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STUDIES OF A CENTRAL NORADRENERGIC SYSTEM IN TISSUE CULTURE

by

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Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of MSc.

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ABBREVIATIONS

BSS	BALANCED SALT SOLUTION
CBLM	CEREBELLUM
DA	DOPAMINE
GABA	GAMMA AMINO BUTYRIC ACID
H2O2	HYDROGEN PEROXIDE
LC	LOCUS COERULEUS
nA	NANOAMPERES
NA	NORADRENALINE
O2-	SUPEROXIDE RADICAL
OH-	HYDROXYL RADICAL
6-OHDA	6-HYDROXYDOPAMINE
PN	PURKINJE NEURON
uM	MICROMOLAR

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ABSTRACT

Explant cultures containing cerebellum and underlying brainstem area were prepared from neonatal mice as described by Hendelman, Marshall, Ferguson and Carriere, (1982), and were allowed to mature for 17-25 days. Purkinje and LC neurons were identified by light microscopy and their health and developmental progress were monitored.

The LC neurons of LC plus cerebellum cultures could be selectively lesioned by incubation for 24 hours with 500uM 6-OHDA. Chemical denervation with 6-OHDA had not previously been documented in vitro. The 6-OHDA was dissolved in an 8mM ascorbate-feed solution to prevent extraneuronal oxidation of the neurotoxin. Treatment with 6-OHDA resulted in vacuolation of the LC region, and numerous ghost cells appeared when examined by light microscopy. Treatment also resulted in a loss of catecholamine fluorescence of the fibers and the LC area which typically exhibit fluorescence. Purkinje neurons did not appear to be affected by 6-OHDA treatment when examined under bright field microscopy.

The effects of iontophoretically applied NA and glutamate on the extracellularly recorded electrical activity of Purkinje neurons were studied in 6-OHDA treated and untreated cultures of either LC plus cerebellum or cerebellum alone.

Seventy-five percent of the neurons tested responded to NA. Of those responding, 90% responded with an inhibition of spontaneous activity. No significant difference was found in the sensitivity to NA in any of the above groups.

Significant differences were found ($p=0.0001$) in the sensitivity to glutamate in 6-OHDA treated as compared to untreated-LC plus cerebellum cultures. The 6-OHDA treated cultures required significantly lower currents for Purkinje neurons to elicit a response to glutamate. This type of supersensitivity, perhaps secondary to denervation of another system, has not previously been described.

With concurrent application of NA and glutamate from multibarrelled electrodes, two types of responses were seen. These responses occurred in all culture types. Of 64 cells studied, 15 exhibited a simple depression of the glutamate response. However, in 49 other Purkinje neurons studied, NA potentiated the glutamate response. In 7 of the 49 potentiated cells, an inhibition of the glutamate response was observed at higher iontophoretic currents of NA. It therefore appears as though these two qualitatively different responses, i.e. potentiation and inhibition of the glutamate response by NA, may be dose dependent. This work supports the concept of a neuromodulatory role for NA in the cerebellum, but further suggests that the influence of NA on other neurotransmitters, i.e. augmentatory or inhibitory, is dose dependent.

INTRODUCTION

The effects of various neurotransmitters in the central nervous system (CNS) have been well documented. Noradrenaline (NA) has predominantly inhibitory effects, as does GABA, while glutamate is primarily excitatory. More recently, though, researchers have been investigating the interactions of NA with other neurotransmitters. Unlike classical inhibitory neurotransmitters, NA applied at low doses has been shown to potentiate synaptic effects and the effects of certain neurotransmitters such as GABA and glutamate. Thus the concept of a neuromodulatory role for NA has emerged (Woodward, Moises, Waterhouse, Hoffer and Freedman, 1979). It has also been well documented that if the neuronal input to a system is removed that system will respond to the decreased availability of transmitter by increasing the number of receptors and consequently its response to that specific neurotransmitter of which it has been deprived (Gnegy and Costa, 1980). This phenomenon is called denervation-supersensitivity (Cannon and Rosenblueth, 1949).

It has been the aim of this research to study NA effects in a tissue culture system, as it provides a unique opportunity for manipulation of variables under strict experimental conditions. This would enable us to

investigate the toxic selectivity on LC neurons when compared with other neuron types and to examine what resultant changes if any occur with respect to NA, glutamate and the potentiation of the NA response.

Neuronal culture preparations have certain limitations, perhaps the most serious being that they are in vitro preparations and not in vivo ones. This gives rise to difficulties in extrapolating back to the whole animal. However, neuronal cultures do offer a number of advantages. They are isolated systems, and therefore, interactions of particular groups of neurons may be examined in the absence of other neuronal, circulatory or hormonal influences, thereby reducing the number of experimental variables. Neurons may be examined in the living state and their developmental progress as well as any experimentally induced changes may be monitored by light microscopy. All recording is done under visual control, providing accurate identification of a given cell, as well as a means of discerning between healthy and unhealthy cells. In addition, one can manipulate various experimental variables. Drugs may be iontophoretically applied to a particular cell. There is of course no blood brain barrier in tissue culture. Components of the extracellular medium may also be varied in order to study their effects. The main disadvantage of the tissue culture system is that it is not an in vivo preparation and thus lacks the intact neuronal and hormonal

milieu. Removal of metabolites as well as delivery of nutrient media may also be somewhat compromised in an in vitro system. The fact that cells are somewhat smaller than in vivo has also presented problems in recording for prolonged periods of time.

The field of tissue culture was initiated by Harrison (1907), at which time there was an ongoing controversy regarding the organization of the nervous system. Ramon y Cajal contended that the nervous system was composed of independent cellular units, whose nerve fibers originated and grew away from a single cell. Held, on the other hand, believed that the nervous system was a syncitium, with neuronal processes serving as cytoplasmic bridges. By using cultured fragments of embryonic tadpole tissue, Harrison was able to confirm Cajal's theory with the observation that neuronal fibers grow out from the soma of individual cells.

Tissue cultures may be divided into two main types, explant cultures and dissociated cultures, and they will be described briefly. Explant cultures are prepared from tissue segments while dissociated cultures are prepared by chemically and mechanically disrupting tissue, and plating out the dissociated cells. The hanging drop culture, pioneered by Harrison, is one in which explanted tissue is placed on a plasma clot. The plasma clot is adhered to a cover slip which is inverted, placed on a depression slide

and is sealed with paraffin (see review by Lewis and Lewis, 1924). An advancement in tissue culture technique which followed was the use of a double cover slip assembly. Initiated by Maximow (1925), this has become known as the "Maximow assembly". Murray and Stout (1947) were the first to use the Maximow assembly for the culturing of neural tissue (see also reviews by Murray, 1965; 1971). This setup permitted long term studies by facilitating the washing and feeding of cultures. The main drawback of this system relates to the depression slide's optical limitation for microscopy.

Another commonly used method of culturing neuronal tissue is the roller tube method (Bornstein, 1973). For these preparations, explants are placed on a plasma clot, or more frequently on a collagen primed glass cover slip, providing a substrate to which the explant can adhere. The coverslip is then inserted in a roller tube and a relatively large amount of nutrient media is added. The roller tube is rotated during the incubation, allowing the explant to be exposed alternatively to the nutrient media for part of the time and to the air/CO_2 mixture for gaseous exchange the remainder of the time. This method of culturing is particularly well suited to the requirement for thin cultures. An additional advantage is that several explants may be prepared on the same coverslip. The main disadvantage of this method is the inherent inaccessibility

of the setup so that the developmental progress and health of the cultures may not be monitored by high powered light microscopy, and some loss of organotypic organization (Hendelman, Marshall, Aggerwal and Wojtowicz, 1978).

Another culture assembly is the Rose chamber (Rose, 1954), which makes use of a rubber gasket to separate two cover slips. This setup has two optical surfaces above and below the living culture, and has been primarily useful for microcinegraphic studies.

With dissociated cultures, unlike explant cultures, the close association and interaction between cells is temporarily interrupted. Following the mechanical or enzymatic dissociation process, the cells reaggregate, often forming a monolayer in which the in vivo topography is not retained. Maximow chambers may be used for culturing dissociated cell preparations. Dissociated cultures are particularly advantageous for intracellular recording studies since they consist of a monolayer of neurons.

Comparative studies of Purkinje neuron development in the mouse in tissue culture and in vivo have been conducted using the Golgi technique (Hendelman and Aggerwal, 1980). These studies indicate that during the first ten days postnatally, development in culture parallels that in the intact animal. However, the Purkinje neurons in tissue culture fail to align and lamination of the cortex does not

occur. Purkinje neurons in tissue culture were also found to have a somewhat altered morphology. Reductions in granule cell populations during the growth of cerebellar cultures have also been noted (Allerand, 1971), and have been attributed to a nutrient-related toxicity (Hendelman, Pilon and Fermin, 1972). Synaptogenesis in mouse cerebellar cultures appears to lag behind in early synaptic development. However, this is followed by a period of accelerated development, resulting in close in vivo and in vitro correspondence by day nineteen (Herndon, Seil and Sidman, 1981). Electrophysiologic studies of neurons in tissue culture have revealed projections from cortex to the deep nuclear area and from the latter to cortex (Hendelman, Marshall, Aggerwal and Wojtowicz, 1978), and these projections are known to exist in vivo (Gould and Graybiel, 1976; Tolbert, Bantli and Bloedel, 1976). It has also been possible to reproduce in vivo electrophysiological results in a tissue culture system (Wojtowicz, Marshall and Hendelman, 1978; Gruol, 1983), giving further validity to this model system.

The present work has been conducted on explant cultures from neonatal mice and include cerebellum plus locus coeruleus (LC), or cerebellum alone. A review of the relevant literature follows.

ORGANIZATION OF THE CEREBELLUM

The cerebellum is composed of two functional parts, the cerebellar cortex and the deep cerebellar nuclei (DN). The cortex is separated from the DN region by white matter which contains afferent, efferent and intracerebellar fibers. The afferents originate from many regions including the dorsal and ventral spinal cerebellar tracts, the external cuneate nucleus, the vestibular nerve and nucleus, the reticular formation, the pons and inferior olive. Most afferents terminate as mossy fibers and contact predominantly dendrites of granule cells. Afferents originating in the inferior olive terminate as climbing fibers and contact Purkinje cell dendrites (Dreclin, 1974). The efferents are axons of the DN and project mainly to the thalamus, red nucleus, lateral vestibular nucleus and the reticular formation. The intracerebellar fibers represent Purkinje cell axons projecting to DN and sending recurrent collaterals to adjacent regions of the cortex, and axon collaterals of DN which project to the cerebellar cortex.

The cortex is composed of three layers: the superficial molecular layer, the central Purkinje layer, and the inner granule layer. Within the molecular layer are found the stellate and basket inhibitory interneurons. Only one cell type, the Purkinje neuron is found in the Purkinje layer, and considerable evidence supports GABA as the transmitter

of these cells. Within the granule cell layer, two cell types are found, the Golgi cells and the granule cells. The Golgi cells are inhibitory, while the granule cells comprise the sole excitatory cell type of the cerebellar cortex and project to all cell types (Eccles, Ito, and Szentagothai, 1967).

THE LOCUS COERULEUS

The LC was first described by Reil in 1809 as a tiny blue streak in the dorsolateral tegmentum of the pons. It was Russell (1955) who, using Nissl-stained sections, first concluded that the cells of the LC comprised a distinct nucleus which had a similar shape and location in a number of species. Until Russell's report, the cells of the LC had been confused with those of the reticular formation, the central grey and the mesencephalic tract of the trigeminal nerve. However, it was not until the advent of the Falck-Hillarp histofluorescence technique that the catecholaminergic specialization of the LC was demonstrated (Falck, Hillarp, Thieme and Torp, 1962).

The LC is the largest and most compact of the noradrenaline-containing nuclei (Anden, Dahlstrom, Fuxe, Larsson, Olson and Ungerstedt, 1966; Olson and Fuxe, 1971). Other noradrenaline-containing cell groups are distributed among several smaller groups collectively known as the

lateral tegmentum. Functionally, the LC has been implicated in such roles as sleep, respiration, and motivation. (Anaral and Sinnamon, 1977; Foote, Bloom, Aston-Jones, 1983). There is as yet no convincing evidence for any single function of this nucleus.

Early studies using retrograde degeneration techniques showed that the LC receives afferent projections from the reticular formation, the central grey, and the sensory and mesencephalic nucleus of the trigeminal nerve (Russell, 1955). More recently, other projections have been demonstrated from the reticular formation (Shimizu and Imamoto, 1970), the fastigial nucleus (Snider, 1975), the raphe nuclei (Pasquier, Kemper, Forbes and Morgane, 1977) and some forebrain and hypothalamic areas (Conrad, and Pfaff, 1976a,b; Saper, Swanson and Cowan, 1976; Swanson, 1976). Projections arising from the forebrain include the medial and lateral preoptic areas, the amygdala, insular cortex and the stria terminalis (Pickel, Segal and Bloom, 1974). Localized horseradish peroxidase injections into the LC confirm that the LC receives projections from all levels of the central nervous system.

The LC is one of the most highly vascularized areas of the brain (Shimizu and Imamoto, 1970). Capillaries have been shown to border directly on the soma and dendrites of the LC neurons (Felten and Crutcher, 1979), and this has

suggested that the LC may receive afferent input through nonneuronal pathways. Hormones or systemically administered compounds may access the LC without having to cross the blood brain barrier (Medina, Grachetta and Shore, 1969). In addition, substances circulating in the cerebral spinal fluid (CSF) may affect the LC neurons due to the close proximity of the LC to the fourth ventricle.

The LC sends projections to all levels of the central nervous system. Projections to the cerebellum, hippocampus, neocortex and spinal cord are well documented. Catecholamine fibers have been observed at all levels of the spinal cord with early histofluorescent techniques (Dahlstrom and Fuxe, 1964). However, a specific projection has been demonstrated recently (Kuypers and Maislay, 1975; Nygren and Olsen, 1977). More recent work has indicated that those LC neurons projecting to the spinal cord are located primarily in the caudal pole of the LC (Satoh, Tohyama, Yamamoto, Sakwanoto and Shimizu, 1977; Guyenet, 1980). Horseradish peroxidase studies have indicated that in the monkey the LC innervates primarily parasympathetic nuclei, while the lateral tegmental group innervates primarily sympathetic nuclei and somatic motor areas (Westlund and Coulter, 1980). Coeruleo-spinal projections have also been demonstrated by histochemical flucrescence techniques in cultures containing dissociated spinal neurons and LC explants (Marshall, Pun, Hendelman and Nelson, 1981).

In the brainstem, the LC projects primarily to sensory and associational nuclei such as the superior and inferior colliculi, the interpeduncular nuclei, pontine nuclei, sensory nuclei of the trigeminal nerve, and the cochlear nucleus (Grant and Redmond, 1981). The LC also sends a substantial projection to the cerebellum (Bloom, Hoffer and Siggins, 1971; Olson and Fuxe, 1971) as has been discussed earlier.

LC fibers ascend primarily via the dorsal bundle (Olson and Fuxe, 1972; Ungerstedt, 1971) and innervate widespread areas of the forebrain (Dahlstrom and Fuxe, 1964; Swanson et al., 1977; Lindvall and Bjorklund, 1974), including the hypothalamus, thalamus, olfactory bulb, septal nuclei and amygdala. As far as is known to date, the LC provides exclusive noradrenergic input to the ipsilateral cerebral cortex (Freedman, Hoffer and Woodward, 1975), the hippocampus (Lindvall and Bjorklund, 1974; Pickel et al., 1974; Segal et al., 1973), and the cingulate gyrus (Pickel et al., 1974).

The LC differentiates very early in fetal life (Taber, 1963; Taber-Pierce, 1966). Peak LC neurogenesis occurs on fetal day twelve but is high from fetal days eleven through thirteen (Lauder and Bloom, 1974). Catecholaminergic fluorescence first appears in the mouse LC on fetal day fourteen (Golden, 1973), and NA has been found to be present

in the soma, dendrites, axons, varicosities and synapses of the LC cells of the rat (see review by Amaral and Sinnamon, 1977).

The LC of the adult rat is approximately 1.0mm rostrocaudally, by 0.5mm dorsoventrally, by 0.25mm wide (Swanson and Hartman, 1975; Grzanna and Molliver, 1980). The LC neurons are 20-40 micra in diameter and show extensive dendritic arborization and axon collateralization (Swanson, 1976; German and Bowden, 1975). These axons are characteristically thin, 0.5-1.5 micra in diameter and unmyelinated (Lindvall and Bjorklund, 1974; Levitt and Moore, 1979). Along the axons are many varicosities with thin intervaricose segments. In addition to NA, these varicosities have been shown to contain dopamine-beta-hydroxylase (Swanson and Hartman, 1975), indicative of a synthetic system for NA. These varicosities are also able to accumulate exogenous 3H-NA (Descarries, Watkins and Lapierre, 1977), and therefore probably also have a specific reuptake system for it. These varicosities may form synapses "en passant", releasing NA onto their target tissues.

LC-PN PROJECTION

The locus coeruleus-cerebellar projection has been well established by histofluorescence techniques (Olson and Fuxe, 1971), as well as by light and electron microscope cytochemistry (Bloom, Hoffer and Siggins, 1971). Early work in the rat cerebellum showed that LC fibers terminated in both the granular and molecular layers (Hokfelt and Fuxe, 1969). It was later shown by Bloom et al. (1971) that the sparse LC innervation of the cerebellum is predominantly to the molecular layer, specifically to Purkinje cell apical dendrites. This finding has been contested by more recent autoradiographic work which showed that the NA terminals are restricted mainly to the proximal dendrites and somata of the Purkinje neurons (Pickel, Segal and Bloom, 1974). A recent study by Kimoto et al. (1981) indicates that NA terminals also make close contact with the dendrites of granule cells.

Considerable electrophysiological evidence has further supported the existence of a coerulear-cerebellar projection. Discrete stimulation of the LC depresses Purkinje neuron spontaneous firing (Hoffer, Siggins, Oliver and Bloom, 1973; Siggins et al., 1976) and this effect can be mimicked by iontophoretically applied NA (Bloom et al., 1971; Lake and Jordan, 1974), as well as by iontophoretically applied cAMP (Siggins, Hoffer, Oliver and

Bloom, 1971). These inhibitions may be blocked by the beta-adrenergic blocker, sotalol (MJ-1999), or by pretreatment with the catecholaminergic neurotoxin, 6-hydroxydopamine (Hoffer, Siggins, Woodward and Bloom, 1971; Hoffer et al., 1975). The mechanisms underlying this inhibition will be examined in detail.

NA-MECHANISMS

Unlike classical inhibitory neurotransmitters (i.e., GABA), iontophoretically applied NA or discrete stimulation of the LC depresses Purkinje neuron discharge by hyperpolarizing the cell membrane with either no change or a decrease in membrane ionic conductance (Siggins, Oliver, Hoffer and Bloom, 1971). It is not known what specific conductance changes are involved with NA, but it has been suggested that conductance changes of such a large magnitude as some of those observed by Siggins, Oliver, Hoffer and Bloom, (1971), are not likely confined to the movement of only a single ion (Woodward, Moises, Waterhouse, Hoffer and Freedman, 1979). This hyperpolarizing effect and associated increase in membrane resistance may also be elicited by iontophoretically applied cAMP. cAMP is thought to be an intracellular mediator of noradrenergic response and its role as a "second messenger" has gained considerable support (Greengard, 1978). The formation of cAMP is catalyzed by

adenylate cyclase and adenylate cyclase activity is known to be very high in rat cerebellum (Weiss and Costa, 1968). Furthermore, a rise in the cAMP content of Purkinje neurons has been demonstrated by cytochemical techniques following LC stimulation or incubation of cerebellum with catecholamines (Kakiuchi and Rall, 1968). The contention that cAMP mediates depressant noradrenergic responses in rat cerebellum was challenged by Godfraind and Fumain (1971), as well as by Lake and Jordan (1974), who failed to observe this effect. More recently, Ganviler (1976), using cultured rat cerebellar Purkinje neurons, observed that while high concentration of cAMP only slightly decreased the firing rate of Purkinje neurons, low concentrations of phosphodiesterase inhibitors strongly enhanced cAMP and NA-induced inhibitions. Phosphodiesterase inhibitors are believed to increase the intracellular concentration of cAMP and therefore this supports the hypothesis that NA and cAMP exert their electrophysiological effects by a common mechanism.

The rather unique membrane changes associated with NA have prompted speculation as to its role in the central nervous system. Recent studies have shown that if the concentration of NA is reduced so that there is either a small or no observable effect on Purkinje neuron discharge, concomitant application of certain neurotransmitters results in a potentiation of their effect. This led Woodward et al.

(1979) to propose a neuromodulatory role for NA in the central nervous system, that is, an ability of NA to inhibit the background discharge of the Purkinje neuron, while enhancing the transmission of convergent synaptic inputs. This selective enhancement by NA of evoked activity over background is referred to as an increase in the signal to noise ratio.

This neuromodulatory effect of NA has been demonstrated for GABA (Moises and Woodward, 1980; Yeh, Moises, Waterhouse and Woodward, 1981), acetylcholine (Waterhouse, Moises and Woodward, 1980; 1981), and for glutamate (Segal, 1982; Moises, Woodward, Hoffer and Freedman, 1979). The facilitation of acetylcholine in the cortex appears to be mediated by alpha receptors, while that of GABA and glutamate appear to be mediated by beta receptors (Waterhouse, Moises, Yeh and Woodward, 1982). Dopamine does not appear to be effective in facilitating responses either to acetylcholine or to GABA (Waterhouse, Moises and Woodward, 1980). NA was unable to facilitate the inhibition produced by glycine (Moises et al., 1979), and was found to have minimal and inconsistent effects on the inhibitions produced by taurine and beta-alanine (Yeh et al., 1981). It is possible that NA may interact only with specific neurotransmitters, or that different amino acids may act on Purkinje neurons in different ways.

Madison and Nicholl (1982) have attempted to explain the proposal that NA increases the sensitivity of various neurons to certain excitatory neurotransmitters. They found that both NA and cAMP block the Ca++ activated K+ conductance in hippocampal pyramidal cells, severely reducing the spike frequency adaptation which normally occurs with depolarizing stimuli. As a result, NA's ability to block this accommodation allows a greater number of spikes to be elicited by a given depolarizing stimulus.

DENERVATION-SUPERSENSITIVITY

Catecholamine depleting drugs and denervation techniques which chronically decrease the availability of NA and dopamine at receptor sites, increase the sensitivity of the cyclic AMP (cAMP) generating system to the respective neurotransmitters and their agonists (Porn and Krishna, 1971; Von Hungen, Roberts and Hill, 1974). This supersensitivity, generally associated with an increase in the density of beta-adrenergic (Sporn, Harden, Wolfe and Molinoff, 1977) and dopaminergic receptors (Burt, Creese and Snyder, 1977), may have important consequences in the development of tolerance to certain drugs and the appearance of withdrawal symptoms following their removal (Irwin, 1961; Tarsy and Baldessarini, 1974; Moller Nielsen, Fjalland, Pedersen and Nymark, 1974).

The earliest evidence of changes in receptor sensitivity following decreased transmitter availability was provided by Canon and Rosenblueth (1949), who showed that the sensitivity of the target tissues is increased following interruption of afferents. They called this phenomenon "denervation-supersensitivity". Since then, several approaches have been used to modify agonist supply to the postsynaptic membrane, and they will be examined briefly.

Responsiveness of a system may be reduced either by decreasing neurotransmitter release from the presynaptic nerve or by decreasing the number of available postsynaptic receptors. Release of catecholamine neurotransmitter may be decreased by:

1. Inhibiting its synthesis (for example with alpha-methyl-para-tyrosine, Moore and Thornburg, 1975).
2. Blocking its storage (for example with reserpine, Baudry, Martres and Schwartz, 1976).
3. Inhibiting its reuptake (for example with cocaine, tricyclic antidepressants) presumably via presynaptic receptors which inhibit release. (Clow, Jenner and Marsden, 1978).
4. Destruction of the presynaptic nerve (either physically or chemically).

Physical techniques for inducing supersensitivity include surgical denervation (Canon and Rosenblueth, 1949), and

electrical lesions (Krueger, Fern, Walters, Roth, and Greengard, 1976; Mishra, Gardner, Katzman, and Makman, 1974), while chemical denervation may be achieved by such selective neurotoxins as 6-hydroxydopamine (6-OHDA) and 6-aminodopamine (Kostrzawa and Jacobowitz, 1974). The number of available postsynaptic receptors may be decreased by the use of specific pharmacological agents, such as propranol, phenoxybenzamine and neuroleptics (Gnegy and Costa, 1980).

One of the most extensively used chemical denervants is 6-OHDA, first described by Tranzer and Thoenen (1967), and subsequently reviewed by Kostrzewa and Jacobowitz (1974). Treatment with this agent results in its intraneuronal accumulation, with the consequent selective destruction of noradrenergic and dopaminergic terminals. When injected intravenously, or intraperitoneally in the adult, 6-OHDA selectively destroys peripheral nerve terminals. When administered intracerebroventricularly or directly into brain tissue, it selectively destroys central noradrenaline and dopamine containing nerve terminals. In sufficiently high concentrations, the cell bodies may also be destroyed (Lanfumey, Arluison and Adrien, 1981). Toxic levels of intracellular concentrations of 6-OHDA have been estimated to be in the range of 25-100 nanomolar (Sachs and Jonsson, 1975; Jonsson and Sachs, 1975). The damage produced by 6-OHDA can be qualitatively assessed by histochemical fluorescence techniques (Malamad, Lahav and Atlas, 1977).

6-OHDA is structurally similar to the catecholamine neurotransmitters (Mishra et al., 1974). It is recognized by dopaminergic and noradrenergic receptors on the nerve terminal and is actively taken up against a concentration gradient. Uptake of 6-OHDA and consequent neurotoxicity may be prevented by such pharmacological agents as cocaine or desmethylimipramine, which act by blocking the reuptake system for NA.

A number of theories have been put forward to explain the mechanism of action of 6-OHDA. 6-OHDA undergoes rapid non-enzymatic oxidation forming 6-OHDA quinones. It has been suggested that these quinones may undergo covalent binding with nucleophilic groups of macromolecules, such as -SH, -NH2 and phenolic -OH, resulting in irreversible damage.

Coupled to the oxidation of 6-OHDA is the reduction of O2. This generates hydrogen peroxide (H2O2), superoxide ions (O2-) and hydroxyl radicals (OH-), all of which have been implicated in the molecular mechanisms responsible for 6-OHDA cytotoxicity. A suggested reaction sequence for the production of O2- and OH- radicals has been proposed by Cohen and Heikkila (1974). It was first noted that the hydrogen peroxide formed during the oxidation of 6-OHDA may itself be responsible for its neurotoxicity (Sachs and Jonnson, 1975). The toxicity may be enhanced by ascorbic

acid, since the oxidation of ascorbate to dehydroascorbate permits regeneration of 6-OHDA from its oxidized quinones, ultimately increasing the toxic effects (Deamer, Heikkila, Panganamala, Cohen and Cornwell, 1971; Heikilla and Cohen, 1972; Jonnson and Sachs, 1975).

More recently, it has been observed that the autooxidation of 6-OHDA also gives rise to the production of superoxide and hydroxyl radicals, both of which are toxic to the neuron (Cohen and Heikkila, 1974). O2- has been shown not only to be neurotoxic, but it also acts as a catalyst for the oxidation of 6-OHDA. This is supported by the finding that neurotoxicity to 6-OHDA may be depressed by superoxide dismutase, an enzyme which has been shown to slow the formation of quinones as well as H2O2 (Cohen and Heikkila, 1977).

An interesting phenomenon is that within the nerve terminals, NA acts as a scavenger of superoxide radicals (Sachs, Jonnson, Heikkila and Cohen, 1975), thus serving a protective function in much the same way as superoxide dismutase. The sensitivity of neurons to 6-OHDA has been shown to be dependant on the stores of NA, such that the autooxidation of 6-OHDA catalyzed by superoxide radicals may be slowed by endogenous or by exogenous application of NA, thus diminishing the neurotoxicity. It was further shown that depletion of the NA stores results in an enhanced neurotoxicity.

To summarize, three hypotheses have been proposed to explain the neurotoxicity produced by 6-OHDA. Each is based on the fact that 6-OHDA is very easily oxidized. The first theory proposes that oxidation products of 6-OHDA may undergo covalent binding with nucleophilic groups of macromolecules, resulting in irreversible damage to the nerve (Saner and Thoenen, 1971; Thoenen, Tranzer and Hausler, 1970; Sachs and Jonsson, 1975). A second hypothesis suggests that the production of hydrogen peroxide during oxidation of 6-OHDA gives rise to neuronal destruction (Sachs and Jonsson, 1975). Finally, the most recent theory proposes that generation of superoxide and hydroxyl radicals are intimately responsible for the observed neuronal toxicity (Heikkila and Cohen, 1972; Cohen and Heikkila, 1977). It is possible that all three proposed mechanisms play causative roles in the degenerative process.

The study of supersensitivity has certain criteria which must be satisfied. First, the lesion must be discrete, so only that system being examined is damaged. Second, depletion of neurotransmitter must be nearly complete, otherwise, supersensitivity cannot be detected (Baudry et al., 1976; Tarsy and Baldessarini, 1974), and third, there must be found a relevant functional change to record as the expression of supersensitivity.

Several approaches have been taken to measure the sensitivity of the catecholamine receptor. Behavioral responses to a catecholamine agonist have been measured, as have the level of spontaneous activity and threshold sensitivity of catecholaminergic neurons. Sensitivity has also been measured in terms of activation of dopamine-sensitive adenylate cyclase (as measured in terms of cAMP production) and in terms of the extent of specific agonist or antagonist binding to the catecholamine receptor.

METHODS

EXPLANTING PROCEDURE AND MAINTENANCE OF CULTURES

Locus coeruleus-cerebellum explant cultures were prepared in the lab of W.J. Hendelman, from newborn Paris RIII mice maintained by the animal centre at the University of Ottawa. Animals were killed by placing them over dry ice in a closed container, followed by removal and cardiac puncture. All subsequent procedures were performed under sterile conditions in a positive pressure hood. The skin and cartilage of the cranium were cut and peeled away, exposing the brain. The cerebellum and peduncular regions of the pons were located and excised. The dura and pia were removed and the excised tissue was sectioned parasagitally so as to include cortical and deep cerebellar nuclei, as well as the locus coeruleus. Each slice or explant was then placed on a collagen-coated cover slip and a drop of nutrient medium was added. The preparation was encased in a Maximow assembly and was incubated in the lying drop position at 35.5 degrees Celcius.

Maintenance of the cultures required that they be washed in a balanced salt solution and fed twice weekly with nutrient medium. This medium was only partially defined and consisted of:

25% chick embryo extract (10cc) (RF50)

25% human adult serum (10cc)

50% minimal essential medium (20cc) The medium contained 1.1% glucose and 1mM glutamine was added. The feed was sterilized by filtration and was stored in a freezer. The progress of the culture was observed by bright field microscopy, using 40X long working distance objectives. Electrophysiological studies were not conducted in regions of the culture which had an apparent thick necrotic zone. Criteria used in selecting cultures for study were that they appeared healthy in that myelin was present (with the absence of beading), and there were no ghost cells or areas of vacuolation and that the required neurons were present. Cultures were used for electrophysiological and morphological studies from day seventeen onward which provided sufficient time for neuronal maturation.

ELECTRODE METHODS

Extracellular electrodes were prepared from borosilicate capillary tubes, 1.5-1.8 O.D. X 100mm (KIMAX-51). Barrels were bent with the aid of a propane torch and six such barrels were then assembled to form a rosette. One unbent capillary tube was then inserted through the middle of the rosette, and heat shrink tubing was employed at the two ends to retain the configuration. The electrodes were then fixed with epoxy resin.

Electrode blanks were positioned in a Stoelting vertical microelectrode puller and the central portion of the electrode was heated and twisted 180 degrees with 1.0 cm diameter tungsten heating coil. Electrodes were then pulled, each blank yielding one electrode. Tip size and shape could be altered by varying heating coil current and magnetic pull settings. Pulled electrode tips were broken back to 4 to 6 micra in diameter, and were stored in a dust-free environment.

On the day of the experiment electrodes were filled with the following solutions with the aid of PE 10 tubing (Clay Adams) attached to a 30 gauge needle. The central recording barrel was filled with 3.0 M NaCl. Two barrels were filled with 0.2 M NA (Sigma) adjusted to pH 5.0-6.0. Two barrels were filled with 0.2 M glutamic acid (Sigma), adjusted to pH 8.0. Two barrels were filled with 1.0 M NaCl, one of which was used as the sum channel for current balancing, the other could be used for testing the effects of current ejection. Filled electrodes were centrifuged for twelve minutes at 4500 rpm at 20 degrees Celsius (C) to achieve filling.

ELECTROPHYSIOLOGY

Electrophysiological experiments were carried out at various times during the day. Some recent evidence suggests that there may be diurnal rhythms in receptor number

(Wirz-Justice, 1984). However, it was unlikely that this would have any effect in the tissue culture system as it lacked hormonal input. Electrophysiological experiments were carried out in a shielded room to minimize extraneous noise. The culture to be studied was removed from the Maximow chamber, and the underside of the coverslip was then adhered to the well of a plexiglass recording chamber with small spots of vaseline at the perimeter of the coverslip. The recording chamber was mounted on the stage of an inverted microscope with long working distance objectives. The floor of the chamber, which was a glass microscope slide, had the optical properties to permit easy viewing of the culture. A total magnitude of 640X was used. Illumination was achieved by a light and condenser mounted above the stage.

The explant was perfused with an oxygenated bicarbonate buffered balanced salt solution (BSS), based on Earl's BSS, which was adjusted to pH 7.35 and contained added glucose as nutrient. The flow rate was adjusted to about 5 mls per minute. The chamber had an inport-outport system to accommodate the gravity feed perfusion system. The temperature of the bath was maintained at approximately 35 degrees C. by means of a heating coil from a 12 volt battery wrapped around incoming glass tubing which contained the BSS. The temperature was monitored via a thermistor probe in the culture chamber.

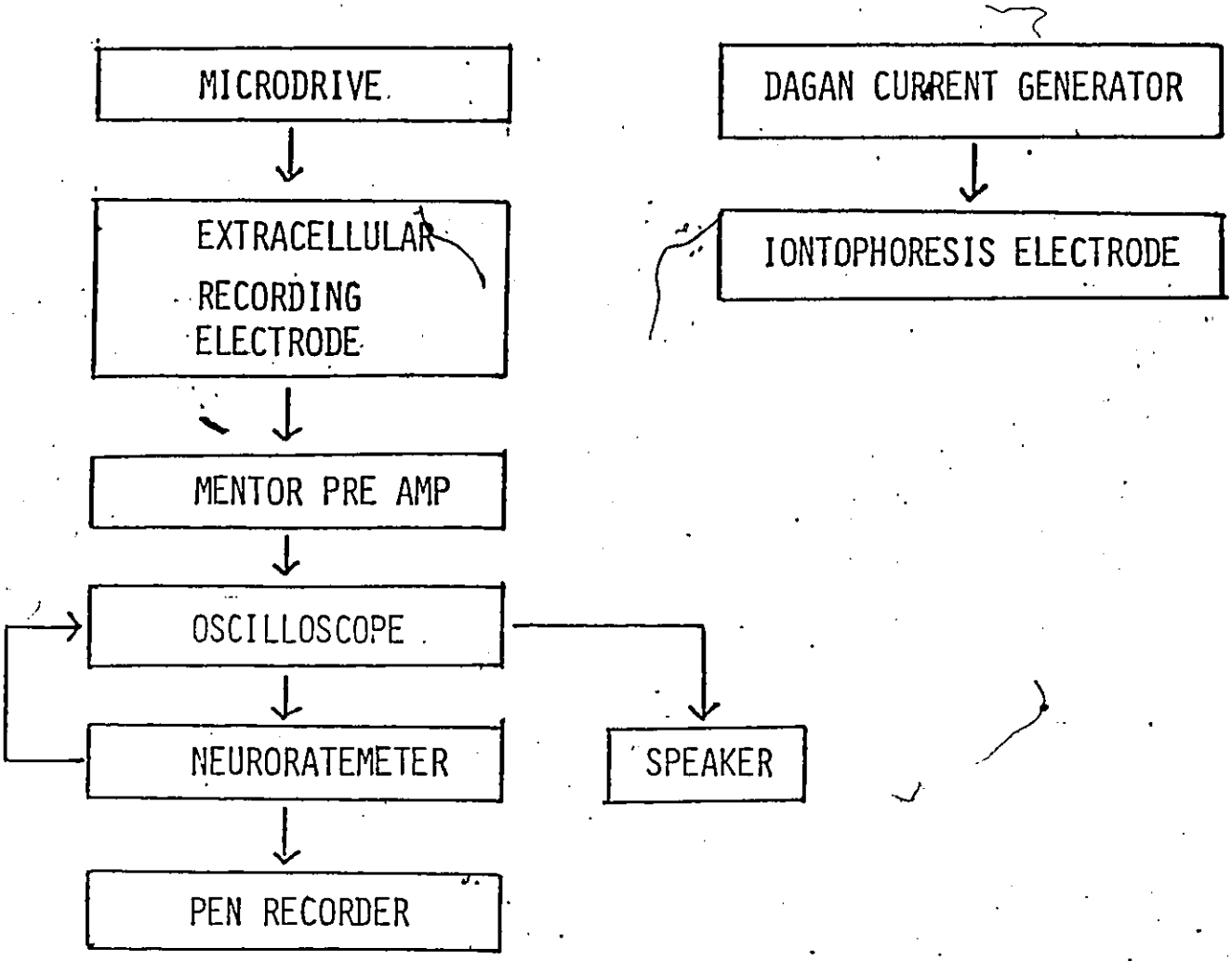

FIGURE 1. EQUIPMENT SETUP

The neurons to be studied were located with the aid of a schematic map drawn earlier, and the electrode was positioned accordingly. Once the cortical area of the cerebellum was located, the electrode was slowly driven down into the tissue to approach a Purkinje neuron. Guided by the amplitude of the spike of EN spontaneous firing, an optimal position of the electrode in relation to the neuron was established. In most cases only one or two neurons were recorded per culture.

EQUIPMENT

The recording equipment setup is shown schematically in Figure 1. The resistance of the recording electrode was measured with the Mentor preamplifier by means of a comparator signal. Recording electrode resistance was between four and ten megOhms. Retaining currents of approximately -30 nA were used for the NA barrels and retaining currents of about +15 nA were used for glutamate. A pulse brightening signal from the neurorate meter was fed back to the oscilloscope to permit detection of those action potentials triggering the neurorate meter. Noise was considerably reduced to about 0.1 mV with the aid of high and low pass filters and a 60Hz notch filter.

CULTURE TYPES

Three main types of cultures were studied electrophysiologically:

1. locus coeruleus-cerebellum co-cultures
2. cerebellum cultures (No LC)
3. locus coeruleus denervated cultures.

In these cultures, the following features of Purkinje neurons were examined:

(i) the level of spontaneous activity

(ii) the response to iontophoretically applied glutamate

(iii) the response to iontophoretically applied NA

(iv) the response to concurrent iontophoretic application of glutamate plus NA.

The LC-cerebellum culture set up has been described earlier. Cerebellum cultures were set up as per the procedure described for LC-cerebellum cultures, with the exception that the locus coeruleus piece was excluded. A third group of cultures was prepared by taking three week old LC-cerebellum co-cultures and exposing them to the selective neurotoxin, 6-OHDA.

6-OHDA INDUCED DEGENERATION IN LC-CEREBELLUM CO-CULTURES

General Objective: To determine the concentration of 6-OHDA which selectively destroys NA-containing cells of the LC in tissue culture. Specific Objectives:

1. To determine the concentration of 6-OHDA which selectively destroys NA-containing nerve terminals.

2. To determine the concentration of 6-OHDA which selectively destroys NA-containing cell bodies and their terminals.

3. To determine the critical incubation time for 6-OHDA induced degeneration to occur.

4. To determine the concentration of ascorbic acid required to prevent the extraneuronal oxidation of 6-OHDA.

Cultures undergoing 6-OHDA induced degeneration of NA-containing cell bodies/terminals were initially assessed by light microscopy.

Since 6-OHDA is easily oxidized, several steps were taken to retard this process before and during incubation of the cultures, and thereby adapt it to use in an in vitro system. An 8mM solution of ascorbic acid, sodium salt (Sigma), was prepared by dissolving it in the nutrient media. The ascorbic acid did not alter the pH of the nutrient media which remained at pH 7.35. The ascorbate-feed solution was then transferred to a nitrogen glove bag in order to provide an oxygen free environment for the addition of the 6-OHDA.

The relatively short period of time that the 6-OHDA was exposed to the air during weighing (about two minutes) did not seem to cause significant oxidation and therefore inactivation. The 6-OHDA was subsequently transfered to the nitrogen glove bag and was added to the ascorbate-feed solution. The mixture was gently agitated, following which the mixture was drawn into a syringe.

Cultures were fed under sterile conditions in the positive pressure hood with the 6-OHDA solution rendered sterile by means of a 0.2 micra milipore filter placed on the end of a syringe. Cultures were incubated in this medium at 35.5 degrees Celsius.

Two types of control cultures were set up. Ascorbate controls were prepared exactly as per the 6-OHDA treatment with the exception that the 6-OHDA was left out. In addition, dopamine-ascorbate controls were set up in which dopamine was substituted for 6-OHDA. Dopamine is structurally similar to 6-OHDA and is easily oxidized. However, it lacks the neurotoxic properties of 6-OHDA and would therefore serve as a good morphological and electrophysiological control for 6-OHDA.

The next day the cultures were washed by three subsequent immersions in BSS and were refed with normal flow serum, the standard feed media. Oxidation of the 6-OHDA solution was observed as indicated by the characteristic brown colouration of the discarded feed.

Cultures were reassessed daily by light microscopy. Between days three and five, cultures were either stained for catecholamines or used for electrophysiology and then stained. Interpretation of results was as follows: the presence of nonspecific fluorescence (no blue) as shown by histochemical-fluorescence techniques, was taken to indicate that NA-containing terminals (specific blue fluorescence observable only in the immediate brain stem region), or brain stem cells and their terminals (total absence of specific fluorescence) had been selectively destroyed in cultures treated with 6-OHDA.

Data Analysis

The frequency of discharge in spikes per second was determined by calibration of the rate meter record. The effect of glutamate alone and NA alone were reported in terms of spontaneous activity. Glutamate was ejected with negative current, usually set to elicit at least a 25% increase in spontaneous discharge. This was considered as a "standard response". Careful attention was paid to the spike amplitude during glutamate application to avoid glutamate-induced depolarization block. The effect of glutamate was analyzed both in terms of glutamate minus (-) spontaneous activity as well as the ratio of spontaneous to glutamate activity for each Purkinje neuron responding. The

reason for using spontaneous/glutamate rather than glutamate/spontaneous activity was to avoid division by zero in cases where the interpulse activity was zero.

NA effects were analyzed in terms of NA minus spontaneous activity as well as the ratio of NA to spontaneous activity.

The effect of glutamate plus NA was analyzed by comparing the difference between the glutamate responses before and during application of NA. This was done in two ways using the following formulae: (Glu + NA)-S - Glu-S and (Glu + NA)-S / Glu-S . If the change of Glutamate-spontaneous was greater by at least 25%, or S/GLU was less by at least 25%, then it was concluded that in a given Purkinje neuron, NA potentiated the glutamate response.

Since the levels of spontaneous activity changed following application of glutamate, the level of interpulse activity just preceding the next glutamate pulse which represented the highest level of background discharge was used as a value for spontaneous activity. Glutamate plus NA data are displayed as descriptive statistics.

Following electrophysiological and denervation experiments, cultures were routinely stained (by a modification of the Bloom and Battenberg method (1976)) to acertain the presence or absence of IC neurons. Glycrilic

acid stains catecholaminergic nerve terminals and in some cases cell bodies which fluoresce bright blue under ultraviolet light.

A 2% glyoxilic acid solution was prepared by dissolving 2.00 grams of glyoxilic acid monosodium salt (Sigma) in 5.0 mls of Ringer's plus 5.0 mls of phosphate buffer (see appendix). The glyoxilic acid solution was filtered with a 2.0 micra milipore filter and was cooled to 2 degrees C. Cultures to be stained were washed in a balanced salt solution and then incubated in the glyoxilic acid solution for ten minutes at 2 degrees C. They were then removed from the solution and excess liquid was removed by touching the edge of the coverslip to a piece of bulbulous paper. Cultures were placed in a porcelain holder and were blown dry for 5 minutes at 37 degrees C., and then baked for ten minutes at 100 degrees C. Coverslips were then mounted on slides with a drop of paraffin oil and were sealed with clear nail enamel. Cultures were examined under the fluorescence microscope using a Zeiss 03 filter set for selective visualization of catecholamine fluorescence. In some cultures a thionine stain was used to exarine for changes in neuronal cell appearance and density.

DRUGS AND SOLUTIONS USED

Balanced salt solution: A 332 mOsm balanced salt solution was used to perfuse the cultures during electrophysiological studies.

10X stock solution was prepared and stored in the refrigerator at 4 degrees C. 1 liter consisted of:

3mM KCl

1mM NaH_2PO_4

0.8mM $MgSO_4$

1.8mM $CaCl_2$

118mM NaCl

On the day of the experiment, the BSS was prepared from the stock solution by taking:

100mls stock

6.00g dextrose as nutrient

2.00g $NaHCO_3$ as buffer

and adding distilled H_2O to total 1000mls. pH was adjusted to 7.35 by bubbling with 95% O_2 and 5% CO_2. Glutamic acid: A 0.2 M glutamic acid solution was prepared by dissolving glutamic acid (Sigma St. Louis Mo.) in distilled H_2O and pH was adjusted to pH 8.0. The solution was stored in the refrigerator at 4 degrees C. for a period not exceeding three weeks. L-Arterenol: A 0.2 M L-noradrenaline solution was prepared by dissolving L-arterenol free base (Sigma) in 0.1 M HCl. pH was adjusted to pH 5.0-6.0. The solution was

filtered through a 0.2 micra milipore filter prior to filling the electrode. Balanced Salt Solution (BSS): for cultures 90cc double distilled deionized water, 10cc Earl's 10X solution BSS, 3cc 7.5% NaHCO3, 3.5 drops of phenol red indicator 0.5%. The BSS was then autoclaved and adjusted to pH 7.4, and was stored in the refrigerator. FEEI: is prepared from sterile solutions in the positive flow chamber. It consists of:

25% chick embryo extract (10cc) (FE50)

25% human adult serum (10cc)

50% minimal essential medium (20cc)

The feed is quick frozen and is stored in the freezer. Glyoxilic acid Ringer's was prepared by dissolving:

860 mg NaCl

30 mg KCl

33 mg CaCl2

in 100 mls of distilled H2O. NaPO4 Buffer: was prepared by adding solution B to solution A until the pH=7.4

Solution A: 8.52g NaHPO4 in 200 mls distilled H2O

Solution B: 2.48g NaH2PO4 X H2O in 60 mls distilled H2O.

RESULTS

CULTURES

During development, the explants spread and thinned out. A necrotic zone usually appeared in the central, thicker portion of the explant, presumably as a result of limited oxygen supply and decreased diffusion of nutrients and metabolites to and from the cells of this region.

The LC cells increased somewhat in size throughout their development in tissue culture. The LC neurons in culture typically appeared in clusters, and other cell types could not be observed. These neurons' somata measured approximately 30 micra in diameter. Under bright field microscopy it was observed that the LC cells had a prominent nucleolus, a pale nucleus, and the cytoplasm, whose limits were not clearly delineated, often contained characteristic refractile granules (see Figure 2).

Purkinje neurons were not mature at the time of explanting. During development these neurons increased in size to about 18-22 micra in diameter (Fig. 3) and were thought to mature by around day seventeen. Quite often, the Purkinje neurons were found associated with long strands of myelin which could be seen radiating down to a deep nuclear area when one was present.

Figure 2. LC neurons in tissue culture. Somata measured approximately 30u in diameter. Note the prominent nucleolus and characteristic refractile granules as seen under bright field microscopy.

Figure 3. Purkinje neurons in tissue culture. Somata measured approximately 18-20u in diameter.

The deep nuclear area contained larger, morphologically distinct neurons interspersed in a reticular network of myelin. Other neuronal elements occasionally present in the explant included some non-LC brainstem nuclei, or the rare mesencephalic neuron. Other cerebellar neurons thought to be present in the tissue culture system such as Golgi cells, granule cells, stellate and basket cells, could not be distinguished with the light microscope. However a thionine stain demonstrated the presence of granule cells in considerable density.

Although other cerebellar neuronal elements could not be distinguished on the basis of light microscopy, only the Golgi cells are similar in size to the Purkinje neurons. Since the Golgi cells number far less than Purkinje neurons (Palay, Chan-Palay, 1974), it is likely that the great majority of larger neurons thought to be Purkinje neurons, were in fact Purkinje neurons. No observable differences could be found between Purkinje neurons set up with or without LC neurons.

Cultures containing the neuronal group characterized as LC by Mandelman et al. (1982), when stained with a 2% glyoxylic acid solution, were found to exhibit a strong catecholaminergic fluorescence. This was indicated by a bright blue colour. The LC area usually exhibited a very bright generalized fluorescence, and only on a few isolated

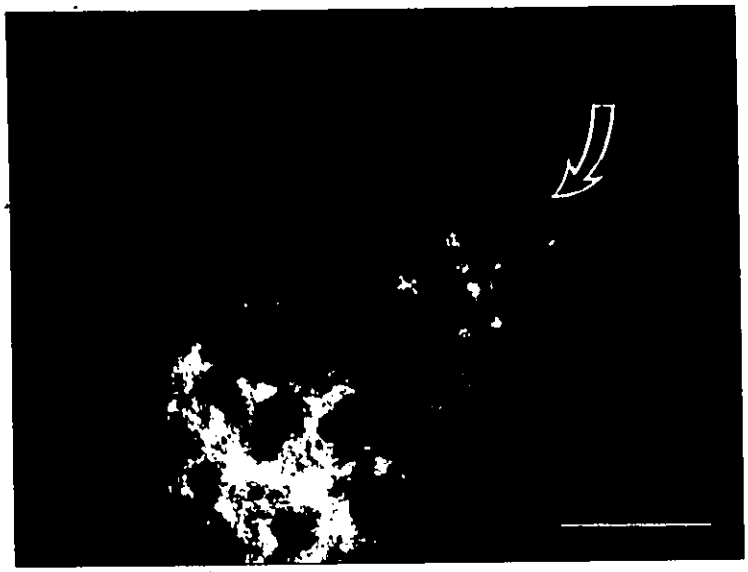

Figure 4. Fluorescence microscopy of LC neurons in tissue culture following specific staining for catecholamines with glyoxcilic acid. Note "Swiss cheese" appearance presumably sihlouettes of non-fluorescing somata.

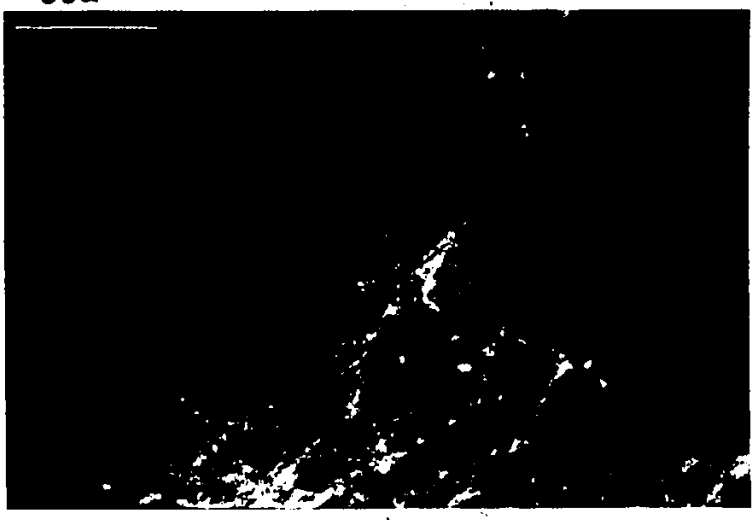

Figure 5. Noradrenergic fibers radiating from LC area in tissue culture.

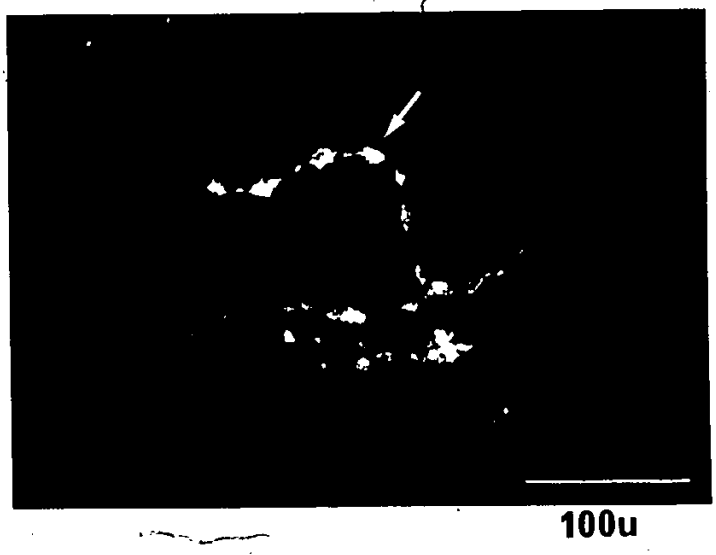

Figure 6. Noradrenergic fibers from LC area. Note characteristic varicosities.

occasions could the somata be visualized. In these instances, the cell bodies flucresced brightly, appeared round, measuring about 30 micra in diameter. On a number of occasions the LC area was characterized by a "swiss cheese" appearance. The holes were presumably sihlouettes of the non-fluorescing LC somata, and were surrounded by brightly fluorescing catecholaminergic fibers (Fig. 4).

From the LC area, dense fibers could be seen radiating in all directions (Fig. 5). It was my impression that the fibers, although propagating in all directions, travelled far shorter distances in directions where no target tissue was present, but for longer distances to successfully reach the cortical area of the explant where the Purkinje neurons were located. Fibers appeared to criss-cross and terminate in the region of the Purkinje neurons, as was determined on the basis of schematic maps drawn earlier. Upon examination of the outlying areas, individual fibers could be seen, due to the decreased density in the periphery of the explant. These fibers were characterized by numerous fluorescing varicosities (Fig. 6).

In those cultures which were set up to contain cerebellum alone (i.e., no LC), neither 6-OHDA treated nor untreated exhibited catecholamine fluorescence.

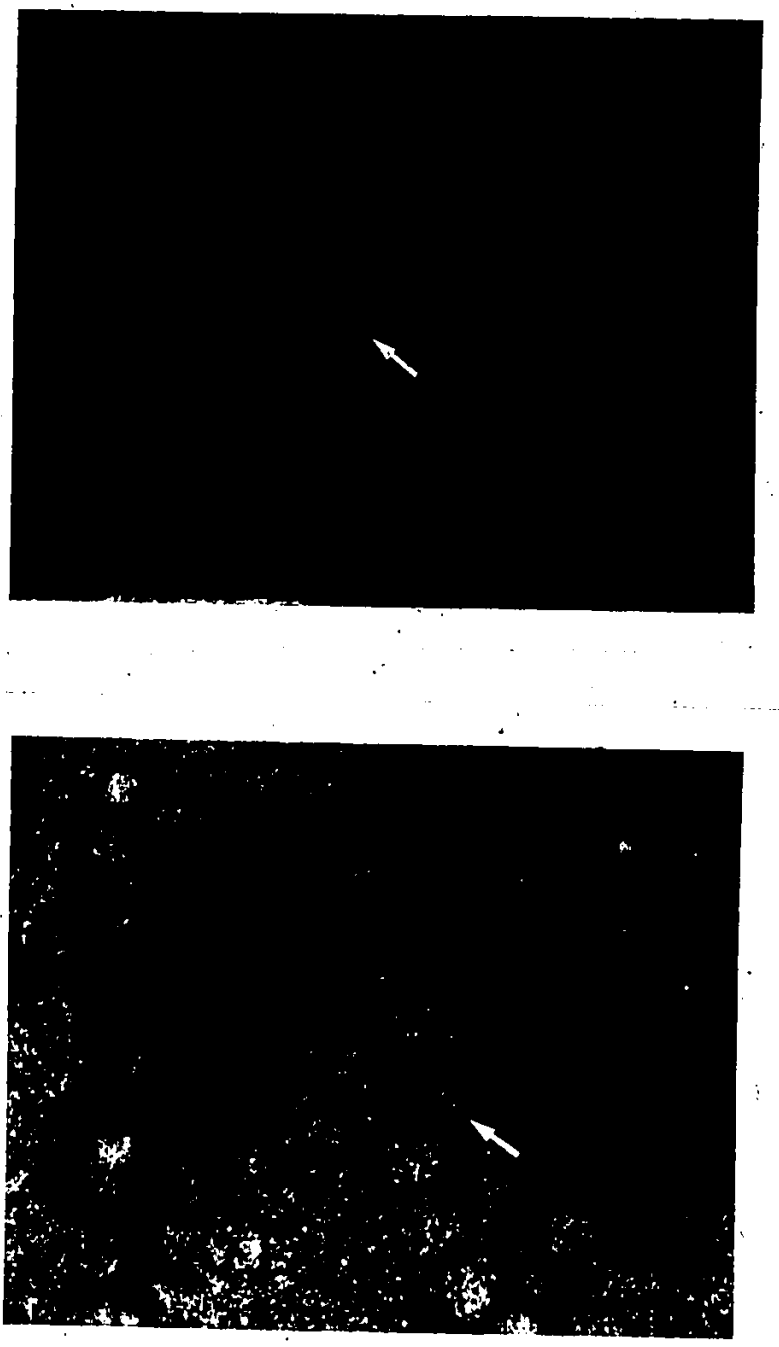

Figure 7. Purkinje neurons before (above) and after (below) treatment with 6-OHDA. The health and size of the neurons appears comparable in the two photographs.

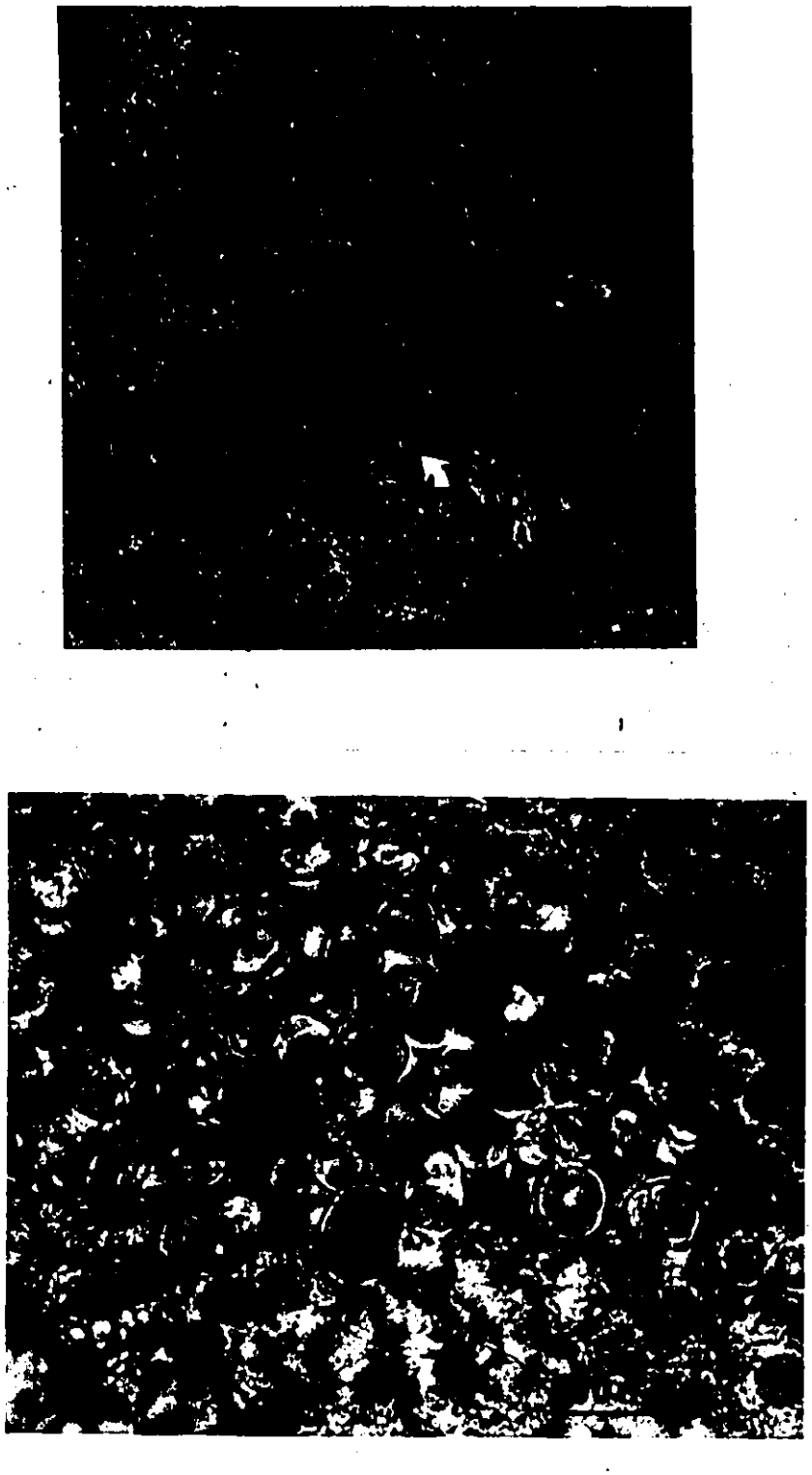

Figure 8. LC neurons in tissue culture before (above) and after (below) treatment with 6-OHDA. Note holes and ghosts, and the absence of LC neurons following 6-OHDA.

6-OHDA

Cultures treated with a 500 uM 6-OHDA/ascorbic acid feed solution exhibited selective destruction of the LC area, leaving the Purkinje neurons apparently unaffected (Garber, Marshall and Hendelman, 1983). Figure 7 shows the PN before and after treatment with 6-OHDA. As can be seen, the health and size of Purkinje neurons before and after treatment appears comparable. Examination of the LC area following treatment with 6-OHDA reveals considerable degeneration. Figure 8 shows the LC area before and after treatment with 6-OHDA in the same culture. Damage to the LC area was observed within 24 hours. Cultures were not examined prior to this time since the visibility of cells in all cultures was substantially reduced within the 24 hour period following washing and feeding with all solutions.

The following changes occured in LC neurons exposed to 500 uM 6-OHDA for 3-24 hours. In some cultures where the LC neurons could still be visualized, the cytoplasmic limits appeared more distinct, and the cell diameters seemed to have been reduced. Some vacuoles were often seen in this area. In the vast majority of cultures, however, considerable neuronal degeneration was evident. Many holes and ghosts were present as were glial elements surrounding the area, and no LC neurons were seen. A thionine stain of these cultures did not reveal any observable difference in

the density of granule cells in treated as compared to untreated cultures.

Different cultures appeared to be affected to different degrees by 6-OHDA. This may in part be due to the very small quantities of 6-OHDA used, in preparing the neurotoxic solutions, giving rise to a variability of about 10% of the final concentration. Also, the amount of feed applied to the culture may vary from application to application. Since cultures were washed prior to being fed with this solution, the amount of BSS they retained may have varied, and therefore, so does the degree to which the feed was diluted. Lastly, there was considerable variation in the size and thickness of the explants, and so it is possible that the deeper layers may not have been exposed to the neurotoxin to the same degree as the superficial ones. All these factors may have contributed to the variability of the effects of 6-OHDA. This variability was observed both by light microscopic and by histofluorescence techniques. It should be noted that preliminary experiments using 100 uM 6-OHDA caused no apparent damage to any cell types, while 750- 1000 uM completely destroyed all cell types in the culture preparation.

Cultures exposed to 500 uM 6-OHDA which contained cerebellum alone, did not exhibit any observable morphological differences as compared to untreated cultures.

FLUORESCENCE 6-OHDA CULTURES

Cultures treated with 500uM 6-OHDA where the LC neurons were still visible by light microscopy, exhibited an altered pattern of fluorescence Although the LC area itself fluoresced, fluorescing fibers were either completely absent, or were extremely sparse. When present, they showed properties indicative of degeneration, ie. fragmentation as well as swelling. However, in the vast majority of 6-OHDA treated cultures, no catecholaminergic fluorescence was observed. Cultures which lacked fluorescence corresponded to cultures in which the LC area appeared destroyed in light microscopic examination.

DOPAMINE TREATED LC PLUS CEREBELLUM CULTURES

Dopamine-treated LC plus cerebellum cultures could not be distinguished from untreated LC plus cerebellum cultures when examined by light or fluorescence microscopy.

PN-SPONTANEOUS ACTIVITY

Extracellular recording from Purkinje neurons demonstrated a number of different firing patterns as well as different frequencies of discharge. The majority (approximately 90%) of Purkinje neurons were found to have relatively regular firing patterns, although a small number

Table 1.

DESCRIPTIVE STATISTICS: GLUTAMATE (±S.E.M)

PREP	LC	NO LC	6-OHDA	LCDA	NO LC 6-OHDA
S	3.86 (0.90)	3.10 (0.60)	4.81 (1.47)	3.92 (1.12)	6.56 (1.45)
nA GLU	39.70 (5.48)	22.38 (3.57)	13.22 (1.82)	49.00 (9.37)	20.73 (2.30)
G-S	11.58 (1.78)	7.03 (1.14)	12.95 (1.85)	13.83 (3.51)	11.21 (2.52)
S/G	0.26 (0.05)	0.36 (0.05)	0.2 (0.05)	0.32 (0.1)	0.34 (0.05)
N	30	29	35	12	22

Table 2.

DESCRIPTIVE STATISTICS: NA (±S.E.M.)

PREP	LC	NO LC	6-OHDA	LCDA	NO LC 6-OHDA
S	8.27 (0.91)	6.2 (1.21)	10.33 (3.44)	11.00 (4.00)	11.10 (2.13)
nA NA	50.88 (6.34)	45.00 (5.58)	36.44 (5.06)	35.00 (5.00)	35.20 (4.68)
NA-S	-5.58 (0.84)	-3.09 (1.42)	-6.47 (3.0)	-5.50 (0.50)	-3.00 (2.67)
NA/S	1.13 (0.88)	0.56 (0.23)	0.45 (0.14)	0.41 (0.26)	0.77 (0.20)
N	34	23	9	2	10

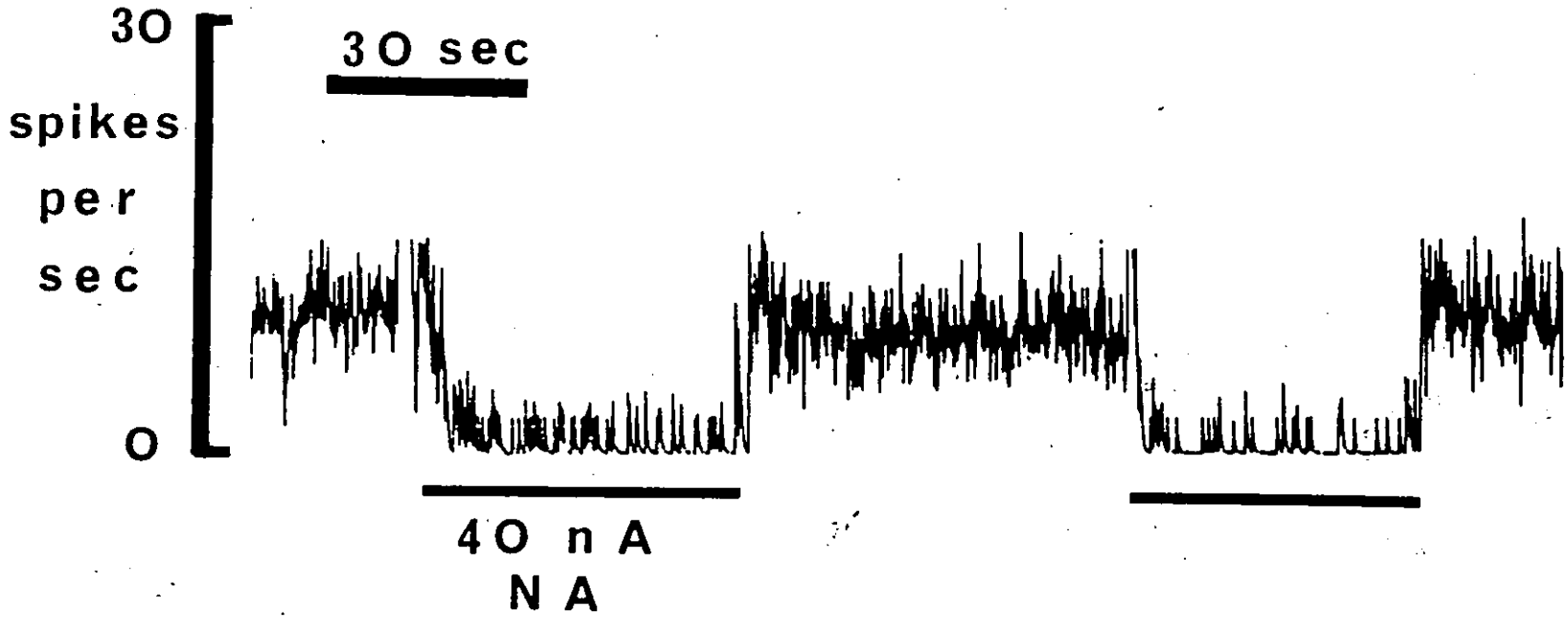

Figure 9. Inhibition of PN spontaneous discharge by iontophoretically applied NA. Continuous ratemeter record shows extracellular responses of a single PN before, during and after two successive applications of NA. Duration of NA application is indicated by the solid line. Current used for the application is indicated in amperes X 10^{-9} (nA) under the solid line.

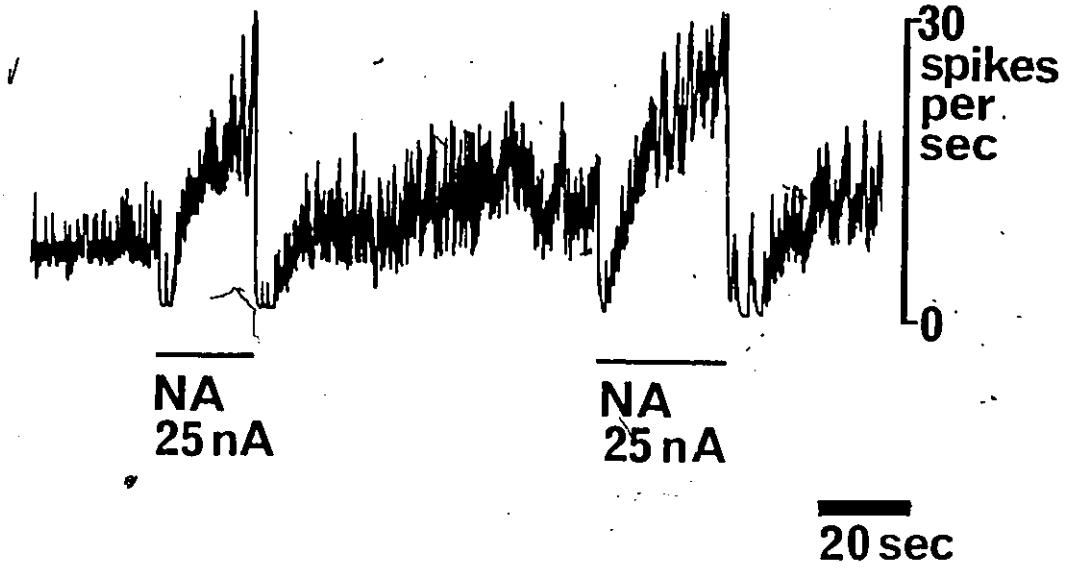

Figure 10. Biphasic response of a single PN to NA. Continuous ratemeter record shows an initial inhibition of short duration followed by a prolonged excitatory phase.

of Purkinje cells exhibited bursting patterns of discharge. A large number of Purkinje neurons fired at low frequency (0.5-10 Hz), although there was a wide distribution of firing rates up to approximately 50Hz. The mean firing rates in the various culture types are shown in Tables 1 and 2.

NA-ELECTROPHYSIOLOGY

Extracellular recording from Purkinje neurons revealed that about 75% of Purkinje neurons tested responded to iontophoretically applied NA, and of those responding, more than 90% showed a decrease in spontaneous activity. The responses were found to be reproducible upon repeated NA application to the same cell.

As seen in Figure 9, a postinhibitory excitation was often observed following termination of NA application. It was unlikely to be a current effect since it often persisted for several seconds, and may therefore represent a membrane effect (Engberg and Ryall, 1966).

Approximately 10% of cells responded either with an excitation, or biphasically to NA, initially with an inhibition of short duration followed by a prolonged period of excitation (Fig. 10). This effect could not be mimicked by Na+ current ejection, and is unlikely to be a hydrogen ion effect since NA pH is between 5.0 and 6.0.

Purkinje neuron sensitivity to NA varied considerably from neuron to neuron, even within the same culture. Apparent sensitivity to a given substance must take into account electrode tip diameter as well as diffusion distance to the neuron. However, even taking these parameters into account, it appeared as though certain Purkinje neurons were more sensitive to NA than others, and that some were almost insensitive.

The duration of the effect of NA varied from cell to cell, and on a few occasions persisted for several minutes following termination of ejection. Almost always, there was a rebound excitation when NA was turned off which persisted for seconds and on a few occasions for at least one minute.

Application of NA often resulted in an increase in the amplitude of the spike, while application of glutamate usually resulted in a decrease in the amplitude of the spike. With application of NA plus glutamate, the amplitude of the spike was usually greater than the amplitude during application of glutamate alone and sometimes greater than the amplitude of the spontaneous activity. The increase in the amplitude of the spike with NA application was observed both when NA depressed spontaneous firing as well as when it had no other apparent effect.

As can be seen from Table 2, there appears to be little difference in the response to NA across cultures. For

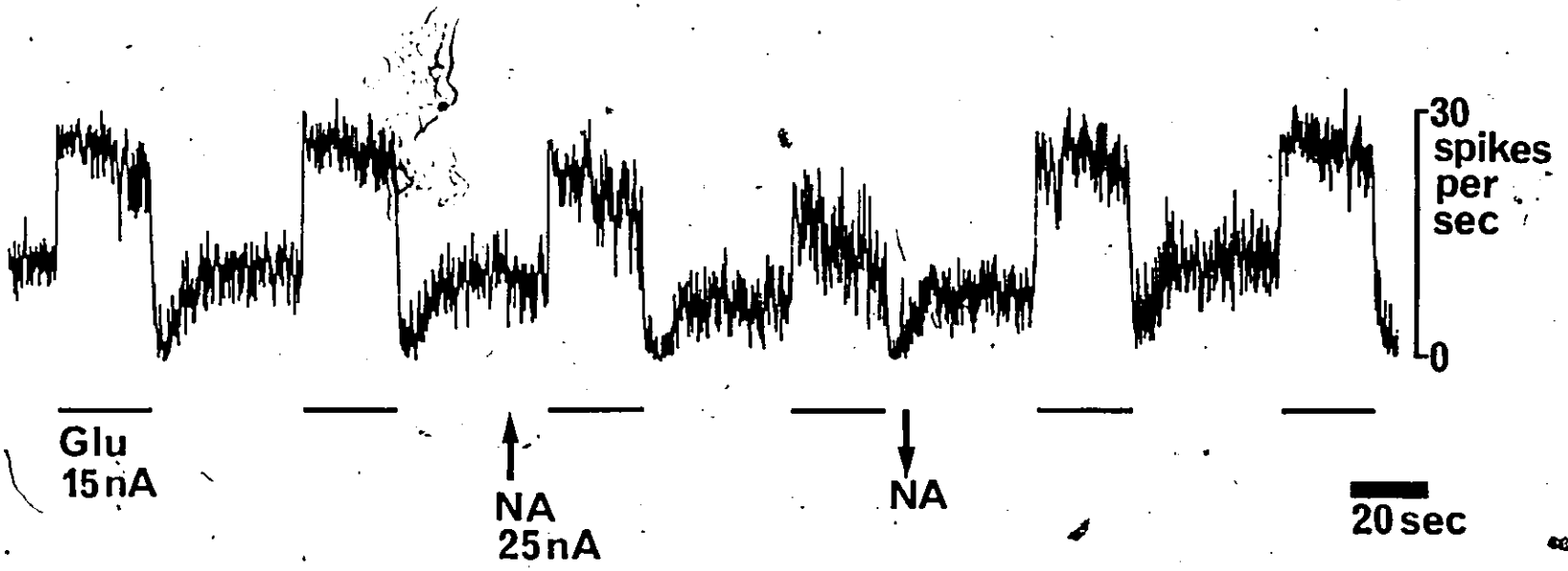

Figure 11. Inhibition of glutamate-induced excitation by NA. The continuous ratemeter record shows the response of a single PN to periodic pulses of glutamate 15nA (solid lines) before, during and after NA administration (↑ indicates NA on; ↓indicates NA off). Recovery of control activity was observed following termination of NA application.

example, the mean response in terms of NA-S (+/- S.E.M.) was -5.58 (0.84), -3.09 (1.42), -6.47 (3.0) spikes/second, for LC plus cerebellum, no LC, and 6-OHDA treated LC plus cerebellum cultures respectively.

GENERAL INTERACTIONS OF NA AND GLUTAMATE

Purkinje neuron spontaneous discharge was reproducibly augmented by uniform current pulses of glutamate passed at regular intervals. The onset of excitation was immediate and the duration of the excitation lasted the length of the glutamate pulse. When NA was applied concurrently with glutamate, two types of responses were seen. In some cases, NA inhibited the glutamate response, while in other cases it potentiated it (Garber, Marshall and Hendelman, 1983). These qualitatively different responses occurred in all tissue culture types. Figure 11 shows a typical inhibition of the glutamate response by NA. The continuous ratemeter record shows the control response of the Purkinje neuron to the two glutamate pulses preceeding the NA application. Iontophoresis of NA revealed a progressive diminution of the glutamate-evoked activity, reducing it to approximately 60% of control. The background activity was also found to be depressed. Recovery to control levels of glutamate excitation was observed with the glutamate pulse following termination of NA application. Potentiation responses

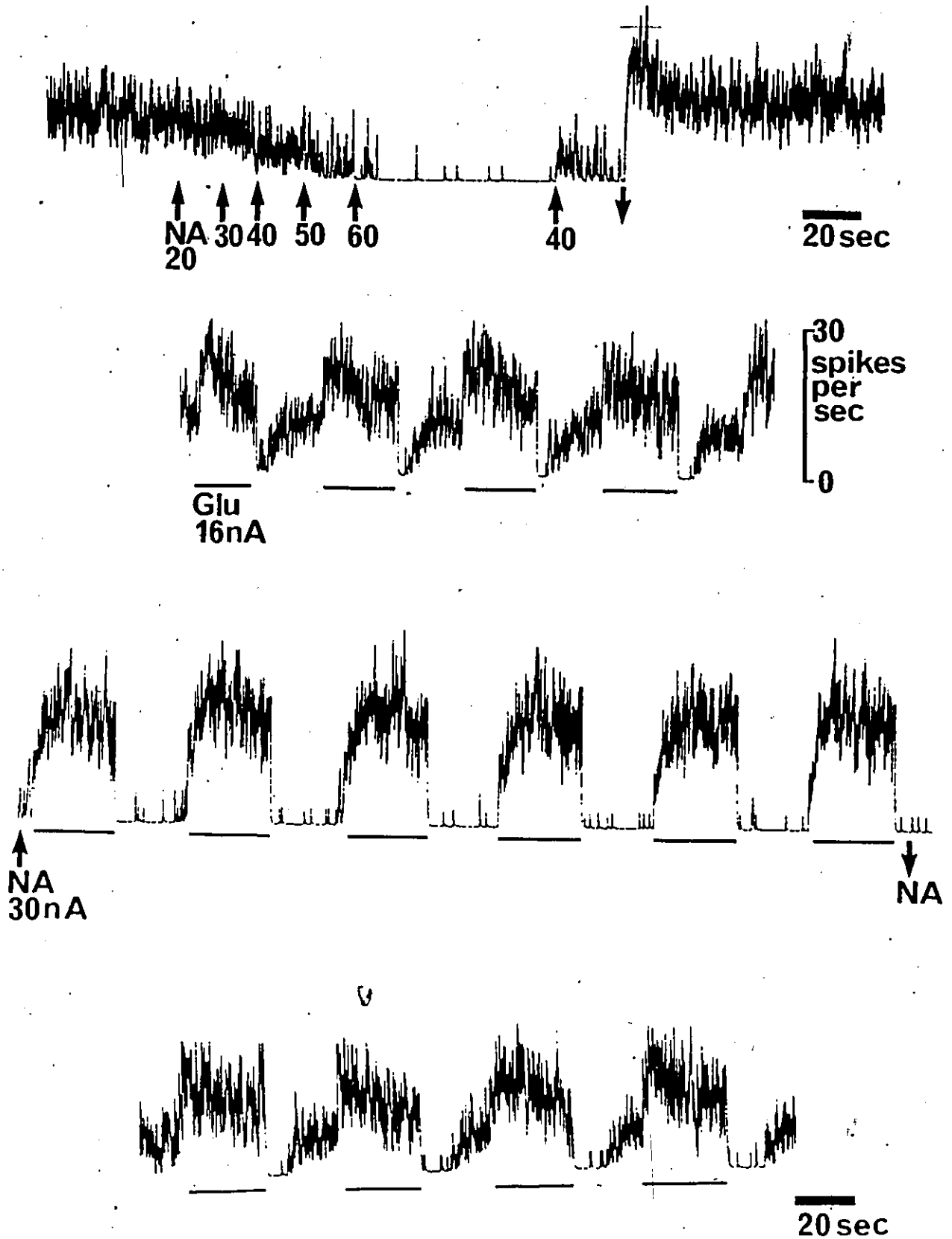

Figure 12. NA potentiation of the glutamate response. The continuous ratemeter record shows the response of a single PN to increasing iontophoretic currents for NA. In the same neuron NA 30nA augments the glutamate response while depressing background activity. (LC plus cerebellum culture).

occurred more frequently than inhibitory responses, and could be characterized by changes in either the glutamate evoked activity, the background discharge, or both. The background level of firing or interpulse activity has been referred to as the "noise", while the evoked activity has been referred to as the "signal". Potentiation of a response is said to occur when there is an increase in the signal to noise ratio.

Three types of potentiation were observed. In some cases, there was little change in the magnitude of the glutamate evoked activity, however, the background activity was considerably depressed (Fig. 12). This reduction in the noise level while holding the signal constant, constitutes an increase in the signal to noise ratio and hence a potentiation. Line 1 of Figure 12 shows a graded inhibitory response of a Purkinje neuron to increasing iontophoretic currents of NA. NA ejected at 20nA did not noticibly alter discharge rate. NA ejected at 30 and 40 nA for short times appeared to have only slightly depressant effects, while NA 60 nA almost completely inhibited spontaneous activity. Termination of NA application resulted in a postinhibitory excitation which persisted for several minutes, and activity eventually returned to pre-NA rates. The second line shows the same Purkinje neuron's response to glutamate alone. Responses to four control pulses of glutamate were consistent in that both the rate and the profile did not

noticeably vary. The downward shift in the glutamate pulse profile was quite typical. Although the amplitude of the action potentials during glutamate application as observed on the oscilloscope visibly decreased, they were still easily distinguished from the electrical noise, and were still able to selectively trigger the neuroratemeter. Therefore, the downward shift in profile throughout the glutamate application was not due to a loss of action potentials as a result of large decreases in amplitude consequent to glutamate induced depolarizations. Quite typically, a postexcitatory depression was observed following termination of glutamate application.

With application of NA, shown in the third line of Figure 12, a marked reduction in the interpulse activity can be seen. Although the maximum level of activity attained during glutamate application is not very observably different from control levels, there is a very noticeable change in the profile during glutamate application with concurrent administration of NA. Rather than a downward shift, the profile was a plateau, and this change in profile was characteristically observed in most cells. This increase in the ratio of the evoked to background activity persisted throughout the NA application and a return to control levels was seen in this cell immediately following cessation of NA application. As shown in the fourth line, both the control rates as well as the downward profile recovered.

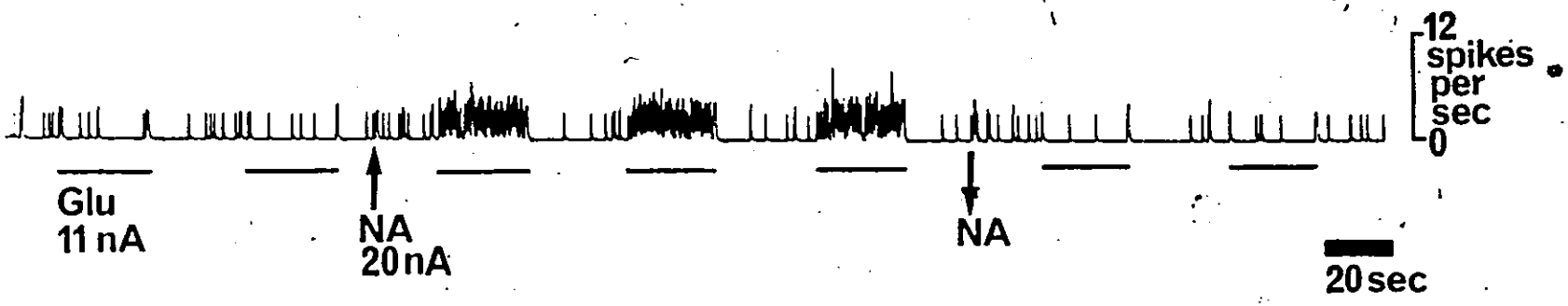

Figure 13. NA elicits a glutamate response. Note that no glutamate response was observed in the absence of NA. NA does not appear to have any effect on PN spontaneous activity at this dose. (LC plus cerebellum culture).

In some cells, NA did not noticeably alter the background activity, however, an absolute increase in the evoked activity was observed. In fact, this effect could be so dramatic as to elicit a glutamate response in the presence of NA where none was present with only glutamate (Fig. 13).

This rather interesting effect of NA was observed when applied concomitantly with glutamate which alone elicited no observable change in rate from spontaneous activity in a few cells. Action potential amplitude during glutamate application was of sufficient magnitude that all action potentials were triggering the ratemeter. As shown in Figure 13, NA augmented the activity during glutamate application such that a difference in the signal and noise became apparent where none was evident before. It is interesting to note that in addition to increasing the rate of firing during glutamate application, NA also gave rise to an increase in the amplitude of the action potentials both when applied with glutamate as well as alone as compared with their respective controls. Termination of NA application resulted in a loss of differentiation between the signal and noise and was comparable to pre-NA activities.

In other cases, NA caused changes in both the signal and noise, increasing the glutamate evoked activity as well as decreasing the background discharge (see Fig. 15). In

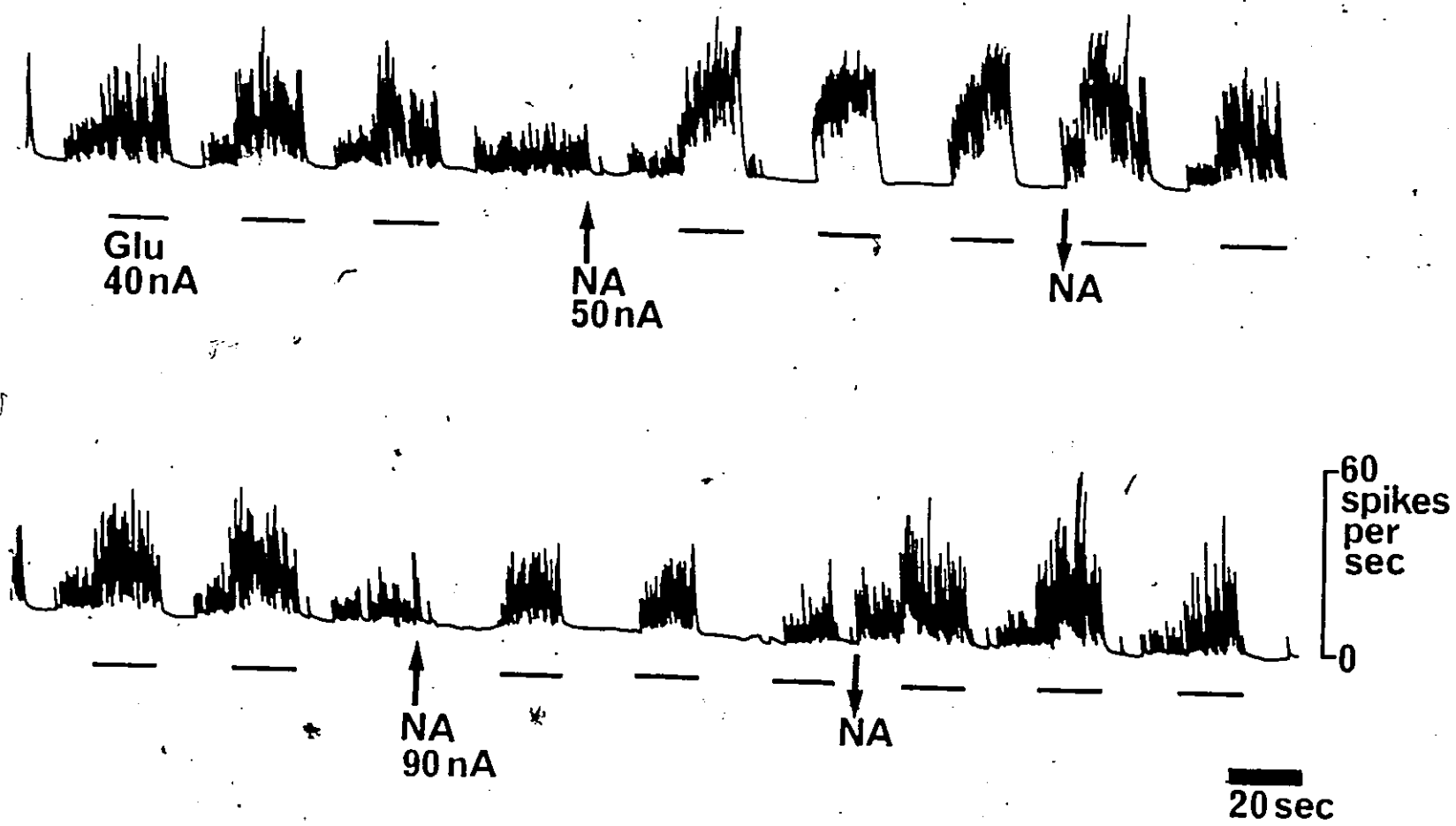

Figure 14. Dose dependent effect of NA on the glutamate response. The continuous ratemeter record indicates that NA 50nA potentiates the PN response to glutamate, while NA 90nA depresses it. (LC plus cerebellum culture)

addition to these three types of potentiation, on a few occasions, a potentiation of the glutamate response was observed following termination of the NA application (e.g. Fig. 15).

DOSE DEPENDENT INTERACTIONS OF NA AND GLUTAMATE

It was very curious that in some Purkinje neurons glutamate appeared to be potentiated by NA, while in others it appeared to be inhibited. With further experimentation, it became apparent that in the same cell, glutamate could be both potentiated and inhibited, depending on the amount of NA applied. This is shown in Figure 14.

The response to three control pulses of glutamate were established following which the level of spontaneous activity was given sufficient time to recover. NA was then ejected at 50 nA. This resulted in a marked increase in the evoked as well as the depression of background activity. NA was turned off and the background as well as glutamate induced activity was allowed to recover at control levels. NA was then applied at 90 nA. This resulted in complete inhibition of spontaneous activity and the glutamate-induced activity was depressed as well. This is most noticeably seen in the third glutamate pulse with continuous NA application. With NA turned off, a rebound resulted and further glutamate-induced activity returned toward control

Table 3.

NA AND GLUTAMATE INTERACTIONS

	INHIBITION	POTENTIATION	INHIBITION/POTENTIATION	N
LC plus CBLM	4	15	2	19
CBLM	4	17	2	21
6-OHDA (LC plus CBLM)	7	17	3	24
TOTAL	15	49	7	64

Table 4.

RESPONSES OF PURKINJE NEURONS TO NA AND GLUTAMATE:

EFFECTS OF 6-HYDROXYDOPAMINE

		LC		no LC	
		control	6-OHDA	control	6-OHDA
GLUTAMATE	Spont.	3.9 (0.9)	4.8 (1.5)	3.1 (0.6)	6.6 (1.5)
	Response	11.6 (1.8)	13.0 (1.9)	7.0 (1.1)	11.2 (2.5)
	nA	39.7 (5.5)	13.2 (1.8)	22.4 (3.6)	20.7 (2.3)
	n	30	35	29	22
NA	Spont.	8.3 (0.9)	10.3 (3.4)	6.2 (1.2)	11.1 (2.1)
	Response (-)	5.6 (0.8)	6.5 (3.0)	3.1 (1.4)	3.0 (2.7)
	nA	50.9 (6.3)	36.4 (5.1)	45.0 (5.6)	35.2 (4.7)
	n	34	9	23	10

levels. It therefore seems quite clear that in this Purkinje neuron, as well as in several others, NA may have qualitatively different effects depending on the amount of NA applied.

The results of NA and glutamate interactions are summarized in Table 3. A total of 64 Purkinje neurons were studied. Of these, NA inhibited the glutamate response in 15 cells, and potentiated the response in the remaining 49 cells. In 7 of these 49 cells, NA could both potentiate and inhibit the glutamate response. The potentiation was always found to occur at the lower iontophoretic current, while the depression of the glutamate response always occurred at the higher iontophoretic current for NA.

NA AND GLUTAMATE INTERACTIONS IN DIFFERENT CULTURE SYSTEMS

LC PLUS CEREBELLUM CULTURES

In 15 of 19 Purkinje cells studied, NA potentiated the glutamate response. Thirteen of these potentiations resulted from an absolute increase in the signal as well as a decrease in background. In one case there was a decrease in the noise only. In four cells NA inhibited the glutamate response. In two cells, NA was able to either potentiate the glutamate response for example as shown in Figure 14, at 50nA NA, or inhibit the glutamate response with 90nA NA.

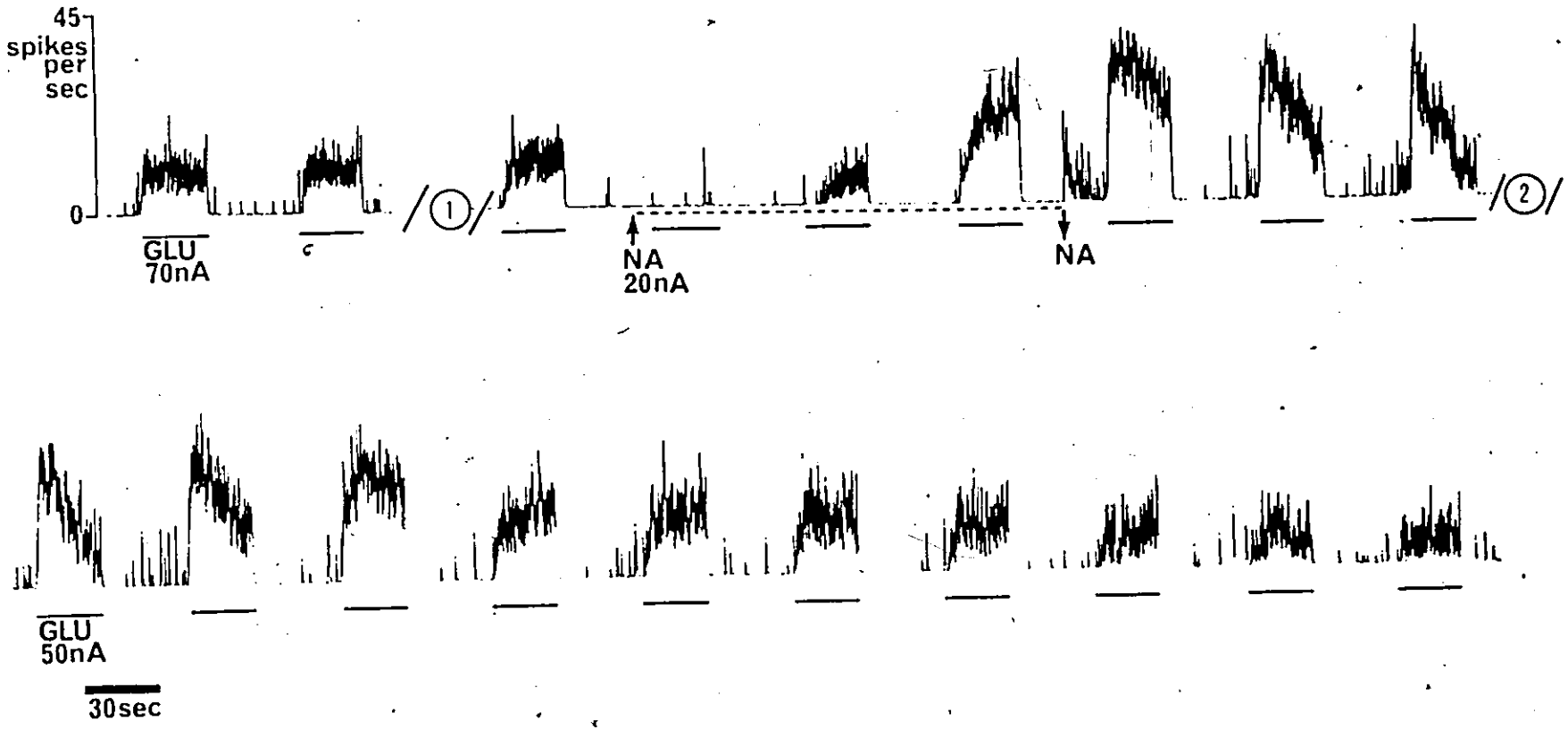

Figure 15. NA potentiates the PN response to glutamate following termination of NA ejection. Note the long duration of the NA effect (approximately 18 min.). (No LC culture)

NO LC CULTURES

A basic question to be considered was whether NA enhancement of the glutamate response was dependent on the presence of LC with cerebellum cultures. The effect of iontophoretically applied NA on the glutamate response in explant cultures containing cerebellum alone did not qualitatively differ from cultures containing cerebellum plus LC. This is demonstrated in Figure 15, which also demonstrates one of the different time courses for the NA effect.

This Purkinje neuron had a low discharge rate characteristic of a great number of cells. The response to three control pulses of glutamate 70 nA are shown. Iontophoresis of NA almost completely inhibited the glutamate-evoked activity associated with the first glutamate pulse, and substantially depressed the glutamate-evoked activity associated with the second glutamate pulse compared with control levels of glutamate-induced activity. However, by the third glutamate pulse, with continuous application of NA, there was a two-fold increase in the glutamate-evoked activity as well as a complete inhibition of the background activity preceeding the third glutamate pulse. Note also the profile of the glutamate response which changed from a plateau to an upward slope. The complete inhibition of background spontaneous activity

persisted until cessation of NA ejection at which time a marked rebound occured. This postinhibitory excitation decreased over the following 10 seconds. Consecutive glutamate pulses were also potentiated, however, a marked change in the profile occurred as a result of a large decrease in amplitude associated with a large increase in firing rate which caused a substantial number of action potentials to be lost. Presumably this decrease in amplitude reflects a glutamate induced depolarization. For this reason, the ejection current for glutamate was reduced to 50 nA. By the third glutamate pulse at 50 nA, all action potentials were triggering the neuroratemeter and the profile which is shown in the ratemeter record plateaued. The response of this Purkinje neuron to 50 nA NA was obtained prior to this run, although it is not shown in Figure 15. The potentiation of glutamate by NA in this neuron was of exceptionally long duration, persisting for almost 15 minutes following termination of NA application.

As indicated in Table 3, NA inhibited the glutamate response in 4 of 21 cultures and potentiated the glutamate response in 17 of 21 cultures. In two cultures NA was able to depress and potentiate the glutamate response at high and low iontophoretic currents respectively.

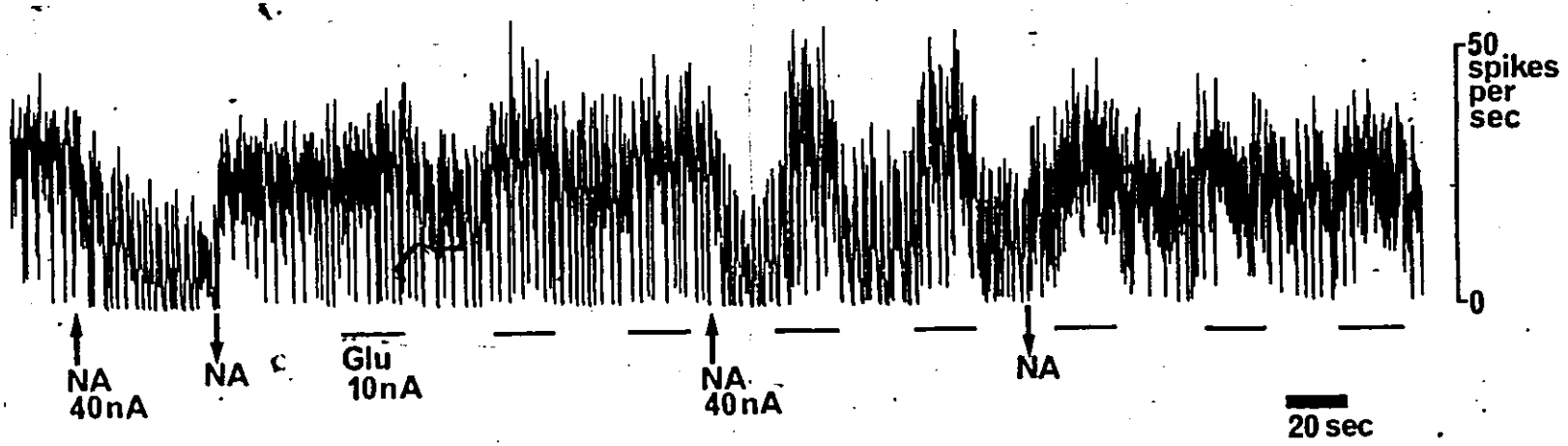

Figure 16. NA potentiates the glutamate response in PN in 6-OHDA treated LC plus cerebellum cultures.

6-OHDA TREATED LC PLUS CEREBELLUM CULTURES

NA applied with glutamate to Purkinje neurons in LC plus cerebellum cultures treated with 6-OHDA caused an inhibition of the glutamate response in 7 of 24 cultures and potentiation in 17 of 24 cultures. In three cultures, NA could either inhibit or potentiate the glutamate response depending on the dose, such that at lower ejection currents, NA was able to potentiate the glutamate response, while at higher ejection currents NA inhibited the glutamate response.

Figure 16 shows the response of a single Purkinje neuron to 40nA NA. In this cell, NA progressively inhibited Purkinje neuron discharge until application of NA was ceased. Following this, control levels of spontaneous activity returned. In this cell glutamate 10nA caused a small increase in firing rate which was not easily discernable from background. Application of 40nA NA following the third glutamate pulse substantially depressed background firing, while the two subsequent pulses of glutamate during NA application caused an absolute increase in the glutamate-evoked activity. The increase in the signal to noise ratio with NA application is quite obvious when compared with the pre-NA profile. Following termination of NA application, the differentiation of signal and noise became less apparent and the profile returned to control.

GLUTAMATE SENSITIVITY

The response to glutamate both in terms of Glu-S, and S/Glu was not significantly different in the LC and 6-OHDA treated cultures (see Table 1). T scores for LC plus cerebellum untreated and dopamine controls were (t40=-0.63,p=0.53; t40=0.66,p=0.51) for Glu-S and S/G respectively. T scores for LC plus cerebellum untreated and 6-OHDA treated LC plus cerebellum were (t63=-0.53,p=0.6; t63=0.84, p=0.40). However, a dramatic difference in the ejection current required for glutamate to elicit a given response was observed between the two culture systems (Marshall and Garber, 1983). The average current required to elicit a standard response in LC plus cerebellum cultures was 39.70nA +/- 5.48 (S.E.M.), as compared with 13.22 nA +/- 1.82 (S.E.M.). The alpha level was adjusted to 0.0125 (0.05/4, the number of t-tests performed), to take into account the multiple t-tests performed on the same data set. In some 6-OHDA treated cultures a marked excitation of spontaneous discharge could be elicited simply by turning off the retaining current. This feature was unique to this culture type.

LC cultures treated with DA as a non-toxic oxidant did not differ significantly from LC cultures in terms of glutamate sensitivity (t40=-0.89,p=0.38). The ejection current required to elicit a standard glutamate response was

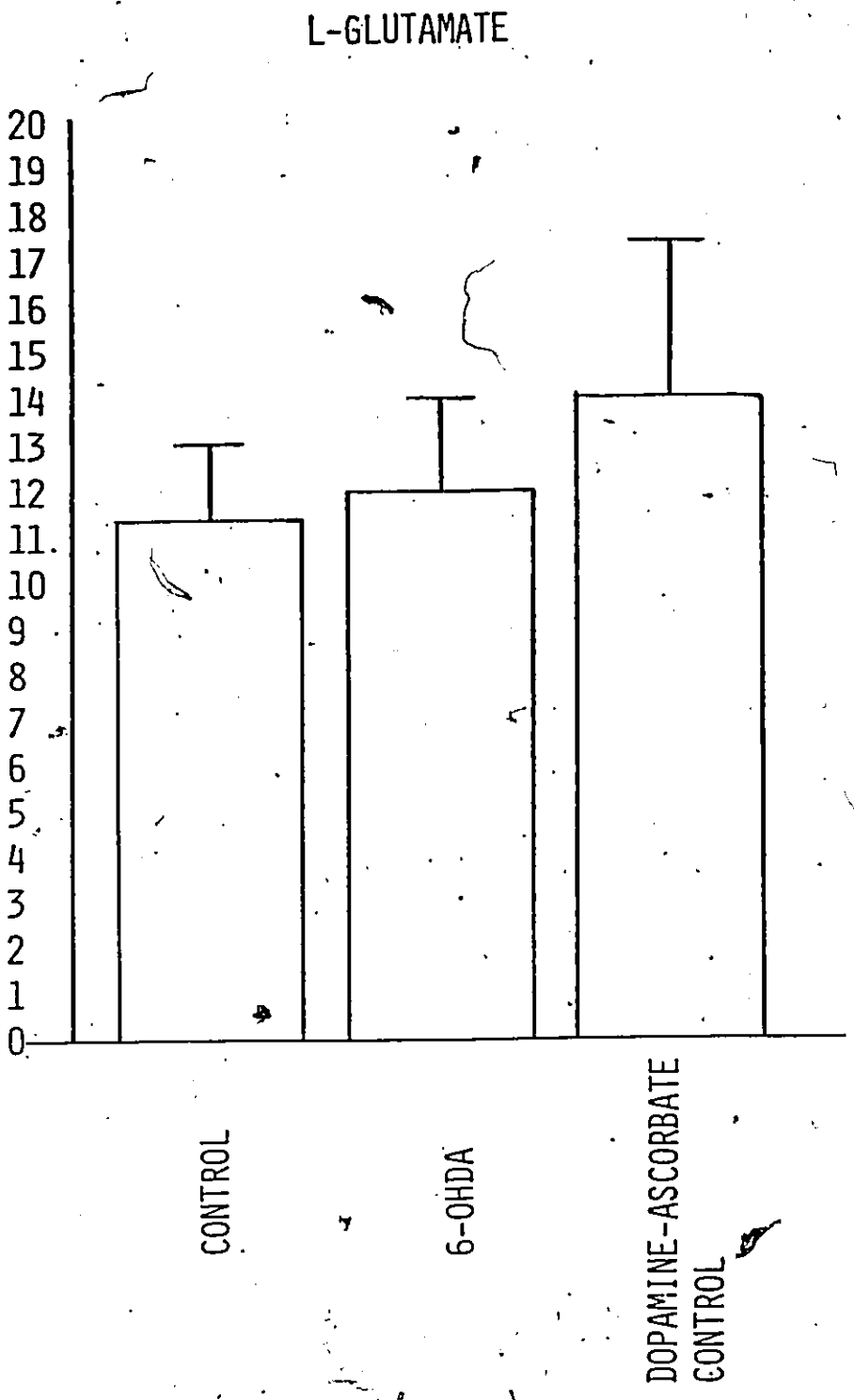

Figure 17.

MEAN LEVELS OF CHANGE IN FIRING RATE (SPIKES/SEC) OF PURKINJE CELLS IN RESPONSE TO GLUTAMATE IN LC PLUS CEREBELLUM CULTURES.

Error bars represent standard errors of the mean (S.E.M.).

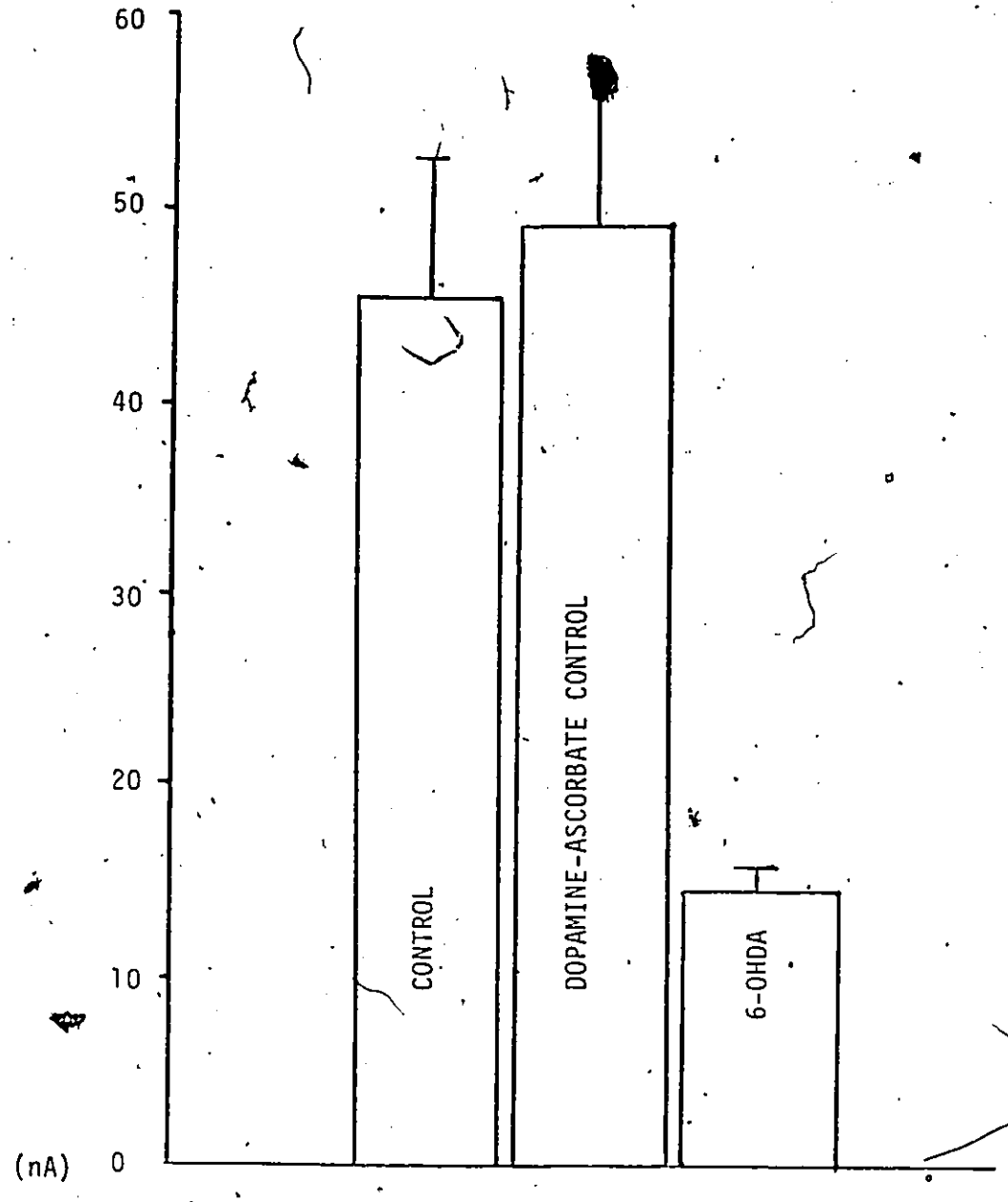

Figure 18. Mean currents used to obtain responses of Purkinje neurons to glutamate in untreated and 6-OHDA treated LC plus cerebellum cultures. Error bars represent S.E.M.

49.00nA +/-9.37 (S.E.M.), and was comparable to that for LC cultures which was 39.7nA. Figure 14 shows the currents used to obtain glutamate responses in LC-containing cultures. Although currents used to elicit responses in the dopamine-ascorbate controls did not vary significantly from untreated controls, considerably reduced currents were able to elicit responses in 6-OHDA treated LC plus cerebellum cultures compared to untreated LC plus cerebellum cultures. This difference was significant ($t_{63}=4.87$, $p=0.0001$). Mean levels of change in firing rate in response to glutamate were not significantly different in these groups (Fig. 17).

Cultures set up to contain cerebellum alone that were treated with 6-OHDA had a sensitivity to glutamate that lay between that obtained from the LC and 6-OHDA cultures, and was comparable to non-LC cultures in terms of glutamate sensitivity.

The results are sumarized in Figure 18. Significantly lower currents were able to elicit a glutamate response in the 6-OHDA treated cultures as compared to controls ($t_{63}=4.87$, $p=0.0001$).

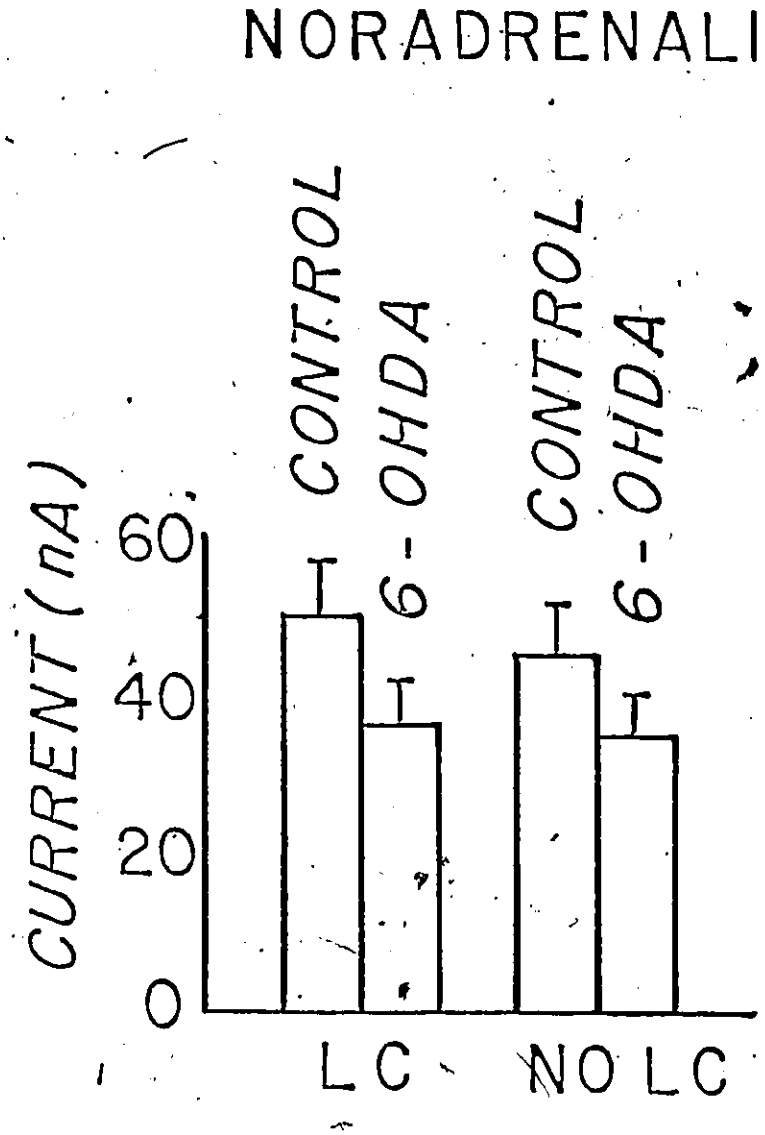

Figure 19. Mean currents used to obtain responses of Purkinje neurons to NA in control (untreated) and 6-OHDA treated cultures.

NA SENSITIVITY

Table 2 summarizes the responses of Purkinje neurons to NA. As is evident from Figure 19, no significant differences in currents required to elicit a NA response were found between 6-OHDA treated and control cultures in either the LC-containing or no LC culture groups. For cultures containing LC, the t-test result obtained for current comparison between control and 6-OHDA cultures was ($t41=1.14, p=0.26$). Responses of these groups to NA was not significantly different ($t41=0.40, p=0.69$; $t34=0.65, p=0.52$) either for NA-S and NA/S respectively. A summary of the effects of 6-OHDA with respect to NA is shown in Table 4. NA was comparably capable of potentiating the glutamate response in 6-OHDA treated cultures (Fig. 16).

DISCUSSION

6-OHDA

As can be seen from the morphology (figures 6 and 7), appropriate concentrations of 6-OHDA appear to cause selective degeneration of noradrenergic cell bodies and/or fibers in the tissue culture system. PN morphology appeared unaltered by 6-OHDA treatment, while LC neurons were lethally affected. It therefore appears that the use of 6-OHDA as a method of noradrenergic denervation will provide a useful method for the study of sensitivity changes in the tissue culture system.

Although some researchers have reported increases in the the spontaneous discharge of Purkinje neurons following treatment with 6-OHDA (Hoffer, Siggins, Woodward and Bloom, 1971), the spontaneous activity of 6-OHDA treated and untreated LC plus cerebellum cultures did not appear to be different (see tables 1 and 2).

NA EFFECTS

The effects of NA on spontaneous activity were predominantly inhibitory, with more than 90% of responses in Purkinje neurons in all culture preparations having the form of a depression of spontaneous activity. This inhibitory effect of NA has been well documented for Purkinje neurons

(Hoffer et al., 1973), as well as for certain other CNS regions (eg. Segal and Bloom, 1974).

In a small number of cells NA appeared to cause an excitation of Purkinje neurons. A small percentage of cells also responded biphasically to NA, initially with an inhibition of short duration, followed by a prolonged excitatory phase. This was unlikely to be a hydrogen ion effect (Marshall and Engberg, 1980), since the pH of NA was maintaind between 5.0 and 6.0. Also, this effect was not mimicked by Na^+ current ejection. It is possible, however, that NA was acting not only on Purkinje neurons, but on inhibitory interneurons as well. This could result in an initial depression of spontaneous activity as a result of NA acting directly on Purkinje neurons. The excitation which followed may have resulted from an inhibitory action of NA on inhibitory interneurons which synapse on Purkinje neurons. A possible candidate is the stellate cell. As indicated by the time course of NA action, the latency for excitation exceeds the latency for inhibition. This period of time could be accounted for by the time required for diffusion of NA to the interneurons as well as time for synaptic relay.

The effect of NA may have been a direct effect on PN since in those cells where NA was excitatory, glutamate was also found to be excitatory. If the excitation produced by

NA was mediated by inhibitory interneurons, one might have expected glutamate to have a depressant effect on spontaneous activity, since this too would be mediated by inhibitory interneurons.

Other researchers have also observed excitatory effects with NA in a small percentage of cells. In cerebellum, NA has been found to exert excitatory effects on neurons in the flocculus, while it depressed the firing of cells in the neocerebellum (Yamamoto, 1967). Generally, it has been considered that the postsynaptic action of NA was exclusively inhibitory and beta-receptor mediated. Any excitations reported for NA were believed to be artifacts due to variations in iontophoretic techniques or anaesthesia (Bloom, 1975; Moore and Bloom, 1979). This view was held for cerebellum as well (Hoffer, Neff and Siggins, 1971).

Recent binding studies have established the existence of both alpha and beta receptor sites in the brain (U'Prichard, Reisaine, Mason and Fibiger, 1980). Szabadi (1980), has proposed that alpha receptors mediate excitatory responses to NA in the brain, while beta receptors mediate predominantly inhibitory responses to NA. Several lines of evidence suggest beta-receptor involvement in Purkinje neuron depression of activity by NA. Isoproterenol causes similar changes in mean spike rates as NA (Hoffer, Siggins and Bloom, 1971b). Also, the NA response can be blocked by

the specific beta-adrenergic antagonists sotalol (Hoffer, Neff and Siggins, 1971), or dichloro-isoprenaline (Freedman, Hoffer, and Woodward, 1975).

The pharmacology of these responses however, remains controversial. In contradiction to the above, other researchers have found that Purkinje neuron excitations were evoked by the beta adrenergic agonist isoproterenol, and reduced by timolol, a beta adrenergic antagonist (Basile and Dunwiddie, 1984). The same investigators found that clonidine, an alpha-2 agonist decreased the firing rate of Purkinje neurons in rat cerebellar slices (Basile and Dunwiddie, 1984).

These contradictory results are particularly confusing, as they have both been demonstrated in media in which synaptic transmission was blocked. This would tend to suggest a direct effect of the pharmacologic agents on the Purkinje neurons. However, it has recently been demonstrated that the K+ concentration of the medium may influence whether the response to NA is an excitation or an inhibition (Basile and Dunwiddie, 1983). These investigators have found that if NA effects are examined in a medium containing 3mM K+ (similar to CSF), NA tends to be inhibitory, while in studies carried out in 5mM K+ (similar to plasma) NA tends to be excitatory.

NA TIME COURSE

There was considerable variation in the time course of NA action on Purkinje neurons. In most cases NA caused a progressive decrease in firing frequency which levelled off and persisted until NA ejection was stopped. In a few cells however, the inhibitory effect of NA decreased with time and appeared to have little or no effect on discharge rates. This may have been due to an adaptation of the Purkinje neuron to NA, or may have been due to NA's action on other inhibitory neurons. In some cases, NA had a prolonged effect which persisted after NA ejection had been terminated. Since the same electrode was used to record activities from subsequent neurons, and this long duration effect was not necessarily observed, the effect was believed to be due to an action of NA on the Purkinje neuron, or on surrounding neurons, rather than to a NA leak from a broken electrode.

Almost always, following termination of NA application, there was a marked increase in Purkinje neuron discharge which persisted for several seconds, and in a few cases for several minutes. This effect was most likely due to NA itself since it could not be mimicked by current ejection. This is in contradiction to Engberg and Ryall (1966), who were able to mimick the NA effect with current ejection.

NA SENSITIVITY

Since it appeared that the LC could be selectively destroyed in cultures exposed to 6-OHDA, it seemed plausable that a supersensitivity to NA might develop in these Purkinje neurons. This supersensitivity should express itself either as an increased response to a given stimulus, or a response of equal magnitude to a lesser stimulus. Studies with 9-amino-acridine propranolol, followed by fluorescence microscopy has enabled localization of beta adrenergic receptors on Purkinje neurons (Atlas, Teichberg and Changeux, 1977). These investigators observed an increased density of fluorescence following treatment with 6-OHDA suggesting an increase in the density of beta receptors. If these changes in receptor density occured in tissue culture as well, and were of sufficient magnitude, it was expected that these changes would express themselves as a supersensitivity to NA as described above.

As was revealed by the descriptive statistics for NA, (table 2) no significant difference was observed between the 6-OHDA treated cultures and the cerebellum plus LC cultures. Although it appeared that a somewhat smaller current was required to elicit the same magnitude of resonse, the differences which exist between these two groups are also not significant. Perhaps the lack of distinction between culture groups with respect to NA sensitivity is inherent in

the culture system itself. Purkinje neurons in our culture systems had quite low levels of spontaneous activity as compared with similar in vivo preparations. For this reason, it has been difficult to compare the responses of Purkinje neurons to an agent which further decreased their already low level of activity. This may account for why supersensitivity to NA was not observed in the 6-OHDA treated group. It is also possible that Purkinje neurons do not display supersensitivity to NA, although a report of enhanced GABA facilitation by NA was reported in the cerebellum following 6-OHDA treatment (Moises, Hoffer, and Woodward, 1980). These investigators termed this a "modulatory supersensitivity", since the mean iontophoretic current required for a potentiation response to NA after treatment with 6-OHDA was significantly reduced.

Another group of investigators, using the selective NA neurotoxic agent DSP4, found no effect either on the number of the affinity of beta-adrenergic receptor sites, as determined by radioligand binding studies in hippocampal cells (Dunwiddie, Mueller, Bickford and Zahniser, 1983).

NA PLUS GLUTAMATE INTERACTIONS

Recent studies have shown that iontophoretic application of NA which alone causes little or no change on Purkinje neuron spontaneous discharge, can potentiate GABA-induced

(Moises et al., 1979; Yeh, Moises, Waterhouse and Woodward, 1979) or electrical stimulation induced (Freedman, Hoffer, Woodward and Puro, 1977) inhibitions of Purkinje neurons. Although much work has been conducted on GABA (Moises et al., 1979; Yeh et al., 1981; Yeh and Woodward, 1983), very little work has been conducted on glutamate or other excitatory neurotransmitters (Moises et al., 1979; Waterhouse et al., 1980; Segal, 1982).

The argument for the role of NA as a neuromodulator has centered on the fact that the potentiation, for example of the GABA response is not a mere summation of two inhibitory effects, even though in much of the work, in addition to the background level of activity being reduced, the evoked activity is reduced as well. The NA-induced facilitation of GABA on Purkinje neurons appears to involve beta adrenergic receptors (Waterhouse et al., 1982), more specifically, beta-1 receptors (Yeh and Woodward, 1983b).

Much work has focused on the ratio of the signal to noise before, during, and after NA application. It has been found that with NA applied at low levels, this ratio can be increased (Woodward et al., 1979).

In this work it has been particularly interesting to make use of two different neurotransmitters with two qualitatively different effects. As was found in many instances, NA was capable of augmenting the glutamate

response, while either holding the interpulse activity constant or decreasing it. The effects demonstrated in this work have been more dramatic than those demonstrated by Moises et al. (1979), and have demonstrated an absolute increase in the signal. This is in contrast to an increase in only the signal to noise ratio by virtue of a relatively more depressed level of spontaneous activity than inhibition of the glutamate response.

It was very curious that in some Purkinje neurons glutamate appeared to be potentiated by NA, while in others it appeared to be inhibited. The first possible explanation that came to mind was that there were two classes of Purkinje neurons, some in which NA was exclusively inhibitory and some in which NA or NA receptors could in some way interact with the glutamate receptor or some other intracellular mechanisms which could enhance the glutamate response. With increasing technical expertise, and the ability to hold Purkinje neurons for relatively long periods of time, it became possible to do several glutamate runs with different ejection currents for glutamate and NA, always holding one constant. It then became clear that NA could both augment, as well as decrease the glutamate response. It therefore appears as though NA may very well modulate responses in the central nervous system, and that two qualitatively different responses may be achieved, and they may depend on the concentration of NA. A reduction in dose dependent response has also been noted by Segal (1982).

A basic question to be considered was whether the NA enhancement of the glutamate response was dependant on the presence of LC with cerebellum cultures. Two approaches were taken to investigate this. First, a comparison was made of the effect of iontophoretically applied NA on the glutamate response in cultures containing LC plus cerebellum and those containing cerebellum alone. Second, it was examined, what differences if any, qualitative or quantitative could be observed in Purkinje neurons following LC denervation with 6-OHDA. Since NA could potentiate the glutamate response in cultures containing cerebellum alone as well as LC denervated cultures, it appears as though the neuromodulatory role of NA is independent of the presence of LC.

Possible mechanisms by which NA may potentiate the glutamate response have been put forward. Madison and Nicoll (1987), have attributed the potentiating effect of NA on glutamate to a noradrenergic block of accomodation. They suggest that NA acts by blocking the calcium activated potassium conductance, reducing spike frequency adaptation with depolarizing stimuli so that the number of spikes elicited by such a stimulus is substantially increased in the presence of NA. In contrast, Segal (1982) has suggested that NA acts postsynaptically to effect a glutamate potentiation. He observed increased excitatory postsynaptic potentials with NA on the glutamate response and this cannot

be explained by an accomodation response as suggested by Madison and Nicoll (1982); nor can it explain NA eliciting a glutamate response when none is present with glutamate alone (see figure 13). It is possible that the mechanism by which NA potentiates the glutamate response may involve interaction of the postsynaptic NA and glutamate receptors (Segal, 1982). It is also conceivable that the dose dependent change from potentiation to inhibition of the glutamate response, may occur as a result of diffusion of NA to more remote receptors on the PN, giving rise to qualitatively different responses, depending on the ejection current used. Such qualitativly different responses resulting from somatic versus dendritic placement of the electrode have been reported by Segal (1982).

GLUTAMATE SUPERSENSITIVITY

One striking, totally unexpected finding was a supersensitivity to glutamate in 6-OHDA treated IC plus cerebellum cultures. Significantly lower currents were capable of eliciting a glutamate response in 6-OHDA treated cultures, and in some cases a glutamate response could be elicited simply by turning off the retaining current. The first possible explanation that came to mind was that the 6-OHDA was not entirely selective for catecholamines and was destroying the granule cell population as well. Denervation

of the granule cells could give rise to an increase number of glutamate receptors on Purkinje neurons, and thereby account for an increased glutamate sensitivity. However, a thionine stain revealed no observable difference in granule cell density for LC plus cerebellum cultures as compared to 6-OHDA denervated cultures.

It was also possible that 6-OHDA might be acting on some other system besides the granule cell population, and that the glutamate supersensitivity might in some way be a result of this. For example, if glutamate is taken up by glial cells and if 6-OHDA is not entirely specific for noradrenergic (and dopaminergic) cells, but destroys glial cells as well, more glutamate would be available to interact with Purkinje neurons and this could give rise to an increased glutamate response.

The second possible explanation that came to mind was that the LC was exerting a tonic inhibitory influence on Purkinje neurons and removal of this system could allow glutamate expression to a greater degree. However, no apparent increase in spontaneous activity was observed in denervated cultures, as would have been predicted by this explanation.

A third possible explanation, is that NA exerts its effects not only on the Purkinje cell population, but also on the granule cell population. Three pieces of evidence

support this hypothesis. First, projections have been demonstrated from LC to granule cells (Kimoto, Tohyama, Satoh, Sakumoto, Takahashi and Shimizu, 1981). Second, the feed medium has been found to contain significant levels of catecholamines (Hendelman et al., 1982). Third, it has recently been demonstrated that NA can enhance glutamate release from granule cells (Dolphin, 1982).

If we have a situation where the LC and cerebellum are present, we have both synaptically released and endogenously circulating NA which is available to cause release of glutamate from granule cells. This glutamate is synaptically released on to Purkinje neurons, maintaining the glutamate receptors at a given level. With noradrenergic denervation, both the LC fibers and the circulating catecholamines are abolished and consequently, so is the glutamate leak from granule cells. The Purkinje neurons then respond to this decreased availability of glutamate by increasing the number of glutamate receptors, and when exogenous glutamate is iontophoretically applied, we observe a greatly enhanced response due to a secondary denervation-supersensitivity.

A possible means of investigating this hypothesis would be to measure and compare the amount of glutamate in the medium from the LC plus cerebellum cultures and that from the cerebellum and 6-OHDA cultures. We would of course

expect to find more glutamate in the cerebellum plus LC cultures.

CONCLUSION

In conclusion then, this research has supported the concept of NA as a neuromodulator, but has demonstrated as well that NA can exert qualitatively different effects on the same cell with concomitant application of glutamate. Furthermore, in the case of NA potentiation of glutamate, in the tissue culture preparation NA was able not only to increase the signal to noise ratio, but was also able to effect an absolute increase or amplification of the signal. Second, a way of denervating with 6-OHDA and documentation of this denervation has been achieved in tissue culture. No quantitative differences were observed with respect to NA in the different culture systems. The effects of glutamate plus NA were not qualitatively different in the different culture systems. Third, supersensitivity to glutamate has been observed in 6-OHDA treated LC plus cerebellum co-cultures, rather uniquely, a supersensitivity secondary to denervation of another system.

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