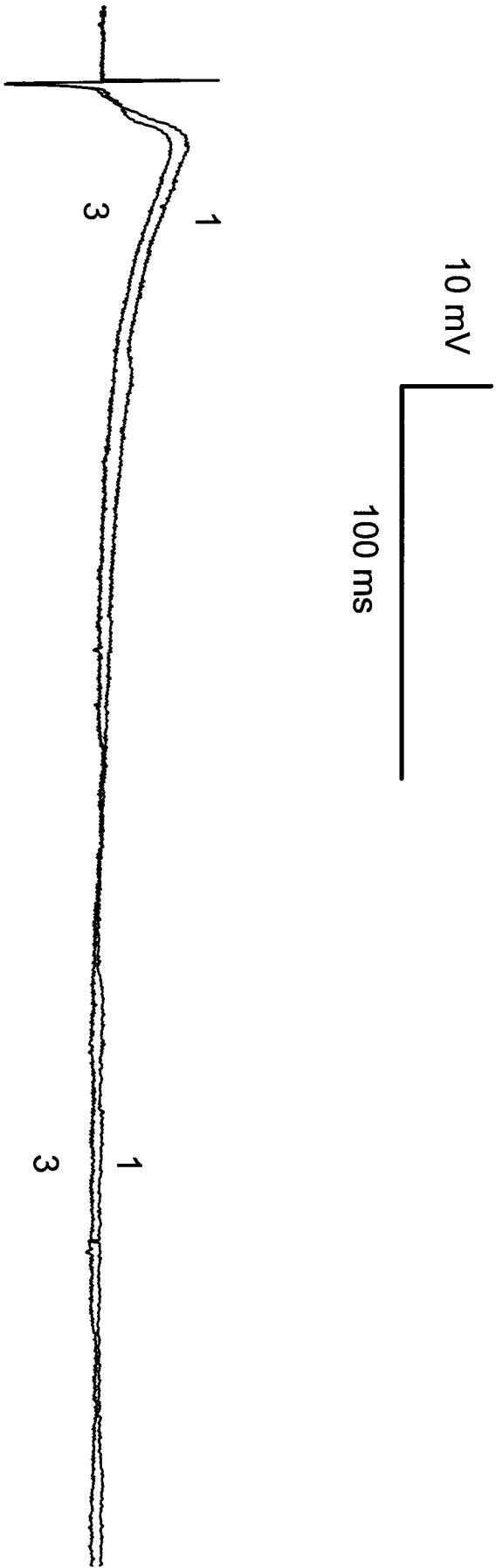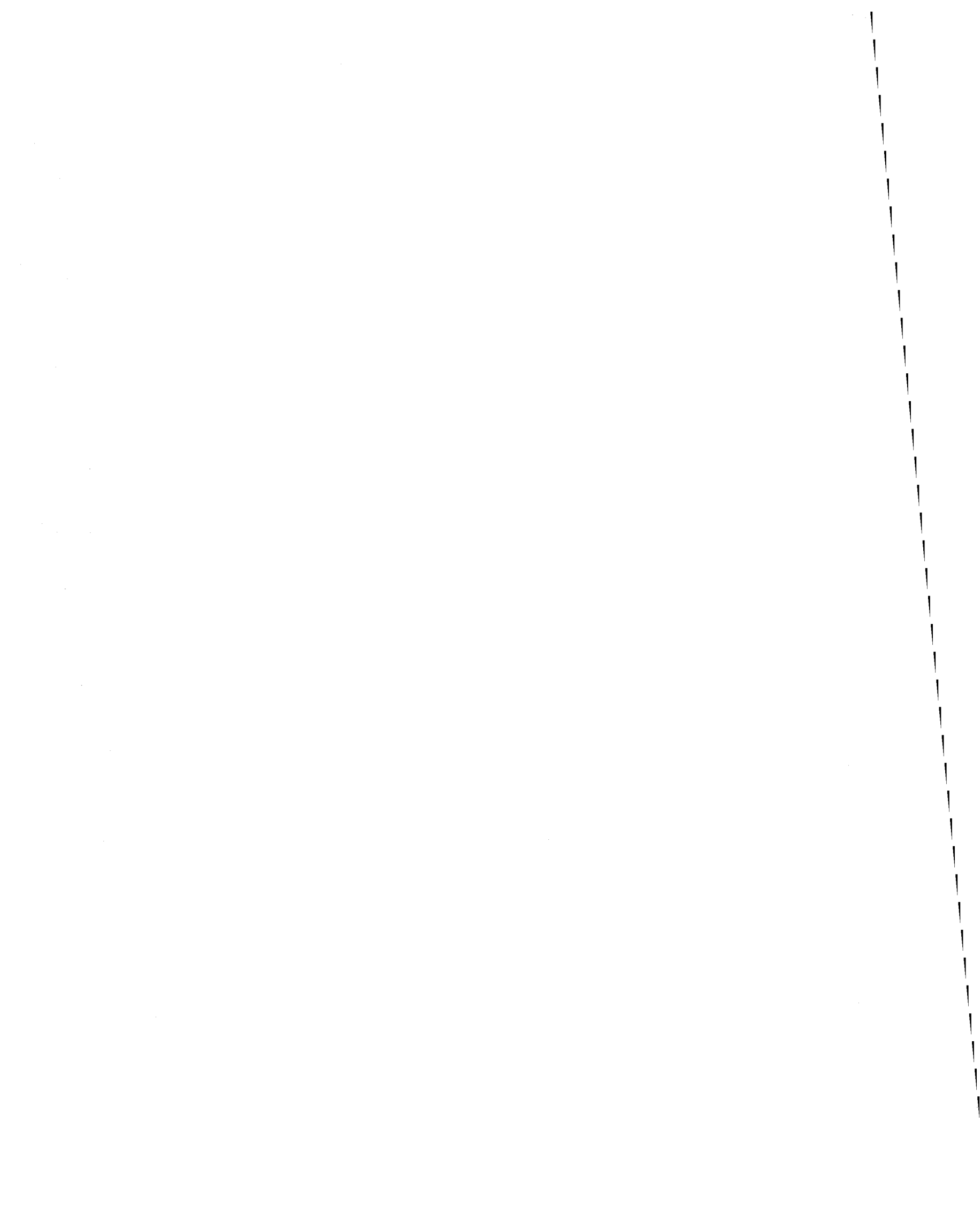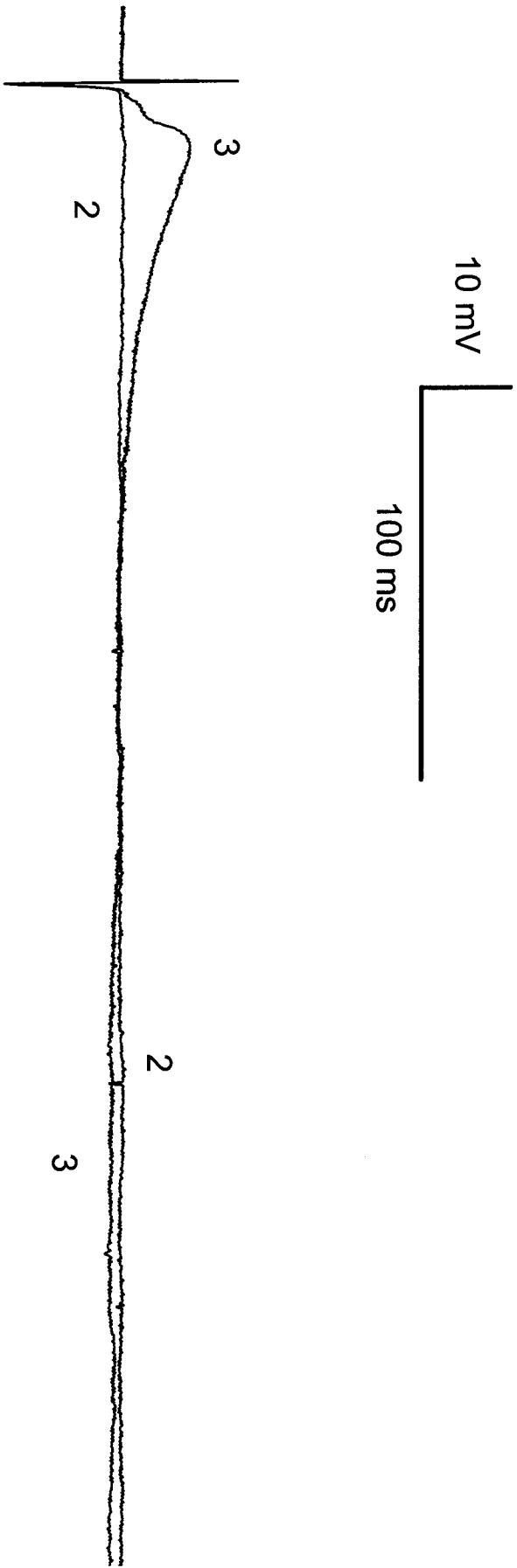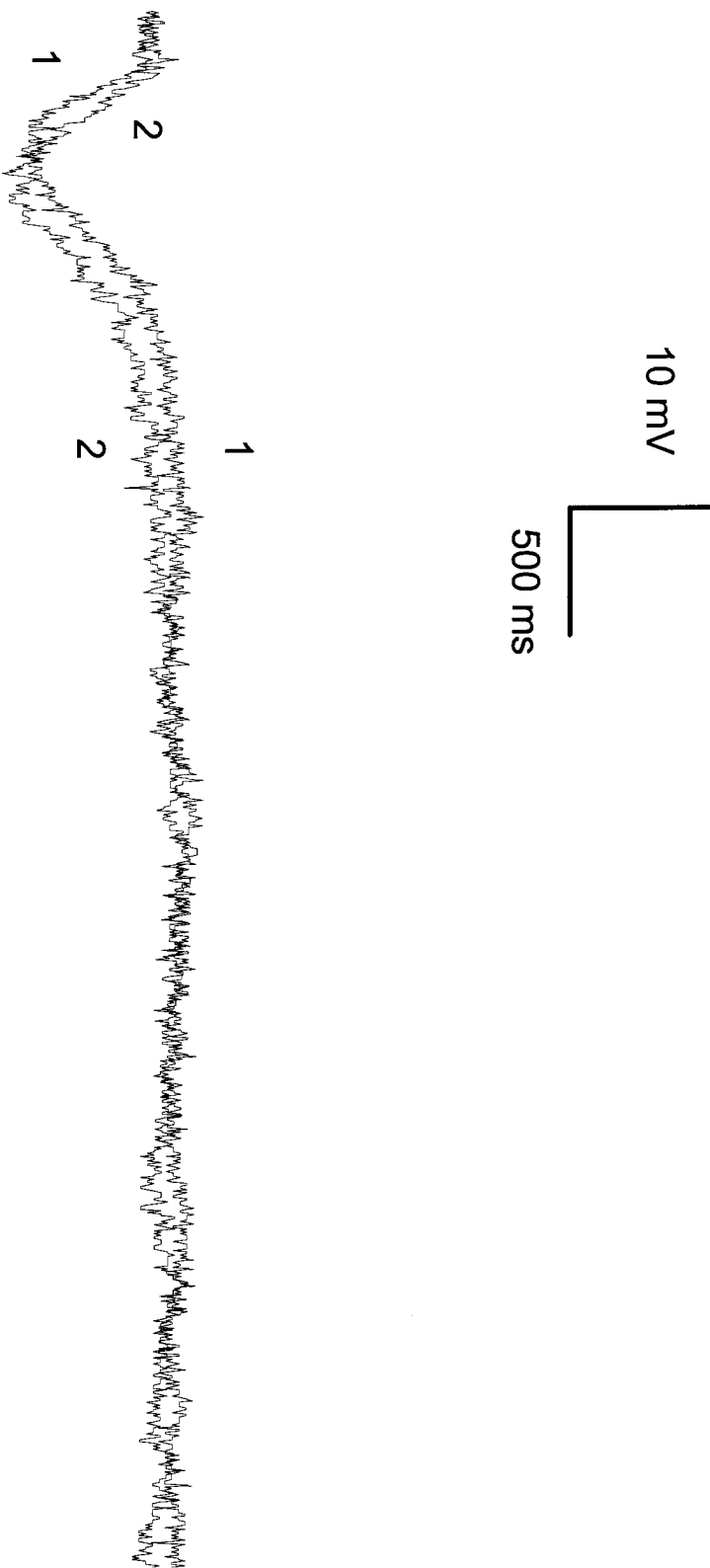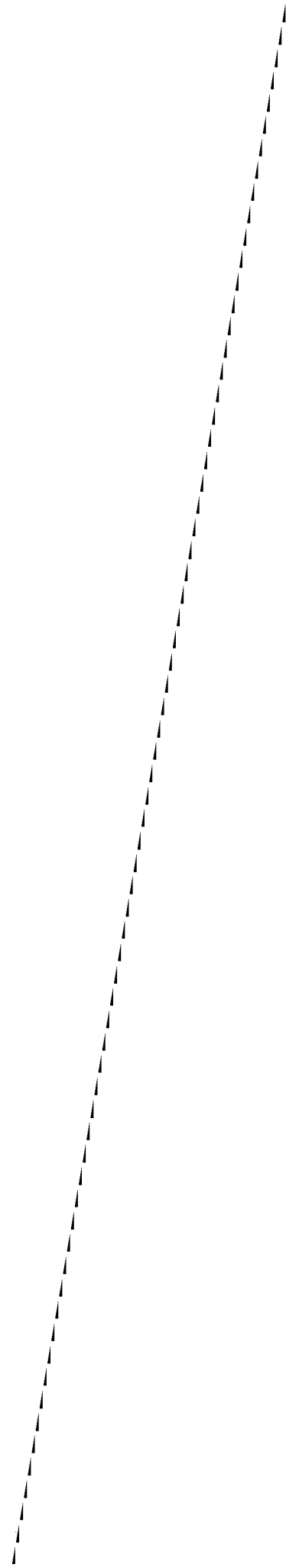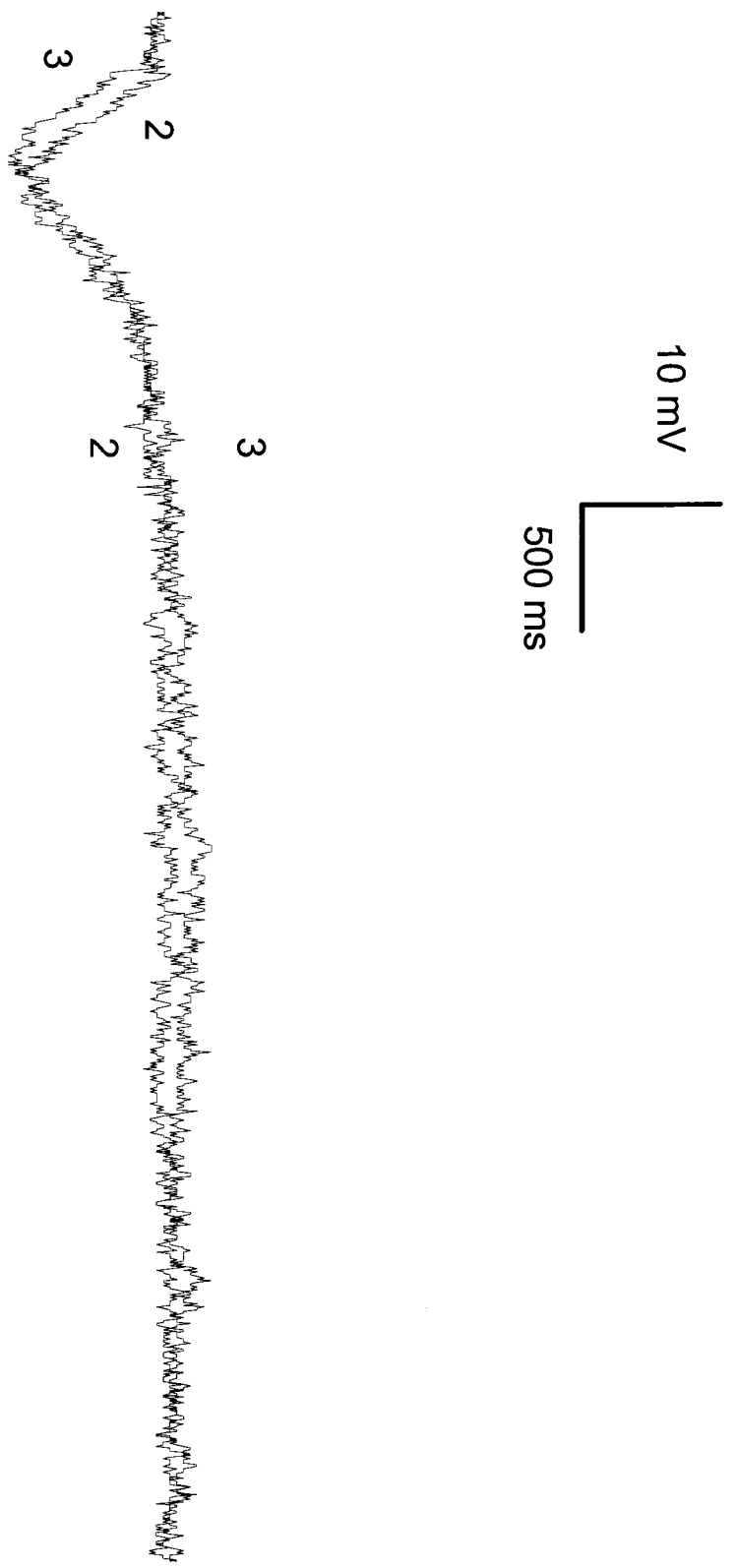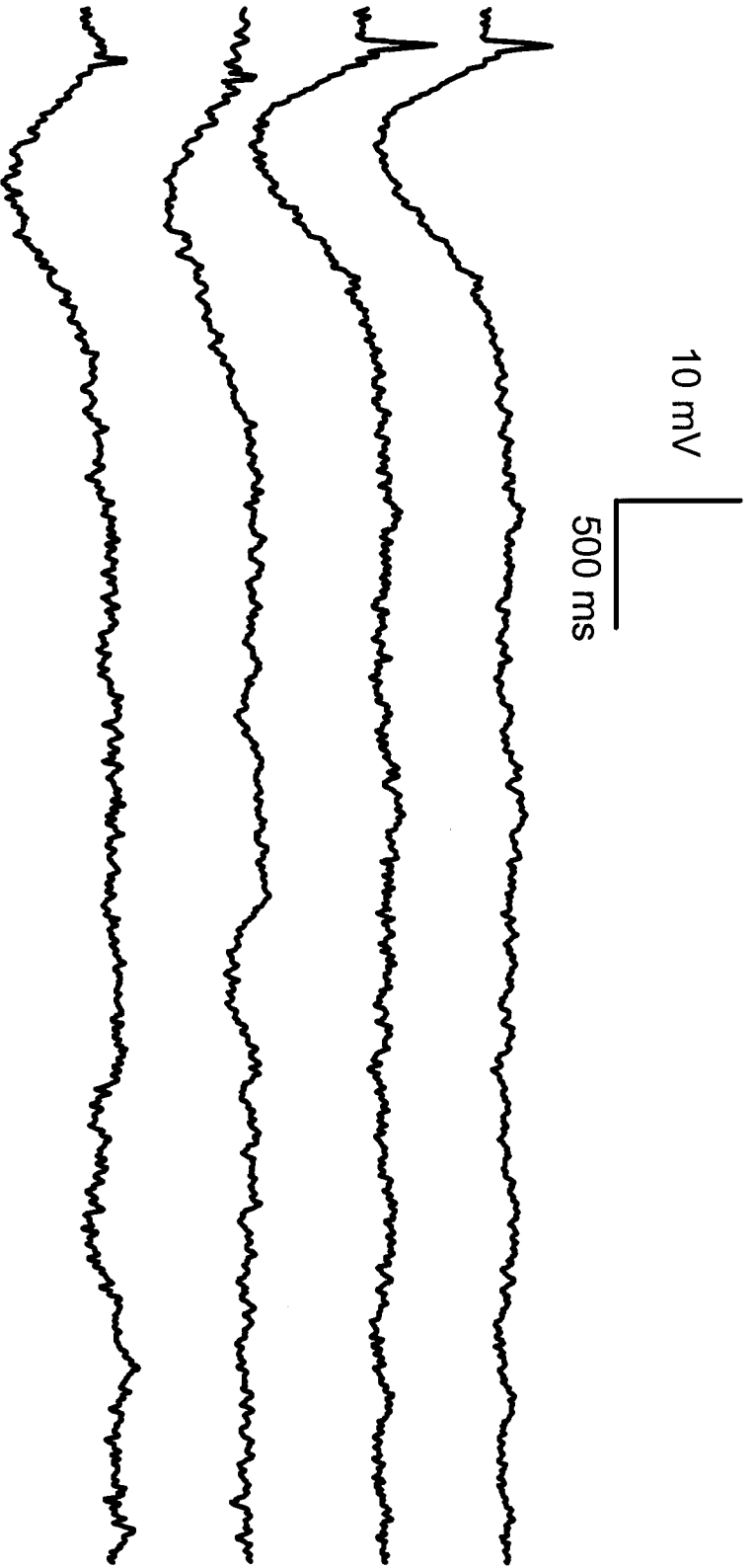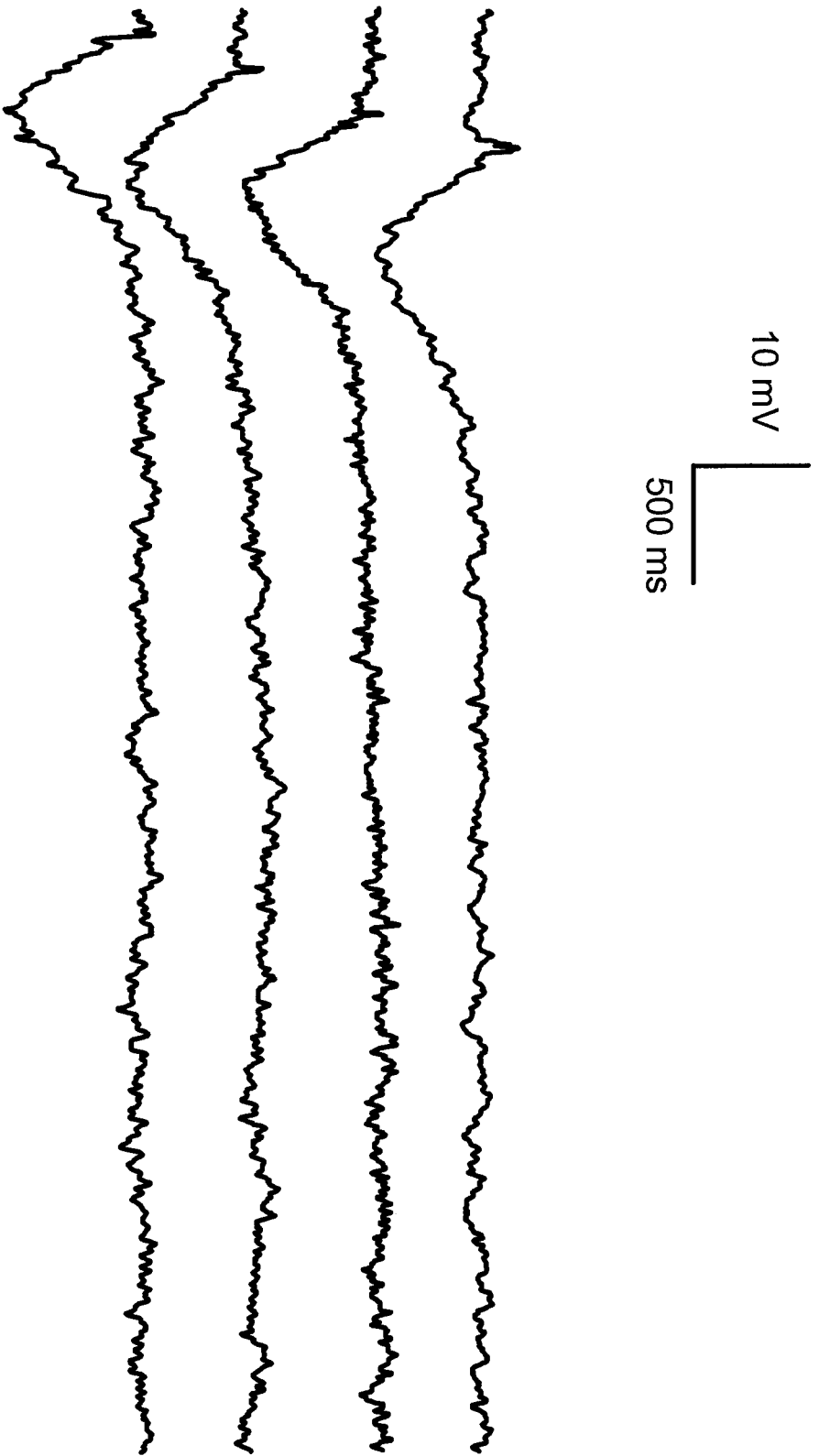
10 mV

500 ms

11c)

10 mV
500 ms
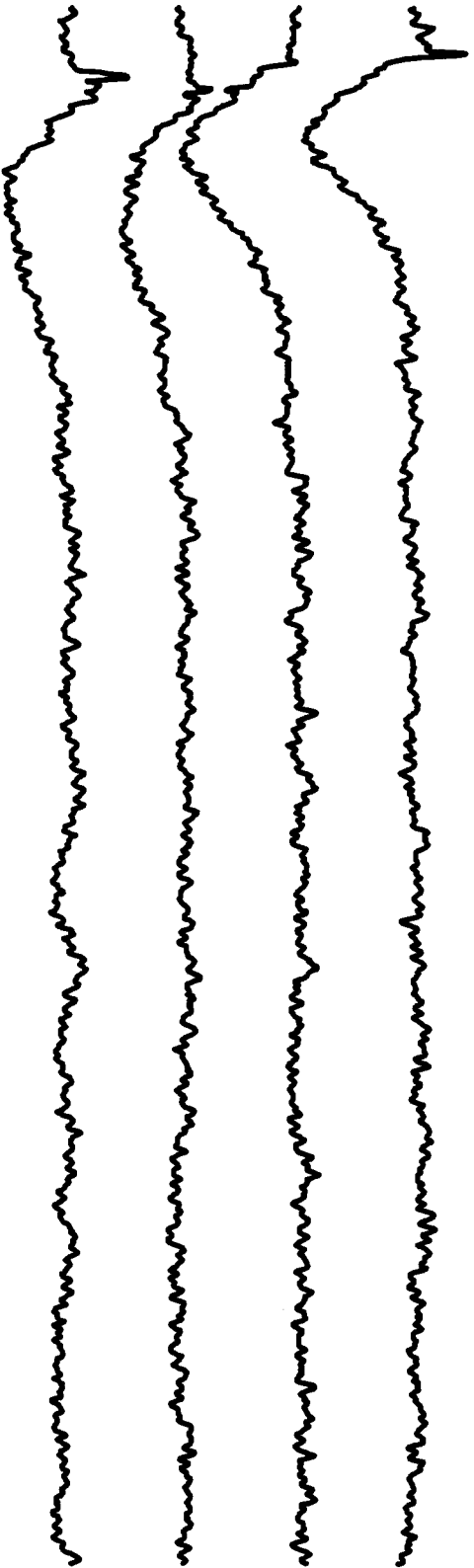
Effect of Estrogen on Early Membrane Resistance

Figure: Bar chart showing Resistance (MOhms) on the y-axis ranging from 0 to 90. Three bars are shown with a legend indicating Control ACSF, Estrogen, and Wash.

- 61.5 MOhms (Control ACSF)
- 59.0 MOhms (Estrogen)
- 70.2 MOhms (Wash)
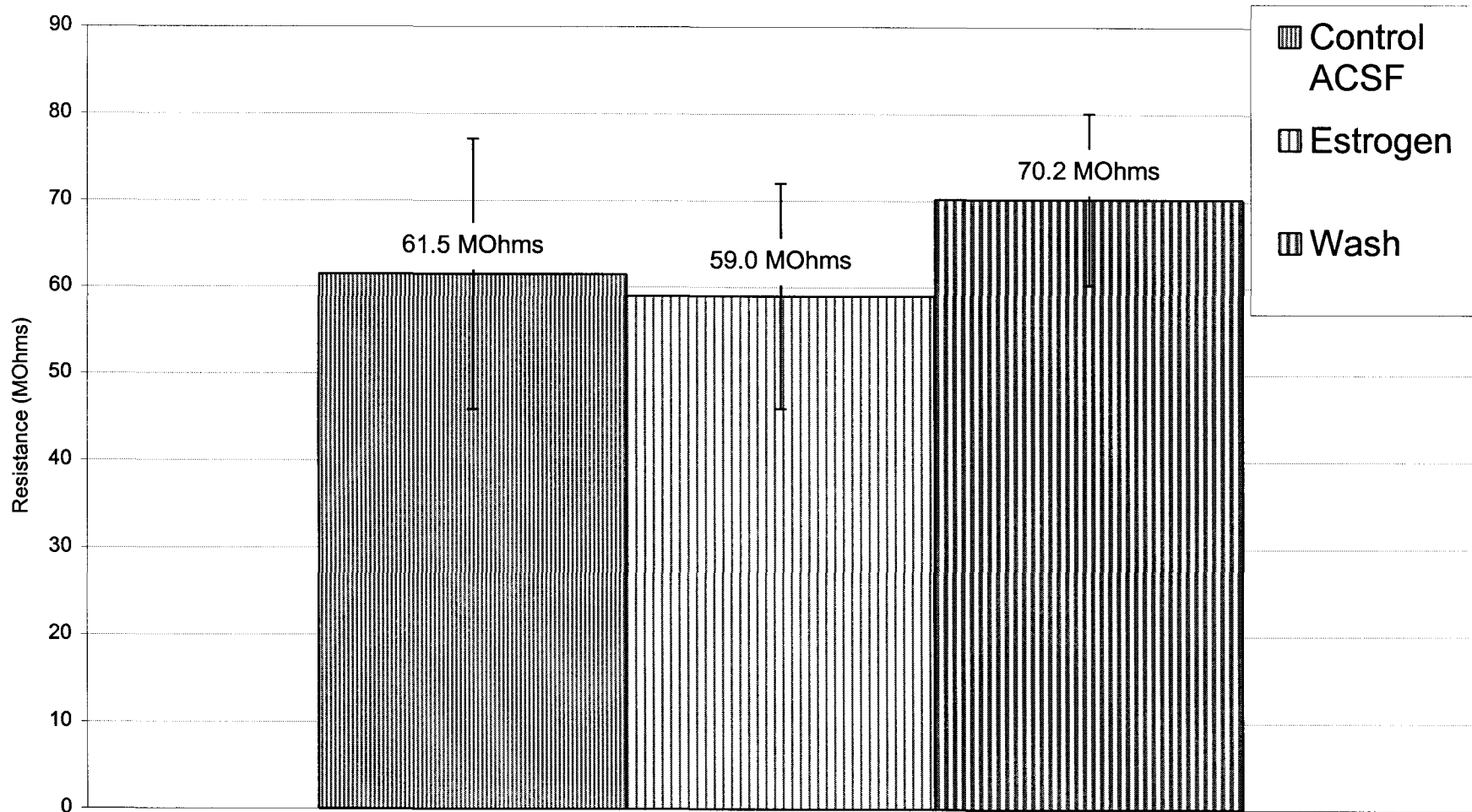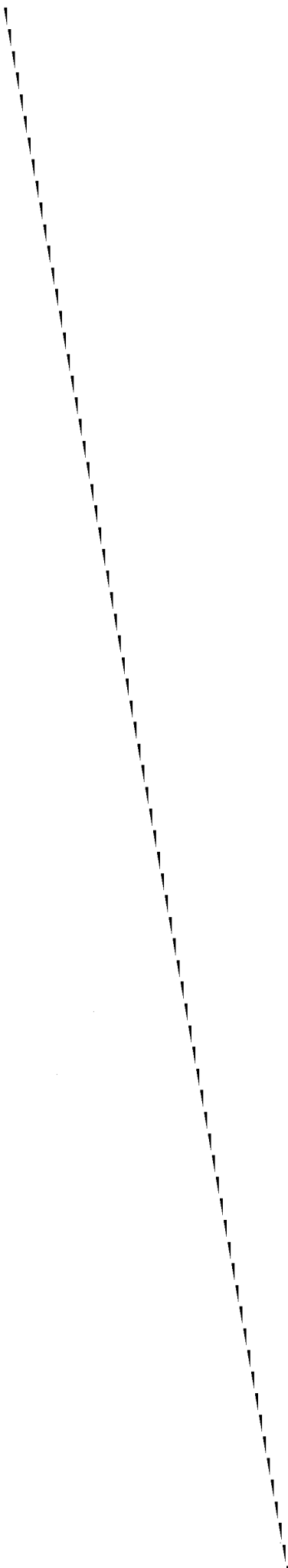
Effect of Estrogen on Late Membrane Resistance

Legend: Control ACSF, Estrogen, Wash

Bar values: 53.5 MOhms, 52.6 MOhms, 63.4 MOhms

14)

-60 mV

Ve

2

1

Vl

*

20 mV

200 ms
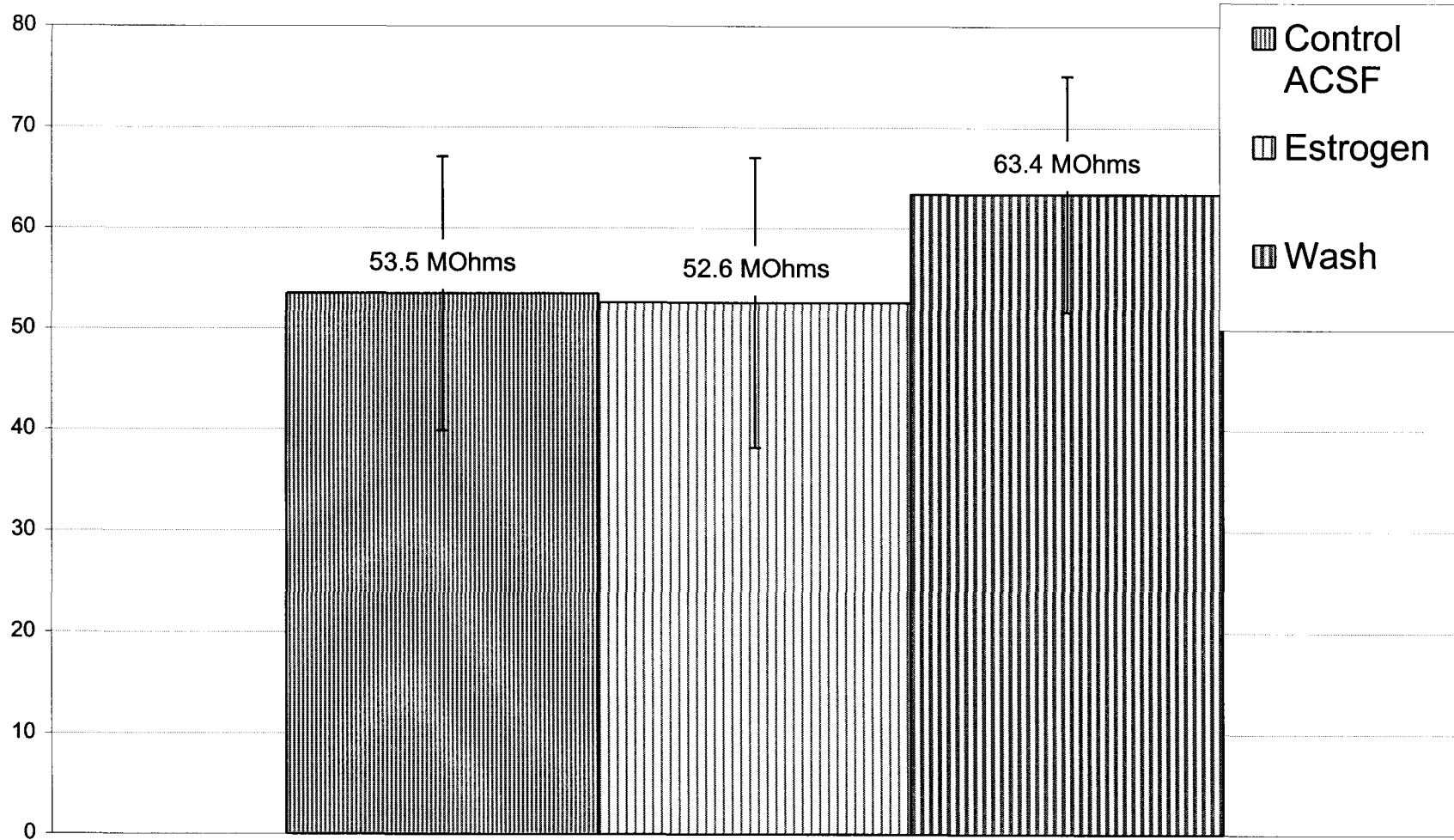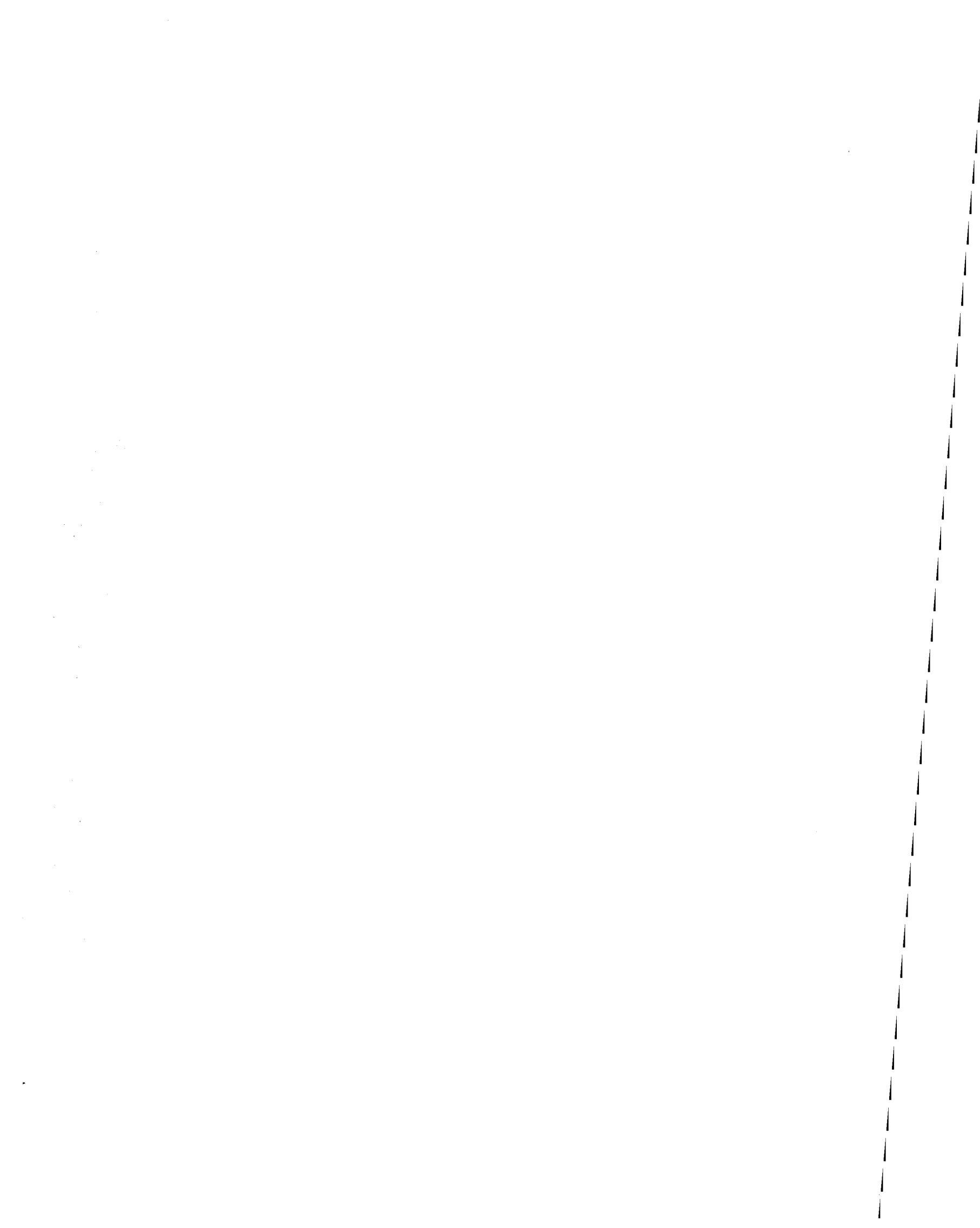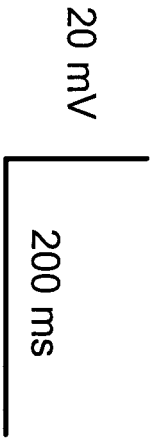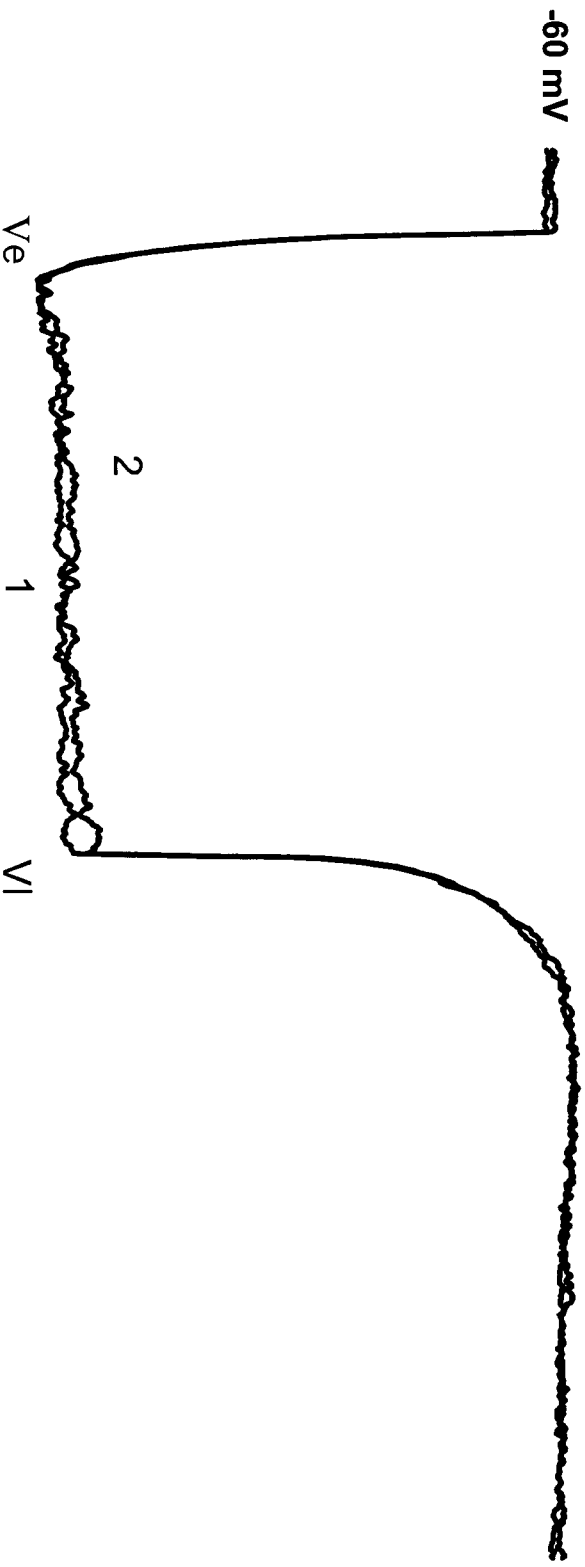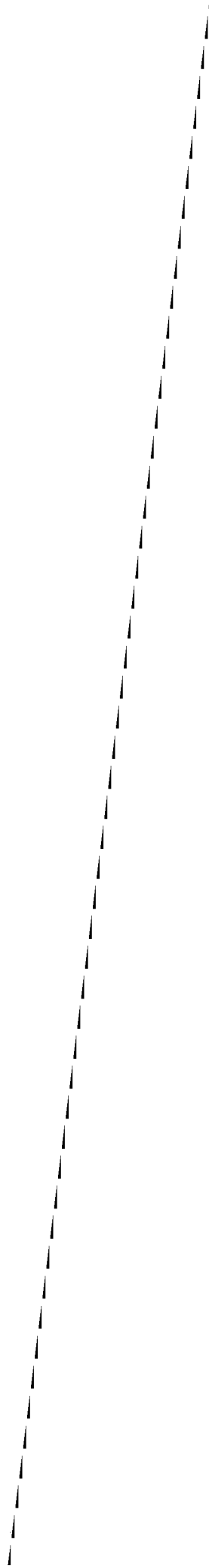
Effect of Estrogen on Resting Membrane Potential
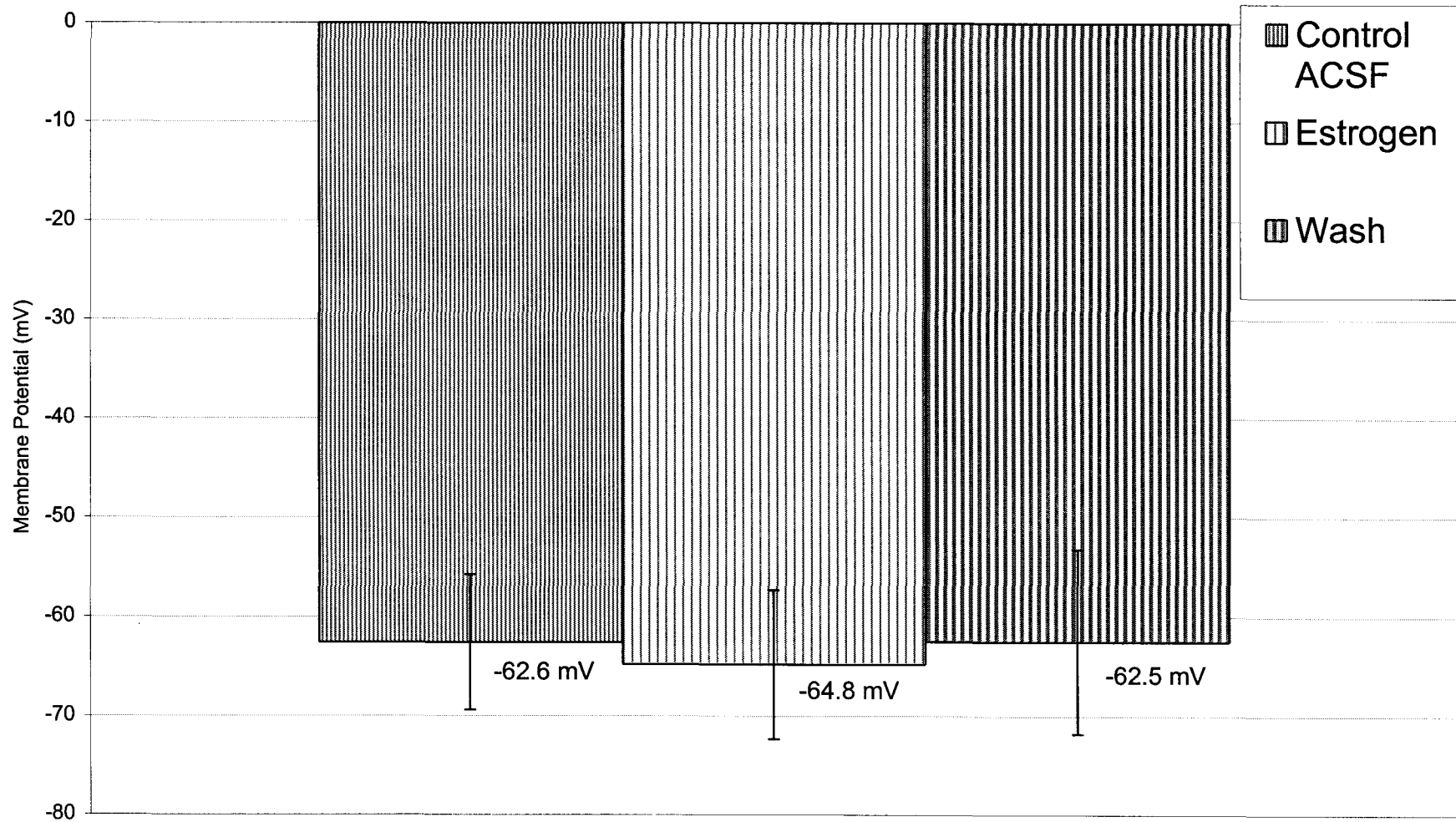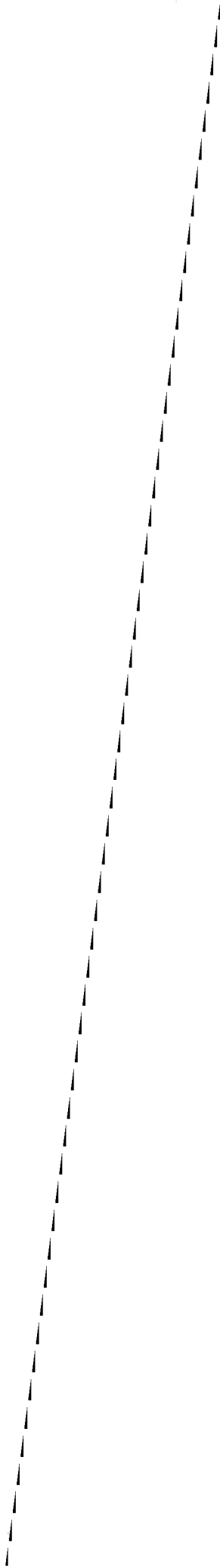
Effect of Estrogen on Number of Action Potentials

17a)

-60 mV

*
*
*
*
*
*
*

20 mV

200 ms

17b)

-60 mV

*
*
* *
* *
*
*

*

20 mV

200 ms
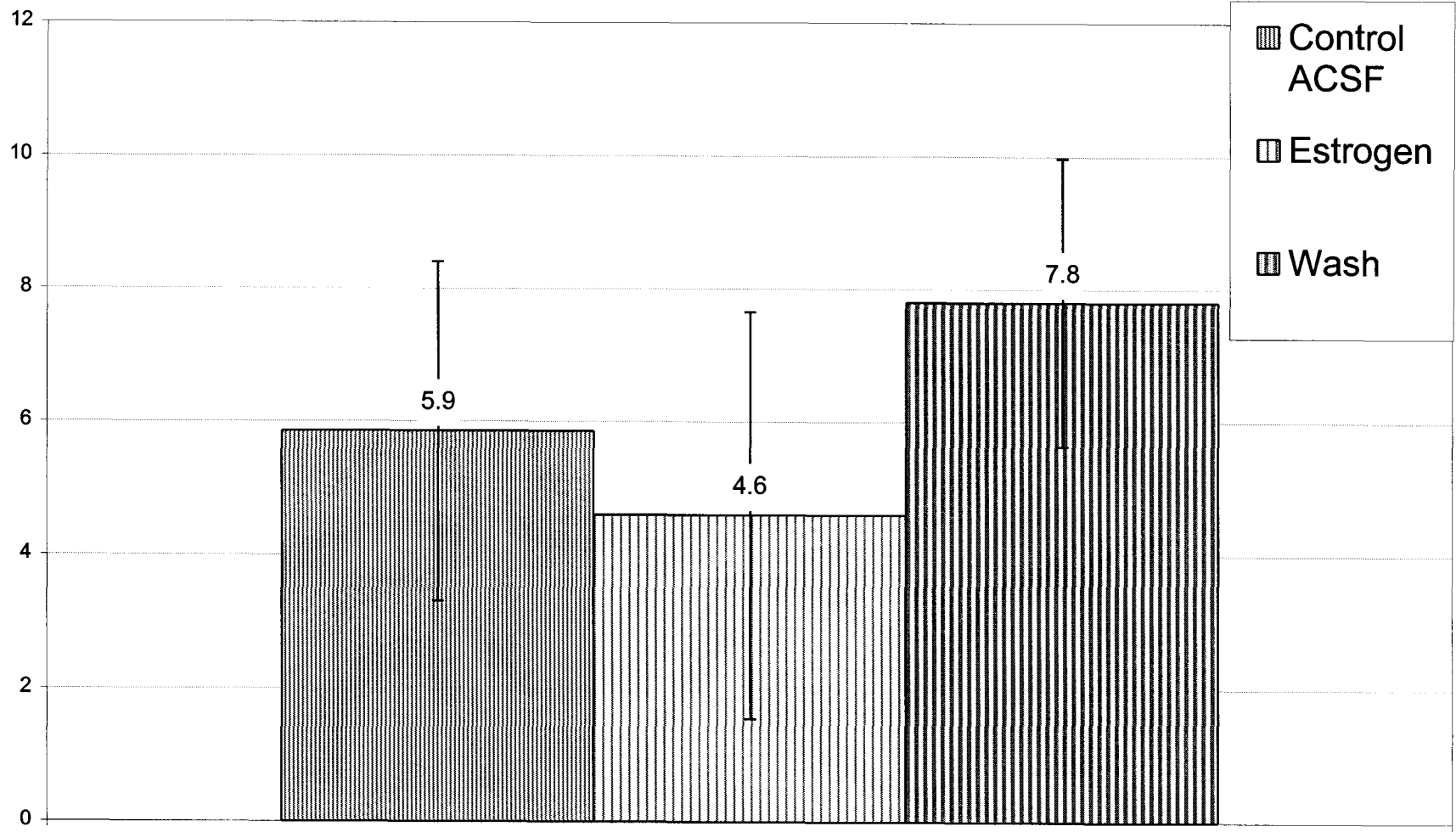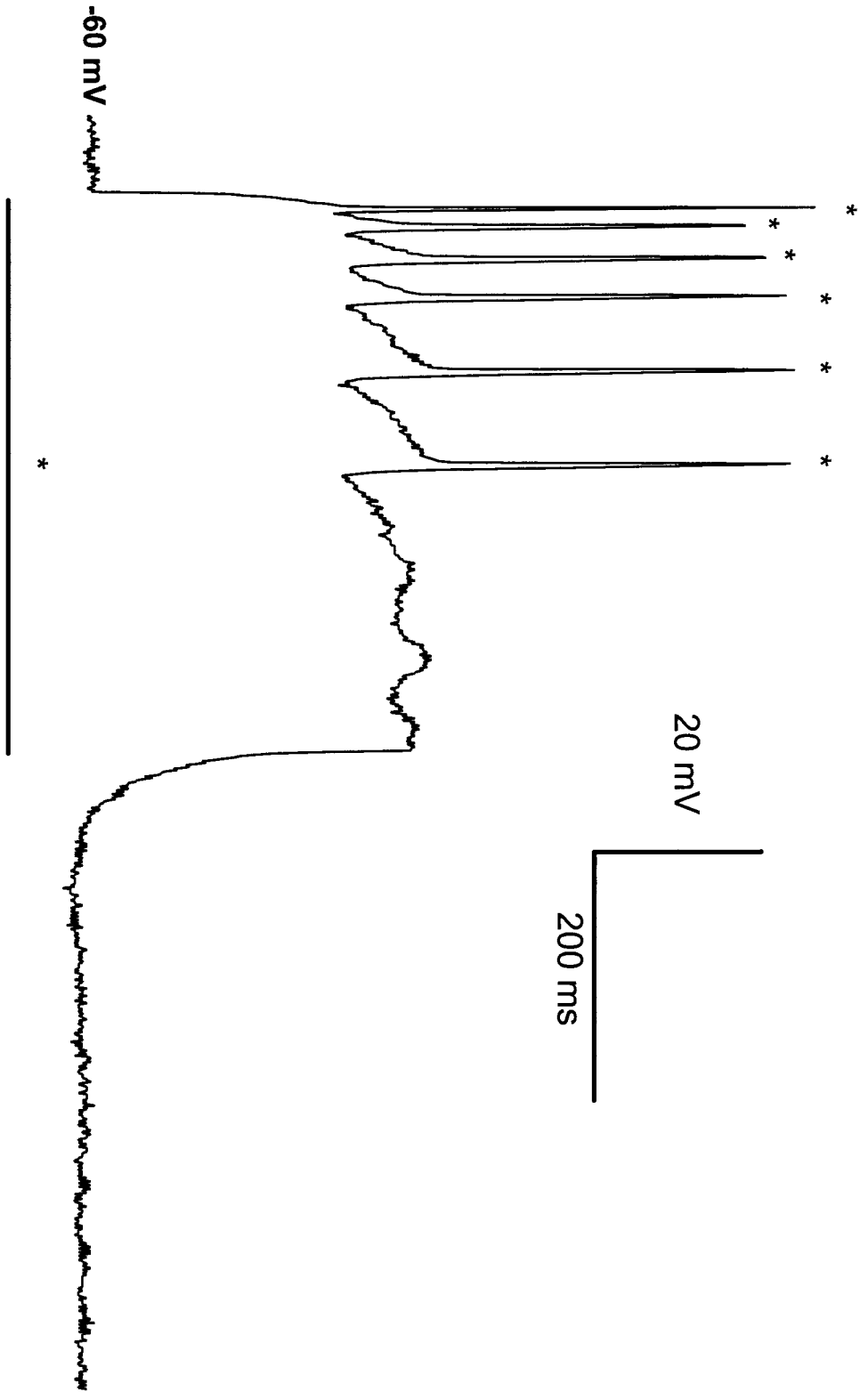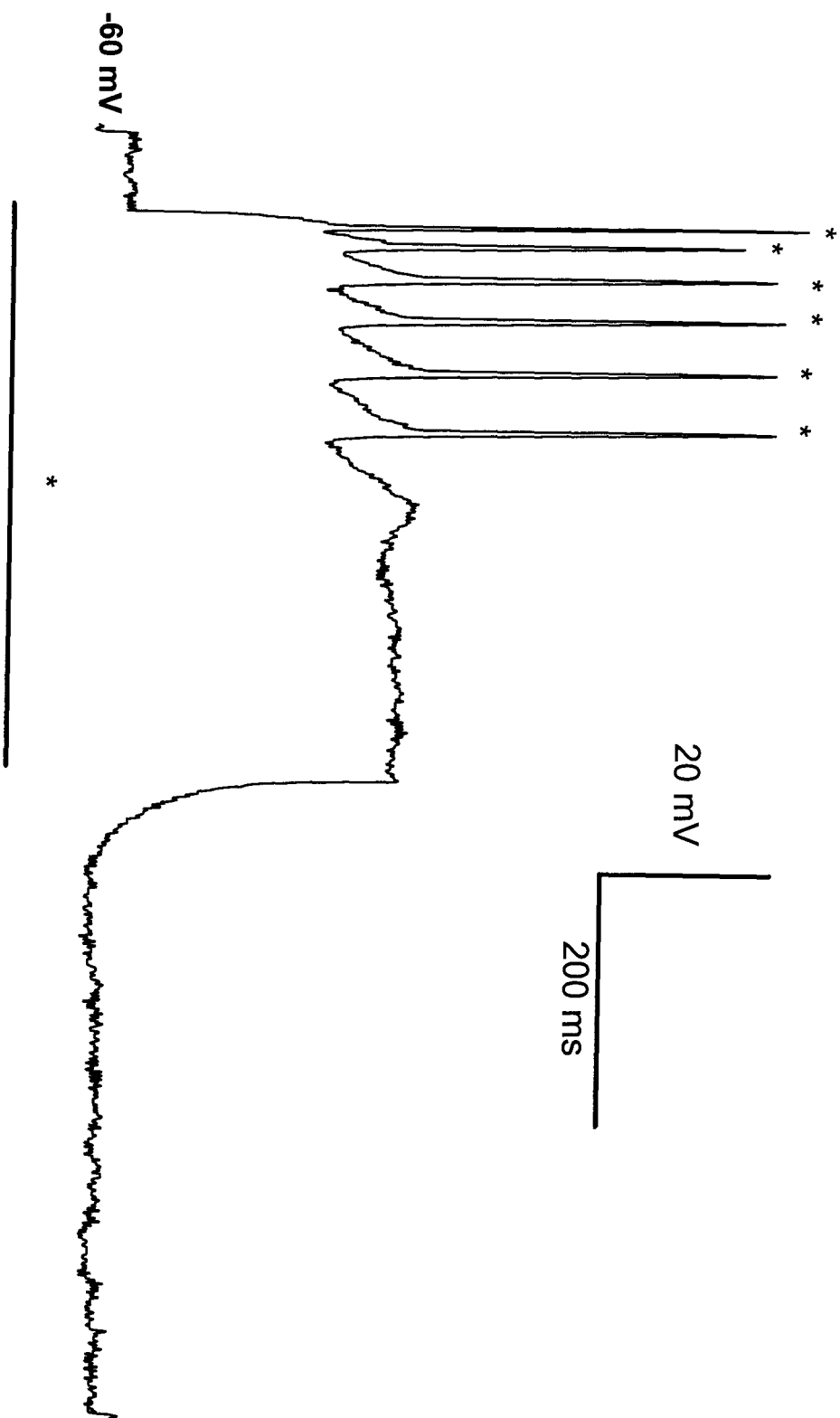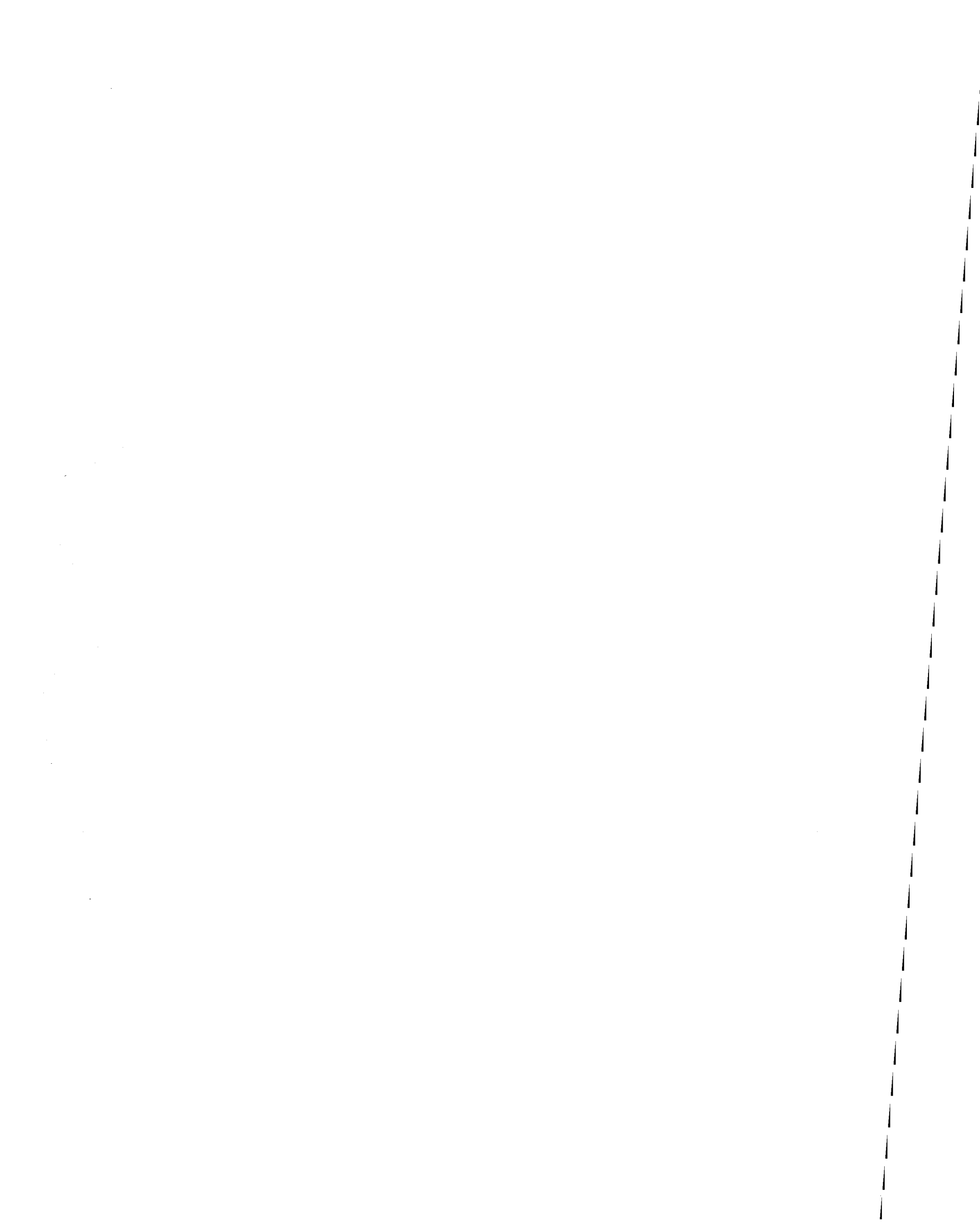
Effect of 4-OHT and Estrogen Blocking Effect of 4-OHT on EPSP Amplitude Modulation
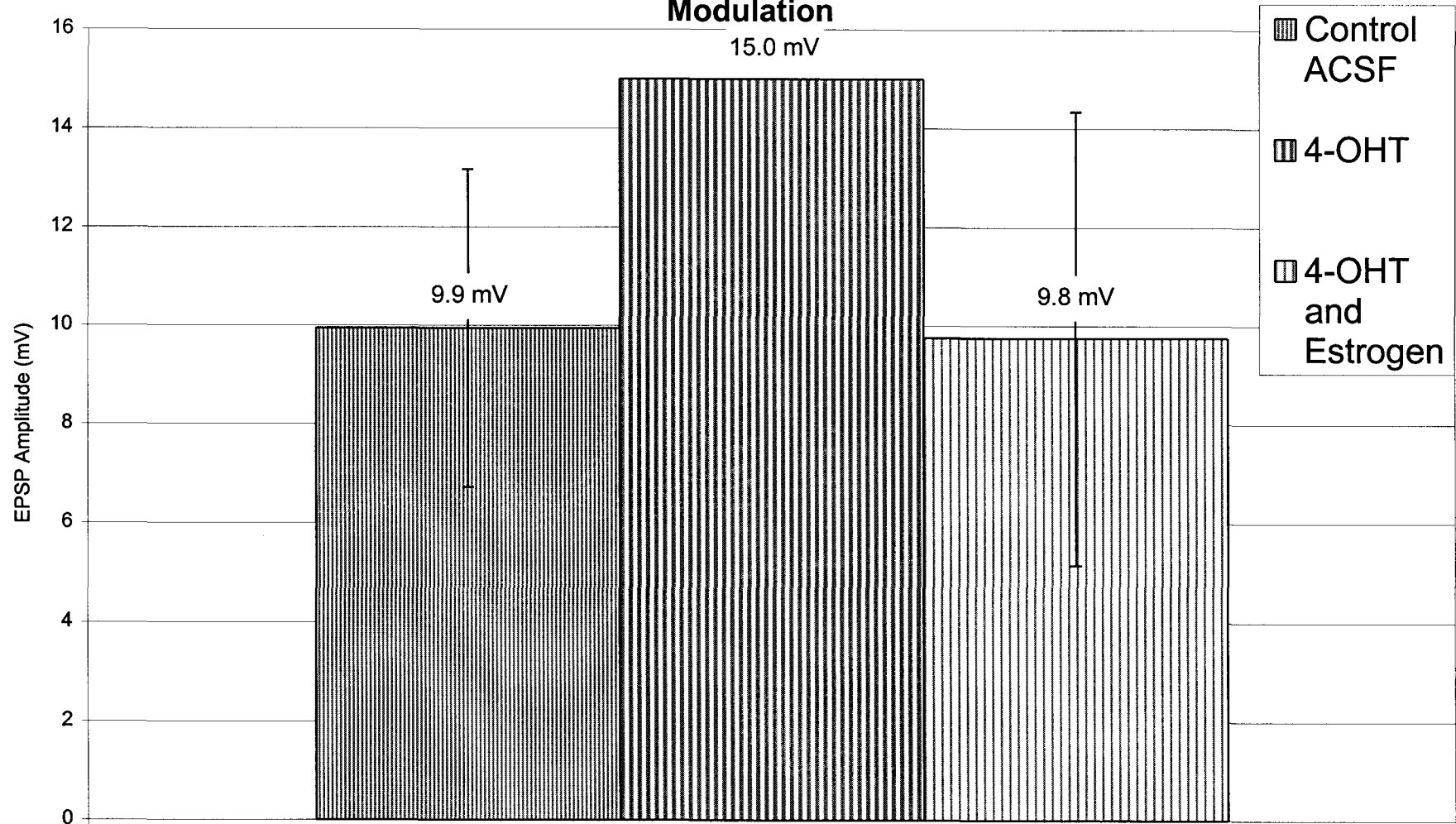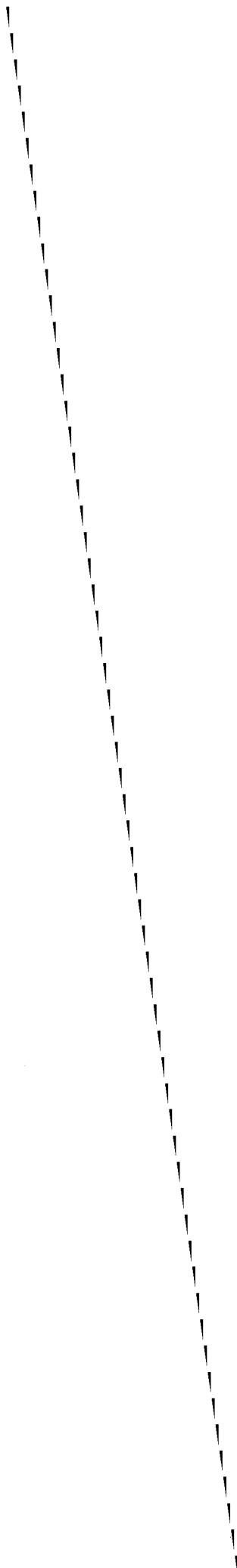
Effect of 4-OHT and Estrogen Blocking Effect of 4-OHT on Early Membrane Resistance
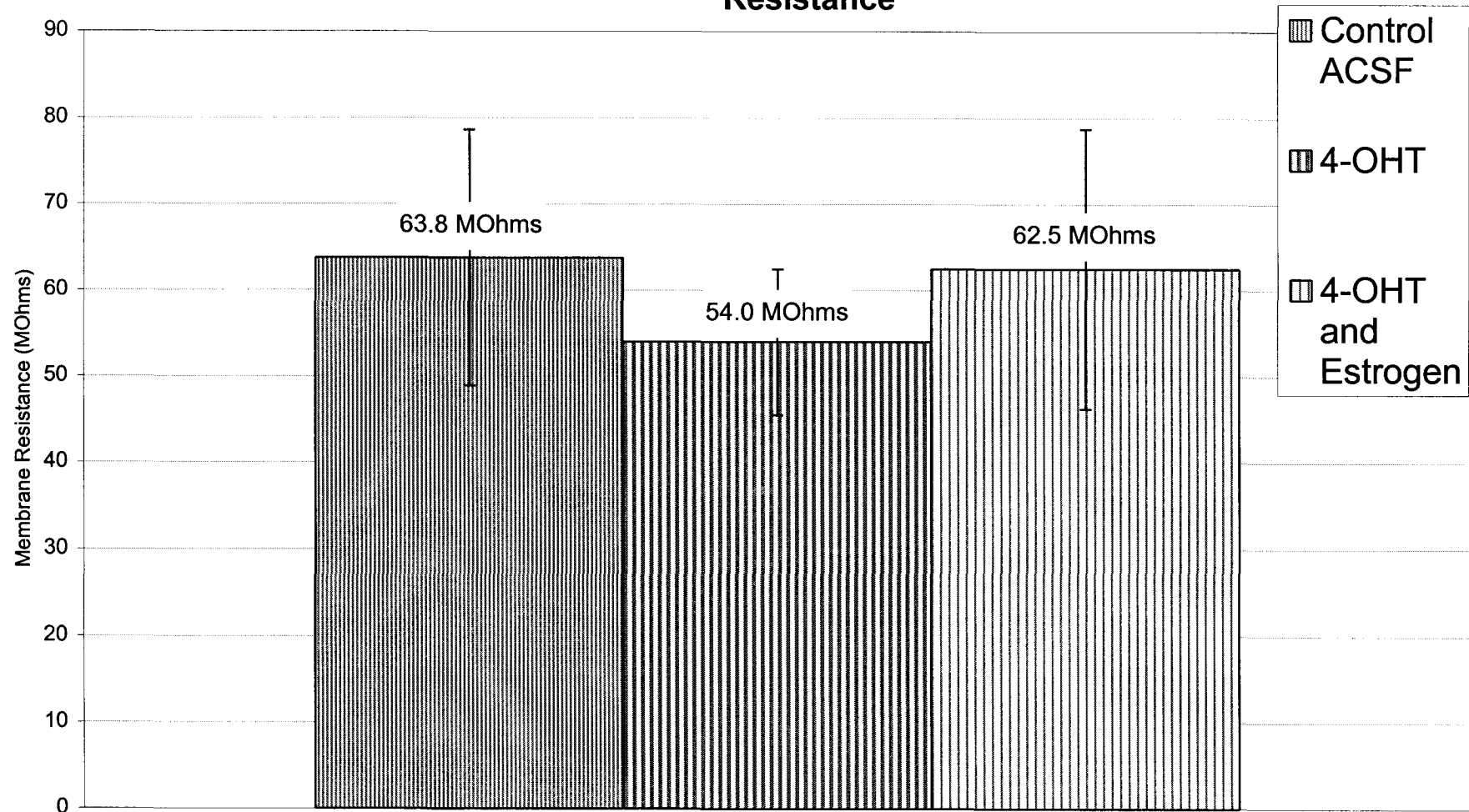
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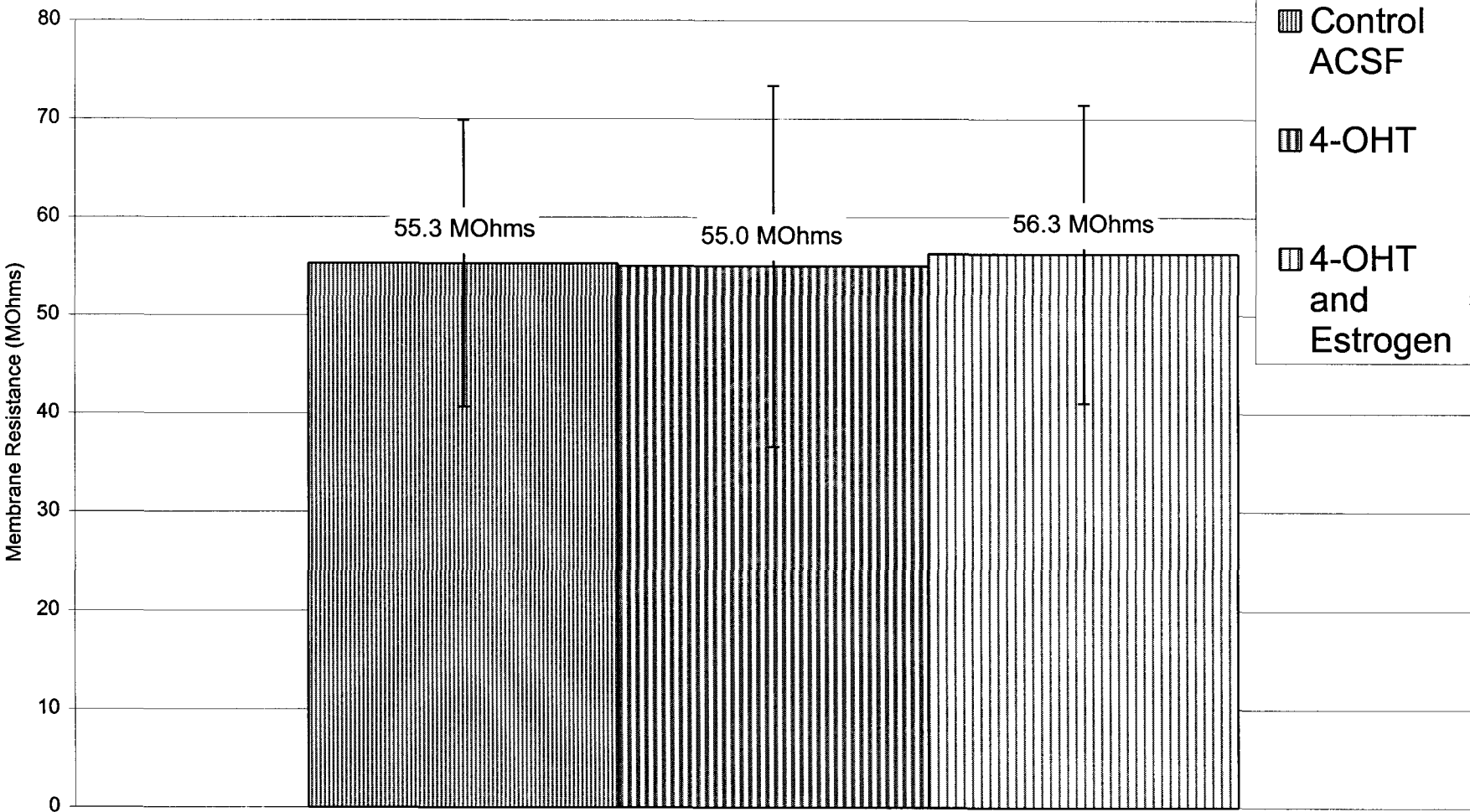
Effect of 4-OHT and Estrogen Blocking Effect of 4-OHT on Resting Membrane Potential
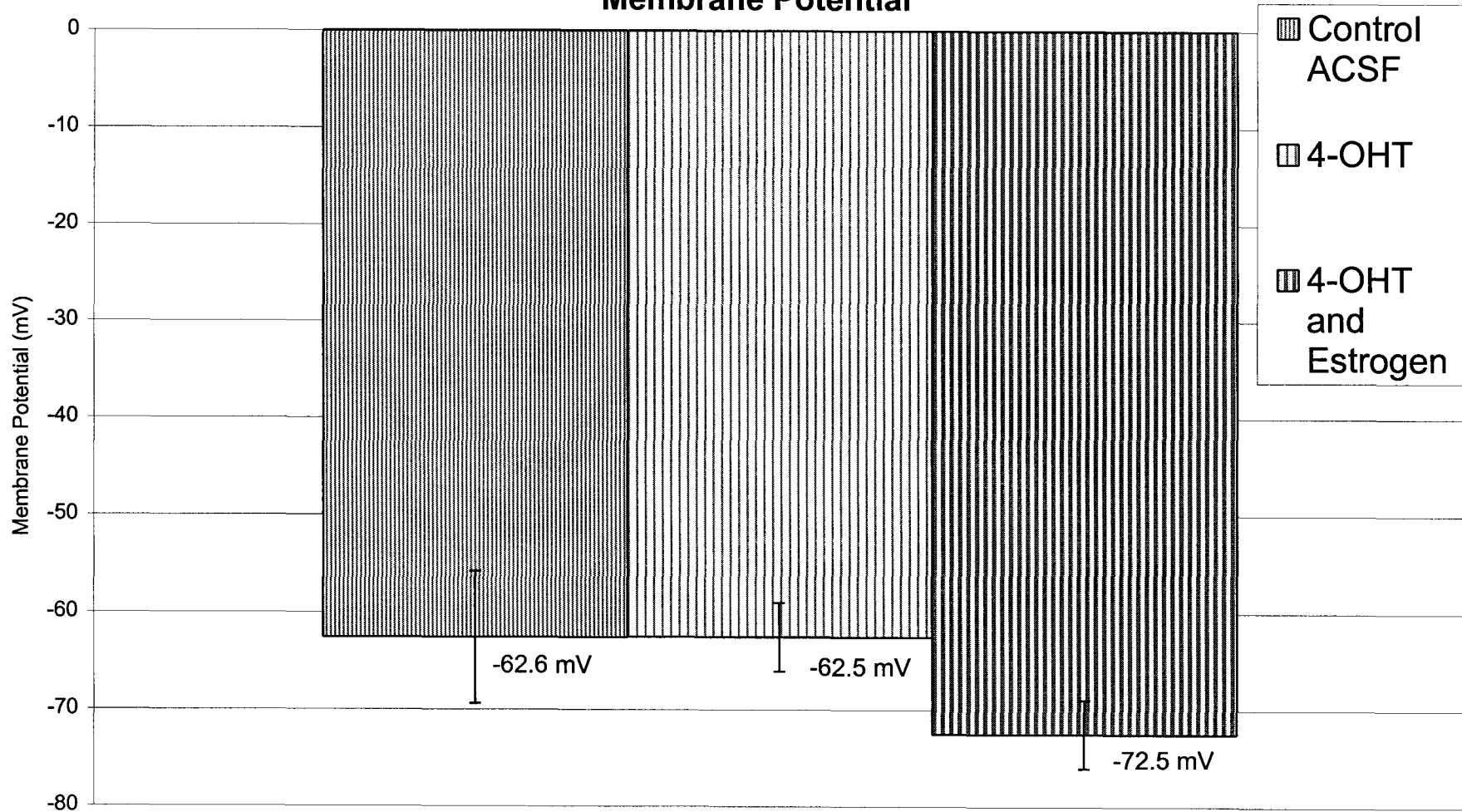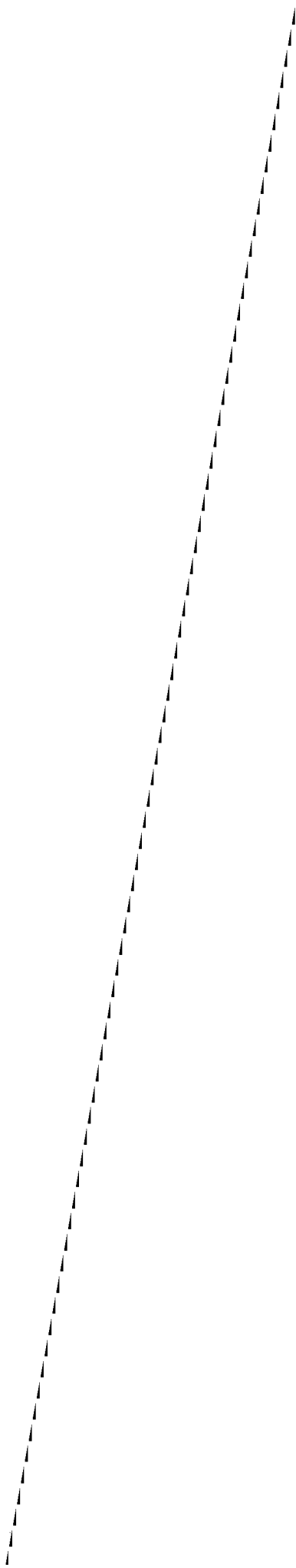
Effect of 4-OHT and Estrogen Blocking Effect of 4-OHT on Action Potential Frequency
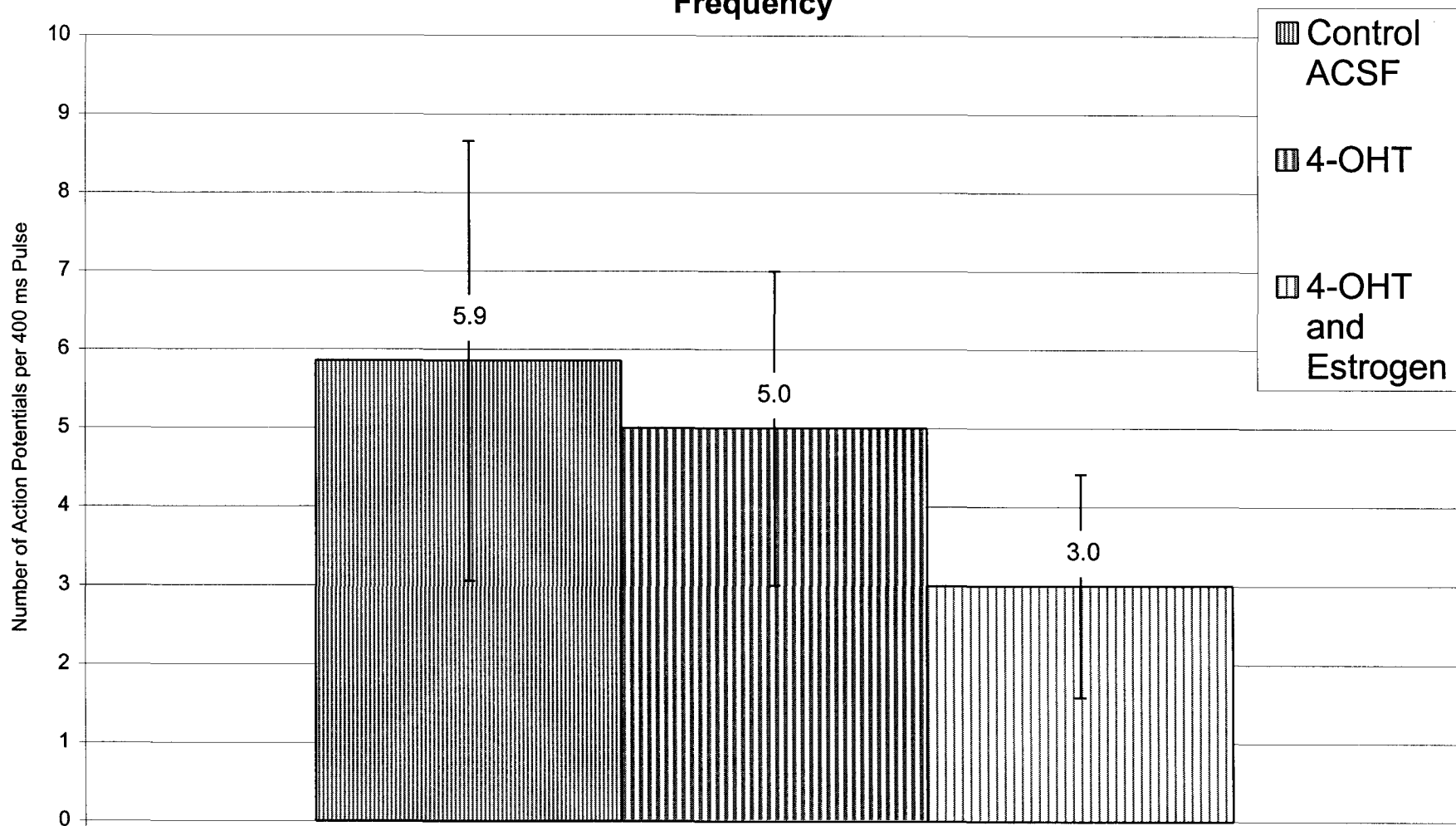
Discussion

The experiments described here investigated the actions of estrogen in the rat BLA, utilizing intracellular recordings from pyramidal neurons of the BLA. This is the major neuronal cell type found in the BLA, where it accounts for over 95% of neurons (McDonald, 1982, 1984: Millhouse and DeOlmos, 1983). Intracellular recordings obtained in this study also closely match properties of pyramidal BLA neurons described in previous studies (Gean and Shinnick-Gallagher, 1988, 1989: Washburn and Moises, 1992a, b, c; Womble and Moises, 1992). In addition, the properties of BLA neurons closely resemble the properties of pyramidal neurons in the rat hippocampus (Wong and Prince, 1981; Madison and Nicoll, 1984) and cerebral cortex (Connors *et al.*, 1982; McCormick *et al.*, 1985; Changac-Amatai *et al.*, 1990).

Direct electrical stimulation of presynaptic afferents to the BLA produces a sequence of synaptic responses in the postsynaptic neuron consisting of an EPSP, IPSP, and a delayed cholinergic response. EPSPs are elicited in the postsynaptic cell by the release of glutamate at the synapse, and subsequent activation of its receptors (Rainnie *et al.*, 1991a; Washburn and Moises, 1992b). There are three known glutamate receptor

types in mammalian neurons: NMDA ionotropic, non-NMDA kainate/ α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) ionotropic, and glutamate metabotropic (Recasens *et al.*, 1991). When activated by glutamate, ionotropic receptors directly activate their associated ion channels and metabotropic receptors activate their associated ion channels through second messenger system (Rainnie *et al.*, 1991a). Stimulation of presynaptic afferents contained in the external capsule (EC) induced the release of glutamate (Rainnie *et al.*, 1991a; Washburn and Moises, 1992b) and evoked EPSPs in the postsynaptic BLA neuron. The EPSPs in figures 4 and 5 are sample recordings showing the effects of stimulated release of glutamate via the EC.

In the present study, estrogen was found to markedly decrease the amplitude of EPSPs recorded from BLA neurons. This finding contrasts other studies in the hippocampus that show estrogen increases EPSPs (Barraclough *et al.*, 1999; Foy *et al*, 1999; SanMartin *et al.*, 1999; Wong and Moss, 1994). However, reductions of EPSP (Valentino and Dingledine, 1981; Dodt and Misgeld, 1986; Dutar and Nicoll, 1988) have been recorded previously in brain regions with properties similar to the BLA upon activation of cholinergic receptors.

Other electrophysiological reports have revealed that field potentials (extracellular) increase upon estrogen treatment in the hippocampus (Foy *et al.*, 1980, 1982, 1983). Field potentials are extracellular recordings of EPSP. Pretreatment with 17 α estradiol was found to block the effects of estrogen treatment, and this suggests involvement of an ER (Foy *et al.*, 1982, 1983). Additional investigations into these effects have uncovered estrogen-induced excitatory effects on NMDA receptors, enhancement of LTP, and increased EPSP amplitude (Foy *et al.*, 1999).

San Martin *et al* found that diethylstilbestrol (DES), a synthetic estrogen, increased EPSP amplitude, but did not effect field EPSPs in the pyramidal layer of the hippocampus. These results may suggest a difference in action between synthetic estrogens and naturally occurring estrogens, or it may be a functional difference between the pyramidal layer and CA1 region. Foy *et al* (1999) showed that estrogen increased both the field EPSP, and the amplitude of EPSPs (intracellular) in the CA1 region of the hippocampus.

In vitro recordings from CA1 hippocampal neurons after 14 days of estrogen treatment has been shown to have no effect on LTP, EPSP amplitude, or neuron excitability (Barraclough *et al.*, 1999). This could suggests that the period of estrogen action ends within days.

Wong and Moss have shown that application of estrogen to CA1 hippocampal neurons causes a rapid and reversible depolarization, increased input resistance, and increased excitability. Similar effects of estrogen on excitability in the hippocampus have been reported by Vardaris *et al.*, (1990). These effects are blocked by 4-OHT. Further investigations by Gu and Moss have shown that kainate currents are enhanced in the CA1 region of the hippocampus by estrogen via a G-protein-linked pathway and show reversibility (1996, 1998). Interestingly, estrogen must be present on both sides of the membrane for this action to occur (Wong and Moss, 1998).

Wong and Moss have shown that estrogen treatment for 2 days increased excitability, prolonged EPSPs, and increased spontaneous activity of CA1 hippocampal neurons (1992). Instant effects of estrogen treatment on ovarectomized female rats showed a rapid (1 min) increase in EPSP amplitude (Wong and Moss, 1992). These actions were reversible and inhibited by a non-NMDA receptor antagonist (Wong and Moss. 1992). The estrogen-induced effect on EPSP amplitude was also seen when glutamate was directly applied to the cell (Wong and Moss, 1992), suggesting that the actions of estrogen were postsynaptic. These findings further suggest that the synaptic alterations seen in BLA neurons by this study may be caused by changes in the glutamate system.

Additional evidence supporting the modulation of neuronal and synaptic functioning in the mammalian brain by estrogen exists. In hippocampal pyramidal cells and extrahypothalamic neurons, estrogen treatment increases the excitatory effects of NMDA and glutamate (Teyler *et al.*, 1990, Smith *et al.*, 1987). The number of NMDA receptors in the hippocampus CA1 region is reduced after treatment with estrogen (Weiland, 1992). Ovariectomy has been found to decrease dendritic spine density on CA1 pyramidal cells *in vivo* and *in vitro* (Woolley and McEwen, 1994; Murphy and Segal, 1996), while estrogen replacement reverses this effect (Woolley and McEwen, 1992). Estrogen increases cell survival rates following glutamate excitotoxic insults, oxidative stress, and beta-amyloid treatment (Behl *et al.*, 1995; Goodman *et al.*, 1996; Green *et al.*, 1996; Singer *et al.*, 1996, 1998). Estrogen antagonists and MAPK inhibitors have been shown to block estrogen's neuroprotective functions (Singh *et al.*, 1999).

IPSPs are elicited in the postsynaptic cell by the release of GABA (gamma-amino-butyric acid) by local inhibitory interneurons. It has been shown that IPSPs in the BLA are initiated by activation of $GABA_A$ and $GABA_B$ (Rainnie *et al.*, 1991b). GABA is the major inhibitory neurotransmitter in the BLA (Rainnie *et al.*, 1991b). Stimulation of BLA

afferents can elicit both an early IPSP and a late IPSP in BLA neurons

(Rainnie *et al.*, 1991b, Washburn and Moises, 1992a, b). Early IPSPs occur

through the activation of the ionotropic $GABA_A$ receptor, and late IPSPs

occur through activation of the metabotropic $GABA_B$ receptor (Rainnie *et*

al., 1991b, Washburn and Moises, 1992a, b). Figures 10 and 11 are sample

recordings showing IPSPs occurring through direct stimulation of an

inhibitory interneuron.

Preliminary data shows the possibility that IPSP amplitude is not

affected by estrogen (Figs. 10, 11). Decreases in IPSP amplitude have been

recorded previously upon activation of cholinergic receptors in brain regions

with properties similar to the BLA (Haas., 1992).

Wong and Moss (1992) found no significant estrogen-induced

difference in stimulated IPSPs or those initiated by GABA treatment.

However, Some changes of IPSP amplitude have been reportedly induced by

estrogen treatment. Canonaro *et al* found that *in vivo* estrogen treatment

reduced the number of chloride channels associated with $GABA_A$ receptors

(1993). However, changes in chloride channels were only found after

treating with estrogen over a relatively long period (>24 hours), and were

not found in the BLA (Canonar *et al.*, 1993). Therefore these results

(Canonaro *et al.*, 1993) should have few implication on the synaptic alteration found in BLA neurons.

Cholinergic responses are elicited in the postsynaptic cell by the release of acetylcholine from presynaptic cholinergic neurons. Their axons run within the EC and are activated following EC stimulation (Washburn ad Moises, 1992a, b). Cholinergic receptors on BLA neurons are activated by acetylcholine release following high frequency stimulation of the presynaptic external capsule pathway (Washburn and Moises., 1992b, c; Rainnie *et al.*, 1991a). Cholinergic input to cells in the BLA arises, for the most part, from regions outside of the BLA (Emson *et al.*, 1979; Woolf and Butcher., 1982; Carlsen *et al.*, 1985), although there is evidence showing small populations of cholinergic neurons in the BLA (Carlsen and Heimer, 1986). The cholinergic axons found in the EC have been traced back to cholinergic neurons residing across a broad region that encompasses the diagonal band of Broca to the ventral palladium (Emson *et al*, 1979; Woolf and Butcher, 1982; Carlsen *et al.*, 1985). Acetylcholine can elicit excitatory responses in BLA neurons via the muscarinic type of cholinergic receptor (Washburn and Moises., 1992a). Cholinergic responses consist of a long, sustained depolarization of the postsynaptic BLA neuron (Washburn and Moises, 1992a). The effect of estrogen on the acetylcholine pathway was

not a focus of this study. However, pursuit of information regarding how estrogen interacts with the acetylcholine pathway remains an area of great interest.

Acetylcholine and glutamate pathways are the two known and described excitatory pathways to the BLA. In the present study, EPSP amplitude and spontaneous activity decreased upon addition of estrogen (Figs. 3, 4, 5). Glutamate is the neurotransmitter associated with EPSP elicitation. Thus, the cause of synaptic activity alteration in BLA neurons is likely associated with the glutamate system.

The receptors involved in the estrogenic alterations of synaptic activity were examined with the use of the ER antagonist 4-hydroxy-tamoxifen (4-OHT). Non-steroidal ER antagonists such as 4-OHT are structurally similar to estrogen with the exception of an alkyl-amino-ethoxy side chain being present (Jordan., 1998). Estrogen and 4-OHT share the same binding site on the ER (Brzozowski *et al.*, 1997). Since they compete for the same binding domain on the ER, 4-OHT was used to test the involvement of the ER in alteration of synaptic transmission. 4-OHT has a binding affinity 339 greater than that of estrogen for the binding site on estrogen receptor ß (ERß) and 178 times greater for ERα (Kuiper *et al.*, 1997). Preliminary data shows that the addition of 4-OHT(60 nM) to the

bathing saline was followed by an increase in EPSP amplitude (Fig. 16).

This may be due to the displacement of chemicals previously attached to the

estrogen ligand-binding site, suggesting that there may be a certain level of

endogenous tonic inhibition. Preliminary data also shows that 4-OHT

prevented the inhibition of excitatory synaptic activity by estrogen (Fig. 16).

This suggests the possibility that ER activation is necessary for the

alterations of synaptic activity seen in this study.

Defining the non-genomic mechanism by which estrogen-induced

alterations in synaptic activity are occurring is speculative. Mainly, this is

due to the limited knowledge of ER function. Genomic mechanisms of

estrogen action are the most clearly understood. However, the genomic

mechanism takes hours from estrogen binding and initiation to completion

and release of post-translationally modified products (Zakon., 1997). In the

case of alteration of synaptic activity by estrogen, the period required to see

effects (20 -30 minutes) is too short to be a product of the genomic

mechanism. Alteration of synaptic activity by estrogen is most likely

occurring via a rapid, non-genomic mechanism. A possible mechanism

through which estrogen may be altering synaptic function in the BLA is via

various protein kinase pathways. Mitogen activated protein kinase (MAPK),

protein kinase A (PKA), and protein kinase C (PKC) are protein kinase

pathways that may serve as potential alternative pathways for estrogen function.

Studies of primary cultured cortical neurons have shown that estrogen induces an increase in MAPK activity within 30 minutes (Singer, *et al* 1999). Estrogen has also been shown to initiate activation of MAPK in neuroblastoma cells (Watters *et al.*, 1997), and non-neuronal cells via non-receptor kinase src (Migliaccio *et al.*, 1996). If the estrogen receptor antagonist ICI 182,780 or a MAPK pathway inhibitor is used in conjunction with estrogen, there is no increased kinase activity in primary cortical neurons (Singer *et al.*, 1999). This implies that estrogen activates MAPK through the activation of an ER in cultured cortical neurons.

MAPK is activated by many intracellular signals, including nerve growth factor (NGF) (Seger and Krebs, 1995). NGF has been suggested to promote survival of neurons in several cases (Kromer, 1987; Hefti, 1986). Estrogen is also believed to modulate neurotrophins (such as NGF) levels (Gibbs *et al.*, 1994; Singh *et al.*, 1995), and neurotrophin receptors (Sorabji *et al.*, 1994a, b; McMillan *et al.*, 1996). This suggests the possibility that estrogen and neurotrophins may participate in a negative feedback mechanism to control the amount of kinase activity mediated by the estrogen receptor, NGF, and other factors.

Another pathway through which estrogen may be inducing its actions is the protein kinase A (PKA) pathway. In guinea pig hypothalamic slices, estrogen weakens the ability of μ-opioids to hyperpolarize beta-endorphin neurons. This action of estrogen takes place within 20 minutes, and it was blocked by the ER antagonists ICI 164,384 and diethylstilbestrol. Furthermore, this action was not prevented by the protein synthesis inhibitor cyclohexamide but was blocked by selective PKA antagonists (Lagrange *et al.*, 1997).

Another possible pathway for rapid actions of estrogen in the BLA is by protein kinase C (PKC). In normal and tumor tissue, estrogen has been shown to increase PKC levels (Drouva *et al.*, 1990, Maeda *et al.*, 1993, Maizels *et al.*, 1993).

Although the actions of the kinases in relation to estrogen action have not been investigated in the BLA, the demonstration of rapid (30 min) estrogen action, with inhibition by an ER antagonist, make them plausible mechanisms for the synaptic activity alteration found in BLA neurons, and potential targets for future research.

Some of the effects of estrogen occurring in the brain have been shown to be associated with NMDA receptors, due to the recent findings of Woolley and McEwen (1994), who showed that the dendritic spine density

changes occurring in the CA1 region of the hippocampus following estrogen treatment rely upon the activation of NMDA receptors. In addition, the competitive NMDA antagonist MK 801 prevents estrogen-induced changes in spine density. Metabotropic and ionotropic NMDA receptor were reportedly affected (Woolley and McEwen 1994). This has implications on our study because BLA neuronal EPSPs rely in part upon the activation of NMDA receptors (Rainnie *et al.*, 1991a).

Blocking of ER by tamoxifen shows that alteration of synaptic behavior may be dependent upon on a specific activating function (AF) of the ER. AF-1 is located in the N terminus and AF-2 is located in the ligand-binding domain of ERα. These are two areas suggested to mediate transcriptional activation of ERα (Tsai and O'Malley, 1994). ERα antagonists inhibit and agonists activate AF-2, but some antagonists have been reported to have agonistic actions (Berry *et al.*, 1990). AF-1 of ERα has been shown to be regulated by growth factors associated with the MAPK pathway, and also may be the cause of agonistic effects of ER antagonists (McEwen, 1997; Kato *et al.*, 1995).

While analyzing the preliminary blocking effect of tamoxifen, it is plausible to hypothesize that the actions of estrogen seen in BLA pyramidal neurons are mediated by the AF-2 region on the ER. Distribution of ERs in

the amygdala (Shughrue *et al.*, 1998; Weiland, 1997) suggests that both ERα and ERβ may be present in the BLA. The ligand binding domains of ERα and ERβ show a 50% homology (Kuiper, *et al.*, 1997), thus the nearby AF-2 regions of the ERs may have similar homologies. Therefore, defining which of the ER subtypes is involved the alteration of synaptic activity in the BLA can help find a more specific location for the actions described here.

Passive and intracellularly stimulated membrane activity recordings did not show significant alteration upon estrogen treatment. Resting membrane potential, early and late membrane resistance, action potential frequency, and action potential accommodation response showed no significant change upon switching the bathing solution to estrogen. This data suggests that the passive currents analyzed in resting membrane potential measurements show no conductance change, and the voltage-gated currents analyzed in the early membrane resistance, late membrane resistance, and action potential accommodation response also show no conductance change. The accommodation response results from the action of a slowly decaying after hyperpolorization current (Womble and Moises, 1993). This Ca^{++}-activated K^+ current was not significantly changed. Action potential frequency allows us to analyze the voltage-gated Na^+ and K^+ currents associated with action potential formation (Kandel *et al.*, 1995).

These currents had no significant change. Therefore, no changes were found in the Na$^+$ and K$^+$ ion currents, which are known to underlie, active neuronal responses, such as action potential firing patterns (Kandel *et al*, 1995).

Voltage recordings from BLA neurons presented in this study revealed significant decreases in EPSP amplitude and spontaneous synaptic activity upon estrogen treatment. This is a rapid (20-30 min.) response. 4-OHT appeared to block this effect of estrogen, therefore suggesting an ER-mediated response. The speed of the response suggests that the ER is not acting by the classical genomic mechanism of ER action.

Voltage recordings analyzing passive and voltage-gated ion channels show no significant change. With the evidence of protein kinase pathways associated with estrogen and ER phosphorylation, it would seem likely that some modulation of ion channels should be occurring. Kinases have been found to effect ion channels in past studies of estrogen action in hippocampal neurons (Gu and Moss, 1996; Kawasaki *et al*, 1994; Wyneken *et al*, 1997; Potier and Rovira, 1999; Ghetti and Heinemann, 2000). The decreases in EPSP amplitude seen in this study are attributed to changes of the glutamate system. Preliminary evidence showed estrogen does not affect IPSP amplitude. Thus, the GABA system does not appear to be affected. Estrogen does not appear to affect any of the membrane properties of the

BLA neurons studied; therefore, ion channels of the studied neurons do not appear to be affected. In conclusion, the accumulated evidence appeared to suggest that estrogen is acting to inhibit the glutamate system. Reduction in glutamate function at BLA synapses supports previous studies that suggest estrogen protects from the glutamate-associated excitotoxicity associated with cerebral ischemia (Hurn PD, Macrae, 2000; Sawada *et al.*, 2000; Rusa *et al.*, 1999; Fung *et al.*, 1999).

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