

CLONIDINE INHIBITS CENTRAL NORADRENERGIC NEURONS IN UNANESTHETIZED CATS

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Within the feline locus coeruleus complex (LCx) there exists a subpopulation of neurons with a distinct behavioral neurophysiological profile, these neurons fire most during waking, less so during slow wave sleep, and fall virtually silent during paradoxical sleep (PS). These 'PS-off' cells have been thought to be noradrenergic, largely because their heterogeneous distribution throughout the LCx parallels the distribution of noradrenergic neurons within the feline dorsolateral pontine tegmentum. The α_2 -adrenergic agonist clonidine has been shown to markedly inhibit the central noradrenergic neurons of the anesthetized rat, regardless of the route of administration. We therefore tested the PS-off cells of the feline LCx with intravenous clonidine, to determine if they too were inhibited by this agent. We found 13/13 LCx PS-off cells to be inhibited by intravenous clonidine (2–4 $\mu\text{g}/\text{kg}$) as compared with saline controls, non-PS-off cells were not inhibited. These data add strength to the argument that the PS-off cells of the feline LCx are noradrenergic.

Clonidine Noradrenergic neurons Locus coeruleus α_2 -Adrenoceptors

1. Introduction

A subpopulation of neurons in the dorsolateral pontine tegmentum of the cat, termed 'PS-off' cells, exhibits stereotypical variation in mean and absolute discharge rate across behavioral states (Hobson et al., 1975; Saito et al., 1977; Sakai, 1980; Sakai et al., 1981; Hobson et al., 1983). In unanesthetized, behaving animals, these neurons typically fire at their highest rates during active waking (AW), at lower rates during quiet waking (QW), still lower during slow wave sleep (SWS), and fall virtually silent during paradoxical sleep (PS).

In the cat, PS-off cells are heterogeneously intermixed with non-PS-off cells throughout the dorsolateral pontine tegmentum. This is in con-

tradistinction to the rat, where PS-off cells appear to be restricted to the nucleus locus coeruleus (Aston-Jones and Bloom, 1981). These observations correlate well with the species-specific distribution of noradrenergic neurons within the pontine tegmentum as demonstrated by both catecholamine histofluorescence (Dahlstrom and Fuxe, 1964; Maeda et al., 1973; Chu and Bloom, 1974a; Jones and Moore, 1974; Palkovits and Jacobowitz, 1974; Poitras and Parent, 1978; Wiklund et al., 1981; Jones and Friedman, 1983) and immunohistochemical localization of the noradrenergic synthetic enzyme dopamine β -hydroxylase (Hartman et al., 1972; Swanson, 1976; Grzanna and Molliver, 1980; Miachon et al., 1984). As a result, several authors have suggested that the PS-off cells of the feline locus coeruleus complex (LCx) (operationally defined in this paper as the nuclei locus coeruleus, subcoeruleus, parabrachialis lateralis and medialis, and Kolliker-Fuse) are noradrenergic (Hobson et al., 1975; Saito et al., 1977; Sakai, 1980; Sakai et al., 1981; Hobson et al., 1983).

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The α_2 -adrenoceptor agonist clonidine has been shown to profoundly inhibit central noradrenergic neurons in anesthetized rats when administered microiontophoretically, intraventricularly or intravenously (Svensson et al., 1975, Cedarbaum and Aghajanian, 1977). One would therefore predict that if feline LCx PS-off cells are indeed noradrenergic they might also be inhibited by clonidine. In the following paper, we report on the responses of feline LCx PS-off cells to intravenous clonidine administration, and the utility of such pharmacological characterization in the identification of central noradrenergic neurons in the behaving cat.

Some of these data have been previously presented in abstract form (Parsons et al., 1983, Reiner and Morrison, 1983).

2. Materials and methods

2.1 Animal preparation

Experiments were performed on 7 adult female cats, ranging in weight from 2–4.5 kg, kept on a 12·12 light/dark cycle. All animals received routine physical and neurological examinations, the animals maintained excellent health throughout the duration of the experiments, which lasted 1–3 months for any given animal. Anesthesia was induced in animals pretreated with atropine sulfate (0.05 mg subcutaneous) by intravenous administration of sodium thiopental (25 mg/kg); the animal was then intubated with a translaryngeal endotracheal tube, and anesthesia was maintained throughout the ensuing surgical procedure using halothane inhalant anesthesia. An intramuscular bolus of dexamethasone (1 mg/kg) was given prophylactically at the start of surgery to reduce the development of cerebral edema.

The cats were mounted in a stereotaxic apparatus and the surgical site prepared for aseptic implantation of an array of up to 23 recording electrodes. Stainless steel screw (0–20) electrodes were implanted bilaterally over the parietal cortex for recording the electroencephalogram (EEG), a single stainless steel screw electrode, placed 1.5

mm lateral to the midline, served as animal ground. Stainless steel screw electrodes were threaded into burr holes overlying the dorso-caudal aspect of each orbit for recording the electro-oculogram (EOG); all screw electrodes were covered with a fast-curing polymer acrylic (Tramix). Stainless steel wire electrodes for recording the electromyogram (EMG) were sutured, bilaterally, into the nuchal musculature. A bipolar macroelectrode was stereotaxically lowered into the right dorsal lateral geniculate nucleus to record pontine-geniculate-occipital (PGO) spikes. All macropotential leads were soldered to a 25 pin female connector (Amphenol, DB25), to which the microwires (below) had been presoldered, and the entire assembly embedded within a base of acrylic atop the animal's head.

An adaptation of the microwire technique described in detail by Harper and McGinty (1973) was used to record from single neurons in the LCx of behaving cats. A miniature microdrive, with 2 cannulas separated in the sagittal plane by 1.5 mm, was stereotaxically implanted at a 30° angle from the vertical, thereby aligning the cannulas parallel to the bony tentorium (Siegel, 1974). The microdrive was lowered until the tip of the rostral cannula was 6 mm above the intended recording site (coordinates used for LCx were AP 3.0, ML 3.0, DV – 3.0, for parabrachial nuclei AP 3.0, ML 4.0, DV – 3.0; for Kolliker-Fuse AP 3.0, ML 5.0, DV – 4.0) and cemented in place with acrylic. Two bundles of formvar-coated, nichrome microwires (six 32 μ m diameter wires per bundle; Stablom 675, stress relieved, California Fine Wire), presoldered to a 25 pin female connector, were then lowered through the 2 guide cannulas to a point 5 mm past their tips, in this manner, the distal ends of the microwire bundles were initially placed 1 mm above the intended recording site. The microwires were glued to the top of the cannulas with cyanoacrylate and covered with a protective varnish (Stoner-Mudge, Shell). The entire assembly was then covered with an inverted 5 ml plastic syringe, with the needle end pointing up and centered over the 0–80 screw in the microdrive, permitting later advancement of the microdrive in steps as small as 37.5 μ m, with a jeweler's screwdriver.

2.2 Data collection

Data was collected by means of low-noise cable (Microdot) attached to a male DB25 plug which matched that embedded in acrylic on top of the animal's head. The low-noise cable was led to a 24 pole commutator (BRS/LVE) which was counter-balanced, permitting the animal relatively unimpeded movement throughout the cage. Macroelectrode signals were fed directly to a polygraph (Grass, 78D); up to 2 channels of macropotential activity were frequency modulated (Vetter FM Converter) and recorded on magnetic tape (Sony, TC-388-4).

The array of 12 microwires was connected to a pair of 12 pole switches which permitted sampling and recording from any microwire without disturbing the animal. The output of this switching box was connected to a differential AC preamplifier (Grass, P-15) where the signal was amplified ($\times 1000$) and filtered (low pass = 300 Hz, high pass = 10000 Hz), a 'silent' microwire from the opposite bundle was used as the indifferent electrode. The preamplified signal was led to a window discriminator (WPI, 120) which provided TTL pulses to (a) deflect a polygraph pen, and (b) trigger a delayed sweep of a digital oscilloscope (Tektronix, 5223), thereby permitting the entire waveform of each action potential to be examined. Constant monitoring of the action potential waveform insured that there was no profound variation in either spike height or configuration over the course of the recording session. Five triggered sweeps of each cell studied were superimposed on an XY plotter to insure the unitary nature of the action potentials being discriminated.

During a typical recording session, the animal was placed in a well-lit, shielded and sound-attenuated cage, equipped with a one-way mirror, with food and water available ad libitum. The animal was connected to the counterbalanced cable assembly and data were recorded across several behavioral states, including at least 2 complete sleep-wake cycles. All data were recorded on polygraph paper, for all neurons, at least 60 s of data for each of 4 behavioral states (AW, QW, SWS and PS) were recorded on magnetic tape for off-line analysis.

2.3 Clonidine administration

Twenty one neurons were tested for their response to administration of the α_2 -adrenoceptor agonist clonidine (Boehringer). These animals were fitted with a 24 g intravenous catheter (Abbocath) in the cephalic vein. The catheter was kept in place for 3 days, during which time patency was maintained with frequent injections of heparinized saline. In a pilot study, we noted that extremely small doses (4–6 $\mu\text{g}/\text{kg}$) of clonidine, administered intravenously, had reliable emetic effects. Therefore, in all animals tested, we first administered clonidine in a series of increasing doses, with at least 20 min between injections, to determine the dose which induced emesis in each animal. Thereafter, in order to prevent aversive associations with clonidine administration, a dosage of clonidine 1 $\mu\text{g}/\text{kg}$ below the emetic dose (sub-emetic dose) was used to test the response of neurons in the LCx to clonidine. In each test, a total volume of 0.6 ml was injected, the first 0.1–0.3 ml (depending upon the sub-emetic dosage appropriate for the individual cat) containing the clonidine and the final volume was made up of heparinized saline. Control injections were made up of 0.6 ml heparinized saline alone. The firing rate of the neuron in response to this protocol was followed for at least 5 min following each injection; selected neurons were followed for up to 20 min following clonidine administration.

2.4 Data analysis

Behavioral states were scored by standard criteria (Ursin and Sterman, 1981) on the basis of EEG, EMG and EOG data, as well as behavioral observations noted at the time of data collection. Mean firing rates across behavioral state of all neurons was determined by off-line analysis of 60 s epochs of neuronal activity in each of 4 behavioral states. The details of the statistical treatment of these data are described elsewhere (Reiner, in preparation).

Neurons were divided according to their state-related neurophysiological profiles (see Results) into PS-off and non-PS-off cells. The response of LCx neurons to intravenous clonidine was quanti-

fied by analysis of the firing rate of individual neurons during consecutive 10 s epochs for a total of 250 s following boluses of the drug. The effects of clonidine vs. vehicle (saline) on individual neurons was plotted over time. Additionally, statistical analysis of the response to clonidine vs. vehicle was performed on the entire population of PS-off and non-PS-off cells, using a 60 s sample of spontaneous activity. Differences between the responses to these 2 treatments were obtained by 2-tailed t-test

2.5 Anatomical localization of electrodes

At the conclusion of the experiments, the animals were deeply anesthetized with pentobarbital, and 0.7 mA of cathodal current was passed through several (2–4) microwires for 1.5 s, thereby depositing a small amount of iron at the end of the electrode tracts. The animals were then perfused transcardially with one pass of heparinized saline (37°C), followed by 5% potassium ferrocyanide in 10% formaldehyde (800 ml). The brains were removed, blocked and cut sagittally into 40 μm thick sections on a freezing microtome. Sections were counterstained with neutral red to enhance visualization of the prussian blue reaction product; the location of the tips of the microwires at the time of recording individual units was then reconstructed on standard sections of the brainstem (Berman, 1968).

3. Results

3.1 State related activity of LCx neurons

A total of 21 LCx neurons were tested for their responses to intravenous clonidine administration. The behavioral neurophysiological characteristics of these neurons were such that they could readily be divided into two groups (1) PS-off cells ($n = 13$) exhibited stereotypical variations in mean and absolute discharge rate across behavioral states, firing at their highest rates during AW (1.55 ± 0.53 , mean \pm S.E.M.), less during QW (1.11 ± 0.47), still less during SWS (0.58 ± 0.33) and falling virtually silent during PS (0.02 ± 0.02); (2) non-PS-off cells

($n = 8$) were found anatomically intermixed with PS-off cells throughout the LCx, but did not exhibit the stereotypical pattern of variation in mean discharge rate across behavioral states seen in PS-off cells (Neurons reported in this study are a subpopulation of 53 PS-off cells and 78 non-PS-off cells whose physiological characteristics and anatomical locations are described in detail elsewhere (Reiner, in preparation); firing rates of the PS-off cells reported herein did not differ significantly from those of the larger population, Student's t-test.)

On the basis of their discharge characteristics during PS, it was clear that a continuum of PS-off cell types existed in the LCx. Thus, we encountered neurons which completely ceased firing during PS, neurons which exhibited isolated action potentials during PS, as well as neurons displaying occasional bursts of activity lasting 1–10 s which interrupted much more prolonged periods of neuronal silence. Our observations that such a continuum might exist originally prompted us to use clonidine to pharmacologically characterize these neurons. On the basis of both our physiological and pharmacological observations (below), we established minimal criteria for inclusion in the PS-off cell category: mean discharge rate < 0.5 Hz during PS, and at least 30 s of complete neuronal silence during a continuous bout of PS lasting 2 min or more. 75% of our sample of LCx PS-off cells had mean firing rates < 0.1 Hz, elevation of the mean firing rates of the remaining 25% was largely due to isolated bursts during PS.

3.2 Anatomical localization of PS-off cells

The reconstruction of recording sites showed that both PS-off cells and non-PS-off cells could be recorded throughout the LCx, including the nuclei locus coeruleus, parabrachialis lateralis and medialis, and Kolliker-Fuse (Reiner, in preparation). It must be emphasized that PS-off cells were almost always found interdigitated with non-PS-off cells throughout the LCx, indeed, PS-off cells and non-PS-off cells were frequently encountered within 275 μm of each other, and it was occasionally possible to record from a PS-off cell and a

non-PS-off cell simultaneously from the same electrode

3.3 Behavioral effects of clonidine administration

Doses of $> 2 \mu\text{g}/\text{kg}$ clonidine invariably resulted in behavioral sedation; this effect occurred within 30 s. The animals would lie down, usually in a sphinx posture, and remain in that position for many minutes. At higher doses ($4\text{--}5 \mu\text{g}/\text{kg}$), cats would initially exhibit behavioral sedation as above, but this was followed in about 100 s by a markedly different pattern of behavior (fig. 1). First, these animals displayed a prodromic phase characterised by mouth and tongue movements, swallowing and circling in the cage. Following this prodrome, cats would vomit 1–3 times, and then return to the sedated behavior noted at lower doses. Thus two distinct syndromes were observed depending upon the dose of clonidine administered, one consisting solely of behavioral sedation, and another consisting of sedation, a prodromic phase followed by emesis, and then behavioral sedation once again. In order to avoid any aversive association with clonidine administration, we routinely used the highest dosage which would not result in emesis (sub-emetic dose) to test our LCx neurons.

3.4 Effects of clonidine upon LCx neuronal activity

The effect of clonidine upon LCx neuronal activity was well correlated with the state-related

neurophysiological profile of these neurons. Thirty seconds following intravenous administration of clonidine, 13/13 PS-off cells were markedly inhibited as compared to saline controls (figs. 2, 3); this inhibition always persisted for at least 5 min and was well correlated with the behavioral sedation noted above. Intravenous saline never reduced the firing of LCx PS-off cells. LCx non-PS-off cells were neither consistently excited nor inhibited by either clonidine or saline administration.

It should be noted that our intravenous injection protocol in unanesthetized cats resulted in mild behavioral excitation, with a concomitant increase in LCx PS-off cell activity. This transient elevation in discharge rate following both clonidine and saline administration can be seen in fig. 2; from a peak at time 0, firing rate gradually returns to baseline levels (following saline injections) over the next 30 s (fig. 2). For this reason, the 10 s epochs prior to and immediately following drug administration were excluded from the group analysis. Rather, the 6 consecutive 10 s epochs following this period of excitation (s 30–90) were used to compute the lumped mean firing rate data (fig. 3). These data show that intravenous clonidine significantly ($P < 0.005$, 2-tailed t-test) inhibited the activity of our population of PS-off cells as compared to saline controls. Of 8 non-PS-off cells tested, only one exhibited even moderate inhibition by clonidine, and the effects upon both individual cells (fig. 2) and the group as a whole

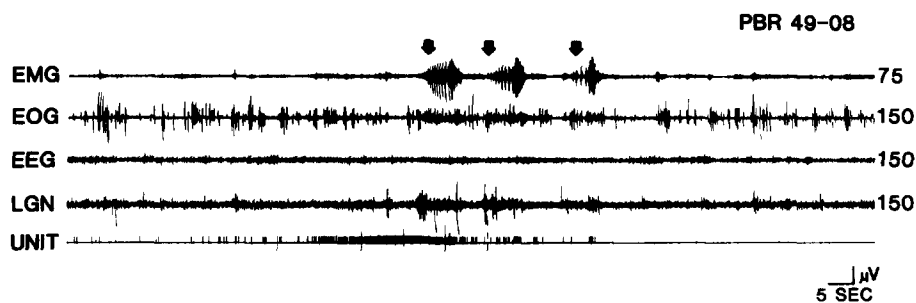


Fig. 1 This figure demonstrates the effects of $5 \mu\text{g}/\text{kg}$ clonidine both upon behavior and LCx PS-off cell activity. A series of 3 emetic episodes, occurring 2 min following intravenous injection, can be clearly seen at the arrows. During the prodromic phase which immediately precedes emesis, PS-off cell activity increases dramatically, while following emesis, the neuron is completely inhibited. Abbreviations: EMG = electromyogram, EOG = electrooculogram, EEG = electroencephalogram, LGN = lateral geniculate nucleus, UNIT = LCx neuronal activity.

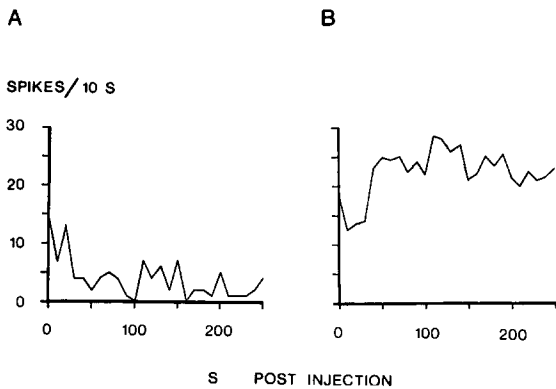


Fig 2 Effects of 3 $\mu\text{g}/\text{kg}$ of clonidine (solid lines) or heparinized saline (dashed lines) upon the firing rates of an LCx PS-off cell (A) and a non-PS-off cell (B) Note the marked inhibition of the PS-off cell as compared to its saline control, and the similar behavior of the non-PS-off cell following both saline and clonidine administration Administration of saline transiently elevated LCx PS-off cell discharge, with a gradual return to baseline over the next 30 s, therefore, we determined the mean rate for each treatment from data obtained from s 30–90 (fig 3)

(fig. 3) suggest that non-PS-off cells were neither inhibited nor excited by clonidine.

Following doses of clonidine large enough to produce emesis, PS-off cells dramatically increased

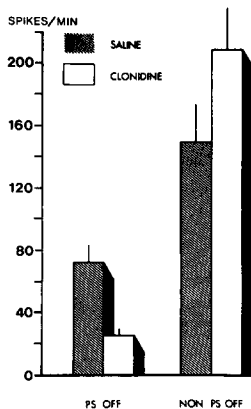


Fig 3 The group mean firing rates of 13 PS-off and 8 non-PS-off cells are shown To avoid any excitatory or inhibitory effects of the handling and injection of clonidine or saline (see fig 2), we computed our mean firing rates from a continuous 60 s sample which began 30 s after the injection As can be seen, clonidine significantly inhibited the activity of LCx PS-off cells as compared to saline controls (*** $P < 0.005$, 2-tailed t-test)

their discharge during the prodromic phase immediately prior to vomiting, and fired at high rates during emesis (fig 1); following emesis, PS-off usually fell completely silent. Both the more moderate inhibition produced by sub-emetic doses and the profound inhibition produced by doses resulting in emesis could be overcome by presenting the cat with a mouse, less arousing stimuli, such as the presence of the experimenter's hand in the cage, however, had no noticeable effect upon either behavior or unit discharge, even though these same stimuli consistently aroused the animals and elevated the activity of LCx PS-off cells in the undrugged state (Reiner, in preparation)

4. Discussion

This study addresses the question of whether or not the PS-off cells of the feline LCx are noradrenergic. The direct demonstration of the neurotransmitter status of physiologically characterized monoaminergic neurons has only recently been achieved (Grace and Bunney, 1980, Aghajanian and VanderMaelen, 1982). In anesthetized animals, these investigators intracellularly injected a marker substance and subsequently processed the brains for histofluorescent demonstration of catecholamine content in their labelled neurons Similar confirmation of the transmitter status of monoaminergic neurons in behaving animals has yet to be accomplished. Rather, assertions of the transmitter status of neurons recorded in behaving animals have all relied upon indirect evidence to correlate neurophysiological indices with neurotransmitter content

Such correlations have necessarily been tenuous when the neurons in question are heterogeneously distributed, as is the case in the feline LCx (Maeda et al, 1973; Chu and Bloom, 1974a, Jones and Moore, 1974, Poitras and Parent, 1978; Wiklund et al, 1981, Jones and Friedman, 1983, Miachon et al., 1984) These studies have shown noradrenergic neurons to be located in many nuclei of the dorsolateral pontine tegmentum, traversing classical cytoarchitectonic boundaries. These dispersed noradrenergic neurons presumably represent the

feline counterpart of rodent cell groups A6 and A7 of Dahlstrom and Fuxe (1964). The fact that noradrenergic neurons represent only a subpopulation of neurons of the feline LCx emphasizes the importance of obtaining at least indirect confirmation of the transmitter status of neurons recorded from this area as a prerequisite to concluding that one has recorded from noradrenergic neurons

In the cat, PS-off cells have been reported in the nuclei locus coeruleus and subcoeruleus (Chu and Bloom, 1974b, Hobson et al., 1975; Sakai, 1980, Hobson et al., 1983), parabrachialis lateralis (Saito et al, 1977, Hobson et al, 1983) and medialis (Sakai, 1980) We have confirmed these observations, and have extended the anatomical localization of PS-off cells to include the laterally lying Kolliker-Fuse nucleus as well (Reiner, in preparation) Thus, PS-off cells have been recorded in all of the anatomical areas where noradrenergic perikarya are found in the feline dorsolateral pontine tegmentum

In all areas examined, PS-off cells comprised only a subpopulation of the neurons recorded, being intermixed with non-PS-off cells of various types. Indeed, the observation that PS-off cells comprise a subpopulation of cells recorded within the LCx of the cat and that this nuclear region contains a subpopulation of noradrenergic neurons have led several authors (Hobson et al, 1975, Saito et al, 1977, Sakai, 1980; Sakai et al., 1981; Hobson et al., 1983) to conclude that the PS-off cells of the feline LCx are noradrenergic. This view is supported by studies in the rat (Aston-Jones and Bloom, 1981) in which the locus coeruleus is homogeneously noradrenergic (Dahlstrom and Fuxe, 1964, Swanson, 1976; Grzanna and Molliver, 1980), for these putative central noradrenergic neurons also exhibit the PS-off phenomenon

In addition to their anatomical localization to areas known to contain noradrenergic neurons, PS-off cells of the feline LCx have physiological characteristics in common with noradrenergic neurons of the rodent locus coeruleus. Central noradrenergic neurons have been described as having slow, regular firing rates in anesthetized rats (Svensson et al., 1975; Bird and Kuhar, 1977, Cedarbaum and Aghajanian, 1977, Andrade and Aghajanian, 1982; Moore and Guyenet, 1983,

Byrum et al., 1984), cats (Strahlendorf et al., 1980) and squirrel monkeys (Segal et al., 1983). Our LCx PS-off cells displayed remarkably regular discharge rates during waking, as determined by interspike interval histograms (Reiner, in preparation).

The α_2 -adrenergic agonist clonidine has been shown to profoundly inhibit the spontaneous activity of noradrenergic neurons in the locus coeruleus of the anesthetized rat (Svensson et al., 1975, Cedarbaum and Aghajanian, 1977), as well as the putative noradrenergic neurons of the A2 (Moore and Guyenet, 1983) and A5 (Andrade and Aghajanian, 1982, Byrum et al., 1984) cell groups. In order to further document the noradrenergic nature of feline LCx PS-off cells, we have characterized the response of these neurons to systemic clonidine administration in behaving animals. We find that systemic administration of clonidine, in doses similar to that used to inhibit locus coeruleus neurons in the anesthetized rat (Cedarbaum and Aghajanian, 1977), markedly inhibits PS-off cells in the feline LCx. Non-PS-off cells were never inhibited in this same fashion by intravenous clonidine, even when located within 275 μ m of clonidine-responsive PS-off cells. These observations have recently been confirmed in preliminary form (Jacobs et al, 1984). The demonstration that the PS-off cells of the feline LCx are inhibited by intravenous clonidine greatly strengthens the case that these neurons are noradrenergic

That the inhibition of central noradrenergic neurons by clonidine is direct is suggested by several lines of evidence. Clonidine, applied intravenously, intraventricularly, or microiontophoretically in the vicinity of rat noradrenergic locus coeruleus neurons inhibits their spontaneous activity, as does iontophoretic application of adrenergic transmitter agents, these inhibitions are blocked by α -adrenergic antagonists, but not by β -adrenergic antagonists (Svensson et al., 1975; Cedarbaum and Aghajanian, 1977) Finally, studies employing autoradiographic localization of p-[³H]aminoclonidine, a marker for α_2 -adrenoceptors, have demonstrated a high concentration of binding sites in the rat locus coeruleus (Young and Kuhar, 1980). Taken together, these results suggest that inhibition of central noradrenergic neurons by

systemic clonidine is probably mediated by direct action upon α_2 -adrenoceptors.

In summary, we have identified a subpopulation of neurons within the feline LCx, all of which exhibit striking changes in spontaneous activity across behavioral states, are anatomically localized to an area containing noradrenergic neurons, and are consistently inhibited by systemic administration of clonidine. Taken together, these data provide compelling evidence that the PS-off cells of the feline LCx are noradrenergic.

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