A Coeruleo-Spinal System in Culture

Abstract. *In combined cultures of dissociated spinal neurons and explants from the region of locus coeruleus, rich catecholamine-containing fiber projections from the explant to the surrounding regions of spinal neurons were demonstrated by fluorescence histochemistry. Electrical stimulation of the explant resulted in slow depolarizing responses in many of the spinal neurons. Cells exhibiting this type of response were also usually depolarized by local application of noradrenaline, whereas other, unresponsive neurons usually were not. The depolarizing responses to electrical stimulation and to noradrenaline were both increased by depolarizing current injection and decreased by hyperpolarizing current. These and other data suggest that the depolarizing responses of the spinal neurons to explant stimulation are mediated by noradrenaline released from axons of locus coeruleus neurons.*

Although it is a relatively small brain nucleus, the locus coeruleus projects axons to widespread areas of the brain (*1*) and is capable of strongly influencing neuronal excitability in these regions (*2*). It has been proposed that this nucleus participates in several diverse functions [for a review, see (*3*)], such as response to stress and control of paradoxical sleep. The neurotransmitter for cells of this nucleus, noradrenaline, has received considerable attention because of its possible relation to affective disorders (*4*).

The actions of the locus coeruleus and its neurotransmitter on cells of the central nervous system are not well understood. Noradrenaline has variously been reported to have excitatory or depressant effects depending on the type of neuron studied, the experimental preparation, and the methods of drug application (*5*). To provide a suitable system for electrophysiological analysis of catecholaminergic actions, we have cocultured explants of locus coeruleus with dissociated spinal cord neuron preparations that have been previously shown to be amenable to electrophysiological studies by means of intracellular recording techniques (*6*).

The cultures were prepared by taking explants of about 1 mm^3 from the region of the locus coeruleus of term or newborn mice; the dissection was designed to include within the explant the ventral portions of the nucleus that project to the spinal cord (*7*). The explants were placed in plastic tissue culture dishes, either on cultures of dissociated spinal cord neurons that had been established about 2 weeks earlier or on a layer of collagen to which freshly dissociated spinal cord cells were added on the following day. The techniques for preparation of the spinal cord cultures have been described (*6*). To ascertain that catecholamine-containing neurons were included in the explant and did send fibers to the surrounding regions of spinal neurons, we stained the cultures using the glyoxylic acid procedure for the fluorescent demonstration of catecholamines (*8*). Over 50 preparations were stained. The age of the explants ranged from 2 to 6 weeks in vitro, and all except four of them exhibited abundant catecholamine fluorescence. Individual fluorescent cell bodies could sometimes be distinguished in the explants at the margins, but usually only a bright, diffuse fluorescence was evident. Fibers radiated from the explant to surrounding regions usually in a linear course for the initial 0.3 to 0.5 mm (Fig. 1a) and then blended into a randomly oriented plexus of fine varicose fibers. The plexus was sparse in some parts (Fig. 1b) and dense in others and extended to a distance of about 2 mm from the explant. By study of the same microscopic fields alternately with phase and fluorescence modes, it could be seen that the fluorescent fibers sometimes congregated about clusters of spinal cord cells (Fig. 1, c and d).

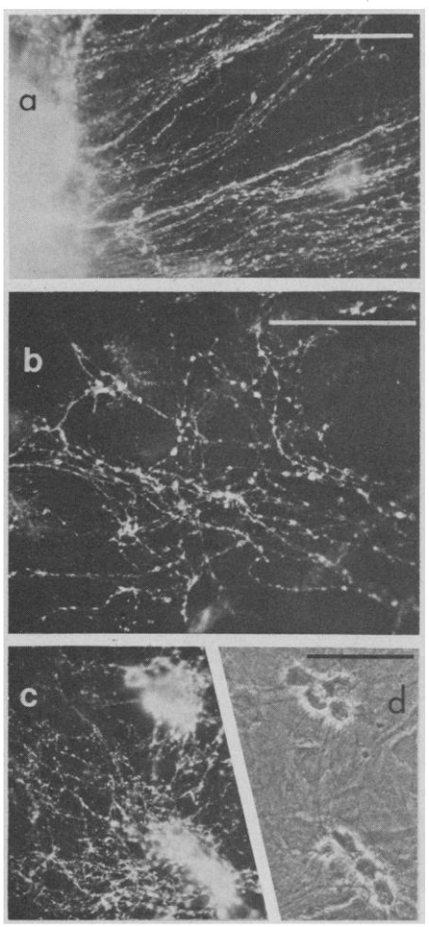

Fig. 1. Catecholamine fluorescence in cocultures of locus coeruleus explants and dissociated spinal neurons. (a) Fibers stream from explant (at left) into surrounding regions. (b) Field about 1 mm from explant showing varicose fibers. (c) Fibers sometimes condense in and around clusters of spinal cord cells as shown in (d), which is a phase-contrast picture of the same field. (Cell morphology is distorted because of heating during the fluorescence histochemical procedure.) Scale bars, 50 μm; bar in (d) also applies to (c).

Electrophysiological studies of the responses of spinal neurons to explant stimulation or to the application of noradrenaline were conducted on cultures in which the age of the spinal neurons in vitro was 4 to 7 weeks (*9*). Efficacy of the explant stimulation could often be confirmed by the observation of short-latency (< 20 msec) postsynaptic potentials or spikes in the intracellularly recorded spinal neurons. In about 40 percent of more than 100 cells tested there were also slow depolarizing responses that reached a peak about 0.7 to 1.5 seconds after the stimulus or stimulus train and had an overall duration of 5 to 20 seconds (Fig. 2a). The amplitude of the responses could be graded by varying the strength (Fig. 2a) or the number of pulses in the stimulus train. In only a few cells, hyperpolarizing or biphasic hyperpolarizing-depolarizing responses were observed, whereas in the other recorded neurons, no responses were observed.

In most of these cells, the effects of noradrenaline applied locally by pressure or iontophoresis were also studied. About 45 percent were depolarized by noradrenaline, smaller numbers showed no response (33 percent) or were hyperpolarized (18 percent), and three cells (4 percent) exhibited a biphasic hyperpolarizing-depolarizing response. A striking observation was the frequent association of depolarizing responses to explant stimulation and to noradrenaline application in the same cell. Thus, of the 38 cells depolarized by explant stimulation, 30 were also depolarized by noradrenaline (see Fig. 2, b, c, and e), whereas only 9 of the 38 cells not depolarized by the stimulation were depolarized by noradrenaline. Application of a χ^2 test to the data showed that this reflects a nonrandom distribution with $P < .001$. It was also found that if spinal neurons were cultured in the absence of the brainstem explants, or with explants that did not contain catecholamine-fluorescent elements, very few of the slow depolarizing

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responses to noradrenaline or to explant stimulation could be elicited.

The depolarizing and hyperpolarizing responses to noradrenaline were different in their time courses. The rising phase of the depolarizations outlasted the noradrenaline application by a few to several seconds, and the duration of the response could be 15 seconds to 2 minutes (Fig. 2b), depending on the mode and duration of drug application. The hyperpolarizations were usually of relatively short duration, and the return to baseline began at about the time the application was terminated. When clear measures of cell input resistance could be obtained during the hyperpolarizations, it was usually found to remain constant or to increase by a small amount; in this regard and in their time course, these hyperpolarizing responses were similar to those reported in spinal motoneurons by Engberg and Marshall (*10*). In most instances, the spinal neurons recorded in the present study were of medium size, not the larger ones in the cultures. Thus few of them were likely to be motoneurons, though beyond that qualification we do not know their identity.

There are undoubtedly other groups of neurons within the brainstem explant that are capable of forming functional connections with spinal neurons. In addition, some spinal neurons appear to project to the explant. Activation of these sets of neurons by the electrical stimulus could also give responses in spinal neurons, and these are probably the sources of the short-latency effects noted above. However, the most consistent responses of spinal neurons to both noradrenaline application and explant stimulation in these cultures were the slow depolarizations, and a relation between them is indicated by the frequent presence of both in some cells, their absence in others, and their absence when catecholamine-containing explants were missing. Other indications of relations between synaptic and noradrenaline responses are listed below.

1) The depolarizing responses to both noradrenaline and explant stimulation were affected in a similar way by intracellularly injected current; that is, they were increased by depolarizing and decreased by hyperpolarizing current (Fig. 2c). Tests of input resistance in which we used small constant current pulses usually showed that there was no change or a small increase during the depolarizing responses.

2) Application of desmethylimipramine, a drug known to block reuptake of noradrenaline into noradrenergic nerve terminals, was found to increase the amplitude of the response of some cells to explant stimulation (Fig. 2d) as well as to noradrenaline. The duration of the responses was not markedly prolonged by this drug as might be expected if the time course were determined by the reuptake mechanism. However, the duration of these very slow responses may be governed mainly by the time course of mechanisms mediating the response, or by diffusion of transmitter into the surrounding bathing medium. Our results suggest that peak concentration of neurotransmitter rather than response time course may be affected by reuptake mechanisms.

3) A specific interaction between the two types of depolarizing response was observed. When a depolarization was induced by locally applied noradrenaline, the depolarizing response to explant stimulation was reduced or abolished (Fig. 2e). This effect was not mimicked by depolarization of the cell by injected current; in fact, as indicated above, the responses normally become larger during depolarizing current injection.

The concordance in about 80 percent of the cells of the depolarizing responses to explant stimulation and to noradrenaline application, the similarity of these responses in time course and effects of altered membrane potential, and the effects of desmethylimipramine all suggest that the stimulus-evoked depolarization is mediated by the release of noradrenaline from locus coeruleus axons. This strong association of responsiveness to both noradrenaline and explant stimulation in individual spinal neurons might indicate that the presence of receptors for this depolarizing response is dependent on innervation of the cell by a noradrenergic input. The depression of the response to explant stimulation during local application of noradrenaline indicates a further assocation of these responses, though its significance is not yet clear. Such a result might be found if the synaptic receptors were occupied by the locally applied noradrenaline or if it acted on presynaptic receptors to depress transmitter release. The latter effect is thought to occur at central noradrenergic terminals (*11*). Either of these possibilities is compatible with the suggestion that the depolarizing responses to explant stimulation result from the synaptic release of noradrenaline.

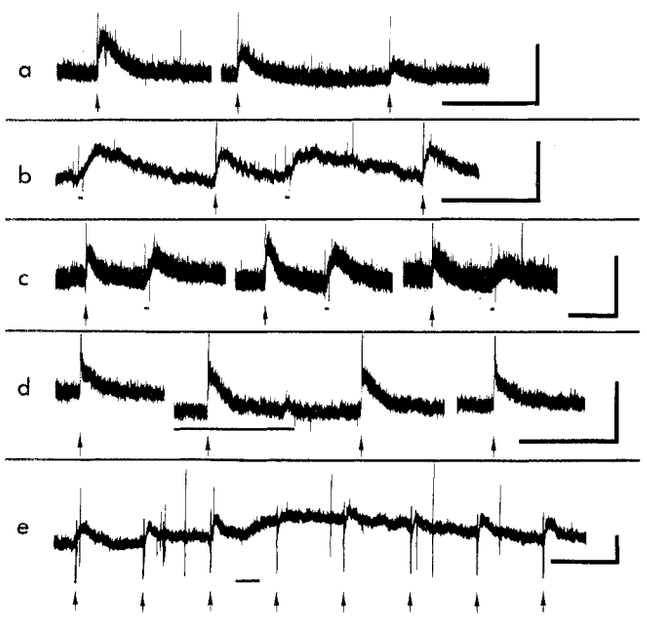

Fig. 2. Polygraph records of depolarizing responses to explant stimulation (arrows) or drug application (bars). (a) Single stimuli delivered through a microstimulating electrode at (from left to right) 20, 15, and 10 V. (b) Alternating iontophoretic applications of noradrenaline (40 nA for 1 second) and explant stimulation (train of five at 25 per second and 20 V). (c) Alternating explant stimulation and iontophoretic noradrenaline application (120 nA for 2 seconds); (from left to right) with no current, during steady injection of 0.15-nA depolarizing current, and during injection of 0.15-nA hyperpolarizing current; injection of a larger hyperpolarizing current abolished the depolarizing response, but caused considerable baseline drift (not illustrated). (d) Responses to explant stimulation before (left), during (center), and after (right) pressure application of 5 μM desmethylimipramine solution; bar under the central section indicates the final part of a 90-second application. The drug application was accompanied by a small hyperpolarization that would by itself tend to decrease the noradrenaline response. (e) Responses to explant stimulation before, during, and after (left to right) depolarization response to pressure application of 10 μM noradrenaline solution. Calibrations to the right of each panel are 4 mV, 20 seconds.

Because the depolarizing responses are increased in amplitude by depolarizing current injections and are usually associated with an increase in input resistance, they are clearly not generated by an increase in sodium conductance. Similar depolarizations have been observed in sympathetic ganglion cells (*12*) and in responses of cerebral cortical neurons to acetylcholine (*13*) and have been attributed to inactivation of resting conductance to potassium ion. This mechanism could also be responsible for the depolarizations we have observed.

The locus coeruleus has been reported to have depressant effects in most studies in vivo (*1*) and in a recent study of cocultured explants of the hippocampus with the locus coeruleus region (*14*). However, in studies of spinal neurons in vivo, both excitatory (*15*) and depressant (*16*) responses have been observed. Iontophoretic application of noradrenaline has also been found to give either mainly excitatory (*17*) or depressant (*18*) responses in spinal neurons. Recently, it was reported that noradrenaline applied to facial motoneurons evokes depolarizations (*19*) similar to those we have found in the cultured spinal neurons. Further data from the same laboratory indicate that neurons of the lateral geniculate nucleus are facilitated by both noradrenaline and by locus coeruleus stimulation (*20*).

Particularly because of the advantages of dissociated neurons in culture for electrophysiological studies, this coeruleo-spinal system promises to be a valuable preparation for the further investigation of noradrenergic actions and mechanisms (*21*).

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9. The cultures were bathed in growth medium containing increased concentrations of calcium and magnesium (usually 3 m*M* for each) during the electrophysiological studies; target neurons were observed through an inverted microscope. Intracellular recording electrodes contained 3*M* potassium acetate. Noradrenaline was applied by iontophoresis from pipettes containing 0.15 to 0.2*M* solution at *p*H 5.3 to 6.0 or by localized pressure ejection of 10^{-3}*M* solutions of noradrenaline in balanced salt solution (*p*H 7.3 to 7.4) containing 10^{-5} to 10^{-4}*M* ascorbate. Other drugs were applied by pressure and dissolved in balanced salt solution at *p*H 7.3 to 7.4. The explant was stimulated electrically by 1.0-msec pulses through a glass pipette filled with 0.9 percent sodium chloride in 1.5 percent agar. The tip was broken back to about 10 μm in diameter, giving a resistance of about 10 megohms.
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22. We thank S. Fitzgerald and E. A. Neale for assistance and helpful discussions. The experiments were conducted at NIH and were supported in part by the Medical Research Council of Canada.

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Response Artifact in the Measurement of Neuroleptic-Induced Anhedonia

Abstract. *Systemic administration of the neuroleptic drug α-flupenthixol attenuated lever-pressing behavior in rats responding for rewarding brain stimulation. The magnitude of this attenuation was dose-dependent and resembled the effects of reward reduction and termination. However, when the operant response requirements of the same rats were changed to nose poking, identical drug treatments produced relatively little attenuation in performance. These data do not support the belief that neuroleptics produce a general state of anhedonia. Rather, the apparent suppression of reinforced behaviors depends at least in part on the kinetic requirements of the response.*

The term "anhedonia" has been used to describe a state in which the reward value of usually reinforcing stimuli is blocked (*1*). Recent reports suggest that such a state can be produced by the administration of antipsychotic neuroleptic drugs (*2*, *2a*). Animals treated with such drugs stop responding for food or brain stimulation in a manner that resembles the behavioral effects of reward termination. Since many neuroleptic drugs block central dopamine receptors (*3*), these observations lend support to the concept of a central dopamine reward system mediating the behavioral consequences of positive reinforcement.

Other investigations, however, have demonstrated that the pattern of responding observed during neuroleptic administration is not equivalent to that seen when reward is withheld (that is, during extinction) (*4*). Many have therefore argued that neuroleptics produce their behavioral effects by interfering with the animal's ability to maintain responding and not with reward per se. We now report that doses of a neuroleptic which produce anhedonic-like effects when rats press a lever for reinforcement have relatively little effect when the same rats are tested with nose poking as the operant response. Our results suggest that even when the dose is high, positive reinforcing events maintain their reward value. It would seem, therefore, that the suppression of reinforced behaviors observed during drug treatment is at least in part a result of the type of response employed in the experimental paradigm.

Adult male Wistar rats were stereotaxically implanted with a bipolar stimulating electrode aimed at the lateral hypothalamus (*5*). Following surgery, the animals were trained (through shaping) to press a lever for 300-msec trains of rewarding 60-Hz sine-wave intracranial stimulation on a continuous reinforcement schedule. The self-stimulation apparatus consisted of four identical Plexi-

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