otides by independent regulatory mechanisms (*19*).

Six-day-old rats were chosen as experimental animals in this study to include potential effects of air ions on neural development. There appears to be a serotonin-dependent adenylate cyclase system in very young animals, which decreases in sensitivity with age (*20*). Our results with serotonin and cyclic AMP may be due to this coupling. It is conceivable, however, that negative air ions could have shifted the apparent developmental stage of the rats in this study by a primary effect on the concentration of some hormone, such as prolactin, sex hormone, or serotonin-derived melatonin. A direct effect of small air ions on prolactin levels was proposed by Olivereau in his study of the effects of air ions on the spontaneous movements of amphibian larvae (*21*). A direct hormonal effect of this kind in our system may have caused the increased cortical weights and decreased cortical levels of a second-messenger cyclic AMP reported here. Comparison of effects of negative and positive ions and different environments on various transmitters should show how the responses are related to the developmental stage of the animal.

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Nigral Dopamine Neurons: Intracellular Recording and Identification with L-Dopa Injection and Histofluorescence

Abstract. *Intracellular recordings in vivo were obtained from dopamine-containing neurons of the rat substantia nigra. These neurons were identified electrophysiologically by antidromic activation and histochemically by* L-*dopa injection and subsequent fluorescence histochemistry. Extracellular spikes and antidromic conduction velocity of the neurons were identical to those previously described for putative dopaminergic neurons. Spontaneous intracellular fast potentials, slow depolarizations during burst firing, and spike prepotentials were observed.*

The nigrostriatal dopaminergic system has been studied intensively because of its participation in the pathogenesis of Parkinson's disease (*1*) and its role in mediating the neurological side effects of antipsychotic drugs (*2*). Mapping of this system by fluorescence histochemical techniques has identified the location of these cells and made it possible to record extracellularly in vivo from the area in which these cells are contained. Dopamine-containing neurons of the substantia nigra exist in a thin band on its dorsal edge (*3*) and are intermixed with neurons that do not contain dopamine (*4*, *5*). Hence, to date, electrophysiological identification of these cells has depended on indirect criteria (*6*). Extracellular administration of L-dopa followed by processing of the brain slices for catecholamine fluorescence (*7*) was one of these criteria, because only the dopamine-containing neurons in this brain region contain aromatic amino acid decarboxylase (the enzyme necessary for conversion of L-dopa to dopamine) and only the dopamine reacts with formaldehyde vapor or glyoxylic acid to form fluorescent compounds. Thus, the increased fluorescence of these neurons after L-dopa iontophoresis (resulting from its uptake and conversion to dopamine) demonstrated conclusively that the recording electrode was in the vicinity of these neurons. However, because the L-dopa was administered extracellularly, it did not allow identification of the specific cell from which the recording was made.

Extracellular recordings of these indirectly identified dopamine-containing neurons have been useful in studying the modes of action of various pharmacological agents, such as the antipsychotic drugs and dopamine agonists (for example, *d*- and *l*-amphetamine and apomorphine), as well as in studying some aspects of their neurophysiological functioning (*8*, *9*). However, the exact membrane mechanisms underlying dopaminergic cell functioning and the effects of drugs on them cannot be inferred from the results of these studies; only intracellular recording can demonstrate the precise synaptic and ionic mechanisms. We report here intracellular recordings from dopamine-containing neurons of the rat substantia nigra. The cells were positively identified as dopaminergic by intracellular iontophoresis of L-dopa at the end of the recording and subsequent processing of the brain for fluorescence histochemistry.

Rats were used as experimental subjects (*10*). Intracellular recordings (*11*) were made from presumed dopamine-containing neurons of the zona compacta region of the substantia nigra (N = 50). The cells were tentatively identified before electrode penetration by their extracellular firing pattern, waveform, rate of

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firing, and location (*5, 7*). Only impaled cells with a stable resting potential greater than −55 mV, spike heights greater than 50 mV, and firing rates below 8 Hz were judged sufficiently stable for electrophysiological recording. Stable resting potentials generally ranged between −60 and −65 mV, although these cells tended to hyperpolarize with extended recording times (more than 20 minutes). The L-dopa was injected into cells with 5-nA ramp depolarizing pulses (approximately 3 seconds on, 5 seconds off) for 5 minutes or longer. Only cells that fired spikes and still exhibited resting potentials greater than −45 mV during the entire injection procedure demonstrated evidence of increased fluorescence, probably a result of diffusion of dopamine from seriously injured cells. After injection, rats were allowed to survive for 10 to 30 minutes before decapitation, and the brains were processed histochemically with glyoxylic acid for fluorescence microscopy (*12*). Only one cell per animal was injected with L-dopa. Injected dopaminergic cells (N = 12) showed markedly increased fluorescence compared to surrounding dopamine-containing neurons (an example is shown in Fig. 1). Four dopaminergic cells were injected with L-dopa and processed for fluorescence after antidromic activation. Injection of L-dopa into zona reticulata cells presumed to be nondopaminergic did not result in glyoxylic acid-induced fluorescence.

Antidromic activation of dopaminergic neurons recorded intracellularly was obtained by caudate stimulation (*13*). Antidromic invasion was established by (i) constant latency, (ii) ability to follow up to 100-Hz stimulation without spike failure, (iii) one spike elicited per stimulus, and (iv) collision of antidromic spikes with directly elicited spikes. A total of 15 dopaminergic cells demonstrated antidromic activation, with an average latency of 11.9 ± 1.1 msec (mean ± standard error), which corresponds to an estimated conduction velocity of 0.54 m/sec (Fig. 2C). This value is very close to that obtained previously for tentatively identified dopaminergic neurons (*5, 14*). In a number of cells (N = 10) antidromic activation elicited a small potential (15 to 30 mV) riding on an inhibitory postsynaptic potential (IPSP) which could be made to collide with a directly elicited spike (Fig. 2D). This potential may correspond to the "initial segment" spike reported previously (*5*), with the full action potential possibly blocked by the IPSP in the soma. This IPSP probably results from orthodromic activation of a striatonigral inhibitory

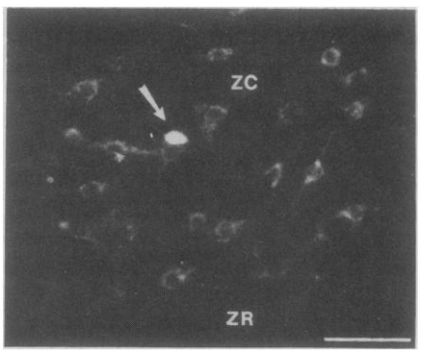

Fig. 1. Fluorescence micrograph of substantia nigra zona compacta. An intensely fluorescent dopaminergic neuron (arrow) intracellularly injected with L-dopa is surrounded by noninjected dopaminergic cells that are less bright and have normal fluorescence. Scale bar, 50 μm. *ZC*, zona compacta; *ZR*, zona reticulata.

feedback pathway [presumably containing γ-aminobutyric acid (GABA)] impinging on the dopaminergic neuron (*15*). Indeed, they appear similar to those reported at the crayfish stretch receptor (*16*) when an antidromic spike invaded the soma during an IPSP. Thus, this small potential could result from (i) failure of an action potential at the initial segment of the axon to invade the soma due to hyperpolarization or (ii) a shunting of the action potential at the soma by the conductance increase associated with the IPSP.

Spontaneous intracellular bursts and fast prepotentials were seen and appear similar to those recorded in hippocampal pyramidal cells (*17*). However, the bursts were noted only in dopaminergic neurons exhibiting a rather fast firing rate (in a more depolarized state), whereas in the hippocampus, depolarizing afterpotentials are seen following single spikes and summate into a burst. Thus, a different mechanism may be involved. The extracellular dopaminergic cell action potential and burst (Fig. 2A), obtained from the cells identified in this report as dopaminergic, are similar to those described previously (*5, 7*). Thus, the initial assumptions concerning the neurochemical identity of these cells is confirmed. In a small number of dopaminergic cells recorded intracellularly (N = 5), small (5 to 10 mV), fast potentials occurred spontaneously (Fig. 2B). In addition, in one-fourth of the cells recorded, a spike was observed to arise from a fast prepotential (Fig. 2B). Whether

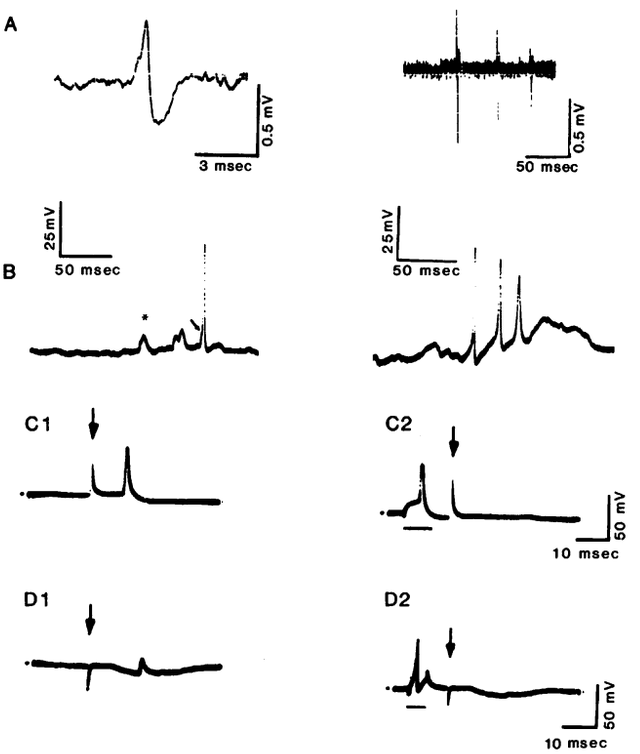

Fig. 2. Electrophysiological recordings from identified DA neurons. (A) Extracellularly recorded spike (left) and burst (right). Recording techniques were as described in (*7*). Potentials within the burst show the progressively decreasing amplitude previously reported as a characteristic of many dopaminergic neurons (*7*). (B) Intracellularly recorded spike (left) and burst (right). Spike shape and duration are similar to those recorded extracellularly from the dopaminergic cell in (A). Fast potentials (asterisk) and spike prepotential (arrow) occur spontaneously. Intracellularly recorded action potentials within the burst show progressively decreasing amplitudes, as in the extracellular recording. Slow, depolarizing potentials follow each spike, with resultant inactivation of spike-generating mechanism. (C1) Dopaminergic cells were antidromically activated from the ipsilateral caudate nucleus (stimulus given at arrow). (C2) Collision of antidromic spike with directly elicited spike. The direct spike was elicited by an intracellular injection of depolarizing current (at horizontal bar) sufficient to bring cell past threshold. (D1) Antidromic activation of an attenuated spike, riding on an IPSP. The IPSP was probably elicited orthodromically from stimulation of a striatonigral fast-conducting inhibitory pathway. (D2) Collision of this attenuated spike with directly elicited spike.

these potentials are generated at the same site or at different sites (for example, at the initial segment or dendrites) is still unknown.

To our knowledge this represents the first report in which intracellular recordings from neurons in the mammalian central nervous system have been obtained simultaneously with evidence for their neurochemical identity. It also represents the first step in the intracellular characterization of brain monoamine neurons recorded in vivo. These techniques should make possible investigations in much greater depth of various aspects of this neuronal system, for example, the function of autoreceptors (*9*, *18*), depolarization inactivation (*19*), burst firing (*7*), effects of afferent inputs on dopaminergic cell membrane properties, and the mechanisms of action of dopamine agonists and antagonists. Data obtained from such studies may further our understanding of dopamine system function in both normal and pathological states.

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11. Glass micropipettes were filled with 3*M* potassium acetate (for electrophysiological recordings) or 1*M* L-dopa methylester in 1*M* lithium chloride (for L-dopa injection) and beveled to an impedance of 35 megohms measured at 1000 Hz with a modification of the method of T. E. Ogden, M. C. Citron, and R. Pierantoni [*Science* **201**, 469 (1978)].
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Bioactive Conformation of Luteinizing Hormone-Releasing Hormone: Evidence from a Conformationally Constrained Analog

Abstract. *An analog of luteinizing hormone-releasing hormone containing a γ-lactam as a conformational constraint has been prepared with the use of a novel cyclization of a methionine sulfonium salt. The analog is more active as a luteinizing hormone-releasing hormone agonist than the parent hormone, and provides evidence for a bioactive conformation containing a β-turn.*

Peptide structures normally exist in solution as an equilibrium mixture of conformers. Backbone conformational constraints are of interest as a means of limiting the number of conformations available to the peptide (*1*). Potential advantages to be realized with the use of these restrictions in biologically active peptides include increasing the potency by stabilizing a biologically active conformer (*2*), decreasing degradation by eliminating metabolized conformers, and improving biological selectivity through elimination of bioactive conformers that give undesired biological responses (*3*). In addition, information can be obtained about the biologically active conformation of the peptide at a specific receptor through the introduction of the conformational constraint (*4*).

Two basic types of conformational modifications have been used in analogs of biologically active peptides which place limits on the possible bioactive conformations. Noncovalent modifications include D amino acids (*5*), *N*-methyl amino acids (*6*), and α-methyl amino acids (*1*). Most frequently applied and successful among these have been the D amino acids. Covalent modifications forming cyclic and polycyclic peptides include as more common examples cyclic amino acids such as proline (*7*) and disulfide bridges (*8*) and cyclization through amide bonds (*7*), all of which are known to occur in nature. β-Lactams appear as naturally occurring modifications in the penicillins and cephalosporins and serve also as reactive agents (*9*). We have been exploring the use of larger ring (five-, six-, and seven-membered) lactams as novel conformational modifications in peptides (*10*) and report here a lactam-containing analog of luteinizing hormone-releasing hormone (LH-RH) more active than the parent hormone.

Since the determination of the sequence of LH-RH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) (*11*), many analogs have been prepared (*12*). Because of its higher potency, one of the useful structural modifications is the substitution of a D amino acid for the glycine residue in position 6 (*13*). For example, the D-Ala^6 analog is 3.7 times as active as LH-RH itself. In contrast, the L-Ala^6 analog has low potency. A second noncovalent constraint resulting from replacement of Leu^7 by *N*-methyl-Leu gave a further increase in activity (*14*). The enhanced biological activity of the D-Ala^6-*N*-methyl-Leu^7 analog is consistent with a β-turn conformation for residues 5 to 8 of LH-RH (*5*). An amino acid of the L configuration in position 6 should destabilize this β-turn, which is the presumed reason for the low activity of such analogs. The presence of a β-turn is predicted also by conformational energy calculations (*15*). The theoretical calculations also predict a stabilization of this conformation in D-Ala^6 analogs and a destabilization in L-Ala^6 analogs (*16*).

The proposed β-turn for LH-RH (*15*) is shown in Fig. 1, **a**. This case seemed ideally suited to the use of a lactam conformational constraint because of the proximity of the pro-*S* hydrogen atom of Gly^6 to the N^{α}-hydrogen of Leu^7. By replacing these two hydrogens with methylene groups and connecting the newly introduced carbon atoms with a

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