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ELECTROPHYSIOLOGICAL STUDIES
OF
LOCUS COERULEUS NEURONS
IN CULTURE

BY

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TABLE OF CONTENTS

FRONT MATERIAL

List of Figures and Tables.....	i
Abbreviations.....	iii
Acknowledgements.....	iv
Abstract.....	v

INTRODUCTION

I) Background.....	1
II) Locus Coeruleus Morphology.....	2
III) Development of the Locus Coeruleus in vivo	6
IV) Afferents and Efferents of the Locus Coeruleus.....	8
V) Noradrenergic Actions in other Brain Regions.....	12
VI) Physiology of Locus Coeruleus Neurons	
1) Resting Activity.....	15
2) Bursting Activity.....	16
3) Postactivation Inhibition.....	17
4) Facilitatory Effects.....	20
5) Non-adrenergic Transmitter Actions.....	21
6) Summary.....	24
VII) Tissue Culturing	
1) Techniques and Caveats.....	25
2) LC Neurons.....	28

METHODS

I) Tissue Cultures.....	31
II) Electrophysiological Recordings	
1) Recording Setup.....	34
2) Intracellular Recording.....	37
III) Iontophoresis.....	39
IV) Electrical Stimulation.....	40
V) Intracellular Staining with Lucifer Yellow.....	40
VI) Catecholamine Fluorescence.....	41
VII) Thionine Staining.....	42

RESULTS

I) Identification and Morphology of Locus Coeruleus	
Neurons in Tissue Culture.....	43
II) Spontaneous and Evoked Activity	
1) Properties of Cultured LC Neurons.....	56
2) Spontaneous Activity.....	60
3) Simultaneous Recordings of LC neurons.....	63
4) Tests for Electrotonic Coupling.....	63
5) Properties of Depolarizations.....	68

RESULTS (cont.)

III) Adrenergic Responses in Cultured LC Neurons	
1) Responses to Noradrenaline.....	72
2) Age-dependence of Noradrenergic Responses.....	78
3) Effects of Adrenergic Antagonists.....	81
4) Application of Adrenergic Agonists.....	91
5) Input Resistance.....	95
6) Potential Dependence of Responses.....	99

DISCUSSION

I) Identification and Characterization of Locus Coeruleus Neurons In Tissue Culture.....	101
II) Electrophysiological Properties of Locus Coeruleus Neurons in Tissue Culture	
1) Comparison of LC Neurons in Different Preparations.....	104
2) Synchronous Bursting of Locus Coeruleus Neurons.....	107
a) Non-synaptic Interactions.....	109
b) Synaptic Interactions.....	112
c) Common Excitatory Inputs.....	114
III) Noradrenergic Responses of Cultured Locus Coeruleus Neurons	
1) Characteristics of Noradrenergic Responses	
2) Calcium Dependence of Responses.....	116
3) Age-dependence of Noradrenergic LC Responses.....	124
IV) Summary and Prognosis.....	134

APPENDICES

A.....	137
B.....	139
C.....	140

BIBLIOGRAPHY.....	141
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LIST OF FIGURES AND TABLES

Figure	DATA PRESENTED	Page
1	Dissection for LC explant tissue cultures.	32,33
2	Schematic of recording chamber.	35
3	Photomicrographs of cultured LC neurons viewed in the living state.	44,45
4	Photomicrographs of catecholamine fluorescence of explant cultures.	46,47
5	Photomicrographs of lucifer yellow injected LC neurons.	49,50
6	Photographic montage of Lucifer yellow injected LC neuron.	51,52
7	Photomicrographs of stained LC explants.	54,55
8	Evoked and spontaneous spiking activity.	57
9	Afterhyperpolarizations in LC neurons.	59
10	Spontaneous rhythmic bursting activity.	62
11	Simultaneous intracellular recordings of two LC neurons.	64,65
12	Signal averages of simultaneously recorded LC cells.	67
13	Spontaneous and evoked depolarizations in LC neurons.	69
14	Effect of modified perfusates on bursting activity.	71
15	Examples of responses of LC neurons to NA.	74,75
16	Dose-dependence of biphasic responses of LC neurons to NA.	77
17	Effects of modified BSS's on biphasic and depolarizing responses to NA.	79
18	Age-dependence of hyperpolarizing and biphasic responses of LC neurons to noradrenaline.	80
19	Effects of adrenergic antagonists on biphasic responses to NA.	82,83

Figure	DATA PRESENTED	Page
20	Combined effects of adrenergic antagonists on biphasic responses of LC neuron to NA.	85,86
21	Dose-dependent antagonism of depolarizing response to NA.	87,88
22	Graph of prazosin inhibition of depolarizing responses to NA.	89
23	Graph of "dose-response" curves and antagonism by prazosin.	90
24	Iontophoretic application of noradrenaline and adrenergic agonists to LC neurons.	93,94
25	Responses to bath application of phenylephrine.	96,97
26	Input resistance measurements during hyperpolarizing response to NA.	98
27	Effects of polarizing LC neurons on the responses to NA.	100

Tables		Page
I	Receptor binding in the LC.	22
II	Amplitude and Duration of Spikes	56
III	Comparison of LC activity in different preparations.	104
IV	Alpha-adrenoceptor agonists and antagonists.	118

ABBREVIATIONS

A	Adrenaline
AHP	afterhyperpolarization
BSS	balanced salt solution
CCK	cholecystokinin
CRF	corticotropin releasing factor
DBH	dopamine- β -hydroxylase
DOPAC	3,4 - dihydroxyphenylacetic acid
DMI	desmethylinipramine
E#	age of embryo in days
epsp	excitatory postsynaptic potential
HRP	horseradish peroxidase
ipsp	inhibitory postsynaptic potential
LC	locus coeruleus
LY	lucifer yellow
NA	noradrenaline
NMDA	N-methyl-DL-aspartate
NPY	Neuropeptide Y
P#	age of neonate in days
TTX	tetrodotoxin
WGA-HRP	wheatgerm-agglutinin horseradish peroxidase
5-OHDA	5 and 6 hydroxydopamine
6-OHDA	
α -MPT	alpha-methylparatyrosine

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ABSTRACT

Mouse locus coeruleus (LC) neurons grown in explant tissue cultures were used to examine the morphology and the electrophysiology of these neurons.

LC neurons can be identified in living cultures. LC neurons stained by intracellular injection of the fluorescent dye, lucifer yellow, have long dendrites and have both somatic and dendritic spines, as has been observed in vivo. In addition, the majority of LC neurons appear to have more than one axon-like process, and these axon-like processes originate from cell somas, proximal and distal dendrites. Explant cultures of LC neurons were often very small fragments of tissue containing less than 50 LC neurons. Other smaller neurons however could usually be found either in or near the LC group of neurons.

Intracellular recordings of LC neurons were performed in cultures, 2 to 5 weeks after they were established. Spontaneous or evoked action potentials varied from 55 to 100 mV in amplitude and the mean duration of spikes was 1.4 ms. Individual spikes were followed by a short (20 - 50 ms) afterhyperpolarization, and a long afterhyperpolarization (up to 500 ms) followed multiple spiking activity evoked by depolarizing current pulses. The long afterhyperpolarization was calcium dependent.

LC neurons in tissue cultures were predominantly quiescent, though a few cells exhibited tonic firing activity. However in more than one half of cells recorded, spontaneous

bursts of action potentials were recorded. Bursts of firing activity appeared to be initiated by the summation of depolarizing events, resulting in a maintained depolarization of up to 15 mV, and evoking firing activity. The end of bursts were characterized by a slow repolarization, although in a few cells a long afterhyperpolarization followed bursts. In simultaneous recordings of pairs of LC neurons, it was apparent that LC neurons were synchronously activated to fire in bursts.

Locus coeruleus neurons do not appear to be electrotonically coupled. Current spread from one neuron to another was not revealed in simultaneous recordings. Furthermore, following injection of lucifer yellow, which has been reported to pass through gap-junctions, dye-coupling did not occur, supporting the view that LC neurons are not electrotonically coupled.

An interaction between LC neurons was revealed after averaging in a few pairs of simultaneously recorded neurons. Small (<300 μ V) depolarizing events in one cell were synchronized with afterhyperpolarizations of evoked spikes in the second cell. These interactions might be explained by the depolarizing influence of an accumulation of extracellular potassium during afterhyperpolarizations.

It appears that synchronous bursting of LC neurons is due to a common excitatory synaptic input acting on all LC neurons. This conclusion is based on the properties of bursts and on the properties of spontaneous and evoked depolarizations, that appear to be equivalent events to depolarizations that summate

to evoke bursts. Bursts and spontaneous and evoked depolarizations are all blocked in perfusates that block synaptic transmission, even when cell excitability is unchanged or increased. Furthermore, the amplitude of evoked and depolarizing events is dependent on membrane potential increasing with hyperpolarization, and decreasing with depolarization. In two cases, the evoked depolarizations were reversed by strongly depolarizing the cell. Therefore, depolarizing events associated with bursting activity and analogous depolarizations have the characteristics of excitatory synaptic potentials.

Responses of LC neurons to adrenergic drugs were also tested. Two types of direct responses to iontophoretically applied noradrenaline (NA) were observed: hyperpolarizations and biphasic responses - a hyperpolarization followed by a depolarization above resting membrane potential. In addition, iontophoretic application of NA often activated an excitatory input onto LC neurons. The most unexpected finding of my studies was an age-dependence of the direct responses of LC neurons to NA. LC neurons grown in tissue culture for less than 20 days only exhibit biphasic responses, whereas those cells grown in culture for more than 26 days only exhibit hyperpolarizing responses. Between 20 and 26 days in culture, neurons exhibited either hyperpolarizing or biphasic responses to NA.

Based on the effects of selective antagonists and agonists, it appears that the hyperpolarizing response is

mediated by α_2 -adrenoceptors, while the depolarizing component of the biphasic response is mediated by α_1 -adrenoceptors. The hyperpolarizing response is selectively blocked by the α_2 -antagonist, yohimbine; the depolarizing response is blocked by the α_1 -antagonist, prazosin. Neither response was affected by β -antagonists. Hyperpolarizations are evoked by the α_2 -agonist, clonidine, and depolarizations are evoked in LC neurons from young cultures with bath or iontophoretic application of the α_1 -agonists, methoxamine or phenylephrine. Therefore, an α_1 -mediated depolarizing response is lost during the maturation of LC neurons in tissue culture.

The α_2 -mediated hyperpolarizations were associated with an increased membrane conductance, and could be reversed by hyperpolarizing recorded cells. On the other hand, no consistent change in membrane conductance during the α_1 -mediated depolarization was noted, though hyperpolarizing the cell increased the amplitude of this response.

An age-dependent change in the responses of LC neurons to noradrenaline may be an important factor in the proposed role of NA and the LC in developmental changes, such as plasticity in the cortex. Synchronous firing of LC neurons in bursts suggests that LC neurons can affect transmission simultaneously throughout the nervous system. Furthermore, bursts of action potentials may be the optimal type of activity for the maximal release of noradrenaline and co-localized peptides. Finally, it will be interesting to determine what role multiple axon systems from single LC neurons may have.

INTRODUCTION

The locus coeruleus is a small noradrenergic pontine nucleus, that projects throughout the nervous system. The role of this nucleus has been elusive though many putative roles have been suggested. Behavioural studies have focussed on the effects of lesions in the area of the LC and on the effects of adrenergic drugs (which may block or mimic the effects of the LC). The physiological effects of noradrenaline and adrenergic agonists at target regions of LC neurons have also been studied. Recently, the activity and the control of activity of locus coeruleus neurons have been investigated with the view that a better understanding of the physiology of these neurons should give an insight into the function of this nucleus. I have been studying the locus coeruleus in explant tissue cultures, to examine some of the mechanisms governing the activity of these neurons for this reason.

I) BACKGROUND

The locus coeruleus (latin for blue location) was first observed in the primate as a "blue streak in the dorsolateral tegmentum of the pons" as early as 1809 by Reil. It was not until 1955, however, that the locus coeruleus was recognized as a distinct nucleus (Russel, 1955). Beginning in the early 1960's, the predominantly noradrenergic nature of the locus coeruleus was realized. Dahlström and Fuxe (1964) demonstrated that LC neurons exhibited catecholamine fluorescence, indicative of catecholaminergic neurons. Since then, the

noradrenergic nature of this cell group has been conclusively demonstrated, based on staining with antibodies directed at dopamine- β -hydroxylase (which monooxygenates dopamine to form noradrenaline) (Swanson, 1976; Grzanna and Molliver, 1980a,b), the absence of immunoreactive staining for phenylethanolamine-N-methyl-transferase (which methylates noradrenaline to form adrenaline) (Hökfelt, et al., 1974), and many other studies (see van Dongen, 1981). A considerable literature on the morphology, physiology and function of the LC has been amassed in the last decade and a half, and the reader is referred to the following reviews for information that is not covered in detail here:

- 1) Amaral and Sinnamon (1977)
- 2) Moore and Bloom (1979)
- 3) McNaughton and Mason (1980)
- 4) Grant and Redmond (1981)
- 5) Foote, Bloom and Aston-Jones (1983)
- 6) van Dongen (1981)

II) Locus Coeruleus Morphology

The locus coeruleus is located ventrolateral to the tip of the fourth ventricle, and is medial to the superior vestibular nucleus and the mesencephalic nucleus of the trigeminal nerve, and lateral to the nucleus of Gudden (Swanson, 1976; Cintra, et al, 1982). Dahlström and Fuxe (1964) distinguished many groups of monoamine-containing neurons, which they systematized as groups A1 to A12 (catecholaminergic) and B1 to B9 (serotonergic). Group A6 corresponds to the location of the LC. Other groups relevant to following discussions are A1 to A5 and A10. Groups A1

which appears to be the same as the adrenergic group C1 (Hökfelt, et al., 1974) and A2 are in the rostral medulla. The adrenergic group C2 are the rostral cells of group A2 (Hökfelt, et al., 1974). Groups A4 and A5 are in the pons; group A4 is in the roof of the fourth ventricle, ventral to the cerebellar nuclei. Finally, group A10 is located in the ventral tegmental area.

As the size and composition of the LC varies amongst species, I will first review findings on the most studied species - the rat. The rat LC is a compact group of approximately 1,600 medium-sized neurons (Swanson, 1976). Two types of LC neurons have been described - multipolar neurons having no major axis, and fusiform or bipolar neurons having a major and minor axis. Swanson delineated two subdivisions of the rat LC: a dorsal division containing mostly fusiform neurons and few multipolar neurons totalling 1,430 neurons, and a small ventral division (approximately 210 neurons) with more multipolar neurons and few fusiform neurons. Neuronal somata in the dorsal division are approximately 20-25 μm in size, while those in the ventral division are 30-40 μm in size, and all appeared to be noradrenergic (Grzanna and Molliver, 1980a,b). Grzanna and Molliver also included the A4 group of noradrenergic neurons and a rostral group of neurons as part of the locus coeruleus, based on dopamine- β -hydroxylase staining.

Both types of LC neurons have 3 - 5 dendrites, which branch once or twice and extend well beyond the limits of the nucleus

(Swanson,1976; Cintra, et al.,1982). Furthermore, LC neurons have dendritic and somatic spines (Swanson,1976; Cintra,et al.,1982; Groves and Wilson,1980a; Shimizu and Imamoto, 1970; Shimizu, et al,1978). In addition to projection axons, LC neurons often have locally ramifying axon branches, which presumably contact other LC neurons (Swanson,1976; Cintra,et al.,1982; Groves and Wilson,1980b; Shimizu, et al.,1978).

Although neuronal elements in the LC are extensively ensheathed by glial processes (Groves and Wilson,1980a), they apparently have specialized contacts. Adherens junctions between LC somatic spines have been described (Groves and Wilson,1980a). Dendrodendritic contacts, that apparently are noradrenergic synaptic junctions between LC neurons have also been described (Groves and Wilson,1980b; Shimizu and Imamoto,1970; Shimizu.et al.,1979)

In further studies, small (10 x 15 μ m), oval neurons have been noted in the rat LC (Cintra,et al.,1982; Shimizu,et al.,1978; Shimizu,et al.,1979). The oval neurons appear to be few in number and are found in the LC, but rarely in the central regions (Groves and Wilson,1980a). Axons arising from these cells ramify within the LC and could not be traced beyond the LC (Cintra,et al.1982). As Grzanna and Molliver (1980a,b) only described medium sized cells (15-30 μ m and larger) which were stained with antibodies directed against dopamine- β -hydroxylase, and Shimizu and coworkers (1979) found that the small cells did not have small or large granular

vesicles which are characteristic of the larger noradrenergic LC neurons, the small oval cells may not be adrenergic.

In the primate, a compact group of approximately 19,000 noradrenergic neurons form the LC, though there may be a few serotonergic neurons (Sladek and Walker, 1979). Some serotonergic neurons have also been found in the rat LC (Steinbusch, 1981). Similarly in the mouse, the LC appears to form a compact group of catecholaminergic cells (Golden, 1973). In the cat, opossum, and apparently in the guinea pig, non-adrenergic neurons are found interdigitated with the noradrenergic LC neurons (Foote, et al., 1983). Finally, in the rabbit, the LC is a loosely organized group of neurons.

It has recently been demonstrated that other neurotransmitters may be co-localized in LC neurons. Enkephalin and synenkephalin (a precursor of enkephalins) -like immuno-reactivity has been observed in monoaminergic neurons in the cat LC (Charnay, et al., 1982, 1985). However, in the rat, enkephalins were found in few neurons (Katchaturian, et al., 1983). Galanin has been localized in high amounts in most LC neurons (Melandar, et al., 1986). In addition, noradrenaline, vasopressin and its associated neurophysin have been co-localized in rat LC neurons, using antibodies directed against vasopressin, neurophysin, and noradrenaline (Caffé, et al., 1983, 1985; Sofroniew, 1985). Finally, neurotensin (Uhl, et al., 1979), corticotrophin releasing factor (CRF) (Merchenthaler, 1984; Swanson, et al., 1983), neuropeptide Y (NPY) (Everitt, et al., 1984), substance P (Shults, et al., 1984)

and somatostatin (Morley,1985) have also been localized in LC neurons but in lesser amounts. Therefore, it appears that LC neurons may release one or more peptides in addition to NA.

III) DEVELOPMENT OF THE LOCUS COERULEUS IN VIVO

Golden (1973) described the origin and development of the mouse locus coeruleus based on catecholamine fluorescence. The locus coeruleus first appeared at embryonic day 14 (E14) and had attained its characteristic postnatal morphology (a close packed group) and position by embryonic day 19 (E19). In the rat, a similar ontogenesis has been described, with catecholamine neurons (based on tyrosine hydroxylase immunocytochemical staining) appearing at E12 and catecholamine fluorescent cells observed at E14 (see Sievers and Klemm, 1982). Based on polyribosome and rough endoplasmic reticulum (RER) morphology and distribution, Sievers and Klemm suggested 5 stages of LC neuronal development. In the first stage (E13-E16), LC neuronal processes are reaching the immature forebrain, and polyribosomes are dense in LC neurons while the RER is poorly developed. The second stage (E17-E20) marks the onset of synaptic transmission by LC neurons, and the RER forms small Nissl bodies. In stage three (E21-postnatal day 7 (P7)), LC axon arbors are expanding with the growth of target tissues, large Nissl bodies are formed, and there is a dense distribution of free polyribosomes. Stage four (P10-P15) is marked by the loss of Nissl bodies, and there is an increased generation of cell processes. During stages 1 to 4, LC cells increase in size reaching a maximal

stages 1 to 4, LC cells increase in size reaching a maximal size (140% of adult size) at approximately P20, and then decrease to adult size between P20 and P30. In the adult stage, Nissl bodies reappear and polyribosomes are found in the RER.

The early development of the LC and the innervation of target tissue such as the cerebral cortex by LC axons before other afferents reach these areas, has prompted suggestions that the LC is important in regulating the development of the central nervous system. Morphological studies on the effects of lesioning the LC early in development have yielded conflicting results. While many authors report no changes in cell morphology or distribution, other authors have observed changes in dendritic morphology and density of synaptic contacts after noradrenergic lesions (see Foote, et al., 1983).

The most extensive work, that implicates LC in the development of the nervous system comes from work on visual plasticity. Changes in the binocularity of visual cortex neurons after monocular deprivation are prevented by cortical lesions of noradrenergic systems affected by cortical infusion of 6-hydroxydopamine (6-OHDA) (Kasamatsu and Pettigrew, 1979); this effect can be reversed by infusion of noradrenaline (Kasamatsu, et al., 1979). This work became controversial when other means of lesioning LC cerebral afferents were used. Similar findings were found by other investigators when cortical 6-OHDA infusions were used, but after DSP-4 noradrenergic lesions (systemic or lateral ventricle

injections) or lesioning of the ascending bundle were used, no changes in visual plasticity were observed (Daw, et al, 1984, 1985). This controversy appears to be reconciled by findings that cortical 6-OHDA infusion may be also damaging acetylcholine systems and that only a combined noradrenergic and cholinergic lesion affects cortical plasticity (Bear and Singer, 1985).

A role of noradrenergic systems in cortical plasticity has also been indicated in a behavioral study. Behavioral effects and effects on brain weight of rearing rats in enriched environments are lost when noradrenergic systems are lesioned in rat pups by subcutaneous injection of 6-OHDA (O'Shea, et al, 1983).

IV) AFFERENTS AND EFFERENTS OF THE LOCUS COERULEUS

The widespread projections of the LC have been investigated using many tracing techniques, including histofluorescence, immunohistochemistry, retrograde labelling and autoradiography. It has been demonstrated that LC neurons project to the cerebral cortex (Lindval and Björklund, 1984; Morrison et al., 1984), thalamus, amygdala (central nucleus), hippocampus, septum, hypothalamus, cerebellum, nuclei in the pons and medulla, and to the spinal cord (for reviews see: Amaral and Sinnamon, 1977; Moore and Bloom, 1979; McNaughton and Mason, 1980; Foote, et al., 1983; Palkovitz and Brownstein, 1983). These projections arise mostly from the ipsilateral LC, though significant contralateral projections exist.

Initial findings on the ultrastructure of cerebral noradrenergic terminals using localization of tritiated noradrenaline taken up by presynaptic terminals indicated that most labelled terminals did not make synaptic contacts (Descarries and Lapierre, 1973; Descarries, et al., 1977). This suggests that noradrenaline (NA) is released from varicosities (boutons en passant) of LC axons as a neurohumor. However, in ultrastructural studies examining varicosities in the cerebral cortex labelled by dopamine- β -hydroxylase or (in young animals) with the false transmitter 5-hydroxydopamine, virtually all noradrenergic terminals formed synaptic contacts (when sampling error is taken into account) (Molliver, et al., 1982). The differences in these studies may be due to observations on different areas of the cortex (i.e. Descarries et al. looked at layers I and II, while Molliver et al. apparently studied all cortical layers).

The projection of single LC neurons is still unclear. It has been demonstrated that single LC neurons can project to multiple target areas: e.g. to the cortex and to the cerebellum (Steindler, 1981, Nakamura, 1977, see also Foote, et al. 1983 and Palkovitz and Brownstein, 1983). A few divisions of the LC have been described based on their target areas, for example projections to the spinal cord appear to primarily arise from the ventral LC and the subcoeruleus (Satoh, et al., 1977; Ader, et al., 1979; see Marshall, 1983). However, further studies are required to determine any topographic divisions of the LC based on projections of individual cells.

It will also be of interest to determine any correlations between the target areas of individual LC neurons and the type of transmitter that is co-localized with each LC neuron.

The afferent input to the LC has also been studied using a variety of techniques. Using horseradish peroxidase (HRP)(Cedarbaum and Aghajanian,1978a; Clavier,1979) and tritiated amino acid tracing techniques (Beckstead,et al.,1979; Conrad,et al.,1974; Kreiger, et al.,1979; Pierce,et al,1976; Saper,et al.,1976,1979; Price and Amaral, 1981), immunocytochemical (Hokfelt et.al.,1974) and biochemical (McRae-Degueuerce and Milon,1983) techniques, the following areas have been proposed to project to the LC:

Insular cortex	Raphè
Amygdala (central nucleus)	Central gray
Hypothalamic nuclei:	Adrenergic nuclei:
preoptic	A1/C1,A2/C2 and A5
dorsomedial	Ventral mesencephalic tegmentum (A10)
paraventricular	Vestibular nuclei
lateral	Nucleus Tractus Solitarius
Bed nucleus of the	Fastigial nucleus of the cerebellum
Stria Terminalis	

A preliminary report has recently been given which suggests that the afferents to the body of the LC nucleus are much more restricted. Areas where numerous cell bodies were labelled included the ipsilateral parabrachial nucleus, vestibular nuclei, nucleus paragigantocellularis, hypoglossi praepositus nucleus and cervical spinal gray matter. (Aston-Jones,et al.,1985,1986). Areas that did not appear to project to the LC included the amygdala, nucleus tractus solitarius, and spinal dorsal horn columns. This study used wheat germ agglutinin

(WGA) HRP, which has many advantages (Sawchenko and Gerfen, 1985). Firstly, WGA-HRP does not diffuse as much as HRP or other labels used previously, and therefore only labelled the input to the body of the LC. Furthermore, as WGA-HRP is more readily taken up and transported, it appears to be a more sensitive tracer than HRP. However, it is possible that WGA-HRP is not taken up by all types of neurons.

In ultrastructural studies on the LC, at least six different types of nerve terminals (based on the morphology of vesicles contained in each terminal) have been observed contacting LC somata and dendrites (Groves and Wilson, 1980a,b). Most terminals (65%) were found on dendrites having diameters of 0.5 to 2.5 μm , and there were no apparent differences in the distributions of contact sites of the different types of terminals. Noradrenergic (axonal collaterals and dendrodendritic) and adrenergic contacts comprise approximately 5% of the terminals and serotonergic terminals comprise approximately 4% of the presynaptic profiles based on labelling with the false transmitter 5-hydroxydopamine. The ultrastructure of terminals on LC neurons suggests that there are multiple inputs to the LC. The origin of many of these terminals is still unclear. It should be noted that as dendrites from LC neurons extend well beyond the nucleus, WGA-HRP may not label inputs contacting these distal dendrites.

V) NORADRENERGIC ACTIONS IN OTHER BRAIN REGIONS

The actions of locally applied noradrenaline and noradrenaline released by stimulation of LC neurons have been investigated in various target areas. Early research indicated a predominantly inhibitory action of NA. However, noradrenaline has diverse effects on neurons acting through at least four types of adrenergic receptor.

Exogenously applied noradrenaline has inhibitory, excitatory, modulatory and/or more subtle effects depending on the brain region and adrenoceptors involved. The inhibitory effects of NA in the cerebellum (and cerebral cortex) may involve the activation of β -adrenoceptors and the production of cyclic adenosine monophosphate (cAMP) (Siggins, et al., 1971, 1973). However, other research suggests that α_2 -adrenoceptors may mediate the cerebellar inhibition (Basile and Dunwiddie, 1984) and that cAMP is not involved in cerebral noradrenergic inhibitions (Collins, et al., 1984a). Membrane mechanisms involved in the inhibition are also unclear, though it has been suggested that an activation of the sodium pump ($\text{Na}^+/\text{K}^+-\text{ATPase}$) is involved (Phillis, et al., 1982, Swann, 1983). However, the pharmacology of this pump activation is mixed and involves direct stimulation of the pump by NA as well as receptor-mediated activation (Swann, 1983). Furthermore this pump may be activated indirectly due to an increase in neuronal activity induced by application of NA. In the cerebral cortex, excitatory effects of exogenously applied NA were initially controversial and dismissed as artifacts due to

pH of iontophoresed NA (Frederickson, et al., 1971). However, other reports did not find an effect of pH on excitatory noradrenergic effects (Stone, 1972). Conflicting results may have been due to differences in the type of anesthesia used (see Szabadi, 1979; Phillis, 1984), the iontophoretic current used and the cortical layer recorded from (Armstrong-James and Fox, 1983).

Excitatory noradrenergic effects in the cerebral cortex, thalamus (lateral geniculate) and raphe nucleus have been associated with α_1 -adrenoceptors (Bevan, et al., 1977; Rogawski and Aghajanian, 1980, 1982; Kayama, et al., 1982; Marwaha and Aghajanian, 1982). However, the receptor pharmacology of this response in the cortex is unclear and α_1 -adrenoceptors, as well as uncharacterized receptors that are sensitive to both NA and dopamine, may be involved (Bradshaw, et al., 1983). It has also been suggested that β -adrenoceptors mediate noradrenergic excitations in tissue slices of the cerebellum (Basile and Dunwiddie, 1984). However, the latter excitations were only obvious when potassium in the perfusate for the slice preparation was high (5.2 mM) (Basile and Dunwiddie, 1983).

Noradrenergic transmission has also been implicated in the modulation of other transmitter actions. In the cerebellum and the cerebral cortex, Woodward's group have repeatedly demonstrated that NA selectively potentiates the effects of some excitatory and inhibitory transmitters (Woodward, et al., 1979; Moises, et al., 1983). Modulation by NA has also been

demonstrated in the hippocampus (Segal,1982). The modulation by NA of inhibitory effects is apparently mediated by β_1 -adrenoceptors (Yeh and Woodward,1983), while facilitation of excitatory responses are mediated by α -adrenoceptors (excitatory facilitation; Waterhouse,et al.,1981), and may involve both presynaptic (Collins,et al,1984b; Dolphin,1982) and postsynaptic mechanisms (Segal,1982).

In addition, Madison and Nicoll (1982,1986a,1986b) have demonstrated that NA-can increase excitatory responses by decreasing the calcium-dependent potassium conductance in hippocampal slices. This effect apparently was mediated by β_1 -adrenoceptors and involved cAMP. This work was performed at low temperatures (29-31°C) and at elevated extracellular potassium levels (5.4 mM), so its relevance to normal physiological function is unclear.

It would therefore appear that the responses of neurons to NA are highly varied and some discrepancies exist. However, if conflicting reports on effects of NA in the cortex and cerebellum are due to the modulation of other transmitter effects by NA, noradrenergic actions might be resolved as follows. α_{2} adrenoceptors are involved in direct inhibition of neurons in the spinal cord (North and Yoshimura,1984), locus coeruleus (see below) and periphery (Brown and Caulfield,1979; Morita and North, 1981), and act as autoreceptors on LC axons terminals to decrease transmitter release. α_1 adrenoceptors are involved in the direct activation of neurons in areas such as the thalamus, cortex,

and periphery, and may be the adrenoceptors involved in the facilitation of excitatory responses in the cortex. Beta adrenoceptors are involved in a direct inhibition of neurons that may involve the generation of cAMP. Beta₁ adrenoceptors are involved in the modulation of other transmitter systems and therefore may potentiate inhibitions of recorded neurons. This effect may be partially mediated by the accumulation of cAMP. In addition, NA may have direct (i.e. not receptor mediated) effects on the Na⁺/K⁺-ATPase.

VI) PHYSIOLOGY OF LOCUS COERULEUS NEURONS

The locus coeruleus has been studied using extracellular recordings, intracellular recordings, and voltammetry. Recordings have been made in anesthetized and unanesthetized animals, in tissue slices and in tissue cultures of LC neurons. Furthermore, recordings have been made from a variety of species, namely the cat, rat, monkey, guinea pig and mouse. As the LC in the cat and possibly the guinea pig and monkey contain non-noradrenergic neurons, I will refer to the activity and responses of LC neurons as those of the noradrenergic LC neurons.

VI) 1) Resting Activity of LC neurons

The activity of LC neurons varies from preparation to preparation, and is dependent on the sleep and waking cycle of the animal, as is evident in extracellular recordings from LC neurons in unanesthetized rats (Aston-Jones and Bloom, 1981a,b) and from noradrenergic LC neurons in unanesthetized cats (Rasmussen, et al., 1986, Jacobs, 1986). During waking states,

LC neurons are spontaneously active, and are activated by sensory stimuli (see below). During slow wave sleep, the activity of LC neurons is decreased, and during paradoxical sleep these neurons are quiescent.

LC neurons recorded in anesthetized animals (urethane-Nakamura,1977, chloral hydrate-Korf,et al.,1974 and Watabe and Satoh,1979) and in tissue slices (Henderson,et al.,1982) exhibit a tonic firing pattern (0.5-5.0 Hz). In anesthetized animals LC neurons only respond to noxious sensory stimuli (Korf,et al.,1974). In addition, in recordings from (urethane) anesthetized neonatal rats, LC neurons are quiescent, but appear to show greater responses to both non-noxious and noxious sensory stimuli (Kimura and Nakamura,1985). Although the activity of LC neurons is altered in anesthetized animals and in tissue slices, these preparations offer many advantages. Intracellular recording is possible (though in vivo this requires mechanical stabilization, Aghajanian et.al.,1983). Furthermore drugs can be bath-applied at known concentrations onto tissue slices.

VI) 2) Bursting Activity of LC neurons

In paralyzed animals (Korf,et al.,1974), and in paralyzed animals recovering from anesthesia (Akaike,1982), most LC neurons exhibit rhythmic bursts of firing activity and in the latter study, it was noted that LC cells appeared to burst in synchrony. Furthermore, in unanesthetized animals, LC neurons burst in response to sensory stimuli of most and perhaps all modalities (Foote,et al.,1980; Aston-Jones and Bloom,

1981a,b; Rasmussen,et al,1986; Jacobs,1986). In addition, it appears that most or all LC neurons in unanesthetized animals are synchronously activated to burst (Aston-Jones,et al,1981b). Aston-Jones and Bloom (1981b) suggested that the synchronous activation of LC neurons may be due to a common excitatory input based on the recordings of field potentials associated with bursts, as these potentials were also present during the otherwise quiescent stage of paradoxical sleep. In tissue culture, LC neurons have also been observed to synchronously burst (Marshall,et al.,1979). My research on the processes involved in synchronous bursting of LC neurons is part of the work reported in this thesis.

VI) 3) Postactivation inhibition of LC neurons

In initial studies on the LC, the effects of stimulating the dorsal noradrenergic ascending pathway (the tract of LC axons projecting to the cortex and other subcortical structures), cerebral cortex, cerebellum or electrical stimulation of sensory systems (e.g. the optic nerve and skin), were investigated. Characterized in extracellular recordings, stimulation of the dorsal bundle antidromically activates LC neurons, which is followed by a period of postactivation inhibition (Aghajanian,et al.,1977; Watabe and Satoh,1979). A postactivation inhibition also follows orthodromic stimulation of LC neurons by sensory stimulation (Cedarbaum and Aghajanian,1978; Watabe and Satoh,1979; Foote, et al.,1980), and has been a subject of some controversy. Two

mechanisms have been proposed to explain the postactivation inhibition: a recurrent noradrenergic inhibition and an opening of calcium-activated potassium channels.

The concept of recurrent inhibition of LC neurons has been established. There is now a preponderance of evidence that α_2 -adrenoceptors mediate a hyperpolarizing response to NA or adrenaline (A) in LC neurons. The α_2 agonist clonidine inhibits LC neurons in extracellular recordings (Svensson, et al., 1975) and hyperpolarizes LC neurons in intracellular recordings in vivo (Aghajanian and VanderMaelen, 1982). The rank order of potencies of adrenergic agonists, also indicates an α_2 -adrenoceptor-mediated inhibition of LC neurons (Cedarbaum and Aghajanian, 1977). Furthermore, LC neurons were demonstrated to be inhibited by epinephrine and activated by blocking α -adrenoceptors in vivo (Cedarbaum and Aghajanian, 1976). In tissue slices, the pharmacological characterization of α_2 -adrenoceptors on LC neurons was completed using various adrenergic antagonists, including the α_2 antagonists, yohimbine and RX 781094 (Idaxozan) (Williams, et al., 1985). Finally, in autoradiographic studies, the LC is observed to have a high density of α_2 adrenoceptors (using [^{3} H]-para-aminoclonidine) and a very low density of α_1 -adrenoceptors (using [^{3} H]-WB-4101) (Young and Kuhar, 1980).

Noradrenaline released from LC neurons onto LC neurons and adrenergic input from areas A1, A2 or A5 may be acting on α_2 -adrenoceptors to inhibit LC neurons. As previously mentioned, noradrenergic (recurrent axon collateral and dendrodendritic)

contacts between LC have been described. As suggested by Aghajanian and Cedarbaum (1977) and Watabe and Satoh (1979) in animal studies, recurrent noradrenergic inhibition of LC neurons has been demonstrated in tissue slices (Egan, et al., 1983). Electrical stimulation of tissue slices evoked an excitatory postsynaptic potential (epsp) followed by a long noradrenergic hyperpolarization. This noradrenaline-induced hyperpolarization of LC neurons has been pharmacologically characterized as an α_2 -adrenoceptor mediated response (Williams, et al., 1985) and due to an increase in membrane potassium conductance (gK+) (Egan, et al., 1983; Aghajanian and VanderMaelen, 1982). Therefore at least one mechanism involved in the postactivation inhibition of LC neurons appears to be the autoinhibition of LC neurons through an α_2 -adrenoceptor mediated increase in gK+.

However, recently Aghajanian, VanderMaelen and Andrade (1983) have demonstrated that LC neurons have a large calcium-activated potassium current ($I_{K(Ca)}$), and have suggested that this current could explain the postactivation inhibition (Aghajanian, et al., 1983; Andrade and Aghajanian, 1984a and 1984b). In one study, the intensity of stimulation of the dorsal bundle was varied and postactivation inhibitions were compared for perithreshold and suprathreshold stimulation of the nucleus. Postactivation inhibition was greatly reduced or abolished in most recorded cells if they were not antidromically activated by dorsal bundle stimulation, and the postactivation inhibition was increased in only half of the

cells tested when the stimulus was increased beyond threshold (Andrade and Aghajanian, 1983b). This work suggests that postactivation inhibition to dorsal bundle stimulation is predominantly mediated by a calcium-activated potassium conductance, though α_2 -mediated inhibition is clearly present in some cells. Recently, Ennis and Aston-Jones (1986) using a similar experimental protocol have demonstrated that both autoinhibitory mechanisms are present in the LC and that recurrent inhibition plays a significant role in autoinhibition. In addition, Ennis and Aston-Jones examined the autoinhibition after stimulation of the contralateral paw and found both mechanisms involved though the inhibition was weaker. However, inhibition following a physiologically relevant activation of LC neurons has not been investigated. It would be of interest to know the relative involvements of α_2 -inhibition and the calcium-activated potassium current in the inhibition following sensory activation of the LC in the unanesthetized animal.

VI) 4) Facilitatory Effects in the LC

In addition to stimulating the dorsal bundle, Nakamura and coworkers (1980) stimulated frontal cortex, cerebellar cortex and midbrain/hippocampus and observed inhibitory effects, and in about one-third of recorded cells an initial facilitation of LC neuron discharge. The onset latency of the postactivation inhibition and the facilitation (when the recorded cell was not antidromically activated) were similar.

Furthermore, both inhibitory and facilitatory effects were reduced by α -methylparatyrosine (α -MPT), and the effects of α -MPT were reversed by an injection of l-dopa, suggesting that noradrenergic mechanisms may be involved in both the inhibitory and excitatory effects. The facilitatory effects observed by Nakamura and coworkers, have not been subsequently investigated, though our research reported here on the effects of iontophoretically applied NA, and on synchronous bursting of LC neurons may be relevant to these findings.

VI) 5) Non-adrenergic Transmitters in the LC

In autoradiographic studies, the LC has been demonstrated to have a high density of opiate receptors (Atweh and Kuhar, 1977) and a moderate density of cholinergic muscarinic receptors (Rotter, et al., 1979). In addition receptor binding for a number of other transmitters has been reported (Table I).

The effects of opiates, acetylcholine and other transmitters on LC neurons have also been investigated. Opiates and related drugs inhibit LC neurons (Korf, et al., 1974, Guyenet and Aghajanian, 1979, Aghajanian, 1982) by an increase in a potassium conductance resulting in a hyperpolarization (Williams, et al., 1982, North and Williams, 1983). Both the noradrenergic and opiate inhibition of LC neurons have been suggested to be mediated by a decrease in cAMP (Andrade and Aghajanian, 1985;

Table I: Transmitter receptor binding in the nucleus Locus Coeruleus

TRANSMITTER	BINDING	TYPE	REFERENCES
Opiates	high	μ	Atweh and Kuhar, 1977 Quirion, et al., 1983
Adrenergic	high	α_2	Young and Kuhar, 1980
Substance P	high		Shults, et al., 1984
Acetylcholine	moderate	m_2	Rotter, et al., 1979
Histamine	moderate		Palacios, et al., 1981
Somatostatin	moderate		Leroux, et al., 1985
Angiotensin II	moderate		Mendelsohn, et al., 1984
CRF	low		Wynn, et al., 1984
CCK	moderate		Van Dijk, et al., 1984
Serotonin	low		Meibach, 1983

Aghajanian and Wang, 1986), and appear to increase the same conductance (North and Williams, 1985). Adenosine (Shefner and Chiu, 1986), glycine (Cedarbaum and Aghajanian, 1978), GABA (Cedarbaum and Aghajanian, 1978b; Guyenet and Aghajanian, 1979) and neurotensin (Young, et al., 1978) have also been reported to inhibit LC neuronal activity.

Acetylcholine excites LC neurons by acting on muscarinic receptors (Guyenet and Aghajanian, 1979; Egan and North, 1985). LC neurons are also excited by the application of substance P (Guyenet and Aghajanian, 1979; Cheeseman, et al., 1983; Nishi, et al., 1982), glutamate (Guyenet and Aghajanian, 1979; Marshall, et al., 1984; Nishi, et al., 1982), Adrenocorticotropin

1981) and by CRF (Valentino, et al., 1983). The substance P excitation may be due to an inactivation of a potassium conductance but is not calcium-dependent (Cheeseman and Henderson, 1984). However, the physiological relevance of these transmitters to the control of the activity of LC neurons is still unclear.

Recently, the activity of the LC has been monitored using voltammetry (Buda, et al., 1983; Gonon, et al., 1983; De Simoni, 1983). In voltammetry, the current required for the oxidation of the two hydroxyl groups on the phenyl ring of noradrenaline (and related compounds (e.g. dopamine)) is used to estimate the concentration of noradrenaline (and related compounds). Oxidation currents measured in the LC in response to +0.1V pulses, appear to be due to the oxidation of the metabolite of NA, 3,4-dihydroxyphenylacetic acid (DOPAC) (Buda, et al., 1983). Changes in the level of DOPAC correlate with changes in LC activity and these authors suggest that the DOPAC level may reflect tyrosine hydroxylase (an enzyme involved in the synthesis of NA) activity and the metabolic activity of LC neurons (Gonon, et al., 1983). Acetylcholine increases the level of DOPAC in awake behaving rats, apparently through muscarinic receptors (De Simoni, 1983). This finding is compatible with electrophysiological studies showing that acetylcholine activates LC neurons through muscarinic receptors.

Finally, the effects of lesioning the ventral tegmental area (VMT-A10) and the raphe nucleus on the levels of NA, DOPAC, dopamine and serotonin determined by HPLC has been

investigated (McRae-Degeurce and Milon, 1983). The levels of dopamine and serotonin decreased in the LC after the VMT-A10 and raphé nuclei were lesioned respectively. However DOPAC levels were increased only by either a lesion of the raphé or the raphé and VMT-A10. These findings suggest that both VMT-A10 and the raphé nuclei project to the LC, and that the raphé has an inhibitory effect on LC activity. It has also been shown that stimulation of the raphé inhibits responses of LC neurons to noxious stimuli (Segal, 1979).

VI) 6) Summary

In summary, the control of activity of LC neurons appears to involve both intrinsic mechanisms and extrinsic inputs. Although our understanding of the locus coeruleus has advanced markedly in the last decade, there are many unanswered questions. The relative physiological involvement of collateral inhibition and a calcium-activated potassium conductance in the postactivation inhibition of LC neurons is still unresolved. Although the raphé, VMT-A10, hypoglossi, and paragigantocellularis nuclei have been demonstrated to project to the LC, other projections have not been confirmed and the projection(s) responsible for the activation of LC neurons in response to sensory stimuli is still unknown. Finally, the mechanism responsible for the synchronous activation of LC neurons to sensory stimuli is just presently being investigated.

VII) TISSUE CULTURING

VII:1) Techniques and Caveats

Culturing of nervous tissue and studies on neuronal tissue cultures has been extensively used in neurophysiology. For reviews on culturing techniques and recent reviews on some studies utilizing tissue cultures, the reader is referred to the following articles and books:

Federoff and Hertz, 1977
 Giacobini, et al., 1980
 Harvey, 1984 -
 Hosli and Hosli, 1978
 Sato, 1973

Tissue cultures of nerve cells were first reported by Harrison (1907, 1910). Since then many techniques of culturing nerve cells have been developed, and there are three main types of cultures: explant cultures, dissociated cultures and clonal line cultures. Explant cultures are produced by dissecting a small fragment (usually 0.5 to 1.0 mm cubic) of tissue, usually from embryonic or neonatal animals. The tissue is then transferred to a Maximow chamber, a roller tube or a special petri dish. Dissociated cultures are made by separating nerve cells either by mechanical or enzymatic dissociation, and the cells are plated out in a Petri dish. Tissue is maintained in culture with either serum-based media or defined media, which may or may not contain antibiotics. Cultured tissue can be maintained for days or weeks *in vitro*, and can be monitored by microscopic viewing throughout this period. Tissue culturing of neurons allows for the investigation of neuronal properties using intracellular and

extracellular recordings, and pharmacological manipulation both chronically before recording and acutely during recording. Furthermore, with the introduction of co-cultures, i.e. the culturing of different nuclei or regions of the brain (which can produce synaptic connections), the interactions of different, often widely separated nuclei can be studied in a highly controlled environment (e.g. Pun, et al., 1985).

However there are a number of questions regarding the validity of applying findings made in tissue culture to the physiology of the animal. Tissue cultures are usually derived from immature cells and are deafferented and deprived of normal targets to varying degrees. Neurons may also be damaged during preparation of cultures, especially during dissociation. Cultured neurons are also maintained in a modified environment for a long period. Many studies in tissue culture have been directed at demonstrating that cultured neurons have similar properties to those reported for equivalent neurons *in vivo* (see Harvey, 1984). Neurons in culture have been demonstrated to have similar morphologies, physiological responses, pharmacological responses, and synaptic connections as neurons *in vivo* (see Crain, 1977, 1980; Hendelman and Marshall, 1980; Spitzer, 1983). However, the most interesting findings are those that were originally demonstrated in culture and subsequently demonstrated *in vivo*.

One of the most striking findings made in tissue culture is from studies on dissociated cultures of sympathetic ganglia. In the adult animal, these neurons are noradrenergic. However,

sympathetic ganglion neurons in culture having noradrenergic properties initially can undergo a transition to cholinergic properties (see Patterson, 1978). The expression of the transmitter phenotype was demonstrated to depend on the presence of factors released by the target tissues. Landis and Keefe (1983) subsequently demonstrated that terminals of sympathetic ganglion neurons on sweat glands undergo a similar change *in vivo*. These terminals originally exhibit catecholamine fluorescence and uptake mechanisms; although catecholamine uptake is maintained past P14, all catecholamine fluorescence is lost. During P7 to P14, these terminals develop acetylcholinesterase activity which is also present in these terminals from adult animals. Therefore a transition from noradrenergic to cholinergic transmission of sympathetic ganglion neurons observed first in tissue culture has been demonstrated subsequently in the animal.

Another example of research on neuronal tissue cultures, that demonstrated new information of neuronal systems, is from work on organotypic cerebellar cultures. A projection from the deep cerebellar nucleus to cerebellar cortex was first demonstrated using electrophysiological techniques in culture (Wojtowicz, et al., 1975). This projection was subsequently demonstrated in the animal (Tolbert, et al., 1976; Gould and Graybiel, 1976; McCrea, et al., 1978) and morphologically in organotypic cell cultures (Hendelman and Marshall, 1980).

Neuronal tissue grown *in vitro*, however, may exhibit some anomalous properties. As many target areas are not included in

the tissue explanted and many of the normal inputs are not present, it is not surprising that some changes in innervation, biochemical properties and physiological responses can occur. Changes observed in tissue cultures include: increased numbers of synaptic contacts between cells; abnormal connections especially when normal target tissues are not present; increased resistances to toxic substances; formation of 'silent' synapses; alterations in transmitter synthesis; and dedifferentiation. These changes are marked in continuous cell lines, and in dissociated cultures (for examples see Giacobini, 1980). However explant cultures retain an organotypic organization that has many similarities to the organization found in the animal. Organotypic or explant cultures therefore have fewer anomalous properties than other culture types.

VII:2) TISSUE CULTURES OF LOCUS COERULEUS NEURONS

Tissues cultures of LC neurons have been derived from fetal (E12 to E18) and newborn mice and from fetal rats. The catecholaminergic nature of LC neuronal cultures has been studied. Catecholamine fluorescent fibers have been repeatedly described growing out from explant cultures (Hosli, et al., 1971; Schlumpf, et al., 1977; Victorov, et al., 1979; Dreyfus, et al., 1979; Pun, et al., 1985; Hendelman, et al., 1982). Measurements of noradrenaline and dopamine levels (indicators of catecholamine synthesis) demonstrated high dopamine and moderate noradrenaline concentrations in the bathing medium (Schlumpf, et al., 1977; Hendelman, et al., 1982) and in the tissue

(Schlumpf, et al., 1977).

The development of dopamine- β -hydroxylase (DBH) and tyrosine hydroxylase (TH) in tissue cultured LC neurons has also been studied (Dreyfus, et al., 1983). Both enzymes developed with a time course similar to that observed *in vivo*, in cultures explanted at E18. However, the measured activity of DBH and TH for cultured LC neurons was considerably decreased if the tissue was explanted at E12, and less markedly decreased in cultures established from E13 and E14 embryos. These findings demonstrate that LC neurons in tissue culture have the ability to synthesize noradrenaline, though the maturation of these neurons may be affected if they are removed at an early critical stage (perhaps involving the differentiation of primordial cells into noradrenergic LC neurons; see development of the LC).

Locus coeruleus neurons have also been cultured with hippocampal, cerebellar, cerebral cortical and spinal tissue. Electrophysiological and gross morphological evidence indicate that the separately explanted LC group can send out processes that innervate the target tissues. In coeruleo-hippocampal (Dreyfus, et al., 1979), coeruleo-spinal (Marshall, et al., 1981; Pun, et al., 1985) and coeruleo-cerebellar (Seil and Leiman, 1985) co-cultures, stimulation of the brainstem explant evoked synaptic responses in the respective target cells. Furthermore, catecholamine fluorescent axonal processes were observed innervating the target tissue (Marshall, et al., 1981; Pun, et al., 1985; Dreyfus, et al., 1979; Hendelman, et al., 1982;

Seil and Leiman, 1985; Rosenberg, et al,1985). Dreyfus and colleagues (1979), however did not find synaptic contacts with membrane specializations in varicosities labelled with tritiated noradrenaline in hippocampal co-explants.

Although these studies have examined some properties of locus coeruleus neurons in tissue culture, relatively little work has been done on the electrophysiology of LC neurons in tissue culture. In intracellular recordings of cultured neurons explanted with cerebellum, brainstem neurons were excited (possibly antidromically), and exhibited long latency ipsp's when the cerebellar cortex was stimulated (Marshall,et al.,1980). As previously noted, locus coeruleus neurons in extracellular recordings also exhibited synchronous bursting activity (Marshall,et al.,1979).

My studies have focused on the responses of LC neurons to NA, mechanisms involved in the synchronous activation of LC neurons, and the morphology of these neurons in explant tissue cultures. The work presented here contributes to the characterization of LC neurons in tissue culture and provides new data and new ideas which are likely to stimulate further research both *in vitro* and *in vivo*.

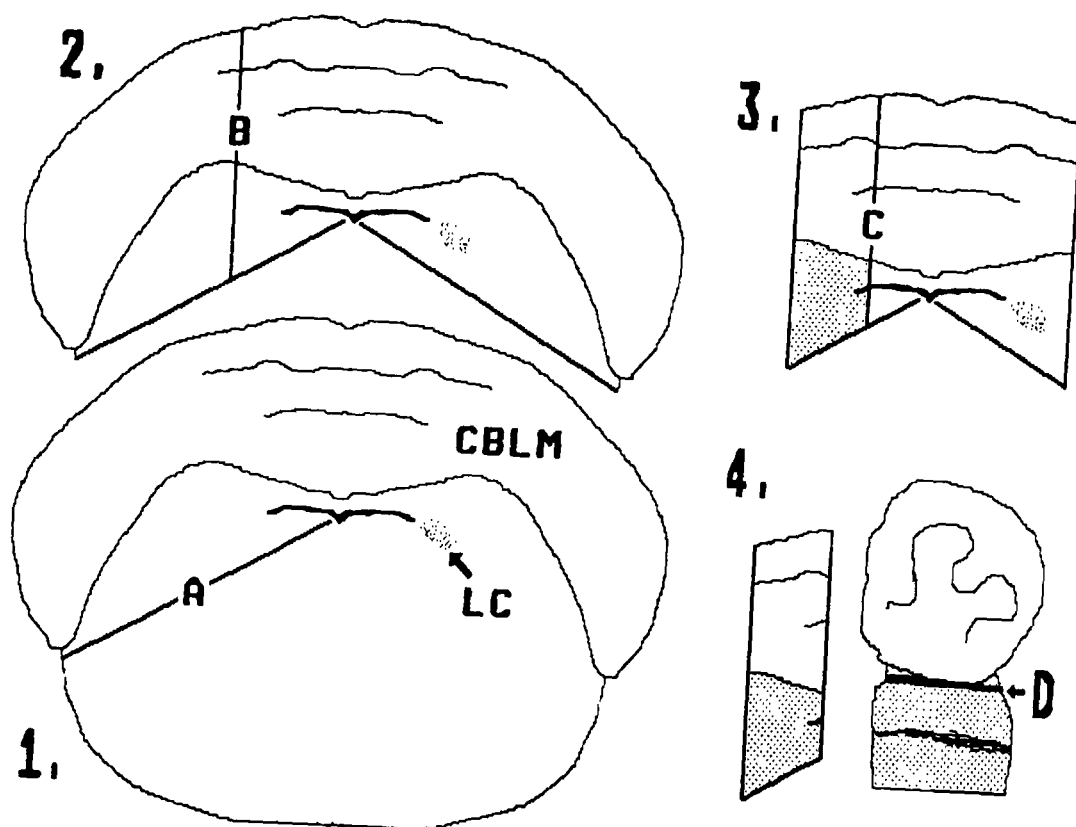
METHODS

I) Tissue cultures

Explant tissue cultures were established by dissecting tissue from neonatal mice (Paris RIII strain) less than 24 hours after birth. Brains were removed from animals in cold induced narcosis, and after cardiac puncture. Brains were transferred to a quadrant petri dish containing balanced salt solution (BSS) with glucose at neutral pH and at room temperature. Under microscopic observation, the pia was removed with fine forceps. A slab of tissue was cut by making a transverse cut with a scalpel (blade #11) parallel with the caudal surface of the cerebellum and a second cut at the rostral edge of the cerebellum parallel to the first cut (see fig 1). Further cuts were made with two razor blade fragments (Gillette francaise traitee) held in blade breakers. The brainstem piece containing LC neurons was dissected by making cuts along lines A, B, C, and D in figure 1. Brainstem pieces were often bisected to produce two smaller pieces containing LC neurons. A cerebellar piece was then trimmed from the medial cerebellum. Brainstem and cerebellar tissue were transferred onto a round collagen-coated coverslip (Goldseal #3347) on a square coverslip (Goldseal # 3600). Using a Pasteur pipette, a drop of nutrient serum-based feeding medium was dropped onto the tissue. The explant was then sealed into a Maximow slide, using a mixture (1:1) of paraffin wax (melting point 58-60°C) and petroleum jelly. This entire procedure was performed with sterile dissection procedures in

Fig. 1:

Schematic drawing of dissection of tissue for LC explant tissue cultures. Slab of tissue - cerebellum and underlying brainstem tissue - is cut at lines A, B, C, and then D, to separate brainstem tissue containing LC neurons. Resulting piece of brainstem tissue was usually bisected before transferring brainstem tissue with medial cerebellar tissue onto collagen coated coverslip. Photomicrograph of slab of tissue dissected is shown below schematic.



a laminar flow hood.

Cultures were maintained in vitro for 2 to 5 weeks, in an incubator maintained at 35°C. Cultures were washed in a balanced salt solution and refed with nutrient medium twice weekly. The culture medium consisted of 50% Eagles minimal essential medium, 25% human serum and 25% chick embryo extract (EE50, Flow laboratories) with 1.1 g% (61.1 mM) glucose and 1 mM glutamine. Cultures were also monitored by microscopic observation at various times during this period. Cultures with clear groups of LC neurons, and LC neurons close to the exposed surface were chosen for electrophysiological studies.

For further details on the preparation of collagen coated coverslips, serum-based media, and balanced salt solutions used in tissue culturing see Appendix 1.

II) Electrophysiological recordings

1) Recording setup

Recordings were performed in a constantly perfused chamber mounted on the stage of an inverted microscope (see fig 2). Perfusion was maintained at a constant rate using a peristaltic pump (LKB microperpex pump). Perfusates were equilibrated with 95%/5% O₂/CO₂ gas continuously bubbled in the perfusate. The perfusate was warmed by heat produced in resistance wire wrapped around the glass inlet tube at the entrance to the chamber. Fluid temperature was constantly monitored using a thermocouple probe in the bath, and was maintained at 34 to 35°C. Perfusates bathing the cultures

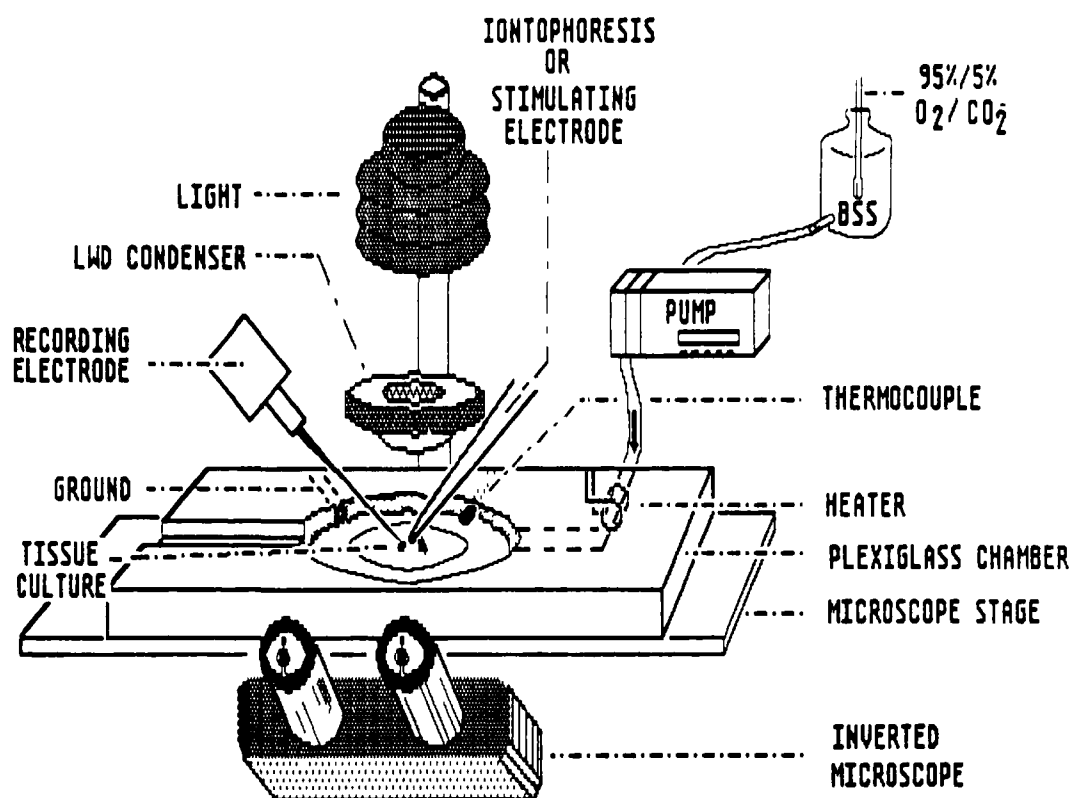


Fig. 2: Schematic of recording chamber for intracellular recording of LC neurons. (LWD - long working distance).

maintained a constant pH between 7.3 and 7.4. The perfusate was drained by gravity from the chamber. The following perfusates (BSS) were used for recording:

Perfusate	NaCl (mM)	KCl (mM)	NaH ₂ PO ₄ (mM)	CaCl ₂ (mM)	MgCl ₂ (mM)	MgSO ₄ (mM)	MnCl ₂ (mM)	NaHCO ₃ (mM)
Normal	118	3	1.0	1.8	0	0.8	0	23.8
0Ca/10MgCl ₂	118	3	1.0	0	9.2	0.8	0	23.8
0Ca/20MgCl ₂	118	3	1.0	0	19.2	0.8	0	23.8
0.5Ca/2Mn	130	3	0	0.5	0.8	0	2.0	12.0
0.5Ca/3Mn	118	3	0	0.5	0.8	0	3.0	12.0
0.5Ca/4Mn	118	3	0	0.5	0.8	0	4.0	12.0
10mM-Mg	118	3	1.0	1.8	10.0	0.8	0	23.8
12mM-Mg	118	3	1.0	1.8	12.0	0.8	0	23.8

Perfusate solution were freshly mixed daily from 10X concentrated stock solutions which contained appropriate amounts of NaCl, KCl, MgSO₄, CaCl₂, H₂PO₄ and MnCl₂. Magnesium chloride, sodium bicarbonate and glucose (to 33.3 mM) were added during preparation. Drugs from freshly mixed stock solutions were also added to perfusates during preparation, or in the case of tetrodotoxin (TTX) from a 1mg/ml stock solution stored at 0-4°C. Stock solutions of drugs were:

Yohimbine ¹	50 µM	- mixed for at least 1 hour.
Prazocin ⁴	25 µM	- mixed at 37°C for at least 1 hour.
Sotalol ²	1 mM	- readily dissolves
Propranolol ¹	1 mM	- readily dissolves
Noradrenaline ¹	0.2 mM	
Clonidine ¹	10 µM	
Phenylephrine ¹	50 µM	
Methoxamine ¹	50 µM	

¹-Sigma; ²-Bristol; ³-Burroughs Wellcome; ⁴-Pfizer.

Drugs were prepared from the hydrochloride salt form except for noradrenaline which was prepared from the free base form.

2) Intracellular recording

Electrodes for intracellular recordings were pulled on a Brown-Flaming puller (Sutter Instruments P-77), that was equipped with a box-type filament. Electrodes were pulled from borosilicate glass pipettes, containing a small glass filament for quick filling (WPI 1B120F; A-M Systems Cat. # 6020). Desirable characteristics of electrodes were that the tips had a slow taper for at least 25 μm from a diameter of 2 μm to the tip and that the shaft had a slow taper to allow for rapid filling with electrolyte solution. Electrodes were filled with either potassium acetate (2M) or with potassium chloride (2M), and had resistances of 70-120 M Ω and 40-90 M Ω with the respective filling solutions.

Intracellular recording electrodes were advanced by an A B Transvertex electromechanical drive system, and individual steps were 2 μm and the rate of stepping was variable. The stepper was mounted on a Kopf stereotaxic X-Y-Z manipulator. A second intracellular electrode or a iontophoresis electrode was positioned using an X-Y-Z hydraulic Narishige manipulator (MO-102R).

Recordings were amplified using either a Dagan 8700 Cell Explorer amplifier or the amplifier section of a Dagan 8500 Preamp-clamp amplifier. Both amplifiers had the capabilities of bridge balancing electrodes with resistances of 0-1000 M Ω , and adequate capacitance compensation to reduce the effects of the capacitance across the micropipette's tip surrounded by BSS. Electrodes were balanced prior to cell impalement by

passing current pulses through the electrode and adjusting the balance and capacitance compensation to produce a "flat" trace. Electrodes were checked for proper balance after impalement by again passing current pulses and adjusted if necessary. As the time constant of the microelectrodes was considerably shorter than the time constant of the membrane, balancing could be achieved by eliminating any high frequency potential shifts during the first and last millisecond of the pulse.

Impalement of LC neurons was achieved under microscopic observation (X 640) that allowed viewing of individual neurons. Often current pulses were passed on approach to the cell to detect an apparent increase in resistance as the electrode contacted the cell membrane. Impalement was often achieved by "ringing" the capacitance compensation: i.e. causing a high frequency oscillation by overcompensating for the electrode's capacitance. Once in the cell, membrane potential was usually stabilized by injecting hyperpolarizing current of less than 0.4 nA.

Recordings were monitored on a storage oscilloscope, recorded on a Gould Brush 2400 chart recorder and taped on a Racal tape recorder for further analysis. Stable impalements were used only if the apparent membrane potential was greater than -50 mV and the spike amplitude either evoked or spontaneous was greater than 55 mV.

Data was also analyzed using a Nicolet model 1200 signal averager or a Tracor Northern averager.

III) Iontophoresis

Iontophoresis electrodes were pulled on a Stoelting vertical puller from four-barrel blanks (Fredreick-Haer 30-32-4). Iontophoresis electrodes were broken back to an outside diameter of about 6 μ m by bumping the tip of the electrode on a glass rod, under (100 X) microscopic observation. Barrels of the electrode were then filled with drug solutions and one barrel was filled with 1M NaCl. Drug solutions used were:

Drug	Conc.	pH	
Noradrenaline (Arterenol-free base, Sigma)	0.2M	5.5	(0.667g NA + 1.5ml H ₂ O + 0.36 ml 1M HCl & titrated to pH 5.5 with 0.1M HCl)
Methoxamine (HCl, Burroughs-Wellcome)	50mM	5.0-5.5	(24.8 mg Meth. added to 2 ml H ₂ O)
Phenylephrine (HCl, Sigma)	50mM	5.0-5.5	(20.4 mg Phe. added to 2 ml H ₂ O)
Clonidine (HCl, Sigma)	50mM	5.0-5.5	(26.7 mg Clon. added to 2 ml H ₂ O)

In iontophoresis (or ionophoresis), substances are retained in and ejected from a pipette with constant current. Ejection of substances was dependent on many factors including size of tip, duration and amplitude of ejection current, duration and amplitude of retention current, and mobility of ion ejected. Barrels containing NA solutions typically had resistances of 30-70 M Ω . Iontophoresis was controlled using a Dagan 6400 microiontophoresis current generator.

We have used iontophoresis solutions of pH 5 to 5.5 to minimize effects of hydrogen ion iontophoresis. Iontophoresis of hydrogen ions have been reported to have direct effects on spinal motoneurons (Marshall and Engberg, 1979). The pH of

iontophoresed noradrenaline solutions has also been reported to affect responses in cortical neurons, with acidic pH's giving spurious results (Fredrickson, et.al., 1971). The extracellular concentration of noradrenaline during iontophoresis has been measured by voltammetry (Armstrong-James, et al., 1980). Measured levels of noradrenaline were complexly dependent on duration and time of ejection, and presumably some of this dependence reflects the rate and, possibly the saturation of noradrenergic uptake and degradation processes.

IV) Electrical stimulation

Stimulating electrodes were fashioned from platinum wire. The tip of the wire was positioned in a solution of 50% nitric acid and 50% sulphuric acid and etched using an AC voltage source of 0-12 V.. After a fine tip was formed, the electrode was insulated to within 40 μ m of the tip. Stimulating electrodes were manipulated into the outgrowth of LC neuronal cultures using a hydraulic manipulator. Constant current pulses were passed through the electrode using a Grass S88 stimulator connected to a Grass (CCU 1 A) constant current generator.

V) Intracellular staining with lucifer yellow

Techniques for the intracellular staining of neurons with lucifer yellow have been described by Stewart (1978). For intracellular injection of lucifer yellow, the electrodes were filled with a 4% lucifer yellow - 1M LiCl solution. Lucifer

yellow was injected into neurons with either a steady hyperpolarizing current or hyperpolarizing current pulses of less than 1.0 nA. After a short incubation period following injection of lucifer yellow, tissue cultures were fixed in a 4% formaldehyde 0.1 M sodium phosphate buffer (see Appendix 2) for at least 2 days. Fixation for shorter periods resulted in high background fluorescence, possibly due to an increase in autofluorescence or due to the leakage and spread of dye from the stained cell. After fixation, tissue was dehydrated in an alcohol series from 50% alcohol to absolute alcohol. Cultures were then cleared in methyl salicylate, and mounted by sealing the culture in paraffin oil (with low autofluorescence) on a slide with nail polish applied along the edges of the coverslip. Cultures were then examined as wholemounts on a Zeiss epifluorescence microscope using an FITC filter set (see Appendix 3).

VI) Glyoxylic Acid Staining Method for Catecholamine Fluorescence

Tissue cultures were routinely stained for catecholamine fluorescence after experiments on the effects of noradrenaline on LC neurons. Tissue was stained for catecholamine fluorescence using a modification of the Bloom and Battenberg (1976) method. Tissue cultures were transferred to a 2% glyoxylic acid, 0.15 M phosphate buffered saline (see appendix 2) at 2-4°C for ten minutes. The tissue was dried in a stream of warm air (30-37°C). The glyoxylic reaction was then completed by heating the tissue in an oven at 100°C for 10

minutes. The tissue on the collagen-coated coverslip was then sealed in paraffin oil onto a microscope slide with nail polish. Tissue was observed with a Zeiss epifluorescence microscope, using a Zeiss 487703 filter set, which has a BP 405/8 excitation filter and a LP 435 barrier filter (for spectra see Appendix 2). Stained tissue was stored in a freezer for further observation.

VII) Thionine staining

Tissue cultures were Nissl stained after most investigations on bursting activity. Tissue was submerged in 80% alcohol for at least 2 days. The tissue was then placed in a 1% thionine solution (containing 1 μ l of acetic acid / 10 ml of solution) for 1 minute, then dehydrated in 95% ethanol and absolute alcohol. Tissue was then cleared in xylene and mounted using Permount.

RESULTS

I) IDENTIFICATION AND MORPHOLOGY OF LOCUS COERULEUS NEURONS IN TISSUE CULTURE

Locus coeruleus neurons can be identified in the living state as described by Hendelman et al. (1982). In living cultures, LC neurons form a compact group of neurons which have large nuclei with single prominent nucleoli (see Fig. 3). These neurons also often have refractile cytoplasmic inclusions and are found adjacent to the ciliated ependymal cells from the fourth ventricle. These neurons are usually 25-30 μm in size, and myelinated axons are usually not found in this region.

The presence of catecholamine neurons in LC explants was routinely verified in experiments on adrenergic responses, by staining with glyoxylic acid. Under fluorescence microscopy, explants containing LC neurons (identified in the living state) consistently had catecholamine fluorescent fibers radiating out into the outgrowth from the explant (see Fig. 4). These fine fibers had prominent varicosities and if the co-explanted cerebellar piece was within 0.5-1.0 mm, these fibers often ramified within this tissue. In explants where the LC area was thin, faintly fluorescent cell bodies could be observed, however, when the LC neuronal group was thicker, only a bright catecholamine fluorescent mass could be observed.

Locus coeruleus neurons were also examined after intracellular injection of lucifer yellow (LY). Injection of LY was achieved by passing constant hyperpolarizing current

Fig. 3:

Photomicrographs of LC neurons in tissue culture viewed in the living state.

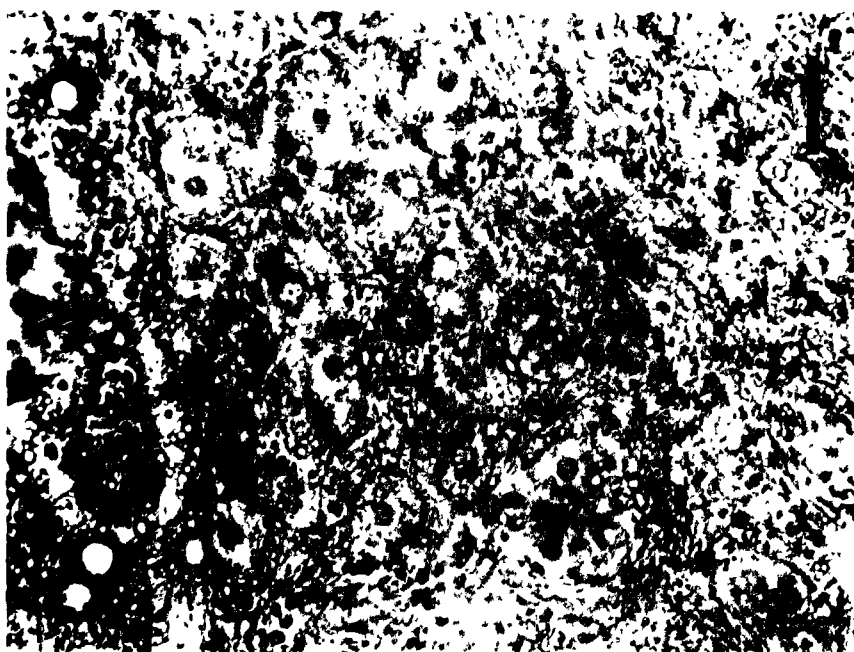
a: a compact group of LC neurons (LC), which are adjacent to the ciliated fourth ventricle ependymal cells (V).

b: LC neurons shown at a higher magnification to illustrate the single prominent nucleolus (n) within the nucleus (N).

Calibration bars: 20 μ m.



b



a

Fig. 4:

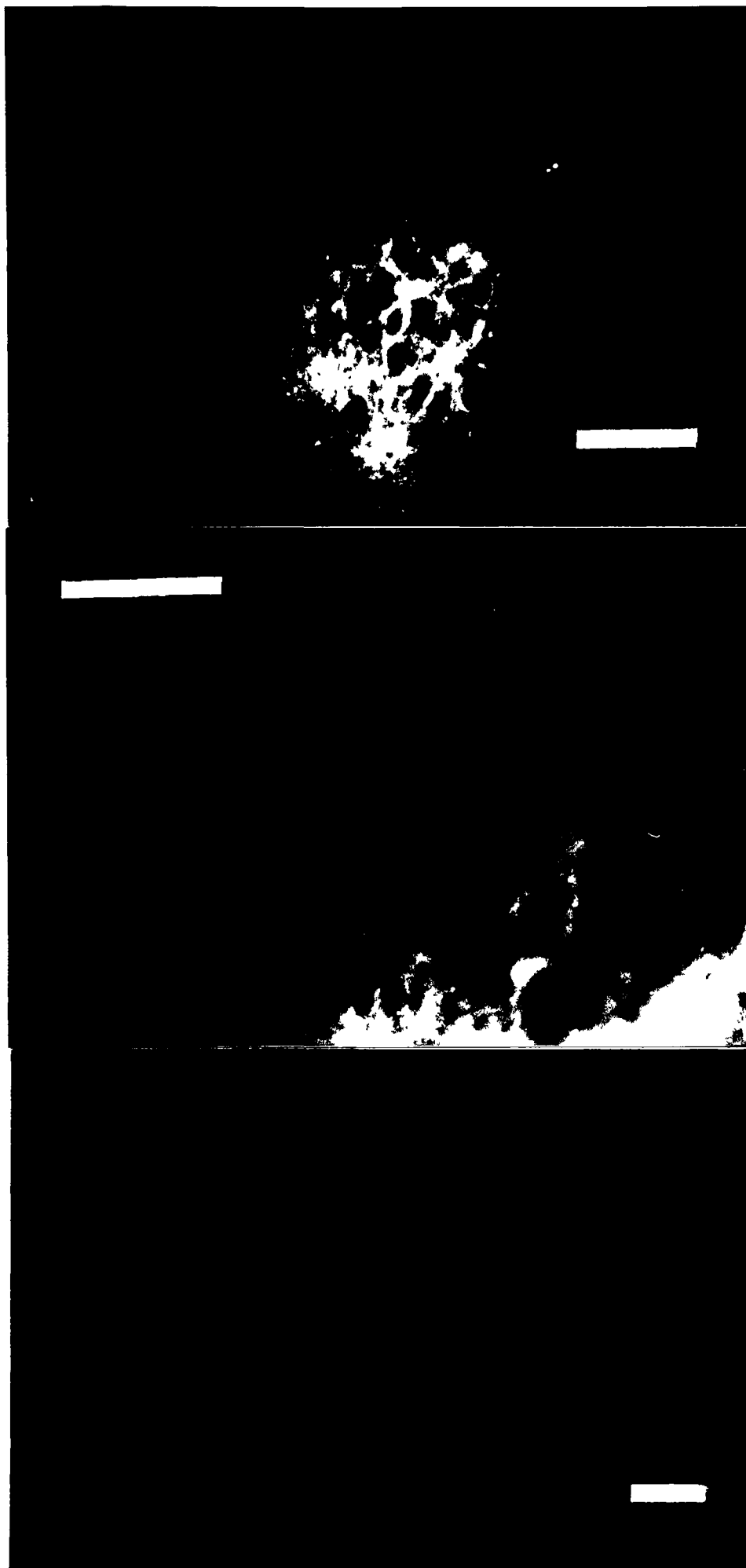
Catecholamine fluorescence of an explant culture containing LC neurons stained using the glyoxylic acid method.

Top - LC neurons form a compact group and fine catecholamine fluorescent fibers radiate out from the explant.

Middle - higher magnification of LC area showing faintly fluorescent cell bodies surrounded by a brightly fluorescent neuropil.

Bottom - Fine catecholamine fluorescent fibers in the outgrowth of the explant exhibiting varicosities (Epifluorescence was viewed with an additional barrier filter in this case).

Calibration bars: top - 100 μm ; middle - 50 μm ;
bottom - 10 μm .



and hyperpolarizing current pulses of less than 1.0 nA upon impalement or in a few cases by only passing hyperpolarizing current pulses (1.0 Hz, 100ms) of less than 1.0 nA. Successful fills were achieved when the cell was stable for at least 15 minutes during injection and exhibited spontaneous or evoked spikes of at least 50 mV. Stained neurons were examined in whole mounts of explants.

LC neurons injected with lucifer yellow were classified as either multipolar (n=18) or bipolar (fusiform)(n=7)(see Fig. 5), based on the soma morphology and origin of dendrites. Neurons were classified as bipolar, if their somas had a major and a minor axis and the dendrites originated only at the two poles of the major axis. Other neurons were classified as multipolar and usually had spherical or kidney-shaped somas. The multipolar cells had 3 to 5 dendrites, whereas the bipolar cells had 2 to 4 dendrites emanating from the two poles of the cell. Dendrites extended for up to 300 μm from the cell body. Lucifer yellow stained cells had both dendritic and somatic spines, and the dendritic spines were very long (up to 10 μm) and delicate, and often were highly branched. Somatic spines were generally shorter, though long branching spines were also observed.

In addition, LC neurons often had multiple axon-like processes. Axon-like processes were long ($>50 \mu\text{m}$), thin ($<< 1 \mu\text{m}$) and smooth (i.e. devoid of spines) processes, and varicosities could often be detected along their distal aspects (Fig. 6). In thin cultures, axon-like processes and

Fig. 5:

Fluorescence of lucifer yellow injected LC neurons.

a. a multipolar neuron;

b. a bipolar neuron exhibiting two axon-like processes (arrows) with separate origins; long fine spines are seen on the dendrites;

c. in addition to injected multipolar LC neuron, two faintly stained cells (arrows) that were not injected are labelled; bright spots at bottom of explant are non-specific autofluorescence.

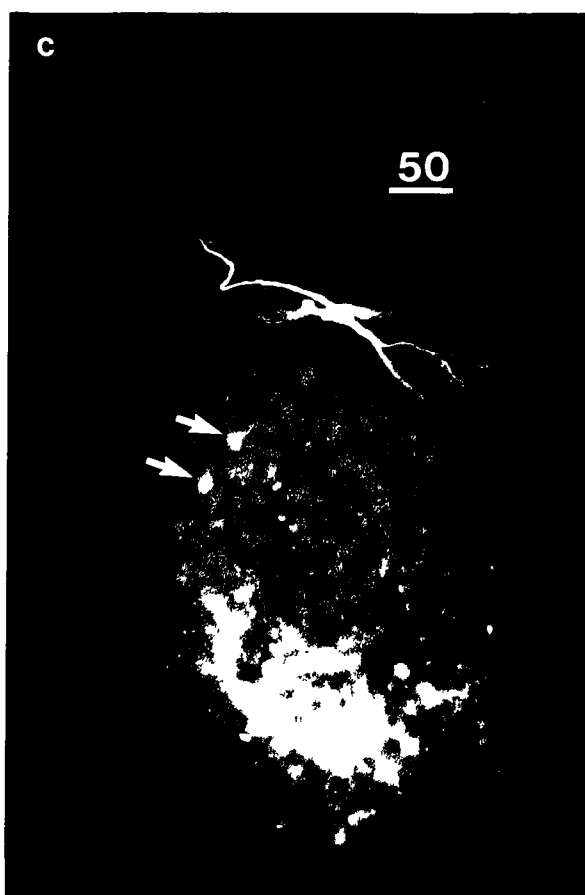
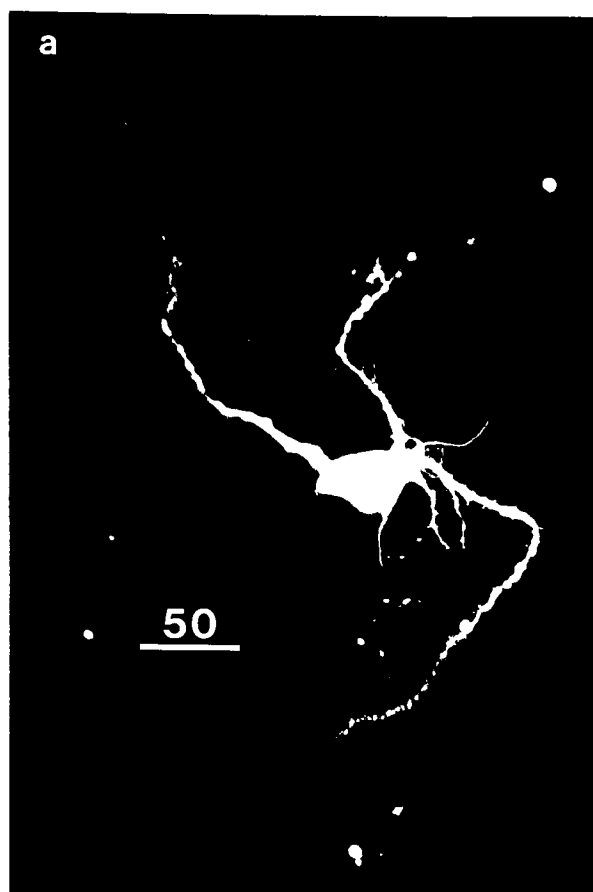
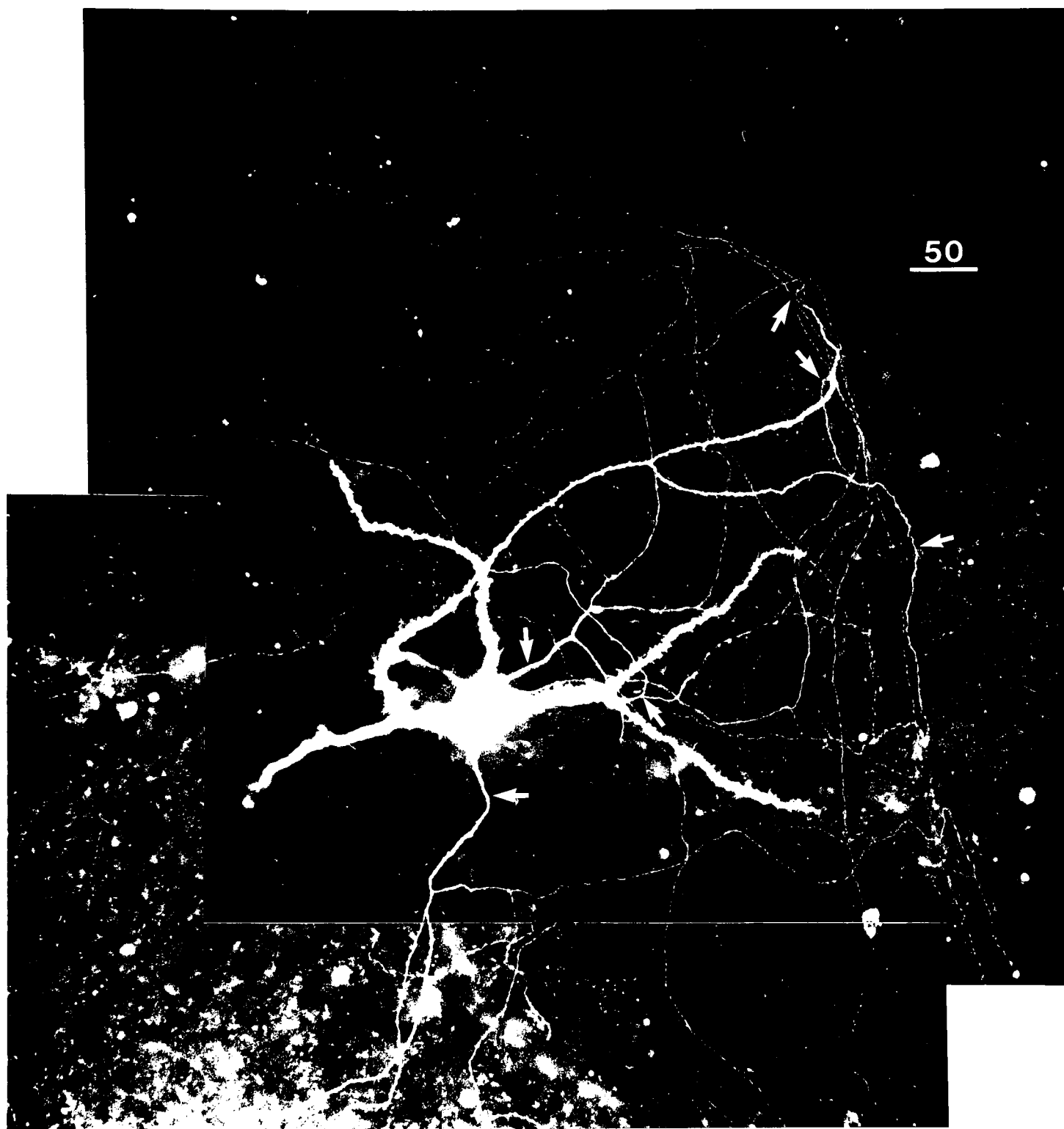


Fig. 6:

Lucifer yellow fluorescence in a multipolar LC neuron. This neuron has at least five axon-like processes with separate origins (arrows). Axon-like processes have prominent varicosities (top and right of photograph). In addition, two axon-like processes originate from the soma, one from a proximal dendrites and others from distal dendrites (e.g. processes originating at top right).



their origins could be clearly discerned. In such explants, it was observed that bipolar cells had one (n=1), two (n=3), or three (n=1) axon-like processes, whereas the multipolar LC neurons had one (n=3), two (n=2), three (n=4) or four (n=1) axon-like processes, with separate origins. Therefore, 4 of 5 bipolar and 7 of 10 multipolar LC neurons had more than one axon-like process. Axon-like processes originated at the cell soma, proximal dendrites or from distal dendrites. Furthermore, axon-like processes could be traced throughout the explant and often far into the outgrowth and often had distinct varicosities.

Thionine stained cultures were observed in whole mounts of cultures. LC neurons formed compact groups of neurons. In cultures with relatively few neurons, individual cells could be resolved and the majority of cells were medium sized (14 X 18 μm to 22 X 32 μm). There were approximately twice as many multipolar cells as there were bipolar cell bodies (cells that were twice as long in one axis as the other). However in addition to the larger LC neurons, smaller neurons (approx. 6-8 μm X 10-15 μm) were often observed in the LC area. In addition, semi-thin (0.5 - 1.0 μm) sections (provided by Dr. W.J. Hendelman) were examined. For semi-thin sections, cultures were pretreated with 5-OHDA, fixed in 1% paraformaldehyde/ 1.4% glutaraldehyde, postfixed in 2% osmium and embedded in araldite for sectioning. In semi-thin sections, small cells could be clearly observed in the LC group and often in the immediate surrounding (Fig. 7). It also

Fig. 7:

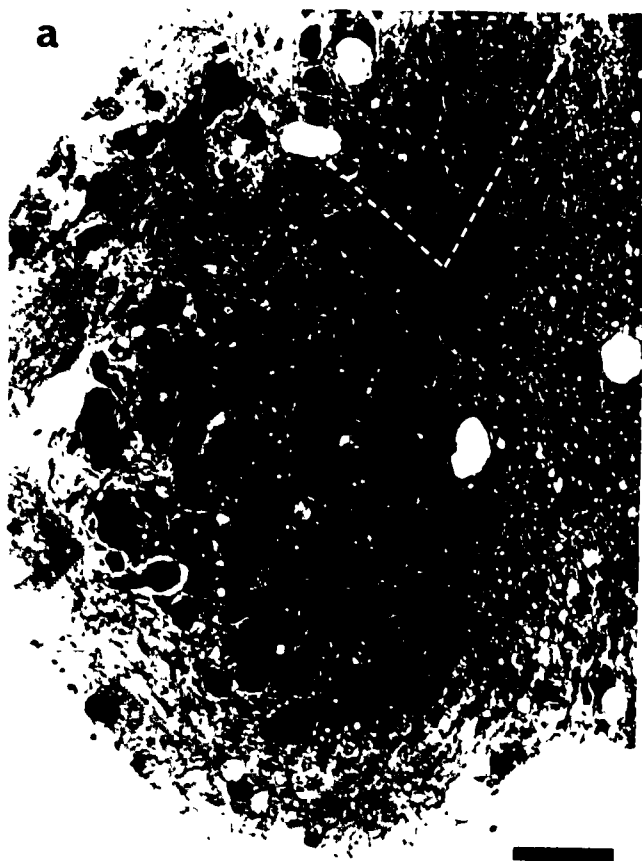
Stained sections and wholemounts of LC explant cultures.

a. semi-thin section of LC explant stained with toluidine blue; area surrounded by white dashed line contains neurons that are smaller than LC neurons found below this group;

b. serial semi-thin section of same LC explant in a., indicating smaller neurons (arrows) within the LC group;

c. higher magnification of small cells indicated in b.;

d. wholemount of LC explant stained with thionin. (Thin sections were generously provided by Dr. W.J.Hendelman).



d

often appeared that these neurons unlike LC neurons had paired or lobed nucleoli.

II) SPONTANEOUS AND EVOKED ACTIVITY OF CULTURED LOCUS COERULEUS NEURONS

1) Properties of Intracellularly Recorded Cultured LC Neurons

LC neurons in explant cultures maintained in vitro for 16 to 36 days were used for recordings. Locus coeruleus neurons were impaled under microscopic visual observation. Apparent resting membrane potentials were in the range of -50 mV to -75 mV, and were usually around -60 mV. Action potentials occurred spontaneously or could be evoked with depolarizing current pulses. Spike amplitudes ranged from 55 mV to 100 mV, and spikes were further examined from tape recordings of 44 neurons. Characterization of spike amplitude and duration was divided into groups on the basis of spike amplitude (with no hyperpolarizing current applied):

Table II: Amplitude and Duration of Action Potentials

Spike amplitude	Mean amplitude ($\pm$ SD)	Mean duration ($\pm$ SD)	n=
< 70 mV	63 $\pm$ 4.0 mV	1.45 $\pm$ 0.3 ms	12
70 - 79 mV	73 $\pm$ 1.0 mV	1.52 $\pm$ 0.2 ms	14
80 - 89 mV	82 $\pm$ 2.3 mV	1.3 $\pm$ 0.2 ms	14
90 - 100 mV	95 $\pm$ 5.7 mV	1.2 $\pm$ 0.2 ms	4
The mean duration of action potentials was 1.4 ms, with a range of 0.9 to 1.8 ms.			

Action potentials, when viewed at a high sweep speed, often had a small shoulder during the repolarizing phase (Fig. 8). This shoulder, which was reflected as a decrease in the rate of fall of the spike, was easily observed in recordings with spikes greater than 70 mV, but usually could not be

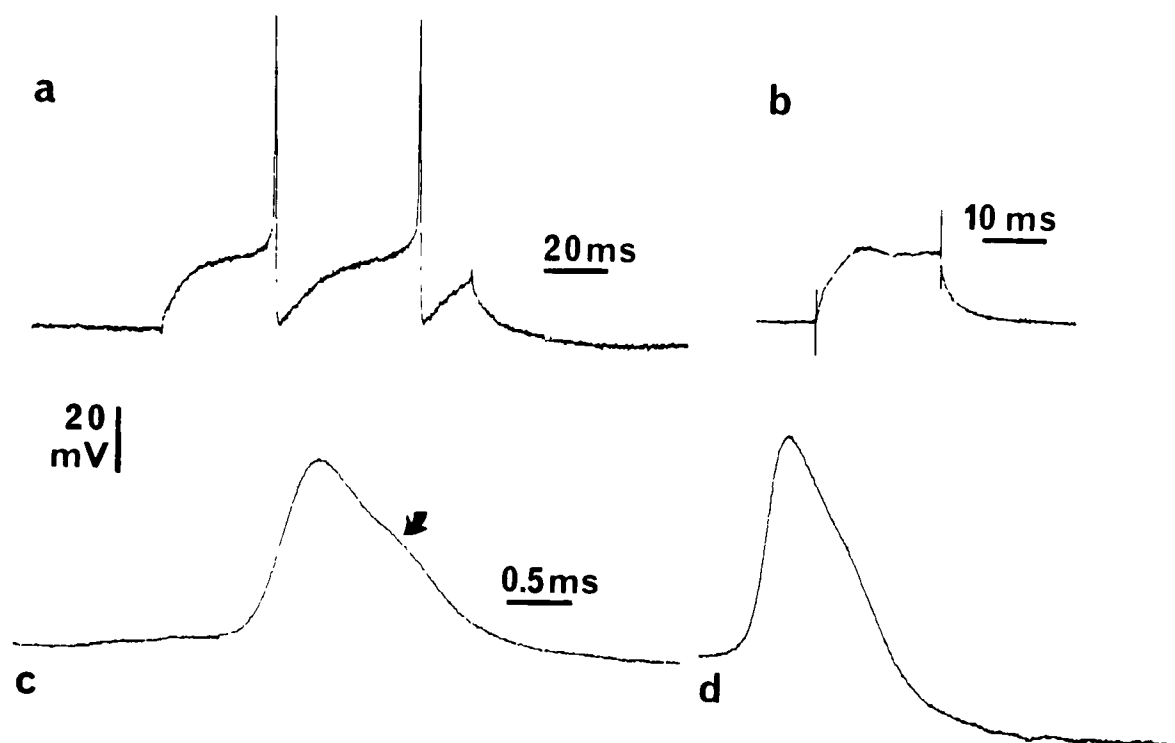


Fig. 8:

Intracellular recordings of LC neurons in culture. Output from digital oscilloscope is shown.

a. spikes evoked by a depolarizing current pulse demonstrating the short afterhyperpolarization (in this case lasting approximately 25 - 30 ms) following single spikes;

b. depolarizing pulse during TTX intoxication evokes a small phasic depolarization;

c. a spike observed during a burst of activity, spike amplitude was 80 mV above resting membrane potential and this spike has a prominent shoulder (arrow) during the repolarizing phase. This example shows the largest shoulder observed in tissue cultured LC neurons.

d. a more typical spike with a shoulder on the repolarizing phase.

detected in cells with spikes of less than 70 mV. Spikes were always followed by an afterhyperpolarization of 5-15 mV that lasted approximately 20 - 50 milliseconds (Fig. 8).

Locus coeruleus neurons could also be stimulated to fire with depolarizing current pulses. With strong (0.5-1.0nA, 100ms) depolarizing current pulses, LC neurons could be activated to fire repetitively, and this firing accommodated during the pulse. With short depolarizing current pulses that evoked at least two spikes, a long-lasting hyperpolarization (lasting up to 500 ms) followed the depolarizing pulse (Fig. 9).

During perfusion with tetrodotoxin (TTX), all spontaneous and tonic activity ceased. LC neurons usually hyperpolarized by up to 5 mV, during TTX intoxication. Depolarizing current pulses did not evoke spiking during TTX intoxication, but there was an (apparently) active response evoked with strong depolarizing current indicated by a small phasic depolarization during the initial 5 - 10 ms of the pulse (Fig. 8). During TTX intoxication, input resistance measurements in 7 cells was $92.7 \pm 19.8 \text{ M}\Omega$ ($\pm$ standard deviation).

Perfusates with varying contents of divalent cations were also used to examine the effects of blocking synaptic transmission. When the perfusate contained no calcium and 20 mM magnesium (Mg), LC neurons depolarized 5 to 15 mV (n=11). With normal BSS containing 12 mM Mg, LC neurons also depolarized slightly (4-10 mV)(n=6). During perfusion with 0.5

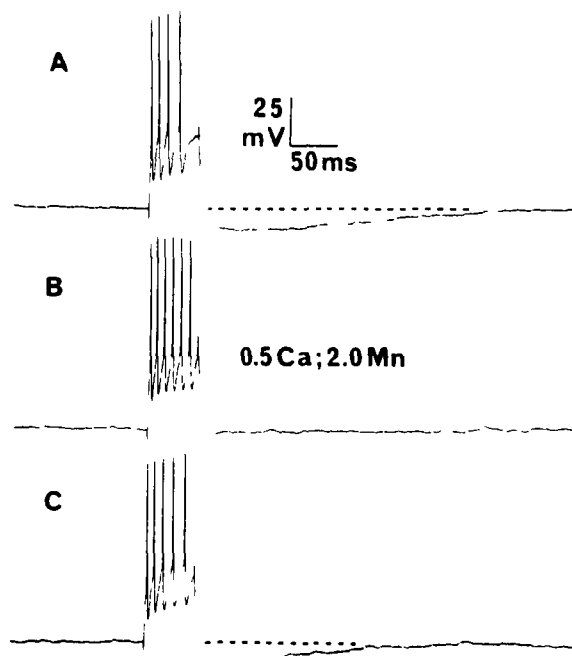


Fig. 9:

Afterhyperpolarizations following bursts of firing activated by depolarizing current pulses.

A. Afterhyperpolarization following burst of firing lasts approximately 300 ms.

B. The long afterhyperpolarization is absent during perfusion with 0.5 mM Ca and 2.0 mM Mn BSS, but the short afterhyperpolarization following single spikes during the current pulse are still present.

C. Long afterhyperpolarization returns during perfusion with normal BSS.

mM calcium and 3 or 4 mM manganese (Mn) containing BSS (n=14), cells depolarized by 5 to 15 mV and the cell membrane became unstable as the membrane potential continually fluctuated. This fluctuation appeared as an increase in "noise" on the chart recording. Finally, in perfusates containing 0.5 mM Ca and 2.0 mM Mn, 10 of 12 LC neurons tested, hyperpolarized slightly then depolarized by less than 5 mV; the other two cells depolarized by less than 5 mV. Spontaneous spiking and bursting activity and excitatory synaptic potentials (epsp's; see below) were absent during the perfusion of any of these modified BSS's, but spiking activity could be evoked.

The afterhyperpolarization following single spikes remained during perfusion of these BSS's. However, the long afterhyperpolarization following multiple evoked spikes was lost during perfusion with low calcium/high magnesium (n=3) or with low calcium/2.0 mM Mn (n=3) BSS's (Fig. 9). The effect of the other perfusates on the long afterhyperpolarization was not tested.

2) Spontaneous Activity of Cultured LC Neurons

Spontaneous activity was usually observed for a short period (5 to 15 min) before drugs were applied or other manipulations were made. The majority of recorded neurons were predominantly quiescent after impalements stabilized and in less than 10% of recorded neurons, cells fired tonically. Although approximately one third of recorded neurons exhibited occasional bursts of activity in this initial period, during overall recording periods (i.e. including during drug

applications and stimulation experiments) more than one half of recorded neurons exhibited spontaneous bursting activity. The duration of bursts of activity ranged from tens of milliseconds to tens of seconds, and bursts occurred sporadically or rhythmically (Fig. 10). Bursts of activity occurred at rates from several bursts per minute to 1 burst every 20 to 30 minutes. Although the rates of bursting were highly variable, the patterns of activity recorded in different LC neurons from the same explant were usually very similar. Furthermore, bursting activity was recorded in LC neurons in explant cultures of all ages (16 to 36 days in vitro).

Barrages of depolarizing events appeared to initiate and continue throughout bursts of activity and a depolarization of 5-15 mV was maintained during bursts of activity (Fig. 10). Individual depolarizing events that were similar in appearance to epsp's were also observed. These spontaneous potentials occurred at a high tonic rate in a few cells, but usually occurred phasically. Barrages of such depolarizations often summated to produce a depolarization that did not evoke spiking activity but had a time course and pattern similar to the depolarizations associated with bursting activity. Spontaneous bursts of activity were usually not followed by an afterhyperpolarization, although afterhyperpolarizations followed individual spikes. In a few cells a long-lasting afterhyperpolarization following bursts was noted. Spontaneous hyperpolarizing potentials (ipsp's) were not observed in the

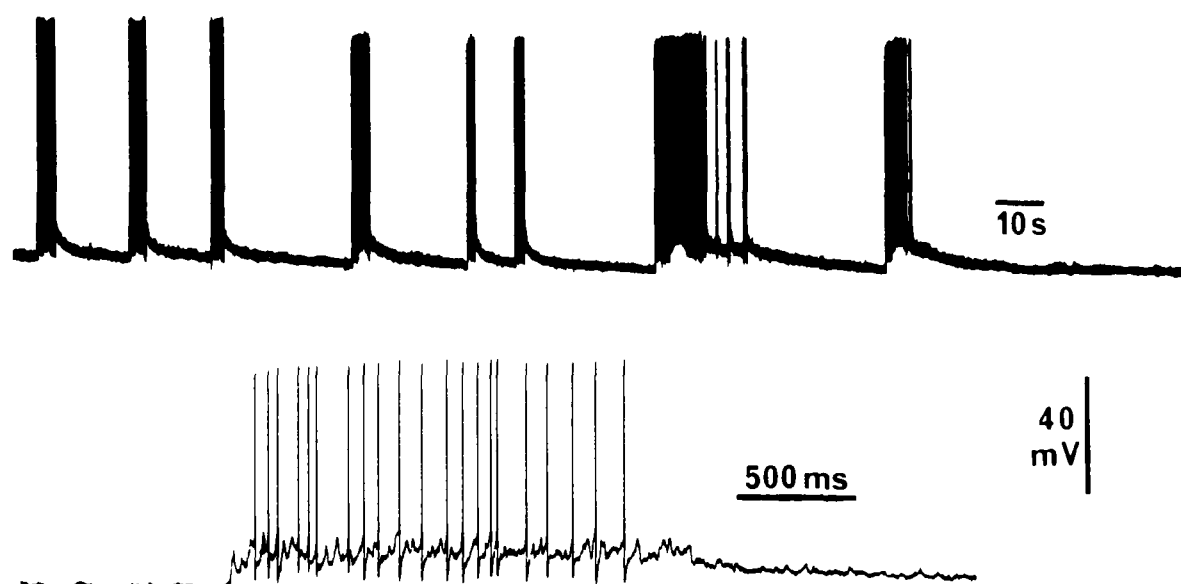


Fig. 10:

Spontaneous rhythmic bursting activity observed in a cultured LC neuron. Bursts of activity are associated with depolarizing potentials, that initiate and continue throughout bursts of activity (lower trace). Note that spontaneous bursts of activity are not followed by a long afterhyperpolarization, though individual spikes are followed by an afterhyperpolarization.

LC neurons recorded in these experiments.

3) Simultaneous Recordings of Pairs of LC neurons

Simultaneous recordings of LC neurons were undertaken to investigate bursting activity, to determine the synchrony of bursts and to test for interactions between LC neurons. Recorded pairs of neurons were within 100 μm of each other. Recordings were used only if both cells exhibited spikes greater than 55 mV and had apparent resting membrane potentials of at least -50 mV. In simultaneous intracellular recordings of pairs of LC neurons, spontaneous bursts of firing activity in one cell were always synchronized with bursts of activity or barrages of depolarizing events in the other cell. Barrages of depolarizations also always occurred synchronously in the two recorded cells (n=13 pairs, see Fig. 11). Synchronous activity was associated with depolarizations and barrages of depolarizing events that had similar time courses in both impaled cells. Furthermore, individual depolarizing events often occurred synchronously in both cells. On the other hand, tonic spiking activity was not synchronized (n=2). In three other pairs of LC neurons no spontaneous activity was recorded.

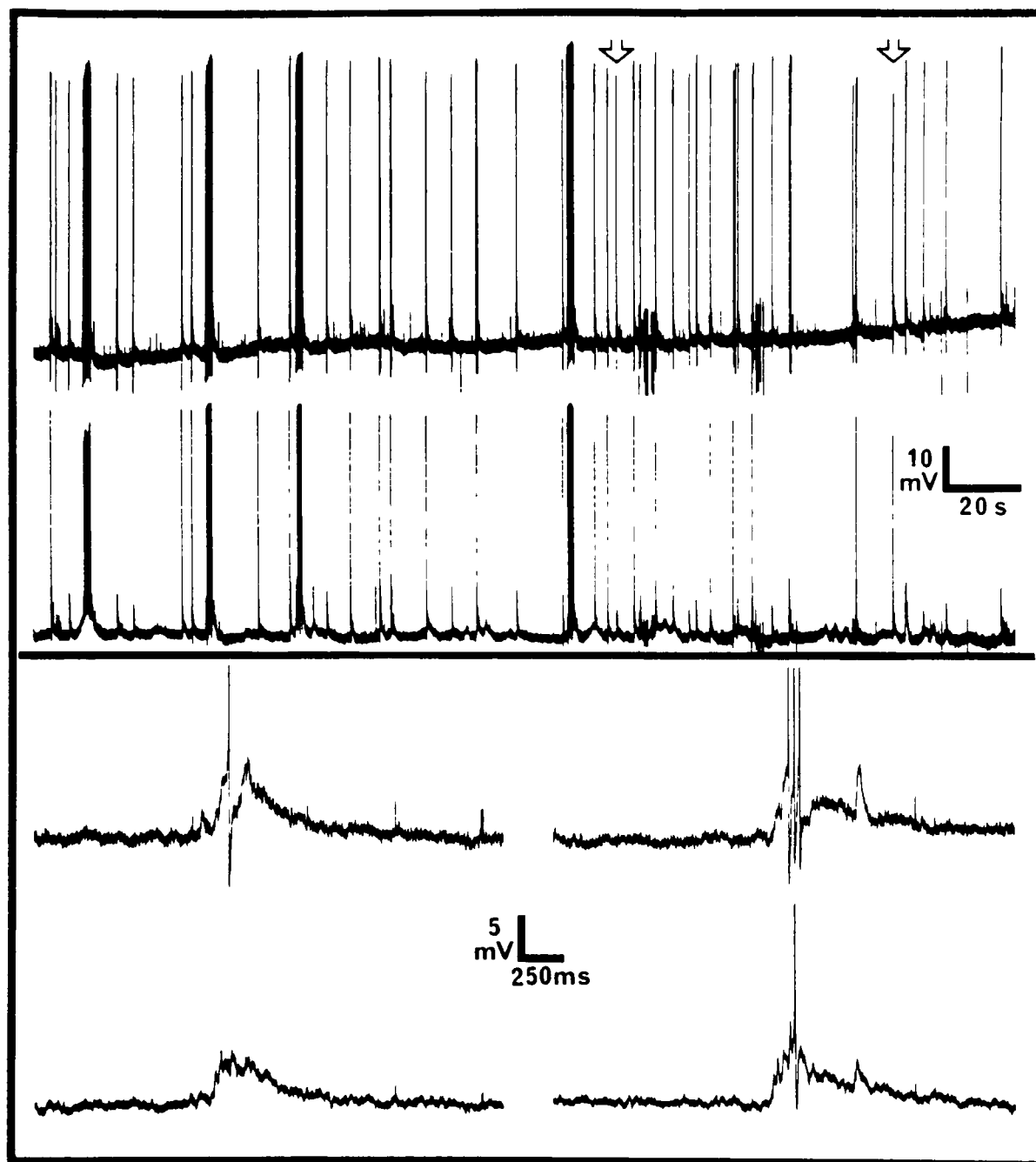
4) Tests for Electrical Coupling

Simultaneous intracellular recordings of pairs of LC neurons were also used to test for electrical coupling between LC neurons by passing current pulses into one cell and monitoring the other cell for any responses. No apparent spread of current from one cell to the other was apparent when

Fig. 11:

Simultaneous intracellular recordings of two LC neurons demonstrating the synchrony of bursting activity.

Synchrony of activity can easily be observed in top trace, especially during strong activation. Synchrony of bursts of activity and barrages of depolarizing events is demonstrated in lower tracings, which are expanded tracings of activity in the top trace at the arrows. Note the synchrony of the late depolarizing potentials in the two recorded neurons shown in the bottom right traces.



hyperpolarizing or depolarizing current was applied to either neuron (n=13 pairs).

If electrotonic coupling occurs at sites remote from the site of recordings (which is assumed to be the cell somas), any passive electrotonically conducted potentials might be highly attenuated. We therefore averaged our data where possible. Even after the data was averaged, hyperpolarizing or depolarizing current pulses to either cell that did not activate spiking activity had no detectable effect on the second recorded cell (n=10 pairs). In six pairs, when depolarizing current pulses evoking spikes were applied to either cell, no interactions were revealed by signal averaging. However, some interaction was apparent in 3 pairs when one cell was depolarized past threshold. In these cases, small (<300 μ V) depolarizing potentials, which were synchronized with the after-hyperpolarization(s) in the stimulated cell (see Fig. 12) were revealed by averaging. When tested in one case, these interactions were bidirectional, and in one case, hyperpolarizing the monitored cell had no apparent effect on the small depolarizing events.

Electrical coupling was also tested by intracellularly injecting the low molecular weight dye lucifer yellow, which has been reported to pass between electrically-coupled neurons (Stewart, 1978). Of twenty-six well stained LC neurons, in only one case was any dye transfer apparent (Fig. 5). In this case, two faintly stained adjacent neurons were found approximately 300 μ m from the injected LC neuron. These secondarily stained

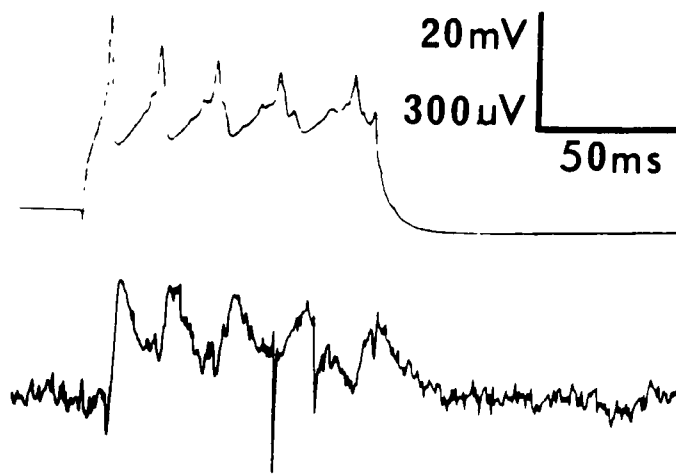


Fig. 12:

Signal averages of 64 traces from two simultaneously recorded LC cells; top: LC neuron stimulated with depolarizing current pulses to give repetitive spikes (note apparent amplitude of spike decreased due to averaging); bottom: small depolarizations observed in second cell that occur during the afterhyperpolarization in the stimulated cell.

cells had no apparent dendritic or somatic contacts with the lucifer yellow injected LC neuron. However, the injected neuron had an axonal process with varicosities that surrounded the secondarily stained neurons, suggesting a transynaptic passage of the dye. Therefore, there was no indication of dye-coupling between LC neurons.

5) Properties of Evoked and Spontaneous Depolarizations

Electrical stimulation of the explant outgrowth with constant current pulses (0.15 ms, 20-100 μ A) through a platinum electrode could evoke two types of depolarizing potentials (Fig. 13). The first type of potential had a very fast rise time (Fig. 13B11) and often evoked a spike in the recorded cell. This evoked potential appeared to be due to the direct stimulation of a process of the recorded neuron since: 1) these potentials could be evoked during perfusion with a synaptic transmission blocking BSS (and therefore were not dendritic calcium spikes) (n=4) and 2) spikes in the recorded cell evoked by depolarizing current pulses collided with this depolarizing potential. The other evoked potentials had a slower rise time and were blocked by perfusates that block synaptic transmission (n=8), and will be referred to as evoked slow depolarizations. Electrical stimulation with single pulses did not evoke any hyperpolarizing responses except those following spike activation (AHP).

Spontaneous depolarizations were similar to the evoked slow depolarizations. Spontaneous depolarizations and evoked

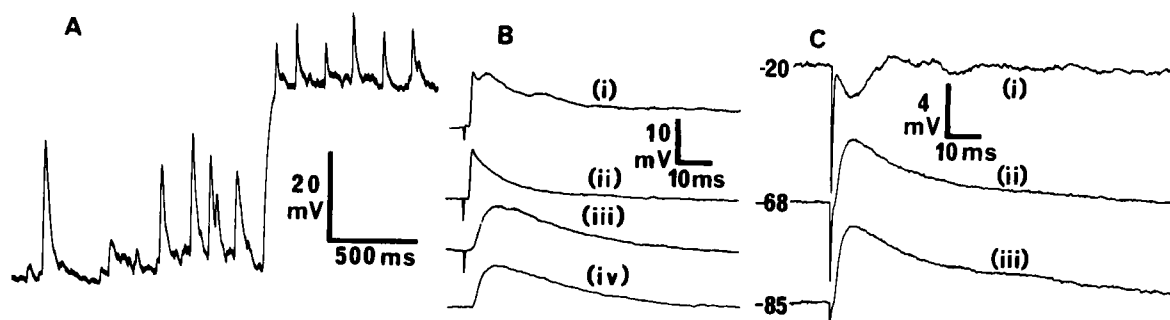


Fig. 13:

Spontaneous and evoked depolarizations in LC neurons.

A. spontaneous depolarizations are considerably larger when the cell is hyperpolarized (beginning of trace) than when it is at resting membrane potential (end of trace).

B. i) complex potential evoked by electrical stimulation of explant which consists of ii) an evoked potential with a fast rise time, and iii) an evoked slow depolarization with a slower rise time and longer duration. The latter evoked potentials are similar to spontaneous depolarizing potentials (iv) recorded in the same cell.

C. Averaged traces of an evoked slow-type depolarization which is increased when the cell is hyperpolarized (iii) compared to at rest (ii) and is reversed during depolarization that inactivated spiking activity (i).

slow depolarizations had similar rise and fall times (Fig. 13Biii & iv), and both were blocked when synaptic transmission was blocked and both are affected similarly by polarizing the cell. As previously noted, spontaneous depolarizations and bursting activity were absent during the presence of synaptic transmission blocking perfusates. However, the threshold for spike generation (as determined by passing depolarizing current pulses) increased, even when the cells depolarized in the following perfusates: low Ca/high Mg, normal BSS with high Mg and low Ca/3 or 4 mM Mn. During perfusion with 0.5 Ca, 2 mM Mn BSS, bursting activity and evoked slow depolarizations were blocked even when the excitability of the recorded neurons increased (Fig. 14). Therefore, the spontaneous and evoked slow depolarizations and bursting activity appear to depend on synaptic transmission.

Electrical stimulation of the outgrowth occasionally could evoke bursting activity in regular perfusates. In a few explants, when the stimulating electrode was placed near apparently smaller cells, bursts could also be activated with single stimuli. Such evoked bursts could not be evoked in the presence of 0.5 mM Ca, 2 mM Mn perfusates, even when the stimulating current was tripled, resulting in direct activation of the recorded cells. Threshold of the cells during the same period only increased slightly (less than 5 mV's).

The dependence of the evoked and spontaneous depolarizations on membrane potential was also tested.

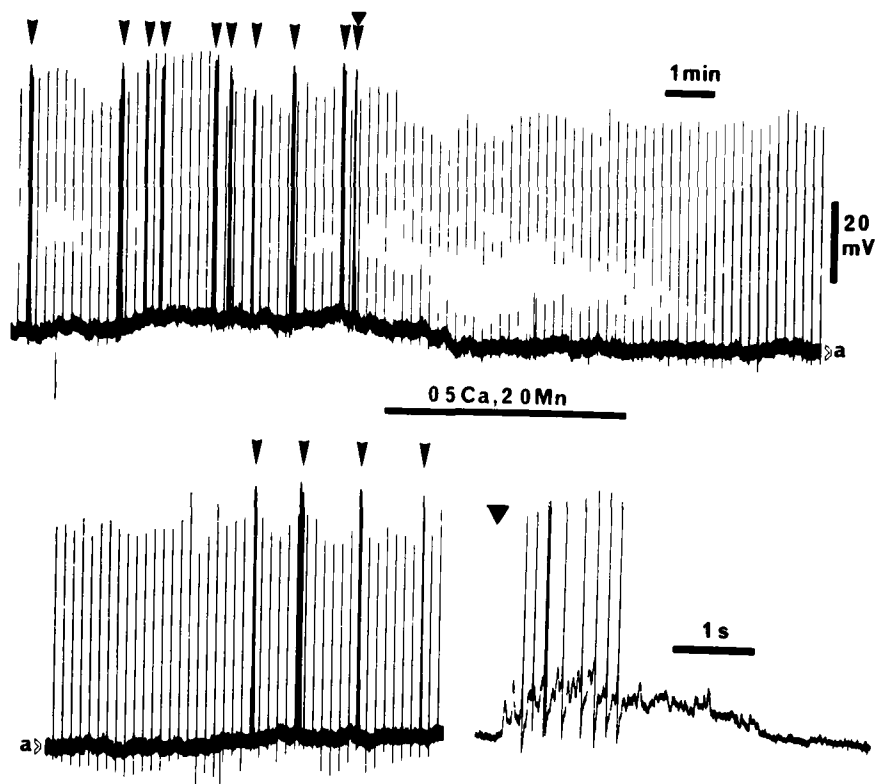


Fig. 14:

Effect of modified perfusates on bursting activity (bursts are indicated by arrowheads; burst with double arrowhead is shown at a faster sweep speed at the lower right). Spontaneous bursting activity ceases during perfusion of 0.5 mM Ca/2.0 mM Mn BSS, whereas excitability (tested using depolarizing current pulses - upward deflections) remained constant or increased slightly. Bursting activity resumed after washout of modified BSS (lower left - continuation of upper trace).

Hyperpolarization of cells resulted in an increase in the amplitude of spontaneous depolarizing potentials (n=5) (see Fig. 13A) and evoked slow depolarizations (n=5). Depolarization of LC neurons resulted in a decrease in the amplitude of both spontaneous potentials and evoked slow depolarization's. In two cases, with strong depolarizing current applied to cells (depolarizing cells to between -30 and +30 mV) the evoked slow depolarization's could be reversed (Fig. 13C). The directly evoked potentials, however could be abolished by strong hyperpolarizing current and were not significantly affected by other manipulations of membrane potential.

Therefore it appears that spontaneous and evoked slow depolarizing potentials have very similar characteristics and are excitatory synaptic potentials (epsp's), as they are blocked in perfusates that block synaptic transmission (even when cell excitability increases), and their dependence on membrane potential is that expected of a synaptic event that increases membrane conductance.

III) Adrenergic Responses in Cultured LC Neurons

1) Responses to Iontophoretically Applied Noradrenaline

For iontophoretic application of noradrenaline (NA), after a stable intracellular recording was achieved, a four barrelled iontophoresis electrode was moved under microscopic observation close to the recorded cell. Noradrenaline was iontophoresed as a cation with iontophoretic currents of 5 to 195 nA of current. Iontophoretic application of NA generally was most effective when NA was ejected for periods of 10 to 20

s with a retention interval of at least 100 s and with currents of ejection between 10 and 90 nA. During retention intervals, NA was retained in the barrel with a backing current of -15 nA. Current from the three iontophoretic barrels was automatically balanced by ejecting a current through the fourth barrel (filled with 1M NaCl) equal to the sum of the currents applied to the three iontophoretic barrels but of opposite polarity.

Iontophoretic application of NA elicited multiple effects on LC neurons. In most cells, an initial hyperpolarization was followed by an excitation (see Fig.15). The excitation appeared as a depolarization, an activation of epsp activity or a combination of these effects. Iontophoresis of sodium chloride acidified to a pH of 5.5 with hydrochloric acid, had no effect. However, this is not an ideal control for the possible effects of hydrogen ion ejection during iontophoresis, as sodium ions have a greater mobility than NA. The responses of LC neurons to NA iontophoresis were examined during perfusion with TTX-containing BSS and perfusates which block synaptic transmission to distinguish direct from indirect responses.

During perfusion with TTX, LC neurons usually hyperpolarized between 5 and 10 mV, and two types of responses to NA were observed: simple hyperpolarizations and biphasic responses (a hyperpolarization followed by a depolarization)(Fig. 15). Biphasic responses were defined as those responses to NA in which the cell depolarized above

Fig. 15:

Examples of responses in five different LC neurons to noradrenaline. Left column, responses to NA before TTX application; middle column, responses during blockade of spontaneous activity with TTX; right column, responses after recovery of activity. Horizontal bars indicate iontophoresis of NA which was ejected with currents of 120, 100, 70, 70 and 90 nA in examples a-e, respectively.

a: LC neuron exhibiting a hyperpolarization and an enhancement of epsp's in response to NA and a monophasic hyperpolarization during TTX application.

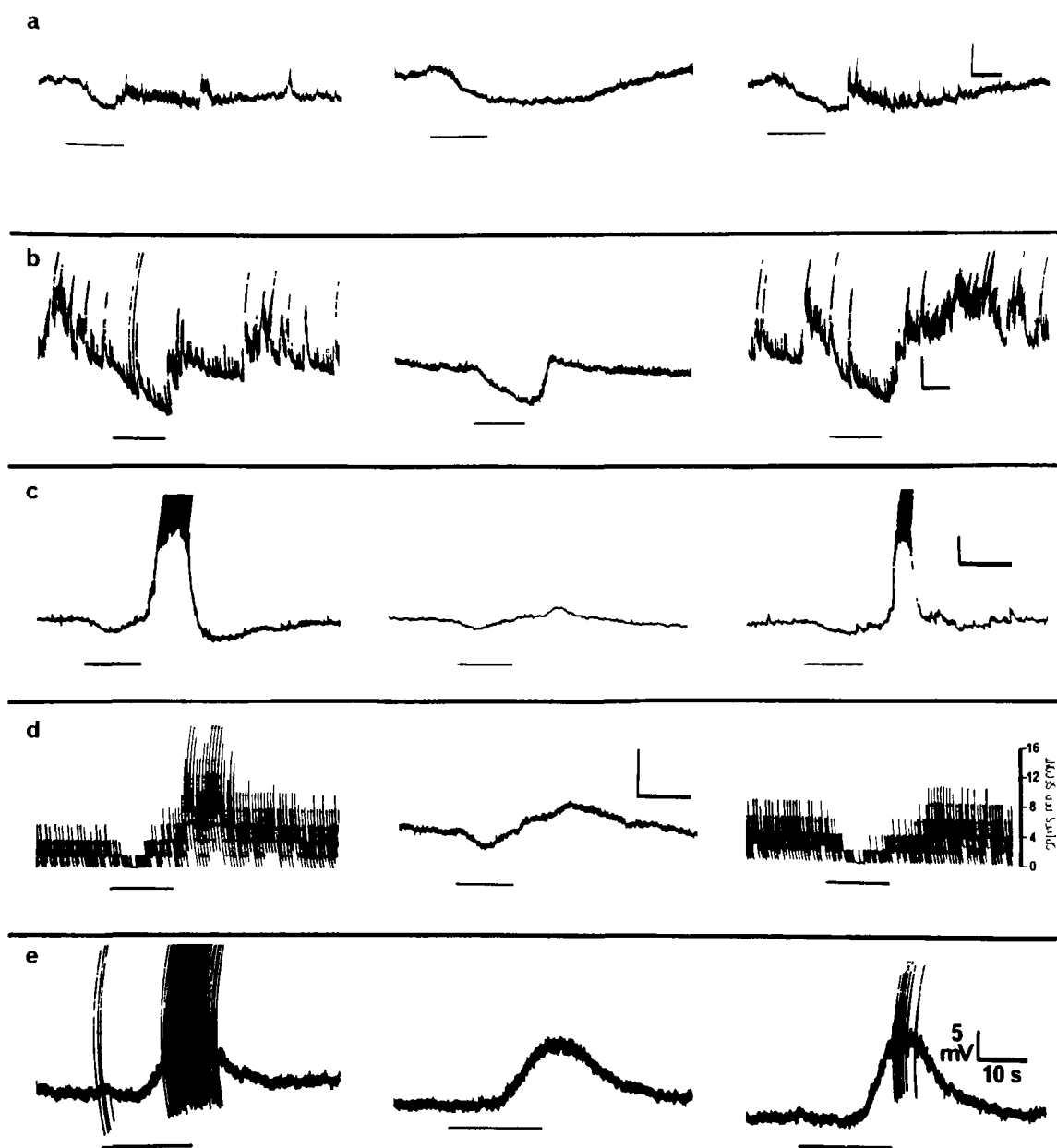
b: LC neuron exhibiting a high rate of epsp activity and a biphasic response to NA with a small depolarizing phase.

c: LC neuron exhibiting a biphasic response to NA; before and after TTX perfusion a burst of activity (spike amplitude was 80 mV) was evoked during the depolarizing phase of the response.

d: an LC neuron exhibiting a biphasic response to NA, and that was spontaneously active before and after TTX application; ratemeter output is shown to demonstrate the biphasic change in firing rate during NA application.

e: depolarizing response to NA again before and after TTX application, action potentials (85 mV) are cut off. Calibration bars are 5 mV and 10 s for all intracellular traces.

Subsequent examples of responses of LC neurons to NA are those observed with TTX in the perfusate with other agents, unless otherwise stated.



resting membrane potential, during the later phase of the response. Apparent epsp activity evoked by NA iontophoresis was lost during TTX intoxication (Fig. 15a). Hyperpolarizing responses generally started within 2 seconds of the iontophoretic application of NA and the amplitude was dose-dependent, reaching maximal responses of approximately 10 mV. The hyperpolarizing responses often lasted for periods of up to 30 s after the cessation of iontophoresis, though some cells quickly repolarized. Biphasic responses were characterized by a similar onset of the hyperpolarizing response, however either during iontophoresis of NA or slightly after iontophoresis ended the cell depolarized above resting membrane potential. This depolarization lasted for 20 to 30 s after iontophoresis ended and could reach maximal potentials of 10 mV above resting membrane potential. Both phases of the biphasic responses were dose-dependent (see Fig. 16).

Noradrenergic responses were also investigated during perfusion with solutions that block synaptic transmission and interfere with calcium influx. In LC neurons that responded with biphasic responses to NA during TTX intoxication, BSS's containing 0 Ca and 20 mM Mg, or 0.5 mM Ca and 3, or 4 mM Mn markedly decreased the depolarizing component of the response and hyperpolarizing responses to NA were predominant (n=9). However during perfusion with normal Ca and 12 mM Mg BSS, which also blocks synaptic transmission, the depolarizing component of biphasic responses was present, although usually

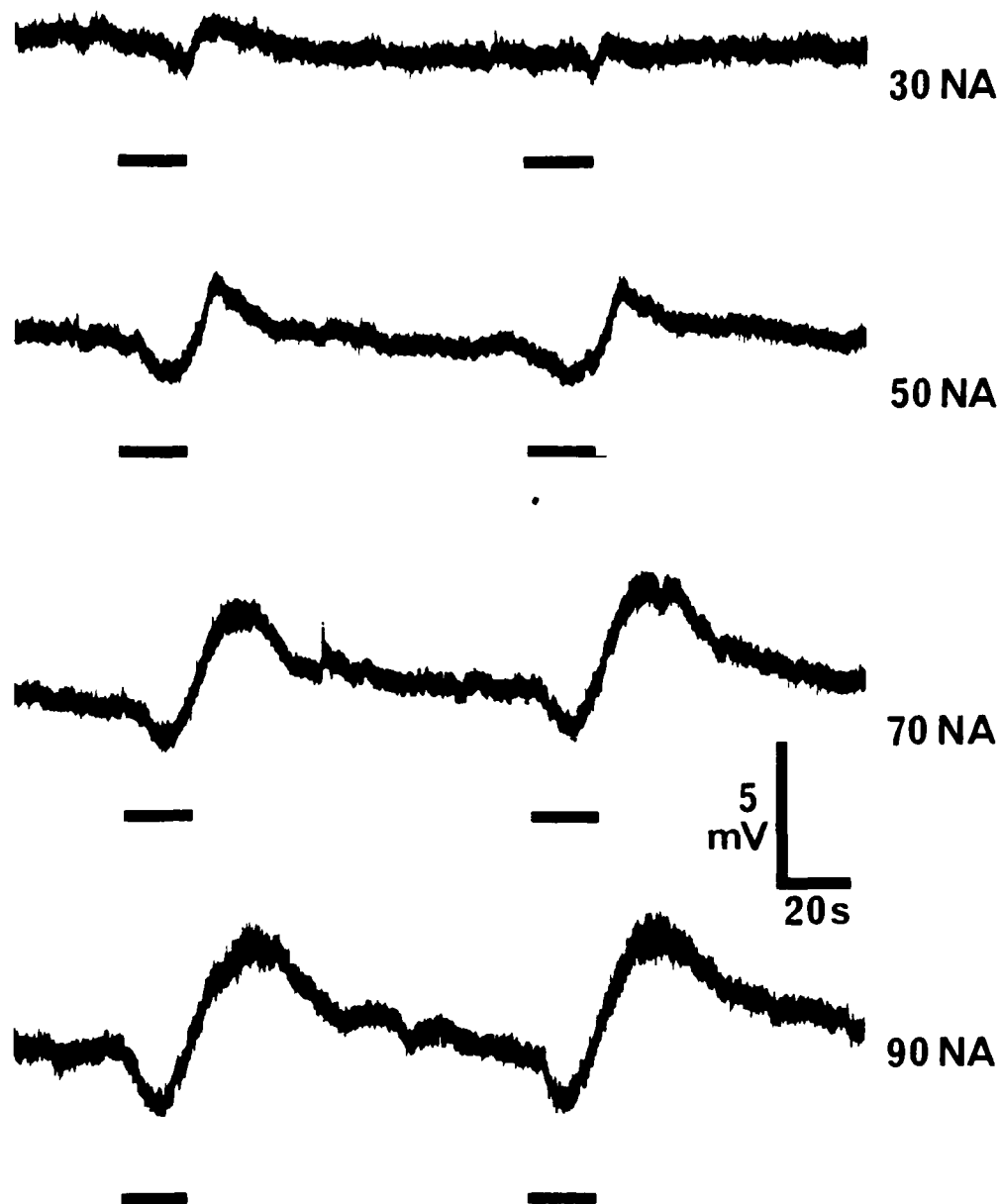


Fig. 16:

Dose-dependence of biphasic responses of LC neurons to NA. Both the hyperpolarizing and depolarizing phases of the response are increased when the iontophoretic current ejecting NA is increased. (30 NA, 50 NA, 70 NA and 90 NA indicate iontophoresis of NA with 30, 50, 70 and 90 nA, respectively)

reduced (n=5, Fig. 17). Depolarizing responses to NA during α_2 -adrenoceptor blockade by yohimbine (1 μ M)(see below) and TTX intoxication were also monitored when 0.5 Ca and 2.0 Mn BSS was perfused. The depolarizing responses to NA were usually maintained in this perfusate, but were often reduced during continued perfusion (n=3, Fig. 17). In one case with continued perfusion, a small depolarizing response was abolished. However, it would appear that at least part of the depolarizing response is a direct response on LC neurons.

Intracellular recordings with electrodes containing potassium EGTA were also attempted. However, LC neurons became very unstable when this calcium chelator was intracellularly injected. Therefore although responses to iontophoretically applied NA were monitored, it was not possible to judge the effects of chelating intracellular calcium.

Therefore, there are three effects of NA on LC neurons:

- 1) a direct hyperpolarizing effect in all neurons,
- 2) in some neurons a direct depolarizing effect,
- 3) in many neurons an indirect effect of NA: an activation of an excitatory synaptic input onto LC neurons.

2) Age-dependence of Noradrenergic Responses

When the direct responses of LC neurons to NA iontophoresis (as determined during TTX perfusion, n=125) were categorized according to the number of days cultures were maintained in vitro, an age-dependence was noted (see Fig. 18). As tissue for LC explant cultures was derived from neonatal mice on postnatal day 1, changes observed in explants

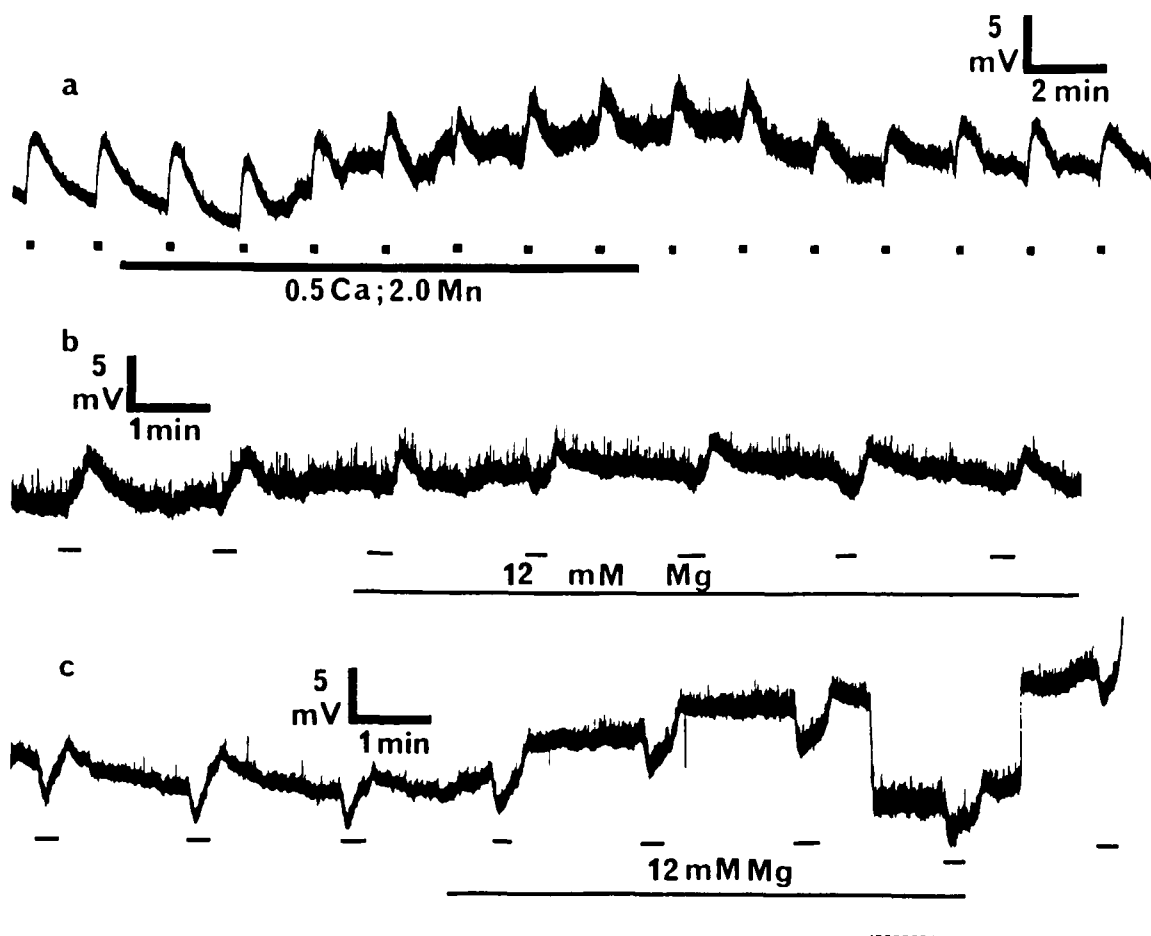


Fig. 17: Effects of modified BSS's on biphasic and depolarizing responses to NA.

a. Depolarizing response (in the presence of $1 \mu\text{M}$ yohimbine) of LC neuron to iontophoretically applied NA (applications indicated by row of boxes) are present in 0.5 mM Ca/2.0 mM Mn BSS, although reduced in amplitude.

b. and c. Biphasic responses to iontophoretically applied NA (horizontal bars) are present during perfusion with BSS containing high magnesium (12 mM) to block synaptic transmission. Depolarizing phase was maintained though often reduced. Repolarizing the cell with hyperpolarizing current (dotted line in C.) had little effect.

(Iontophoretic current of application was 100, 50 and 70 nA in examples a - c, respectively.)

**AGE-DEPENDENCE OF RESPONSES OF LC NEURONS
TO NORADRENALINE**

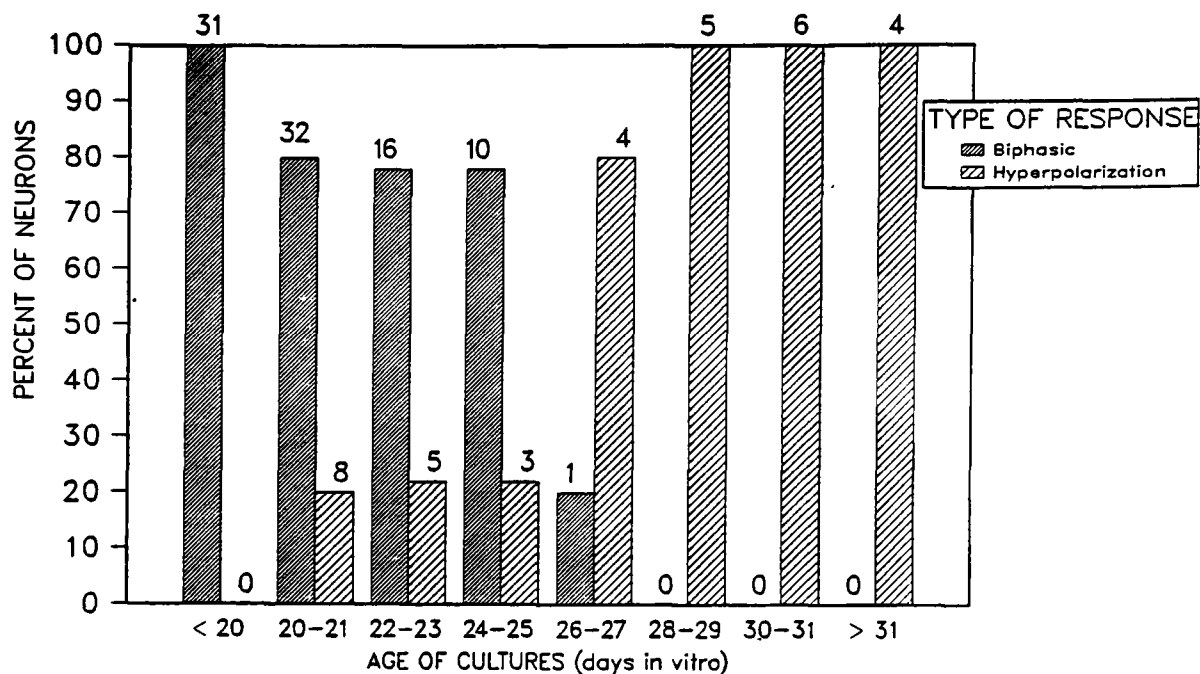


Fig. 18:

Occurrence of hyperpolarizing and biphasic responses of LC neurons to noradrenaline in cultures of different in vitro ages. The number of neurons in each group is indicated above each bar.

likely will be related to postnatal developmental changes. LC neurons recorded from cultures grown in vitro for 27 to 36 days, responded to NA with only a simple hyperpolarizing response in the presence of TTX (n=16). However, in cultures that were maintained for less than 26 days in vitro, biphasic and hyperpolarizing responses to NA were observed in LC neurons. Furthermore, in neurons grown for 16 to 18 days (n=2) and 18 to 19 days (n=29) in vitro, only biphasic responses were observed.

3) Effects of Adrenergic Antagonists

Adrenergic receptor antagonists were bath applied during TTX intoxication. The α_2 adrenergic antagonist, yohimbine, selectively blocked the hyperpolarizing response either in cells that exhibited simple hyperpolarizing responses or biphasic responses (Fig. 19)(n=24) at concentrations of 100 nM to 1 μ M. In cells with biphasic responses, during α_2 blockade with yohimbine, LC neurons responded to NA with a simple depolarization that often appeared to start within a few seconds of the start of iontophoresis. The antagonism of the hyperpolarizing response by yohimbine was reversible, and was usually reversed after 30 minutes of washout. Furthermore, the hyperpolarizing response to NA was not reduced by the α_1 -antagonist, prazosin (tested at up to 1 μ M).

On the other hand, prazosin selectively blocked the depolarizing component of biphasic responses (Fig. 19)(n=18)

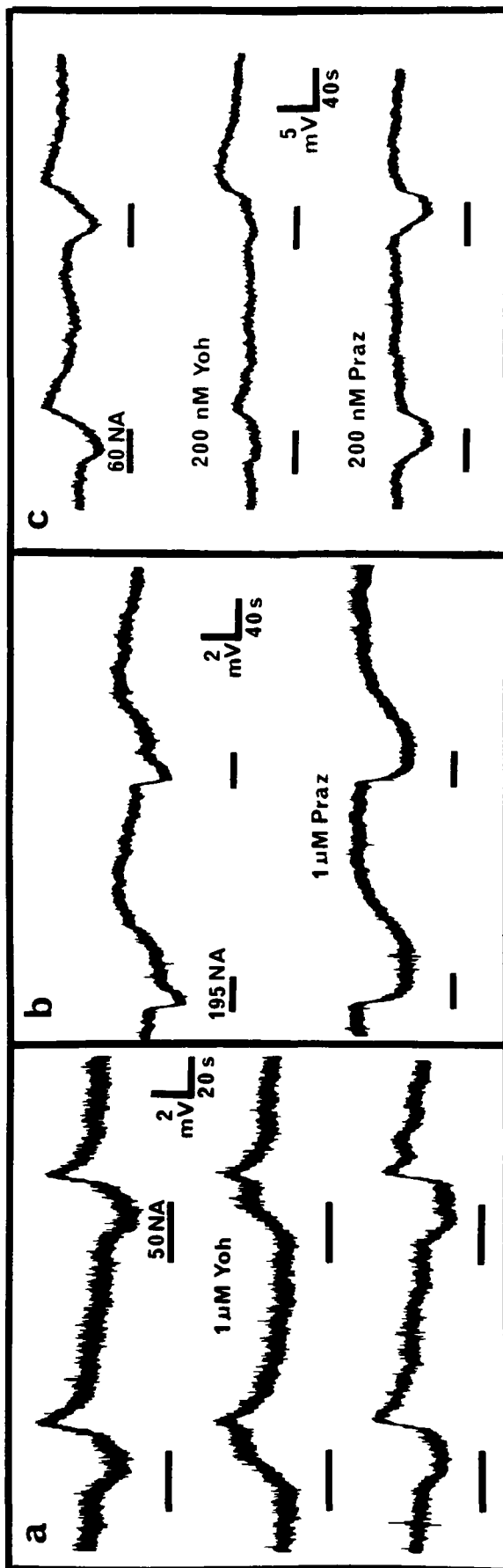
Fig. 19:

Application of adrenergic antagonists by bath perfusion during recording of biphasic responses to iontophoretically applied noradrenaline. Iontophoretic applications are indicated by bars below recording traces.

a: biphasic response to NA (upper trace); during perfusion of 1 μ M yohimbine, hyperpolarizing phase is blocked leaving only a depolarizing response (middle trace); the effect is reversed after washing by control perfusate (lower trace).

b: upper trace shows control biphasic response to NA; prazosin (1 μ M) selectively blocks the depolarizing phase of the response (lower trace).

c: biphasic response to NA shown in upper trace shows selective reduction of hyperpolarizing phase during perfusion with 200 nM yohimbine (middle trace); subsequent perfusion with 200 nM prazosin resulted in a selective loss of the depolarizing phase of the response (lower trace).



at concentrations of 100 nM to 1 μ M. LC neurons exhibited long-lasting simple hyperpolarizations in the presence of prazosin. Concurrent application of yohimbine and prazosin blocked all responses to NA (see Fig. 20). Furthermore, the antagonism of the depolarizing responses to NA by prazosin was dose dependent. The depolarizing response to NA in the presence of 1 μ M Yohimbine was partially antagonized by prazosin at concentrations less than 100 nM (Fig. 21 and 22). Prazosin also shifted the "dose-response" curve of the depolarizing response to iontophoretically applied NA in the presence of 1 μ M yohimbine to the right (Fig. 23). As depolarizing responses to NA during perfusion with prazosin were not as large as maximal responses in the absence of prazosin, it appears that the antagonism by prazosin was not surmountable. However this might reflect the limits of iontophoretic application as the maximal single channel iontophoretic current was used. It should also be noted that the "dose-response" curves are plotted as the response versus the logarithm of iontophoretic current and the iontophoretic current as it can not be assumed that there is a linear relationship of iontophoretic current to noradrenaline concentration in the tissue. The antagonism of the depolarizing response was reversible at low concentrations (<100 nM), however at higher concentrations a recovery was often not observed.

Corynanthine had no effect on the biphasic responses at 1 μ M (n=2), but in one case, when tested at 10 μ M antagonized

Fig. 20:

Effects of adrenergic antagonists on biphasic responses of LC neuron to NA.

- a. Response to NA is dose-dependent;
- b. Yohimbine (500 nM) blocks the hyperpolarizing component of the response;
- c. Prazosin (500 nM) in combination with yohimbine completely blocks responses to NA;
- d. Yohimbine antagonism is reversed after washout, and
- e. Prazosin blockade takes much longer to reverse and only a partial recovery is observed.

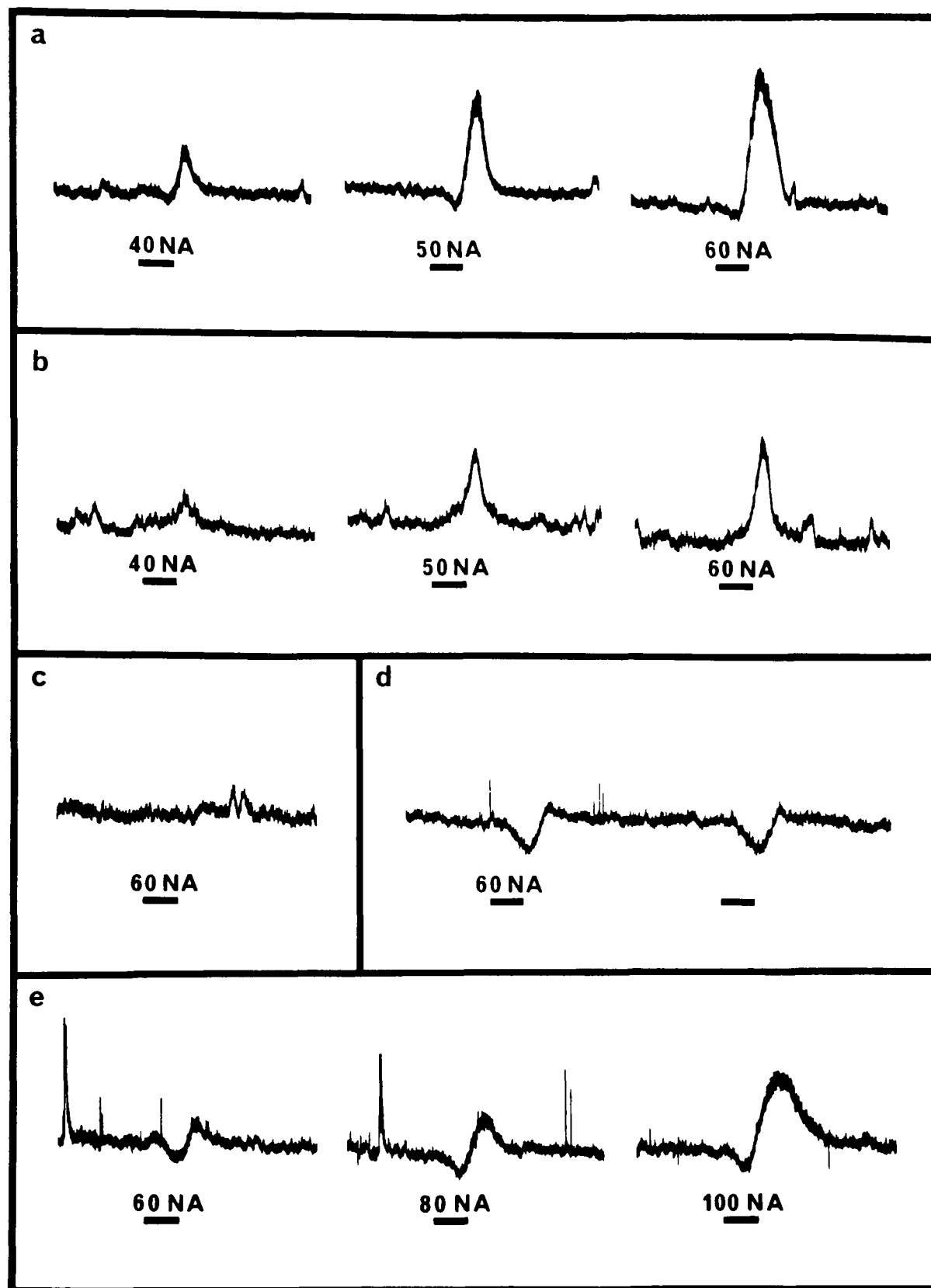
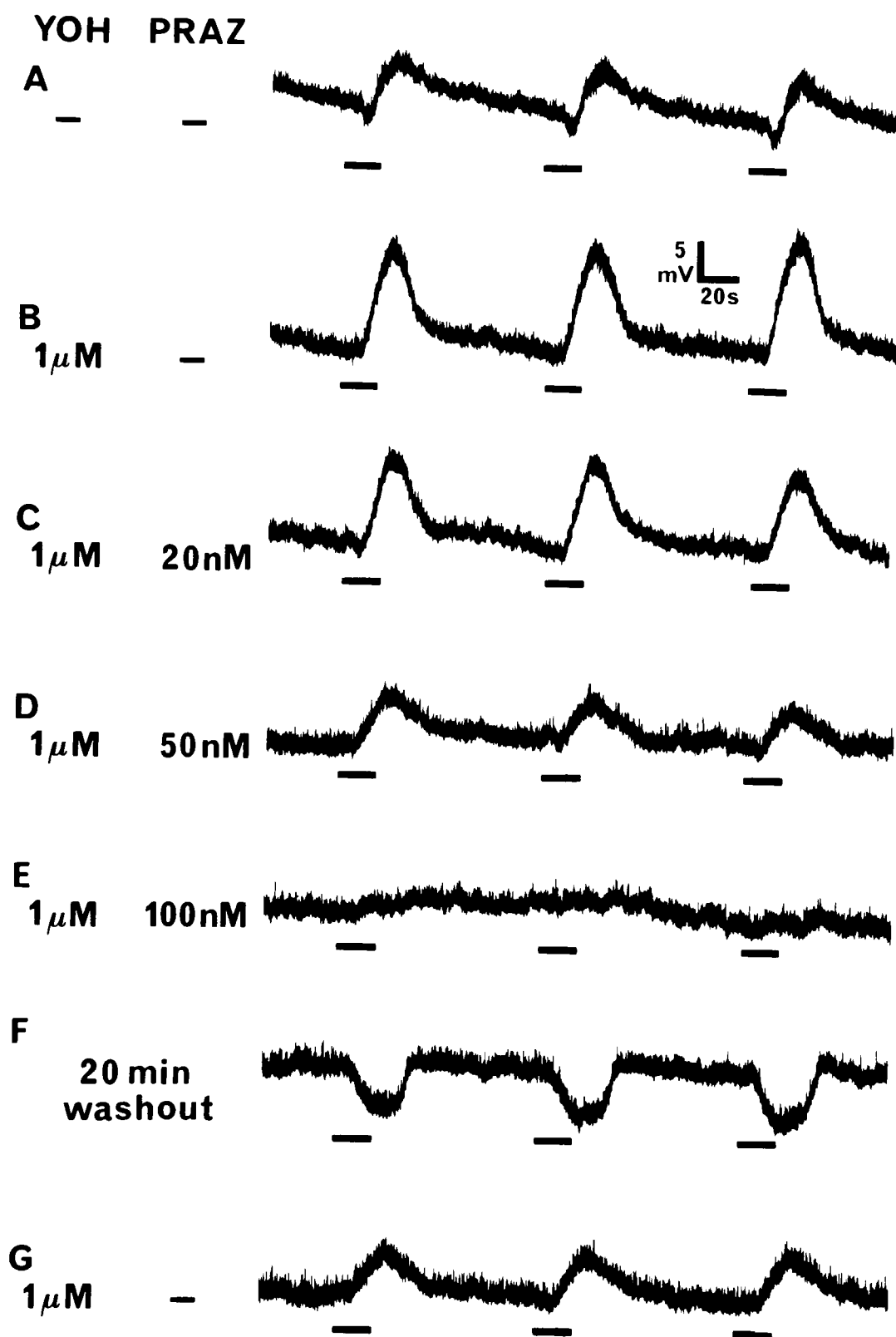


Fig. 21:

Dose-dependent antagonism of depolarizing response to NA.

Depolarizing response to NA (in the presence of 1 μ M yohimbine - b) is increasingly antagonized by 20, 50 and 100 nM prazosin (c - e). The faster washout of yohimbine results in a simple hyperpolarizing response to NA shortly after washout of drugs began (f). Reversal of prazosin blockade is evident during subsequent perfusion with yohimbine (g)(after 34 min. of washout). (NA was applied with an iontophoretic current of 40 nA)



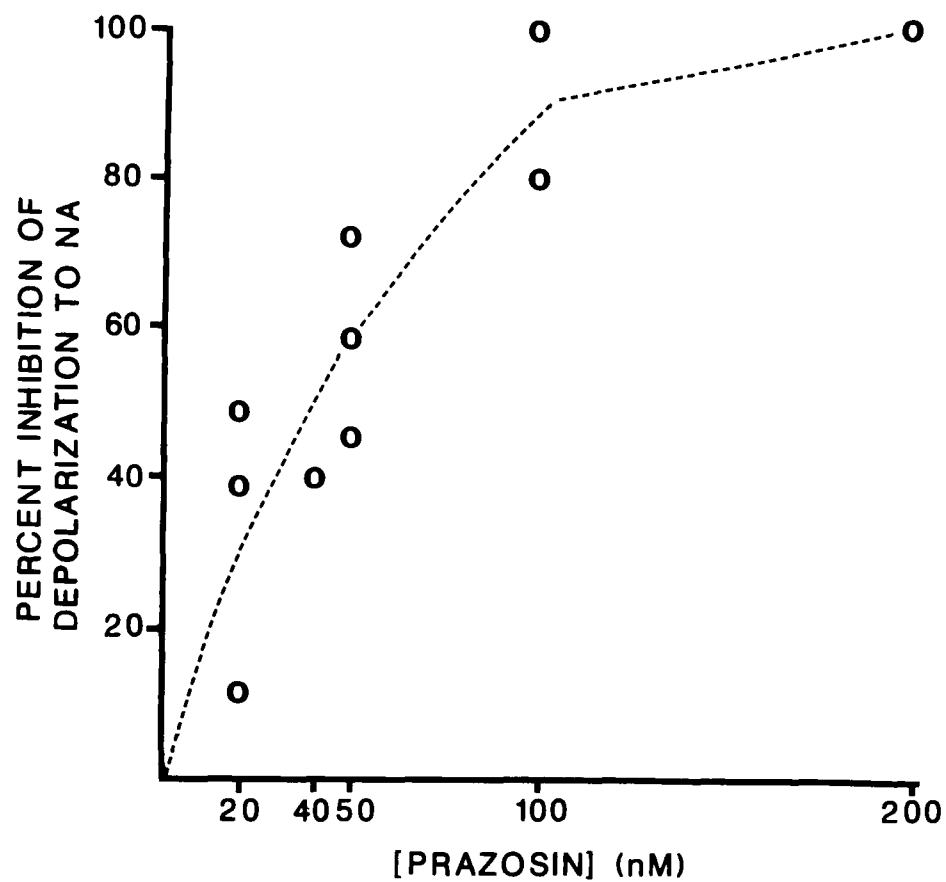


Fig. 22:

Prazosin inhibition of depolarizing responses to NA (in the presence of $1 \mu\text{M}$ yohimbine) expressed as percent inhibition. Data from experiments on four cells.

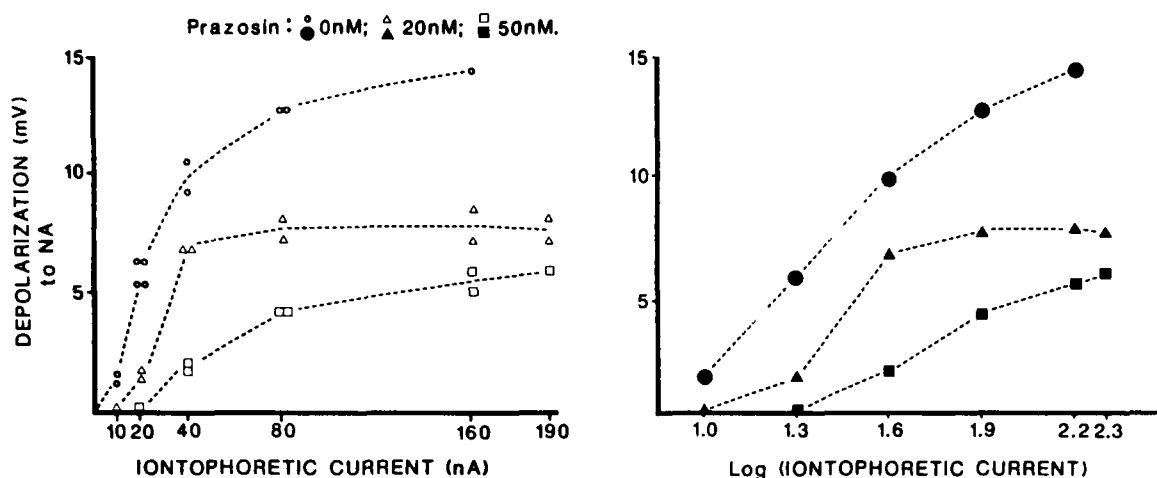


Fig. 23:

Effects of prazosin on the "dose-response" curves of depolarizations of an LC neuron to iontophoretically applied NA (in the presence of 1 μ M yohimbine). The same data is presented in two forms. At the left, the amplitude of individual responses (open symbols) in the presence of 0, 20 or 50 nM prazosin are plotted against the current of NA iontophoresis. At the right, the means of responses at each given iontophoretic current and prazosin concentration (solid symbols) are plotted against the log of the iontophoretic current in nA. In both representations, prazosin shifts the dose-response curve increasingly to the right at 20 and 50 nM; The depolarizing responses at maximal iontophoretic currents (190 nA) during prazosin application are smaller than the maximal amplitude of the depolarizing response without prazosin.

the depolarizing component of biphasic responses to NA. Finally, the beta(β) adrenoceptor antagonists, propranolol and sotalol at concentrations of 1 μ M had no effect on LC neuron responses to NA (n=4).

Bath application of yohimbine (200 nM - 1 μ M) without TTX did not produce any noticeable changes in bursting activity or membrane potential in most cells (n=8). However, in one case although tonic activity was continually decreasing, during application of 1.5 μ M yohimbine the rate of firing during bursts of activity appeared to increase slightly.

Prazosin application without TTX at 200 or 500 nM produced varying effects in young (n=5) and old cultures (n=2 cells). In young cultures (<21 days in vitro, DIV), two LC neurons hyperpolarized, fluctuations in membrane potential stabilized in one cell, and two cells were unaffected during α_1 -adrenoceptor blockade. In older cultures (29 DIV), one cell hyperpolarized and the other cell was unaffected by prazosin perfusion. Furthermore, bursting in one cell subsided during perfusion of prazosin, but bursting activity was present in two LC neurons during receptor blockade with 200 nM prazosin.

4) Application of Adrenergic Agonists

Clonidine, a partial α_2 agonist, and phenylephrine and methoxamine, α_1 agonists were applied either by iontophoresis or bath application (see Starke, 1981, for review of agonist selectivities). Clonidine produced a selective and long-lasting (up to 5 minutes) hyperpolarization, when iontophoresed onto cells that had either simple

hyperpolarizing or biphasic responses during TTX intoxication (n=5)(see Fig. 24). Iontophoresis of phenylephrine and methoxamine, on the other hand, produced selective and long-lasting (up to 5 minutes) depolarizing responses in cells, that exhibited biphasic responses to NA (n=3 and 14 respectively)(see Fig. 24). Responses of LC neurons to all the agonists lasted much longer than the responses to NA. Furthermore, when long applications of clonidine were applied during pulsatile applications of NA that evoked biphasic responses, the responses to NA became predominantly depolarizing during the clonidine induced hyperpolarization (Fig. 24).

Noradrenaline and the agonists phenylephrine and clonidine were also bath applied during recording of some LC neurons. Drugs were perfused for between 30 seconds to 2 minutes to ensure complete mixing in the bath. Noradrenaline (2 to 100 μ M), applied to young cultures (ie < 23 days in vitro) elicited long depolarizations, that lasted up to 7 minutes (n=4). When prazosin was used to block α_1 -adrenoceptors in one of these cells, noradrenaline evoked a long hyperpolarization. Clonidine hyperpolarized LC neurons, but in one case at 4 μ M, this hyperpolarization was preceded by a small depolarization. Phenylephrine applied at concentrations between 6 and 50 μ M selectively evoked depolarizations in LC neurons (n=3). These depolarizations lasted for up to 5 minutes and were dose-dependent, reaching peak amplitudes of 10 mV. In addition, bath application of

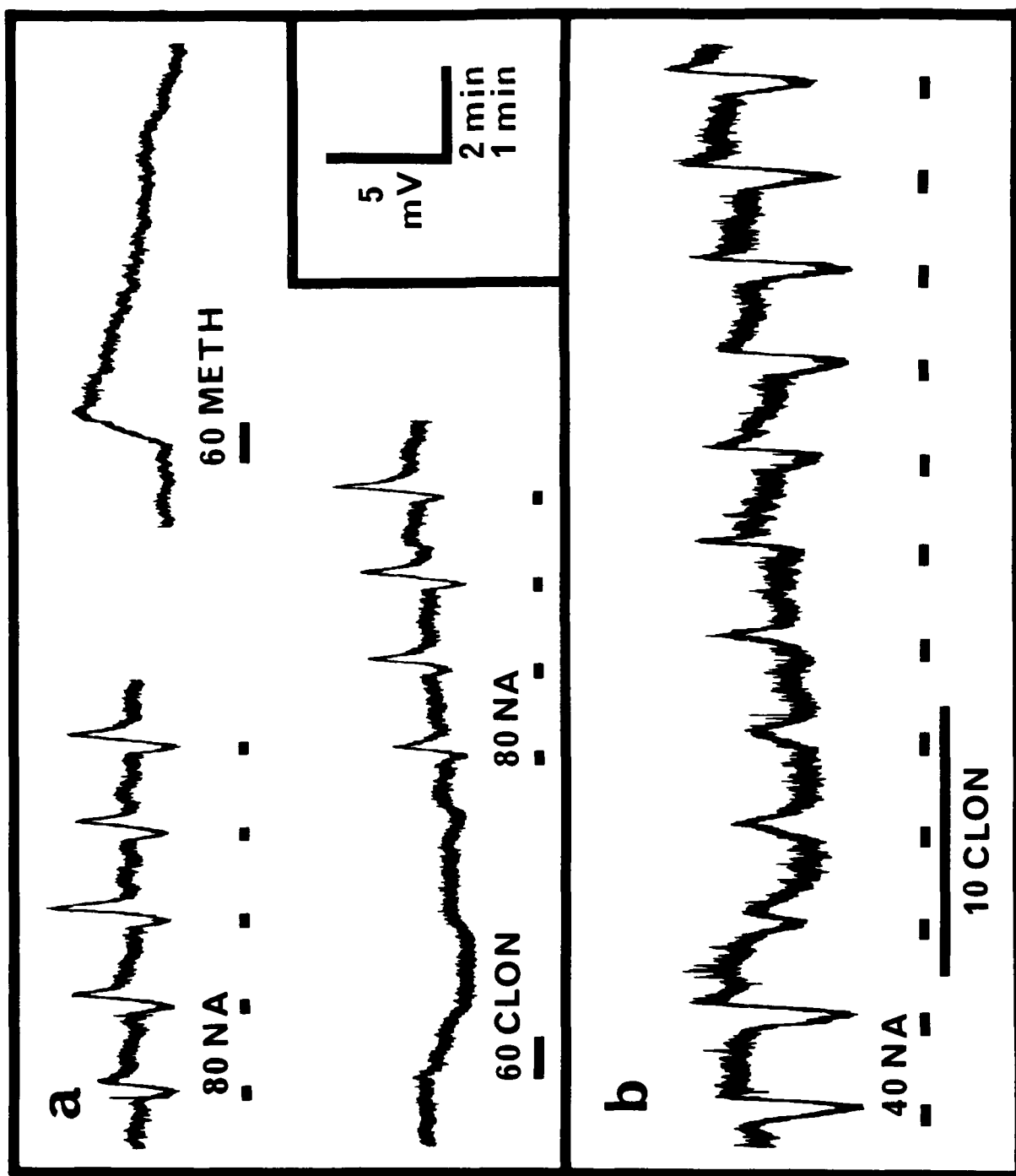
Fig. 24:

Iontophoretic application of noradrenaline and adrenergic agonists to LC neurons.

a: NA evokes biphasic response (upper left) while methoxamine gives a depolarization with no evident hyperpolarizing phase (upper right); clonidine application results in slow hyperpolarization with no apparent depolarizing phase.

b. application of clonidine during continuing series of NA applications; response to clonidine is a slow hyperpolarization; NA responses appear as simple depolarizations during maximal clonidine response and gradually return to control biphasic pattern.

Time calibration bar (inset) represents 2 min for a and 1 min for b.



phenylephrine without TTX evoked a slow depolarization and appeared to evoke bursting activity (n=1)(Fig. 25).

5) Input Resistance Changes During Responses to NA

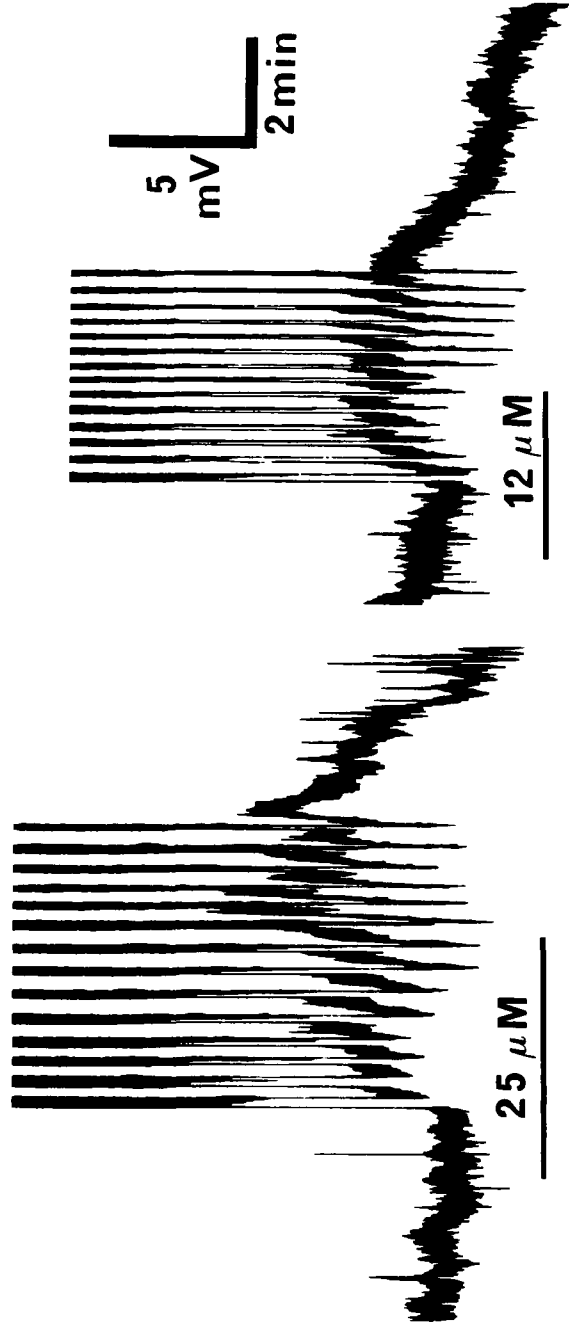
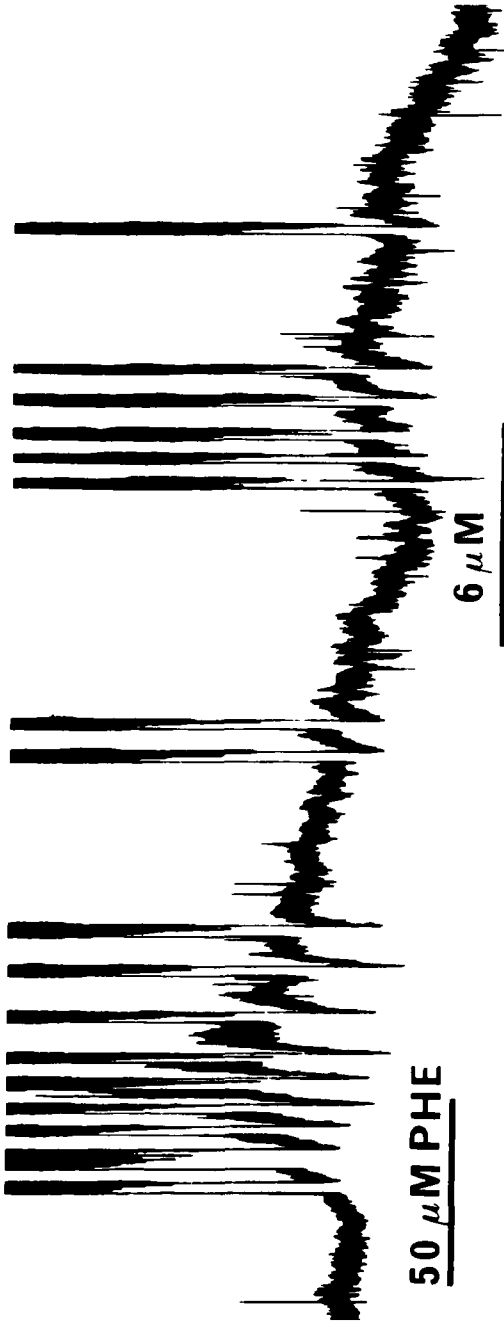
Input resistance was measured by repetitively passing hyperpolarizing current pulses with the recording pipette balanced and measuring the change in membrane potential. Input resistance could then be calculated using Ohm's law (i.e. V_m/I). Rectification properties of the membrane were compensated for by polarizing the membrane to potentials reached by the response with steady polarizing current. The reliability of input resistance measurements was increased by averaging responses during a control period, during each phase of the response and during polarization of the membrane to potentials reached by the noradrenergic response.

Input resistance decreased during the simple hyperpolarizing responses to NA and during the hyperpolarizing phase of biphasic responses in LC neurons to NA. This was only apparent when rectification was accounted for (n=6)(see Fig.26). Although measurements were obtained during the depolarizing phase of biphasic responses to NA, the input resistance changes could not be attributed to the mechanisms involved in the depolarizing phase, as conductance changes involved with the hyperpolarizing component likely were still present.

Input resistance measured during the depolarizing responses to methoxamine or during α_2 adrenoceptor blockade

Fig 25:

Responses of an LC neuron to bath application of phenylephrine. Bursts of firing activity occur on the slow depolarization evoked by phenylephrine perfusion. The amplitude of the depolarizations are dependent on the concentration of phenylephrine applied. At 50 μ M phenylephrine, the depolarizing response is slightly smaller, this might be due to phenylephrine acting on α_2 -adrenoceptors at this concentration. The number and length of bursts during phenylephrine application also appears to have a relationship to the concentration of agonist applied.



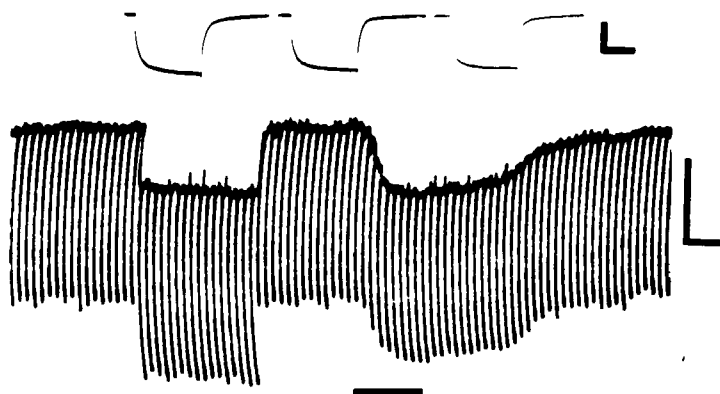


Fig 26:

Input resistance measurements taken during TTX perfusion. The averages of 8 responses to constant hyperpolarizing current pulses (0.2 nA, 100 ms) during steady hyperpolarizing current injection, during resting conditions, and during the hyperpolarization evoked by iontophoresis of noradrenaline are shown above the related period in the chart recording. Averages are photographs of oscilloscope tracings. When the resistance during steady hyperpolarizing current and during the noradrenergic hyperpolarization are compared, a decrease in input resistance (of 8%) during the hyperpolarization to noradrenaline is observed. (NA was applied with an iontophoretic current of 80 nA)

Calibration bars: chart recording: 10 mV, 10s; averages of responses: 10 mV, 50 ms.

with yohimbine, increased between 3 and 12% in five cells, but in two cells, there was a decrease in input resistance of less than 4% during the depolarizing response. Therefore, there was no consistent change in conductance measured during the depolarizing responses to NA.

6) Membrane Potential Dependence of Responses

The hyperpolarizing response to NA could be reversed by hyperpolarizing LC neurons (n=3, Fig. 27). The depolarizing component of biphasic responses was enhanced by hyperpolarization, but this simply could have been due to the effects of hyperpolarization on the hyperpolarizing response. However, during α_2 -blockade with yohimbine (1 μ M), depolarizing responses to NA were increased by hyperpolarizing cells and decreased by depolarizing cells (n=3, Fig. 27).

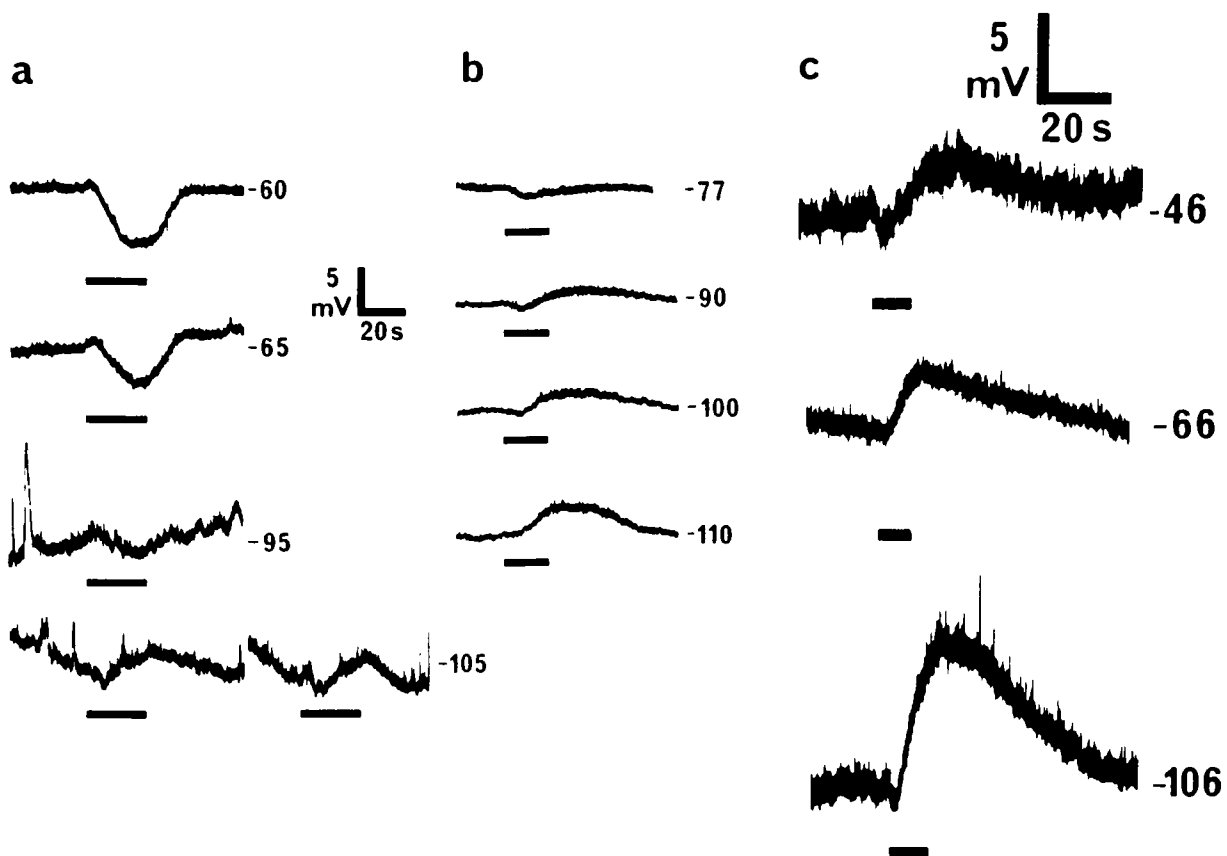


Fig. 27:

Effects of polarizing the cell on the responses to NA.

a. The hyperpolarizing response to NA (70 nA) is increased by depolarizing the cell (to -60) from resting membrane potential and is decreased and reversed by hyperpolarizing the cell (to -95 and -105 mV).

b. In a cell exhibiting a biphasic response, hyperpolarizing the cell apparently increases the depolarizing phase of the response; however this observation could also be explained by the decrease in the hyperpolarizing phase of the response. (100 nA NA)

c. However, depolarizing responses to NA in the presence of 1 μ M yohimbine are increased by hyperpolarizing the cell (to -106 mV, in this case) from rest potential (-66 mV) and is decreased by depolarizing the cell (-46 mV). (100 nA NA)

DISCUSSION

I) Characterization of Locus Coeruleus Neurons In Tissue Culture

Locus coeruleus neurons have been previously described in explant tissue cultures (Hendelman, et al.,1982). In brainstem explants, neurons which have a large single nucleolus in their nuclei, refractile cytoplasmic inclusions and form a compact group of medium sized neurons, have been characterized as LC neurons. This identification is based on the following observations: 1) explants containing these neurons originate from pontine brainstem tissue at the tip of the fourth ventricle - the location of LC neurons *in vivo*; 2) catecholamine fluorescence is exhibited by explants containing these neurons, and in some cases, LC neurons identified in the living culture have been shown to exhibit catecholamine fluorescence; 3) fine catecholamine fluorescent fibers with varicosities emanate from brain stem explants with these neurons 4) noradrenaline and dopamine synthesis is evident in cultures with LC explants (Hendelman, et al.,1982), 5) lucifer yellow injections reveal that these neurons have somatic and dendritic spines and long dendritic processes as has been noted in Golgi studies and ultrastructural studies on LC neurons *in vivo* (Shimizu and Imamoto,1970; Shimizu, et al.,1978; Swanson,1976), and 6) these neurons are consistently inhibited by NA through an α_2 -adrenoceptor mediated inhibition. These unique morphological and physiological characteristics of LC neurons distinguish LC neurons from other neuronal groups in this region. In these

studies, we have characterized these neurons in more detail.

1) Morphological Characteristics of Cultured LC Neurons

My observations on the morphology of LC neurons using lucifer yellow staining support findings from cultured LC neurons intracellularly stained with horseradish peroxidase (Marshall, et al., 1985). Our most striking finding is that many LC neurons apparently have multiple axons, some of which emanate from proximal and distal dendrites. An earlier observation on cultured brainstem neurons tentatively identified as LC neurons, also noted that these neurons have multiple axons (Hendelman and Marshall, 1980). In Golgi staining of LC neurons *in vivo*, although axons are stained, it is usually not possible to discern origins of axons. However, Swanson (1976) indicated in one of his figures that a Golgi stained LC neuron had two axons - a projection axon and a locally ramifying axon - with separate origins. Other neurons have been described to have multiple axons and axons originating from dendrites (e.g. Golgi cells in the cerebellar cortex - Palay and Chan-Palay, 1974 and neurons in the spinal trigeminal nucleus - Gobel, 1975).

As LC neurons have widespread projections, it is of interest to determine if LC neurons, *in vivo*, have multiple axons, and if so, do different axon systems project to different target areas. Only one study *in vivo* (Swanson, 1976) has suggested that LC neurons have multiple axons. If LC neurons have multiple axons (some emanating from distal dendrites), that project to different brain regions, what is

the significance of such an arrangement. Although it appears that LC neurons act as a homogeneous group, we do not know what is occurring on distal dendrites. If spike transmission is modified by the dendrites, although all LC somata and axons originating near the soma may be simultaneously activated, activity in axons emanating from distal dendrites could be markedly different. Individual LC neurons then could be divided into functional units - the area near the cell soma involved in integrating information for axons arising near the cell soma; and distal dendrites that pattern the activity for axons arising on distal dendrites.

2) Other Neuronal Cells in Culture

Smaller neurons in and around the LC explant were observed in thionine stained cultures. However in the living state it was difficult to detect these neurons. No small cells were injected with LY. This is likely due to the relative difficulty in penetrating smaller cells and the preponderance of medium-sized LC neurons. Although impalements were achieved under microscopic observation, it was not always clear which neuron was impaled. The LY injections suggest that intracellular recordings in LC cultures is predominantly, if not exclusively, from the medium-sized LC neurons.

II) Electrophysiological Properties of Locus Coeruleus

1) Comparison of LC Neurons in Different Preparations

Spontaneous firing activity of LC neurons as described in the introduction is dependent on the preparation (see Table II). In our tissue culture preparation, as in vivo, LC neurons exhibit synchronous bursting activity. Bursting activity recorded in unanesthetized animals appears to be in response to sensory events and habituates (Aston-Jones and Bloom, 1981a,b; Rasmussen, et al, 1986; Foote, et al, 1980). In paralyzed animals recovering from anesthesia (Akaike, 1982), and unanesthetized paralyzed animals (Korf, et al., 1974), synchronous bursting of LC neurons also occurs. In anesthetized animals, LC neurons exhibit bursting only in response to noxious stimuli. However, LC neurons in tissue

Table III: Comparison of activities and responsiveness of locus coeruleus neurons in different preparations

PREPARATION	TONIC FIRING	BURSTING ACTIVITY	PROBLEMS
ANESTHETIZED ANIMALS	0.5-5.0 HZ	In response to noxious stimuli	Effects of anesthesia
NEONATAL ANESTHETIZED ANIMALS	None	In response to most sensory stimuli	Effects of anesthesia
UNANESTHETIZED ANIMALS	Varies with sleep-waking cycle	In response to sensory stimuli & changes in state	Intracellular recordings unfeasible
TISSUE SLICES	0.5-5.0 HZ	None observed	Deafferented, Tissue damage
TISSUE CULTURES	In a few cells	Long spontaneous bursts	Deafferented, Modified environment.

slices do not exhibit bursting activity. Cultured LC neurons were often quiescent for long periods of time, which is unlike the tonic activity observed in recordings of adult LC neurons in tissue slices and in anesthetized animals, and the varied activity of LC neurons in unanesthetized animals (except for the quiescent activity observed during paradoxical sleep). However recordings from anesthetized neonatal animals suggests that the tonic spontaneous activity of LC neurons at younger ages may be very low (Kimura and Nakamura,1985). Although most groups have used chloral hydrate as an anesthetic, Nakamura has used urethane, and LC neurons in urethane-anesthetized animals are spontaneously active in adult animals (Nakamura,1977) but not in 1 day old animals (Kimura and Nakamura,1985).

In addition to spontaneous firing activity, spontaneous depolarizing events, that appear to be epsp's are also recorded in cultured LC neurons. Spontaneous epsp's have not been reported in intracellular recordings in tissue slices (Henderson,et al.,1982) or in anesthetized animals (Aghajanian and VanderMaelen,1982), though synaptic depolarizations could be elicited by stimulation (Henderson, et al.,1982; Egan, et al.,1983). Spontaneous ipsp's were recorded in LC neurons explanted as a single explant with cerebellum (Marshall,et al.,1979), but were not observed in my studies. These ipsp's may be due to cerebellar inputs to the LC. As these regions were explanted as separate pieces in my studies, cerebellar outputs likely do not reach LC explants (as no myelinated

fibers are found in the LC), perhaps explaining the absence of ipsp's in my studies.

Action potentials and evoked activity in tissue slices and in tissue cultures also have similarities and some differences. The mean duration of action potentials in tissue slices from adult animals were 1.4 ms (Williams, et al., 1984) while from young animals were 1.7 to 2.2 ms (Marshall and Williams, 1986). In tissue culture, the duration of spikes ranged from 0.9 to 1.8 ms with a mean duration of 1.4 ms. In both preparations, a "shoulder" on the repolarizing phase of individual spikes has been observed though it was not observed in all cultured LC neurons, and when it was observed in cultured LC neurons it was not as prominent as those observed in tissue slices. In tissue slices, this "shoulder" appears to be due to a calcium spike (Williams, et al., 1984). TTX intoxication in tissue slices does not interrupt LC firing though the maximal rate of rise and the amplitude of the spike are decreased. However, in tissue cultures, TTX intoxication results in an abrupt cessation of all activity (i.e. spontaneous epsp's, bursting and tonic activity) and only with strong depolarizing current injection can any active response be elicited. Furthermore, the afterhyperpolarization following individual spikes appear to be much longer in tissue slices than in tissue cultures. The longer duration of afterhyperpolarizations in tissue slices may reflect a calcium-activated potassium conductance component of this afterhyperpolarization. As the afterhyperpolarization

following single spikes was much shorter in tissue culture, and the shoulder on the repolarizing phase of spikes was less prominent in tissue culture, it appears that cultured LC neurons do not have as robust a calcium component to individual spikes as compared to LC neurons in tissue slices.

Activation of LC neurons in culture, and in vivo (Aghajanian, et al, 1983; Andrade and Aghajanian, 1984a) with depolarizing current injection can also activate a long AHP lasting up to 500 ms. In vivo, this has been characterized as due to a calcium activated potassium conductance (Aghajanian, et al., 1983). A similar calcium-dependent AHP has also been described in slice preparations (Williams, et al., 1984). The long AHP following multiple evoked spikes in cultured LC neurons also appears to be a calcium-dependent potassium conductance, as this hyperpolarization was blocked by perfusates that interfere with calcium influx.

2) Synchronous Bursting of LC Neurons

Synchronous bursting activity of LC neurons has been observed in recordings in vivo and in culture. However, it has only been possible to study synchronous bursting using intracellular recordings in tissue cultures. This research has been presented (Finlayson and Marshall, 1985) and has been submitted for publication (Finlayson and Marshall, 1986b).

The synchrony of the activation of LC neurons in culture is clearly demonstrated in simultaneous intracellular recordings of pairs of LC neurons. Bursts of firing activity

or barrages of depolarizing potentials were always synchronized in simultaneously recorded cells, and individual depolarizing events also often occurred synchronously in the recorded cells. On the other hand, tonic firing activity was not synchronized.

Many mechanisms have been suggested to explain the synchronous bursting of other neurons such as hippocampal and cortical neurons. The mechanisms suggested include :

- 1) excitatory synaptic input driving all cells synchronously (Johnston and Brown, 1981; Miles and Wong, 1983), and
- 2) synchronous excitation of cells by ephaptic conductance or by electrotonic conductance of depolarizing potentials (Andrew, et al, 1982; Jefferys and Haas, 1982; Knowles, et al, 1982; Richardson, et al, 1984; Snow and Dudek, 1984, 1986; Taylor and Dudek, 1982; Turner, et al, 1984), and
- 3) synchronous release of neurons from a common inhibitory input, and
- 4) extracellular accumulation of ions such as potassium, which simultaneously increases the excitability of all neurons (Konnerth, et al, 1984).

I have tried to determine the nature of the depolarizing potentials that appear to initiate and continue throughout bursts of LC neurons by testing for electrotonic, ephaptic and other interactions between LC neurons, and determining any dependence of spontaneous and evoked depolarizing events on membrane potential and synaptic transmission.

My evidence indicates that LC neurons are synchronously activated by excitatory synaptic events, that summate to evoke the depolarization associated with bursts. Furthermore, the depolarization of cultured LC neurons may be enhanced by

elevation of extracellular potassium during bursting activity. These conclusions are based on the following findings.

a) Non-synaptic interactions

Tests for electrical interactions between LC neurons indicate that LC neurons are not electrotonically coupled, based on simultaneous recordings of LC neurons and intracellular injection of lucifer yellow. Intracellular injections of lucifer yellow (LY), a low molecular weight fluorescent dye was used to test for coupling between LC neurons. Lucifer yellow can apparently pass from injected cells to electrotonically coupled neurons by passing through gap junctions. This phenomenon, known as "dye-coupling", may be affected by experimental procedures. Constant hyperpolarizing current used for dye injection may prevent dye-coupling, presumably by closing gap junctions due to the development of a transjunctional potential (Spray, et al, 1979). However, we have used optimal conditions for the demonstration of dye-coupling and have not detected any dye-coupling between LC neurons. In the only case where cells were secondarily stained, the dye apparently passed between the cells by a transsynaptic route.

Simultaneous intracellular recordings were also used to test for electrical coupling between LC neurons. In simultaneous recordings of cultured LC neurons, no current or voltage spread from one cell to another was apparent, as injection of polarizing current pulses into either cell did not alter membrane potential of the other cell. However,

ephaptic or electrotonic conduction of potentials at dendritic sites remote from the recorded cell somas would give rise to potentials that would be highly attenuated when they reached the soma. Therefore, I also averaged data to reveal any small potentials that might represent current or voltage transmission between cells. After averaging, no potentials in the monitored cells were observed when hyperpolarizing or depolarizing current pulses were injected into the other cells.

However, in three simultaneous recordings, when one cell was sufficiently depolarized by current pulses to evoke spikes, small depolarizing events were revealed by averaging. These small depolarizations occurred synchronously with the afterhyperpolarization of evoked spikes in the stimulated cell. However, there was not any interaction apparent at the beginning or end of the depolarizing pulses. Ephaptically or electrotonically conducted potentials would have the same polarity as the potential inducing them. As these small depolarizations are of the opposite polarity to the afterhyperpolarizations, they do not appear to be electrically conducted events. Furthermore, if electrical conduction of potentials had occurred, potential changes due to hyperpolarizing or depolarizing (not evoking spiking activity) current injection should have also evoked responses in the monitored cell.

However, these small depolarizations might be explained on the basis of accumulation of extracellular potassium.

During the afterhyperpolarizations(s) of a spike(s) in the activated cell, potassium is released and any accumulation of potassium in extracellular spaces could depolarize nearby neuronal elements. Such depolarizing events, due to potassium accumulation released during the AHP of another neuron, have been demonstrated to occur between cockroach neurons (Yarom and Spira, 1982). With repetitive activity, this depolarization would increase, and with simultaneous activation of all cells, such depolarizations may be larger and could enhance and prolong the depolarizations associated with bursts. However, these depolarizations do not appear to be the depolarizing events that initiate bursts of activity, as they are too small to be those potentials. Furthermore, no LC cell recorded fired before depolarizations initiating a burst were observed.

It is interesting to note that spontaneous bursts were usually not associated with a prolonged afterhyperpolarization (individual spikes were followed by a short afterhyperpolarization), but stimulated activity with depolarizing current pulses did evoke a long afterhyperpolarization. Two possible reasons may explain this observation: 1) mechanisms involved with the long afterhyperpolarization may be modulated during bursting activity or 2) the long afterhyperpolarization was masked by ongoing activity, such as epsp activity. An accumulation of extracellular potassium would also reduce an afterhyperpolarization, which is due to an increase in potassium conductance. However this requires further

investigation.

B) Synaptic Interactions

The nature of spontaneous depolarizations and depolarizations evoked by electrical stimulation of the explant was examined. Stimulation of the explant evoked two different types of depolarizing potentials - one potential with a fast rise time and another potential with a slower rise time that was similar to spontaneous depolarizations. The fast rise time evoked potentials appeared to be potentials due to the direct stimulation of neuronal elements of the recorded cell, as these potentials were present during perfusion with solutions that blocked synaptic transmission, and since they collided with spikes evoked at the cell soma by depolarizing current pulses. Furthermore, the directly evoked potentials could be blocked by hyperpolarizing the cell; presumably this blocks spike transmission at a site remote from the cell soma, so that the potential fails to reach the soma. The other evoked potential and spontaneous depolarizations appear to be excitatory post synaptic potentials (epsp's).

Spontaneously occurring single depolarizations appeared to be the same depolarizations that summate to evoke bursting activity. These spontaneous depolarizations often occurred at higher frequencies, summing to produce a larger and longer depolarization. The spontaneous depolarizing potentials and evoked slow depolarizations, which appear to be very similar, have the characteristics of epsp's that are mediated by an

increase in ionic conductance. These events are not reversed ipsp's, as they were observed in LC neurons recorded with either potassium chloride or potassium acetate electrodes. In addition, these potentials had very similar time courses.

Both of these events are similarly affected by polarization of the cell, increasing and decreasing in amplitude with hyperpolarization and depolarization, respectively. Furthermore, the evoked slow depolarizations could be reversed with strong depolarization of the cell. Therefore, the spontaneous depolarizations and evoked slow depolarizations have the characteristics of synaptic potentials mediated by an increase in membrane conductance, with a reversal potential between -30 and +30 mV.

Bursting activity and both spontaneous depolarizations and evoked slow depolarizations were also blocked by perfusates that block synaptic transmission. However, during perfusion with these perfusates, thresholds for spike generation usually increased, although cells often depolarized. Therefore, the loss of spontaneous depolarizations could have been due to either blockade of synaptic events or due to a decrease of cell excitability. However, when 0.5 mM calcium, 2.0 mM manganese BSS was used to block synaptic transmission, spontaneous depolarizations and bursting ceased and slow depolarizations could not be evoked, even though cell excitability increased or remained the same. Research on evoked responses in the spinal cord also has demonstrated that low calcium/ 2.0 mM Mn blocks synaptic

transmission, but does not increase firing threshold (Bagust and Kerkut, 1980).

Furthermore, it appears that electrical stimulation of the explants may be stimulating neuronal elements that are responsible for the spontaneous depolarizations that summate to initiate synchronous bursts. With single suprathreshold stimuli ($>100 \mu\text{A}$) or multiple stimuli applied to the explant, bursts of firing activity could sometimes be elicited. These bursts were blocked by perfusion with 0.5 Ca/2.0 Mn BSS, even when the intensity of stimulation was increased 2 or 3 fold, and direct stimulation of cells still occurred. Therefore, bursting is blocked by blocking synaptic transmission. Together with the effect of polarizing current on depolarizations, this evidence indicates that LC neurons are synchronously activated by a common excitatory synaptic input.

c) Common excitatory inputs - *in vivo* and *in culture*

Aston-Jones and Bloom (1981a,b) have also suggested that synchronous bursting activity of LC neurons in unanesthetized animals was due to a common excitatory input onto LC neurons. They reported field potentials that were associated with bursting activity. Similar field potentials were also observed during paradoxical sleep, when LC neurons are quiescent, indicating that these field potentials were not due to a population spike, but possibly a population epp.

The question that then arises is: "What is the source of this common excitatory input?". Synaptic connections between LC neurons have been described. However, it is unlikely that

these contacts are responsible for the common excitatory input. First of all, noradrenaline released by LC neurons is inhibitory to LC neurons in adult animals. Although we have observed noradrenergic excitations in cultured LC neurons, we observed these only in younger cultures and we observed bursting in all age groups. Blocking the excitatory α_1 -adrenoceptors with prazosin also did not block spontaneous bursting. Furthermore, in simultaneous recordings, stimulation of one cell does not evoke synaptic potentials in other cells (the potentials I observed after averaging were far too small to be related to the depolarizing potentials), ruling out LC interactions as the basis of excitatory effects.

However, the cells that are responsible for the excitatory synaptic input in tissue culture must be found in or near the LC at least at birth, as they are explanted with the LC. In addition, LC explants originate from a highly restricted region of tissue, often with less than 50 LC neurons and no other apparent neuronal groups, and still usually exhibit bursting activity. Thionine staining of such cultures reveals that there are neurons in and around the LC, that are smaller than most LC neurons. Such small neurons within or near the LC explant might be responsible for the common excitatory input.

Where would such neurons be found *in vivo*? As noted in the introduction, small oval cells are found in and around the LC group in Golgi stained material. It is possible that the small oval neurons that had locally ramifying axons within the

LC, are the common excitatory input onto LC neurons, and are relaying sensory activation from another neuronal group(s). It is important to determine if these neurons are indeed non-adrenergic as suggested by Shimizu (1979) and if so what transmitter(s) do these neurons use.

III) NORADRENERGIC RESPONSES OF CULTURED LOCUS COERULEUS NEURONS

1) Characteristics of noradrenergic responses

I have investigated the effects of noradrenaline on cultured LC neurons using intracellular recordings, iontophoretic and bath application of noradrenaline and adrenergic agonists, and bath application of adrenergic antagonists and modified balanced salt solutions. Much of this data has been published (Finlayson and Marshall, 1984,1986a).

Iontophoretic application of noradrenaline (NA) onto cultured LC neurons elicited two types of responses: a simple hyperpolarization and a hyperpolarization followed by an excitation. The excitations appear to be direct depolarizations, barrages of depolarizing events (epsp's) or a combination of these effects. Excitations of LC neurons by NA have not been previously reported from electrophysiological studies.

The responses of LC neurons during tetrodotoxin intoxication were either simple hyperpolarizations or biphasic responses - a hyperpolarization followed by a depolarization. Both components of this response were dose-dependent

increasing in amplitude as ejection of NA was increased by increasing iontophoretic current applications. In addition, without any blocking agents, NA iontophoresis also activated barrages of epsp's, and therefore may be exciting other neurons included in the cultures. This excitation of excitatory neurons by noradrenaline may explain the recurrent facilitation of activity of LC neurons evoked by cortical stimulation (Nakamura, et al.,1980). As this facilitation was lost when noradrenergic transmission was inhibited by depletion of NA (Nakamura,et al.,1980), this facilitation may be due to LC collateral actions on intrinsic excitatory neurons.

The hyperpolarizing and depolarizing responses to NA have been examined using selective agonists and antagonists, and appear to be mediated by the two subtypes of α -adrenoceptors. Subtypes of α -adrenoceptors were first recognized in studies on the sympathetic nerve-muscle junction (for review see Starke,1981). Two distinct α -adrenoceptors were identified on the basis of differential effects of antagonists and agonists on the "presynaptic" (α_2) receptors (measured by the outflow of transmitter - NA) and "postsynaptic" (α_1) receptors. Table III gives the relative affinities and potencies of the agonists and antagonists for the two subtypes of α -adrenoceptors.

Yohimbine has been demonstrated to be a highly selective competitive antagonist of α_2 -adrenoceptors (Weitzell, et al.,1979). Prazosin, the most selective α_1 -antagonist, also

Table IV: Relative Potencies and Affinities of
Antagonists and Agonists for α -adrenoceptors

RECEPTOR		ANTAGONISTS	
α_2		rauwolscine, yohimbine >> corynanthine, prazosin	
α_1		prazosin >> corynanthine, yohimbine > rauwolscine	
		AGONISTS	
α_1	potencies affinities	phenylephrine > clonidine clonidine > phenylephrine	
α_2	potencies affinities	clonidine >> phenylephrine clonidine >> phenylephrine	

is a competitive antagonist (Constantine, et al., 1973; Timmerman, et al., 1980).

The two subtypes of α -adrenoceptors have also been demonstrated in the central nervous system, using the selective agents characterized in the periphery. Selective binding, antagonism and agonism on central α -adrenoceptors by the above agents have been demonstrated in the following reports:

Tanaka and Starke, 1980 - α_1 & α_2 adrenoceptors;
Hedler, et al, 1981 - yohimbine diastereomers;
Timmermans, et al, 1981 - yohimbine distereomers,
prazosin, clonidine;
Marwaha and Aghajanian, 1982 - α_1 and α_2 antagonists
Greengrass and Bremner, 1979 - Prazosin binding.

The hyperpolarizing response to NA in cultured LC neurons is similar to the α_2 -mediated inhibition of LC neurons observed in tissue slices and *in vitro*. The hyperpolarization of cultured LC neurons by NA is blocked selectively by the α_2 antagonist, yohimbine, and is mimicked by the selective α_2 agonist, clonidine. Furthermore, the hyperpolarizing response

to NA was not affected by prazosin (up to 1 μM), propranolol (1 μM) or sotalol (1 μM). Unlike in other studies, our input resistance measurements are not contaminated with changes in neuronal activity as they were made during TTX intoxication. However, as in tissue slices, the hyperpolarization to NA in cultured LC neurons is associated with a decrease in input resistance i.e. an increase in conductance. Therefore it would appear that cultured LC neurons have α_2 -adrenoceptors that are similar to receptors on adult LC neurons.

The depolarizing component of biphasic responses observed during TTX intoxication appears to be a direct response (see below) of LC neurons to NA mediated by α_1 -adrenoceptors. The depolarizing response was dose dependent, and was blocked by the selective α_1 antagonist, prazosin and was not affected by yohimbine (up to 1 μM), propranolol (1 μM) or sotalol (1 μM). The selectivity of the blockades of the hyperpolarizing responses by α_2 antagonists and the depolarizing responses by α_1 antagonists was indicated by the absence of antagonistic effects of yohimbine and prazosin on the depolarizing and hyperpolarizing responses, respectively.

The prazosin antagonism of the depolarizing response was dose-dependent, producing a partial antagonism of the response when applied at less than 100 nM. Prazosin application also shifted the dose (iontophoretically applied NA) - response (depolarizations during α_2 -adrenoceptor blockade) curve to the right. It was not possible to completely surmount the antagonism by prazosin with

iontophoretically applied NA. This might be due to the inability to apply sufficient amounts of NA by iontophoresis to overcome the antagonism, as maximal currents of iontophoresis were tried. It is also possible that the inability of iontophoretically applied NA to overcome the antagonism of the depolarizing response by prazosin is due to a mixed interaction (competitive and non-competitive) of prazosin with the receptors involved. A mixed action of prazosin could explain difficulties in reversing the blockade of prazosin. It is not possible to calculate a dissociation equilibrium constant for the prazosin antagonism from iontophoretic experiments, as the tissue concentrations of NA at various iontophoretic currents is unknown. However, the highly selective antagonism of the depolarizing response to NA by prazosin indicates that α_1 -adrenoceptors mediate this response.

Further evidence that noradrenergic depolarization is mediated by α_1 -adrenoceptors comes from the fact that the α_1 agonists, phenylephrine and methoxamine, applied either by iontophoresis or in the bath, selectively depolarized young LC neurons. The longer depolarizations to iontophoretically applied methoxamine or phenylephrine than to NA may reflect the fact that NA is actively taken up by cells whereas the agonists continue to act until they are washed out of the tissue. The longer durations of the responses to agonists may also be due to duration of receptor occupancy and other aspects of ligand receptor interactions. The long

depolarizations of LC neurons to bath applied NA, likely reflects the slow washout of NA from the bath.

2) Calcium dependence of responses

The adrenergic hyperpolarizations and depolarizations of LC neurons appear to be direct effects. Both of these responses were present during TTX intoxication, which blocked the indirect activation of epsp activity by NA, as well as spontaneous and evoked synaptic activity. Although the depolarizing component was blocked in some cases by perfusates that block synaptic transmission, the response was maintained (although sometimes reduced) during perfusion with normal calcium, high magnesium BSS or with 0.5 mM Ca and 2.0 mM Mn BSS, both of which also block synaptic transmission. This indicates that at least a part of the depolarizing response to NA in LC neurons is direct. Perfusates that contain low calcium, and manganese or high magnesium may affect the depolarizing responses in at least two ways. Cell membranes became highly unstable during perfusion with these perfusates, and this may have affected the depolarizing response. Secondly, these perfusates may block a calcium conductance of LC neurons, that is involved in the depolarizing response to NA. A calcium dependence of the depolarizing response would also explain the reduction of this response by normal calcium, high magnesium perfusates and the further reduction and occasional abolishment of depolarizing responses to NA with continued perfusion of 0.5 mM Ca and 2.0 mM Mn perfusates.

Therefore, the noradrenergic depolarization in LC neurons may be calcium dependent. I have tried to demonstrate a calcium dependence of the depolarizing response by intracellularly injecting EGTA, a calcium chelator. However, no information was derived from these experiments, as LC neurons became highly unstable when EGTA was injected. It should also be noted that the noradrenergic hyperpolarization of these cells is not dependent on calcium as it was not affected by any of these perfusates.

It has now been documented that α_1 -adrenoceptors mediate depolarizations and excitations of neurons in many regions. The effect of altering divalent cations on these responses has been investigated in the excitation of raphe, parasympathetic and spinal neurons. In parasympathetic neurons, noradrenaline also elicits direct biphasic responses in many cells (Nakamura, et al., 1984; Akasu, et al., 1985). However in these cells, low calcium/high magnesium or high magnesium and manganese perfusion had no effect on the α_1 -mediated depolarization but blocked the α_2 -mediated hyperpolarization. In the raphe, the α_1 agonist, phenylephrine suppresses the transient potassium conductance (I_A) (activated by a depolarizing step from -70 mV to -30mV), but this suppression was not blocked by 1 nM(?) cadmium, a calcium channel antagonist (Aghajanian, 1985). In neonatal lateral horn neurons in spinal cord slices, α_1 -mediated depolarizations were decreased in 5 of 12 cells but were not affected in the remaining cells by either low calcium, high magnesium or TTX

containing perfusates (Ma and Dun, 1985). Therefore, in the lateral horn, α_1 -responses appear to be direct and indirect, with the indirect responses being blocked by TTX intoxication and by low Ca/high Mg perfusates). Finally, in hepatocytes, it has been demonstrated that α_1 -adrenoceptor activation by phenylephrine induces repetitive transient intracellular calcium rises, as measured with the dye, aequorin (Woods, et al., 1986). The actions of α_1 -adrenoceptors appear to be complex, involving calcium in some tissues, and possibly indirect activation of excitatory input. It is also curious that α_2 -mediated actions in parasympathetic neurons were calcium dependent unlike α_2 -mediated responses in other areas such as the LC. It should also be noted that noradrenaline induces biphasic and depolarizing responses in cultured dissociated spinal neurons co-cultured with LC explants (Marshall, et al, 1981). Although the receptor mediating this depolarization has not been completely characterized, it is blocked by the α -adrenergic antagonist piperóxane (Pun, et al, 1985).

Activation of excitatory inputs onto recorded neurons by NA has been noted in many studies, including the aforementioned study on lateral horn neurons. An activation of excitatory inputs onto substantia gelatinosa neurons (North and Yoshimura, 1984), and onto hippocampal neurons (Rosenberg, et al., 1986) have also been noted. I have also noted an activation of excitatory input onto LC neurons.

Input resistance measurements in TTX treated cultured LC

neurons did not reveal any consistent changes in membrane conductance, during depolarizations to methoxamine or depolarizations to NA in the presence of yohimbine. In both neonatal lateral horn cells (Ma and Dun, 1985) and parasympathetic neurons (Akasu, et al., 1985), the α_1 -mediated depolarizations were associated with decreases in membrane conductance. The differences in my findings in cultured LC neurons and these studies is uncertain. Resistance measurements may be affected in all these neurons by conductance changes due to the α_2 -mediated responses, and by the location of the receptors (i.e. somatic or dendritic). These factors may have influenced my measurements for conductance changes.

The effect of polarizing the membrane on the depolarizing response (increasing with hyperpolarization and decreasing with depolarization) of LC neurons to NA, suggests that the depolarizing response is mediated by an increase in conductance. Therefore, it is possible that the above mentioned factors may have affected our conductance measurements. However, this requires further investigation.

3) Age-dependence of noradrenergic LC responses

The most interesting finding in my thesis research is that the depolarizing responses were age related, being lost after the tissue was grown *in vitro* for more than 26 days. As LC neurons in older cultures only exhibit direct hyperpolarizing responses to NA, and LC neuronal responses from adult tissue are only hyperpolarizing responses, LC

neurons in older cultures may represent relatively mature cells that can be compared to adult LC neurons. Therefore the loss of the depolarizing response may represent a developmental change in the responsiveness of LC neurons to NA. It is also important to note that the time frame for this developmental change may be considerably different *in vivo*.

There are a number of possible explanations for the loss of a response in neurons to exogenously applied transmitters:

- 1) receptors may be lost due to a loss of or the absence of the appropriate innervation,
- 2) receptors may be lost due to changes in levels of circulating hormones or other circulating factors during maturation,
- 3) receptors may still be present but the response is suppressed, possibly due to an uncoupling of the receptors from ion channels or membrane enzymes or a loss of driving force (e.g. due to changes in cellular ionic composition),
- 4) redistribution of receptors from their original site (e.g., the cell soma) to other regions of the cell (e.g. distal dendrites) (Mueller, et al., 1984),
- 5) selective neuronal death of cells exhibiting the responses.

The loss of the depolarizing response in cultured LC neurons does not appear to be due to a loss of cells. Marked losses of cells in LC regions, that would be expected if neurons with α_1 receptors were lost during development, were not observed. Furthermore, all neurons recorded from cultures that were less than 20 days *in vitro* (n=32) had biphasic responses to noradrenaline, suggesting that all cultured LC neurons in these age groups had α_1 -adrenoceptors.

Are α_1 -adrenoceptors present in adult or neonatal LC nucleus? An autoradiographic study using the α_1 antagonist, WB-4101, indicated that α_1 adrenoceptors in the adult LC are just above background levels, whereas high densities of α_2 -adrenoceptors (labelled with para-aminoclonidine) were found in the adult LC (Young and Kuhar, 1980). In a more recent study, another proposed α_1 ligand, HEAT (2-*N*-(4-hydroxyphenyl)-ethylaminomethyl-tetralone) was used to study the development of α_1 adrenoceptors in the brain (Sargent Jones, et al., 1985). This study indicates that α_1 -adrenoceptors appear as early as postnatal day 15 in the rat LC, and that these receptors were present until at least day 45. The selectivity of the two ligands - WB-4101 and HEAT, does not appear to underly the differences in these studies. Although WB-4101 labels sites that are not α_1 -adrenoceptors it also binds to α_1 -adrenoceptors (Unnerstall, et al., 1982). HEAT binding was displaced by either 1.0 μ M prazosin or 10 μ M phentolamine (a non-specific α -adrenoceptor blocker), suggesting that sites labelled by HEAT are α_1 -adrenoceptors (Sargent Jones, et al., 1985). The affinities of these two ligands, however could be the difference. HEAT may have a greater affinity for α_1 -adrenoceptors, and therefore label areas with relatively low levels of these receptors. Unfortunately, autoradiographic studies have not resolved the location of receptors. It is possible that α_1 -adrenoceptors in the adult animal are on the small oval cells, which may be non-adrenergic. A quantitative study on α_1 -adrenoceptors in the LC is required to determine

possible changes in these receptors during maturation.

Physiological evidence of the presence of α_1 -adrenoceptors has also been reported. Injection of phenylephrine or noradrenaline into the LC induces an α_1 -adrenoceptor mediated hypotension in cats (Sinha, et al, 1984). However, the cat LC contains both noradrenergic and non-adrenergic neurons, so the cells responsible for these effects is unclear.

However, in a recent study, the responses of LC neurons in tissue slices from young rats (8 to 26 days) to noradrenaline and adrenergic drugs have been investigated (Marshall and Williams, 1986). Unlike LC neurons from older animals, young LC neurons were depolarized by the α_1 agonist, phenylephrine. Furthermore, the response to noradrenaline was a hyperpolarization, which quickly decreased after a maximal potential was reached - i.e. there was a "sag" in the response. This "sag" was blocked by the α_1 antagonist prazosin, and was more pronounced in slices from younger animals. However, it was also noted that a small α_1 component of adrenergic responses could also be detected in some LC neurons from adult animals, when the responses to NA were compared before and after treatment with prazosin (J.T.Williams, personal communication). Therefore, as an α_1 -adrenergic response of young LC neurons has now been observed in a different preparation, it appears that LC neurons normally have a substantial α_1 -adrenergic response at a young age.

Although it appears that α_1 -adrenoceptors are present in the LC nucleus at an early age, the maturation and location of these receptors is unclear. Our data are compatible with a loss or a substantial reduction of these receptors from LC neurons, which would explain the absence of binding of WB-4101 in adult animals. However, it is also possible that these adrenoceptors become preferentially located on distal dendrites during maturation, so their effect is not usually observed at the soma. Either an incomplete loss of, or a redistribution of these receptors could explain the relative absence of the α_1 -adrenergic component of adrenergic responses observed in adult LC neurons.

The development and regulation of α_1 -adrenoceptors have been studied in other central nervous system tissues. The effects of denervation of noradrenergic systems have consistently demonstrated a denervation supersensitivity of α_1 -adrenoceptors (cerebral cortex: Reader and Briere, 1983; Sutin and Minneman, 1985; lateral geniculate nucleus: Menkes, et al., 1983). Conversely, hyperinnervation by noradrenaline has been reported to produce a decrease in α_1 -adrenoceptors (Sutin and Minneman, 1985). The effect of acute and chronic desmethylimipramine (DMI) treatment on α_1 -adrenoceptors has also been investigated. Based on behavioral effects of phenylephrine and clonidine, DMI (which is a noradrenaline uptake blocker) treatment resulted in an increased α_1 -adrenergic response and a decreased α_2 -adrenergic response (Plaznik, et al., 1984). Other studies have

focused on the ontogeny of these receptors. In the pineal gland, α_1 -adrenoceptors increase from E19 to P10, then decrease to adult levels by P20 (Sugden and Klein, 1985). In the hypothalamus, α_1 -adrenoceptors decreased in number after birth, but in the cortex increased after birth (Johnson, et al., 1984). Sargent Jones et al (1985) found that α_1 -adrenoceptors decreased in the bed nucleus of the stria terminalis, the pineal, and in the globus pallidus, beginning 5 to 15 days after birth. Furthermore, these receptors completely disappeared in the globus pallidus.

Developmental decreases as noted in other tissues is of interest to the developmental changes in the responses of LC neurons to NA. The loss of depolarizing responses in LC neurons to NA could be due to similar decreases in α_1 -adrenoceptors. It is also of interest to note that α_1 - and α_2 -adrenoceptors appear to be differentially regulated, as is evident in effects of DMI treatment on these receptors. Therefore, α_1 -adrenoceptors may be down-regulated by NA, while α_2 -adrenoceptors may be upregulated.

4) Loss of receptors or responses in other systems

A number of examples of responses or receptors that are lost during maturation have been recently reported, and this phenomenon may occur in many tissues during development. For example, in the rat retina, both "on" and "off" ganglion cells in the immature eye (7-9 weeks) are depressed by both glycine and GABA (Ikeda and Robbins, 1985). However, in 18 to 22 week

old rats, "on" retinal ganglion cells are only inhibited by GABA and "off" retinal cells are only inhibited by glycine. In the adult animal, GABA is the transmitter that inhibits "on" cells, while "off" retinal ganglion cells are only inhibited by glycine. Therefore, it appears that non-functional receptors are lost from these two cell types. Another example of loss of responses in neurons comes from work on Rohon-Beard cells in the developing xenopus spinal cord. These neurons are depolarized by glycine at Stage 26, but are not affected by glycine at Stage 43 (Bixby and Spitzer, 1982). Furthermore, the responsiveness of rat Purkinje neurons to excitatory amino acids changes with age (Crepel, et al, 1982). In 5-6 day old rats, Purkinje neurons are sensitive to N-methyl-D-L-aspartate (NMDA), but in adult rats, these neurons are much less sensitive to NMDA. A similar change was not observed for other excitatory amino acids.

It has also been demonstrated, that receptors are lost by biochemical and autoradiographic methods. In the chicken retina a subtype of the dopaminergic D₁ receptor is lost during maturation (Ventura, et al, 1984). Finally, glutamate binding in the globus pallidus is present in young rats (7 days) and human infants (6 weeks), but is not present in the adult of either species (Greenamyre, et al, 1984). Therefore receptors or at least responses of specific receptors are lost during development, but what is the significance of these losses.

A number of possible reasons for the loss of receptors

(or responses) can be suggested:

- 1) retraction of neuronal innervation and the concomitant loss of transmitter x, may result in the loss of receptors for that transmitter (possibly the case for pallidal glutamate receptors),
- 2) failure of appropriate innervation with transmitter x, resulting in loss of unused receptors (possibly the case in retinal ganglion cells),
- 3) the receptors may develop as vestiges of phylogeny, serve no function and are lost during development,
- 4) the receptors may play an important role during development, and are subsequently lost.

5) Physiological significance of loss of α_1 -responses

In the case of LC neurons where noradrenergic innervation obviously persists after development, α_1 -adrenoceptors may have an important role early in development. As the LC has been repeatedly suggested to be involved in regulating development of neuronal areas, the presence of α_1 -adrenoceptors early in development may be important for such a role.

I have tried to determine any physiological influence of α_1 -adrenoceptors on the activity of LC neurons in "young" cultures, by monitoring activity before, during and after blockade of α_1 receptors with prazosin. Although, in a few cases, LC neurons hyperpolarized during prazosin application, changes in activity were not observed in all cells. Therefore, I could not demonstrate any physiological function of α_1 -adrenoceptors in tissue culture. However, observations in other studies may be explained by the presence of α_1 -adrenoceptor mediated responses at this stage of

development.

In behavioural studies, the effect of the α_2 agonist clonidine on motor activity has been investigated. In adult animals clonidine has a sedative effect producing a hypoactivity, whereas in young rats, clonidine induces a hyperactivity (Reinstein and Isaacson, 1977). Both of these effects of clonidine on behaviour are antagonized by alpha adrenergic blockers (Nomura and Segawa, 1979; Nomura, et al., 1980). Non-selective α -adrenoceptor antagonists and the α_2 -antagonist, yohimbine, block both effects of clonidine, so both effects appear to involve α_2 -adrenoceptors. However, selective α_1 -antagonists have not been tested, and it is possible that α_1 -adrenoceptors are involved in the clonidine induced hyperactivity (Nomura and Segawa, 1979). As these effects of clonidine are thought to involve central mechanisms, they might be explained by clonidine acting on α_1 -adrenoceptors in the LC of young rats. However, the pharmacology and location of these drug effects need to be established before a reasonable conclusion can be made.

In extracellular recordings of LC neurons in anesthetized neonatal rats (Kimura and Nakamura, 1985), these neurons appeared to be activated by a broad range of stimuli, unlike cells recorded in anesthetized adult animals. In addition, the bursts of activity elicited appear to have a longer duration than in adult animals. These comparisons, however, are complicated by differences in anesthetics used by different groups. Nakamura (1977) used the same anesthetic

(urethane) to anesthetize adult animals, and did not find the robust activation of LC neurons by sensory stimuli. However, in urethane anesthesia, it may be possible to activate LC neurons by more sensory inputs, as in one case a hand clap activated an LC neuron in an adult rat (Nakamura, 1977). Therefore, it would appear that LC neurons in neonatal animals, have a prolonged response to sensory stimuli. It will be interesting to test if noradrenaline and α_1 -adrenoceptors are involved in prolonging the sensory response.

IV) SUMMARY AND PROGNOSIS

I have demonstrated a number of new characteristics of locus coeruleus neurons using an *in vitro* model - explant cultures of LC neurons. The most striking finding is a developmental change in the noradrenergic responsiveness of these cells, which may reflect a substantial loss of α_1 -adrenoceptors. Secondly, I have presented evidence that LC neurons are activated by an intrinsic excitatory input that may be functioning *in vivo*, to synchronously activate LC neurons to burst in response to particular sensory events. In addition, LC neurons may have multiple axon systems, some originating distal from the soma. Furthermore, I have demonstrated that cultured LC neurons have many similar characteristics to LC neurons *in vivo*.

Many putative roles have been suggested for the locus coeruleus, based on lesioning and psychopharmacological tests. Many of these putative roles have been reviewed (Amaral and Sinnamon, 1977; Aston Jones, Foote and Bloom, 1983). However, recently the physiology of the LC has been emphasized, when roles for this small nucleus have been considered. The literature on the physiology of the locus coeruleus has added much to our understanding of the roles of this nucleus.

The LC now appears to have minor involvement or no involvement in previously postulated functions. For example, it had been suggested that the LC is especially important in the onset and maintenance of paradoxical sleep. However, as LC neurons are quiescent during this stage of sleep, the LC has at most a permissive role in paradoxical sleep.

The activity of LC neurons, as recorded in unanaesthetized animals, suggests that this cell group is involved in arousal, attention and/or vigilance (Foote, et al, 1980; Aston-Jones and Bloom, 1981a,b; Jacobs, 1986). The most striking feature of the activity of the LC is the synchronous activation of these neurons to fire in bursts by sensory stimuli. Bursts of activity of LC neurons may be the physiologically significant activity of this nucleus, for a number of reasons.

Aston-Jones, Segal and Bloom (1980) have demonstrated that the conduction velocity of LC axons is a function of previous discharge, with a slowing of conduction velocity following each spike. Based on the magnitude of changes in

conduction velocity at different spike discharge frequencies, these researchers suggested that short fast discharges likely would be the most effective in releasing NA at the target regions. Therefore, bursting activity would be the most effective discharge pattern for noradrenergic release.

Furthermore, in a recent study on peripheral noradrenergic neurons, it has been demonstrated that burst discharges release peptide transmitters that are colocalized in these neurons (Lundberg, et al, 1986). As noted in the introduction a number of peptides that may be cotransmitters of LC neurons have been localized in these neurons. It is thus quite likely that LC neurons release both NA and one or more peptides from their terminals. If, as in the periphery, peptides are preferentially released by burst discharges, bursting may also serve to enhance release of these transmitters from LC neurons. Finally, the synchronous activation of LC neurons to burst suggests that all of the varied regions to which the LC projects will be affected by noradrenergic transmission at about the same time. Therefore, synchronous bursts of activity of LC neurons may cause the optimal and simultaneous release of NA and cotransmitters of LC neurons at its widespread target regions.

Therefore, it will be important to understand the intrinsic and extrinsic mechanisms involved in burst initiation and the modulation of bursting activity (such as the inhibition of bursting during paradoxical sleep). My research indicates that intrinsic neurons are involved in

research indicates that intrinsic neurons are involved in initiating bursting activity, and activity in these intrinsic excitatory neurons may be facilitated by noradrenergic transmission from LC neurons. Bursting activity is also affected by auto-regulation of LC neurons by noradrenergic autoinhibition and a calcium-activated potassium conductance.

The age-dependent adrenergic depolarizing response which I have observed, may enhance bursting activity of LC neurons in response to repetitive sensory stimuli in young animals. An enhanced responsiveness of LC neurons might be involved in putative roles of the LC during development, such as noradrenergic influences on plasticity in the visual cortex (Kasamatsu and Pettigrew, 1979, Kasamatsu, et al, 1979).

Further research on the mechanisms involved in bursting activity, including intrinsic regulation and pathways involved in relaying sensory information to the LC, will aid in our understanding of this small nucleus and its role in brain function.

Appendix 1: Tissue Culture Methods

I) Preparation of Collagen Coated Coverslips

1) Preparation of collagen

Collagen was partially purified from rat tail tendons. Each rat tail was removed from a guillotined rat and was cleaned then frozen (for 24 Hrs). Each thawed tail was partially sterilized by placing it in 80% ethanol for 15 min. Using sterile rongeurs and towel forceps, tendons were removed and placed into sterile water. Using needle forceps, the tendons were teased apart. Separated tendon fibers were then placed in 150 ml of sterile 1% glacial acetic acid solution, and refrigerated at 4°C for 48 hours. After 24 hours, if dissociation of fibers was incomplete, 1 to 2 ml of sterile 1% acetic acid solution was added and the solution was refrigerated for the remaining 24 hours. The dissolved fiber - acetic acid solution was then centrifuged at 40,000 rpm at 4°C for 1 hour. The supernatant was separated from the gelatinous pellet. The supernatant was then dialyzed against sterile distilled water, in a proportion of 10 ml supernatant to 1.5 L distilled water, for 4 to 5 hours at 4°C. Dialysis tubing was Spectrapor #4 with a molecular weight cutoff of 12,000 to 14,000.

2) Priming of Coverslips

Three drops (from a bent-tip pipette; 50 µl/drop) of dialyzed collagen were spread on each round coverslip (Goldseal #3347, 22 mm, thinness #1). Collagen was then reconstituted on the surface of each coverslip by exposing

them for 3 minutes to ammonium hydroxide vapours (14 coverslips at a time in a 150 mm diameter covered petri dish, suspended above a 150 mm round filter paper with 8 drops of ammonium hydroxide). The ammonium hydroxide was then removed by rinsing the coverslips thrice in distilled water (with 14 μ M phenol red). Each rinse was 10 minutes. The collagen coated coverslips were then stored in BSS with 10% horse serum for up to 2 days before using.

II) BSS for Tissue cultures

Balanced salt solution (BSS) for tissue culture was prepared by mixing triple distilled water, Earle's 10X (sterile) BSS and sodium bicarbonate (sterile 7.5% solution) in the ratio of 90:10:3. Phenol red was added (to 14 μ M), and the BSS was bubbled with CO₂ to adjust the pH to 7.4.

III) Culture Medium

Chick embryo extract and human serum were thawed and separately centrifuged (25 ml/tube) at 40,000 rpm for 1 hour and at 4°C to remove lipids. Eagles minimal essential medium (MEM), chick embryo extract, human serum and glucose solution (1.11mM) were then combined in the ratio of 10:5:5:1, and glutamine was added (to 1 mM)(100 μ l of 200 mM glutamine solution/42 ml). Tubes containing 1 ml of the feed medium were quickly frozen in dry ice and acetone or alcohol, and kept frozen until used.

Appendix 2: Staining Solutions

I) Phosphate Buffer Solution

A solution of 0.3 M sodium phosphate, dibasic (Na_2HPO_4 ; 8.52g/200 ml distilled water) was titrated with 0.3 mM sodium phosphate, monobasic (NaH_2PO_4 ; 2.48 g/60 ml distilled water) to a final pH of 7.4.

II) Fixative for Lucifer Yellow Staining

Formalin (10 ml), phosphate buffer solution (33 ml)(see above) and distilled water (57 ml) were mixed.

III) Glyoxylic Acid Solution

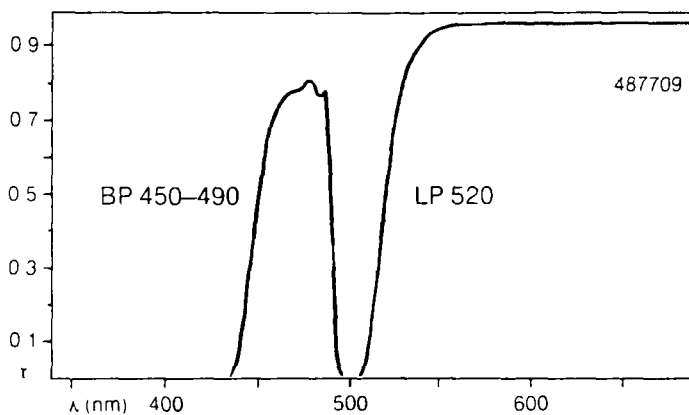
Ringer solution (5 ml) and phosphate buffer solution (5 ml)(see above) were combined and 0.2 g of glyoxylic acid (sodium salt monohydrate, Sigma) was added. After the glyoxylic acid was dissolved, the solution was filtered (.22 μm mesh, 25 mm wide filter) into a precooled Columbia dish.

Salt	Ringer for glyoxylic acid stain		[] of ions in stain
	Stock Ringer mg/100ml	Solution []	
NaCl	860	147 mM	73.5 mM
KCl	30	4.0 mM	2.0 mM
CaCl_2	33	2.2 mM	1.1 mM
Glyoxylic acid			270 mM
Phosphate buffer			150 mM

Appendix 3: Fluorescence

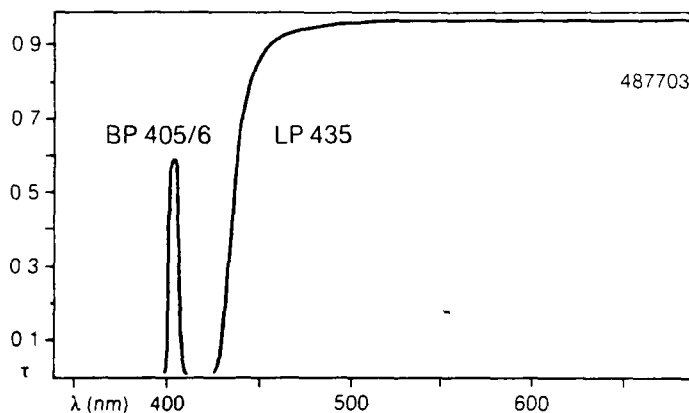
1) Filters used for Lucifer Yellow Fluorescence

Lucifer yellow is excited maximally with 426 nm light and has a fluorescence that is maximal at 540 nm (for spectra see Stewart, 1978). The FITC filter set (see spectra at right) is an adequate filter set for excitation and observation of lucifer yellow fluorescence.



2) Catecholamine Fluorescence

Glyoxylic acid induced catecholamine fluorescence has been reported to be maximally excited at 415 nm and maximal emission is at 475-480 nm (for spectra see Lindvall, et al, 1974, p212). The catecholamine fluorescence filters (487703) have the excitation/barrier spectra as shown at the right.



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