have a direct effect on the renal action of PTH, an agent which by itself has been implicated in the control of vitamin D metabolite hydroxylation (*15*).

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Failure to Confirm Cyclic AMP as Second Messenger for Norepinephrine in Rat Cerebellum

Abstract. *Microiontophoretic applications of adenosine 3′,5′-monophosphate (cyclic AMP) to spontaneously active, electrophysiologically identified Purkinje cells of the rat cerebellum failed to mimic the strong depressant action of norepinephrine on the same cells. These findings, in combination with a reevaluation of other studies, cast doubt on the hypothesis that cyclic AMP mediates the depressant actions of norepinephrine in the cerebellum.*

Bloom and colleagues have proposed that adenosine 3′,5′-monophosphate (cyclic AMP) mediates the inhibitory action of norepinephrine (NE) on cerebellar Purkinje neurons (*1–3*). However, the failure of others to replicate their findings in the cerebellum (*4*) and the lack of evidence for the mediation by cyclic AMP of the depressant effects of NE on neurons of the cerebral cortex (*5*) suggest that the evidence for the role of cyclic AMP in the actions of NE needs further scrutiny.

One type of so-called "interlocking" (*2, 3*) evidence has been derived from studies with pharmacological agents such as methylxanthines, prostaglandins, and nicotinate, with the implicit assumption that the results obtained arise because of direct and specific manipulations of cyclic AMP metabolism (*2, 3*). This assumption is untenable in view of the multiple and diverse actions of the pharmacological agents that could interact with NE through mechanisms unrelated to cyclic AMP (*5*). In fact, in the brainstem, where cyclic AMP depressed many of the neurons also depressed by NE (*6*), these pharmacological tools failed to provide any decisive evidence that the similar effects of cyclic AMP and NE were causally related rather than merely coincidental.

Because the data from studies with methylxanthines, prostaglandins, and nicotinate are not unequivocal and are at best only suggestive as to the involvement of cyclic AMP in the mediation of NE's effects on neurons, the evidence crucial for this hypothesis is the demonstration that applications of cyclic AMP mimic the effects of NE. Accordingly, we have attempted to replicate the crucial experiments of Bloom and colleagues (*1–3*), who reported strong depressant effects of NE and cyclic AMP on rat cerebellar Purkinje neurons, and to give considerable attention to the precise repetition of their experimental paradigms including species, anesthesia, techniques for applying drugs, and identification of neurons.

Experiments were performed on 12 male hooded rats (250 to 500 g), 6 of which were anesthetized with halothane and N_2O and 6 with methoxyflurane and N_2O. Spontaneously firing Purkinje cells were identified in the cerebellar vermis by antidromic activation from the deep cerebellar nuclei or by their characteristic climbing fiber bursts (Fig. 1). Extracellular action potentials were recorded by the central barrel (2*M* NaCl) of seven-barreled micropipettes whose outer barrels were filled by centrifugation immediately before use with the following drugs: L-norepinephrine bitartrate, 0.2*M*, *p*H 5.0 (Sigma); adenosine 3′,5′-monophosphate, 0.5*M*, *p*H 6.0 to 7.5 (Calbiochem); aminophylline, 0.2*M* (Sigma); and 2*M* NaCl. Drugs were applied into the vicinity of neurons by microiontophoresis, and their effects were assessed from rate meter records of extracellular action potentials and from oscilloscope displays of the potentials, which were often photographed.

We have overcome the objections (*2, 3*) to the work of Godfraind and Pumain (*4*) by (i) using spontaneously firing, electrophysiologically identified Purkinje neurons of gas (nonbarbiturate) anesthetized rats and by (ii) employing a continuous automatic balancing current (*7*) in addition to conventional current controls with each neuron. Iontophoretic applications of aminophylline prior to tests of cyclic AMP were utilized to minimize enzymatic degradation of cyclic AMP by phosphodiesterase. These doses of aminophylline produced profound depression of Purkinje cell firing. In order to eliminate the possibility of additive effects (*5*), the cell was allowed the

Table 1. Actions of norepinephrine and cyclic AMP on Purkinje cells.

Anesthetic	Number of cells					
	Norepinephrine			Cyclic AMP		
	Excited	Depressed	No effect	Excited	Depressed	No effect
Halothane	0	21	3	5	3	12
Methoxyflurane	2	10	1	5	3	3
Combined data	2	31	4	10	6	15

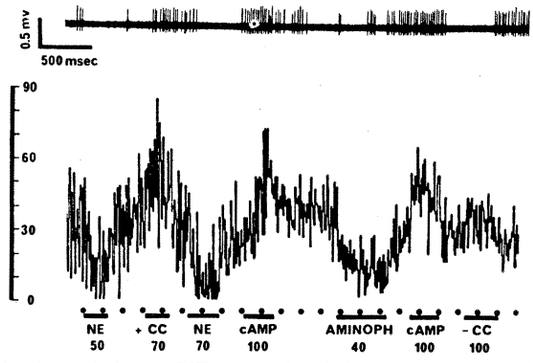

Fig. 1. Action potentials and drug responses of a Purkinje cell in a rat under halothane anesthesia. (Top) Action potentials from a spontaneously active Purkinje cell in the cerebellar vermis, showing climbing fiber burst responses. (Bottom) Rate meter tracings from the Purkinje neuron shown above. The ordinate shows the firing rate in action potentials per second. The dots along the abscissa denote 30-second intervals. Drug applications in nanoamperes are indicated by horizontal bars; NE, norepinephrine; +CC, anodal current control; cAMP, cyclic AMP; Aminoph, aminophylline; and —CC, cathodal current control. The current balancing circuit (*7*) was in use throughout.

minimal time required to recover its control firing rate before cyclic AMP was tested (Fig. 1).

The effects of NE and cyclic AMP were examined on 37 Purkinje cells (Table 1). Although 31 of 37 Purkinje cells were depressed by NE (manifested as an increased duration of pauses between spike clusters), only 6 of 31 of these cells were depressed by cyclic AMP, even when the testing of cyclic AMP was preceded by a period of application of the phosphodiesterase inhibitor aminophylline (Fig. 1). The Purkinje cell climbing fiber responses were more resistant to depression than the simple spikes (*1*). About one-half of the Purkinje cells were not influenced by cyclic AMP (Table 1). Before a cell was rated as "no effect" several doses of cyclic AMP ranging from 100 to 200 na were tested, usually preceded by aminophylline applications. About one-third of the Purkinje cells were genuinely excited by cyclic AMP, while only two cells were excited by NE. Figure 1 shows one such genuine excitation by cyclic AMP. The automatic balancing current (*7*) was in use, and applications of cyclic AMP produced increased firing of this Purkinje neuron although a cathodal current (— CC) through a second NaCl barrel produced no effect on the cell firing. The second NaCl barrel (in addition to the NaCl barrel used for neutralizing the current at the electrode tip) allowed us to assess whether the balancing current circuit was indeed eliminating electrotonic effects on the cells, and to evaluate these electrotonic effects when they occurred rather than to "discard all data wherein the automatic neutralization of tip potential . . . does not meet our satisfaction" (*3*).

A chi-square test of the distribution of the effects of NE and cyclic AMP revealed that the responses of these Purkinje cells to NE were significantly different ($.001 < P < .005$) from their responses to cyclic AMP. There were no significant differences between the responses of Purkinje cells to drugs in rats under halothane compared to cells in rats under methoxyflurane anesthesia.

It should be emphasized that great care was taken to replicate the protocol of Bloom and colleagues and to overcome the objections to the studies of Godfraind and Pumain raised by Siggins *et al.* (*2, 3*). Nevertheless, our results, in agreement with those of Godfraind and Pumain (*4*) show little evidence for the mimicking of the depressant action of NE by cyclic AMP, thus giving no support to the hypothesis that cyclic AMP mediates the effects of NE on rat cerebellar Purkinje cells.

It cannot be denied that there is a large volume of evidence which demonstrates stimulatory effects of exogenous catecholamines on adenylate cyclase in slices and homogenates of brain tissue (*5* and references therein). However, in additional studies, these actions have been shown to occur in presynaptic terminals (*8*) and in glial cells (*9*), as well as in neurons (*10*), and hence are not exclusive to transmitter effects on postsynaptic neurons. In addition, the technology used in these in vitro studies has dictated the use of long (in terms of synaptic events) incubations; consequently, the assumption that the increases in cyclic AMP subserve subsynaptic membrane responses to transmitter is a tenuous one. At present the most direct and unambiguous technique for examining postsynaptic responses of neurons to putative transmitter substances is the microiontophoretic application in vivo of compounds close to neurons while recording their electrophysiological characteristics with the same micropipette.

The experiments of Siggins *et al.* (*11*) to show the effects of stimulating presynaptic NE-containing fibers on cerebellar neuronal cyclic AMP are inconclusive since they failed to demonstrate that the changes occurred only in specific cells receiving the noradrenergic innervation. This is the case since the experimental conditions were such that some cells (granule cells) were maximally reactive in the control condition and no difference could be distinguished (noting that the assay is only qualitative) after the nerve stimulation.

We have used the microiontophoretic technique to examine the responses of rat cerebellar Purkinje cells to NE and to cyclic AMP and have failed to replicate the evidence crucial to the hypothesis that the depressant effects of NE on Purkinje cells are mediated by cyclic AMP (*1–3*). The technical objections (*2*) which were used to discount the negative findings of Godfraind and Pumain (*4*) do not apply to our findings. Inasmuch as our results contradict those from Bloom's laboratory (*1–3*) and confirm those of Godfraind and Pumain (*4*) they present a serious challenge to the attractive hypothesis that cyclic AMP is the second messenger for NE in the cerebellum.

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