Pharmacologic therapeutic window of pramiracetam demonstrated in behavior, EEG, and single neuron firing rates

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Summary. Following oral or intravenous administration, a representative cognition activator drug, pramiracetam sulfate, is shown to have a pharmacologic therapeutic window at three different levels of study: learned behavior, gross EEG activity of the frontal cortex and hippocampus, and firing rate of single hippocampal neurons.

Key words. Pramiracetam sulfate; cognition activator; nootropic; therapeutic window; learning; quantitative EEG; single CA-1 neuron firing.

Although there is a paucity of experimental literature on the subject, the concept of a 'therapeutic window' has traditionally meant that a drug has a range of doses which produces an optimum mix of a high probability of the therapeutic effect and a low probability of toxic effects. However, the new class of drugs known as cognition activators (or nootropics) is essentially entirely free of toxic effects even at doses far in excess of those required for the therapeutic effect. Thus, the question arises whether cognition activators possess a therapeutic window in the absence of toxic effects, meaning that the pharmacologic effect simply weakens and even disappears as the optimum dosage is exceeded.

In the present report we show that the new cognition activator drug pramiracetam sulfate[1-4] does indeed act in this manner. Moreover, and quite surprisingly, we have been able to demonstrate the therapeutic window of the drug at three different levels of study: i.e. learned behavior, EEG activity of the brain, and firing rates of individual hippocampal neurons. In what follows, all drug doses are expressed in terms of the active drug moiety.

Behavioral therapeutic window. In our behavioral study, we used a modified form of the hidden platform/water maze test[5], modified by us to make it suitable for one-trial spatial learning.

Our hidden platform/water maze consists of a 56 cm by 91 cm rectangular sink filled 27 cm deep with water opaqued with India ink; a small round wire-mesh platform 17 cm in diameter is placed in one corner of the tank hidden 2.5 cm beneath the water surface. A rat placed in the maze will quickly learn to escape by finding and climbing onto the platform located in the corner of the sink diagonally opposite the start position. The rat will learn not only to recognize the vicinity of the platform when they reach it, but also will learn to swim towards it from a distance despite the absence of cues from the submerged platform. The general room illumination is set very dim, and virtually all lighting within the swim maze is provided by a GE-88 miniature lamp (2A, 6V, DC) positioned 47 cm above the center of the water surface.

The subjects used were naive male Long-Evans rats weighing between 260 and 310 g. Test groups consisted of 20 animals dosed orally with various doses of pramiracetam (or vehicle) 60 min before testing. Each rat was tested under only one of these treatment conditions.

The testing of the rats under a treatment condition required two trials run 24 h apart. In trial 1, the training trial, a rat is placed at the start position with his head facing toward the corner. A clear plexiglas screen is positioned around the submerged platform to act as a barrier forcing the rats to swim and investigate other maze areas. After 1 min has elapsed the screen is removed and the animal is allowed 3 min to find the 'safety' of the platform. Those animals failing to find the platform within the allotted 3 min are placed onto the platform and cued for 10 sec. In trial 2, the animals are similarly placed at the start position but with the screen removed. The time required by the rat to escape the water by climbing onto the safety platform is recorded.

In calculating the mean completion time, the four highest and four lowest time scores are discarded, and only the remaining 12 animal times are averaged. By dropping the four highest and four lowest times, rats which locate the platform only by chance and rats that had learned very little from the initial training trial are eliminated.

The findings are presented in figure 1, which plots the mean time taken by each treatment group in trial 2 to find the safety platform. The 'range of control values' demarks the highest

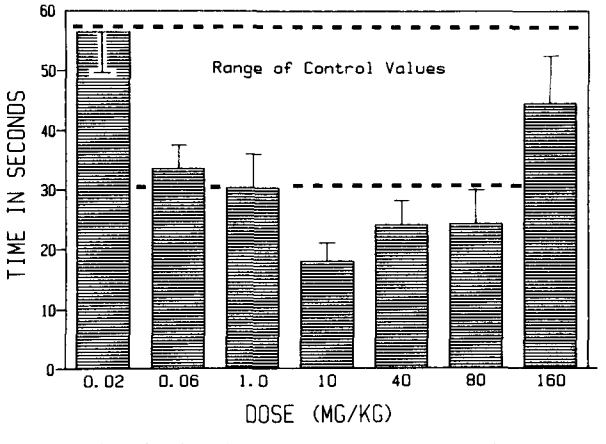

Figure 1. Effect of various doses of pramiracetam on one-trial learning of a modified Morris water maze task. Twenty male adult Long-Evans rats tested per dose. Drug treatments were administered orally 60 min prior to testing. Mean time taken by the 12 mid-performing rats under each dose is graphed along with the standard error of the mean. The 'Range of Control Values' shows the highest (worst) and lowest (best) mean scores found in ten separate replications of the vehicle condition.

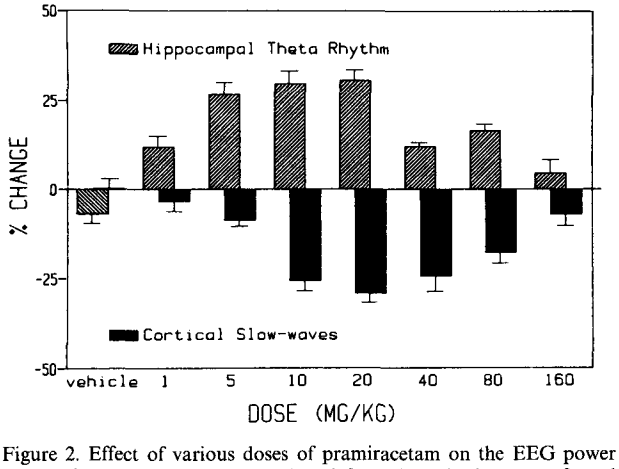

Figure 2. Effect of various doses of pramiracetam on the EEG power spectra from dorsal hippocampus and frontal cerebral cortex of aged Fisher-344 male rats. Doses were administered orally 90 min prior to data collection, which is the time of peak action of pramiracetam in this test. Findings are graphed as mean percent change from vehicle control condition. Four rats tested per dose, twelve different rats serving in the experiment as a whole.

(worst) and lowest (best) mean scores achieved in ten separate replications of the vehicle condition. The findings show a clear therapeutic window which closes completely at 160 mg/kg, a dose which is free of any behavioral toxicity or autonomic effects[1,2].

Electroencephalographic therapeutic window. Our second study was carried out at the level of gross electrophysiological activity of the brain. The quantitative EEGs from the frontal cerebral cortex and dorsal hippocampus of aged rats were used. Our method has been described in detail elsewhere[3].

In brief, all tests were conducted on 12 male Fisher-344 rats supplied by Harlan Laboratory of Indianapolis, Indiana. These animals, weighing between 300 and 400 g, were 20–25 months old at the time of electrode implantation. [Even though aged rats are costly and difficult to procure, they show the activating EEG effects of nootropic drugs much better than young rats because of the generalized slowing of EEG frequencies seen in aging[6-8]].

Permanent recording electrodes were implanted bilaterally in the dorsal hippocampi (in the dentate region), and epidurally on the frontal cerebral cortices. The hippocampal electrodes were made from 30-gauge platinum wire with 0.5 mm of insulation scraped off at the tip. The frontal cortical electrodes consisted of a stainless steel screw 1.0 mm in diameter screwed into the skull and brought into contact with the surface of the dura mater. The animal was grounded through a similar stainless steel screw secured to the occipital bone of the skull.

At the time of recording, a commutator device enabled the rat to move freely about the floor area of a test chamber 28.5 cm long, 21.5 cm wide, and 45 cm high. Movement artifacts were eliminated by passing the insulated leads through a tygon tube filled with a saturated NaCl solution (electrically grounded).

The recording system used was a 12-channel Grass polygraph, with outputs connected to a 12-channel tape recorder. The analog signals were first electronically summed and then led through a spectrum analyzer and averager (Nicolet model 446) and finally displayed by a digital oscilloscope and plotter with quantitative options. In the EEG spectra electronically placed windows were set as follows – in the hippocampus: 4–8 Hz; in the cortex: 1–13 Hz.

The test sessions were always at least 180 min in length, with EEG samples taken every 30 min. These samples consisted of 32 successive 8-sec epochs analyzed with 400-line resolution. Each individual EEG spectral power analysis therefore represented 256 sec of the EEG tracing. Each EEG test session was run at about the same time in the afternoon.

Pramiracetam was placed into solution in deionized water, and pure deionized water served as the placebo control substance. Treatments were administered by oral intubation, four rats serving under each dose condition of the drug.

The main findings are summarized in figure 2, which presents the effects obtained 90-min posttreatment, the time that pramiracetam produces its maximal arousal effects on old rat EEG. The findings in figure 2 show how each dose of pramiracetam affected EEG voltage output from the hippocampus and the frontal cortex. The basic unit of measurement was millivolts root-mean-square (in the 4–8-Hz band for the hippocampus, and the

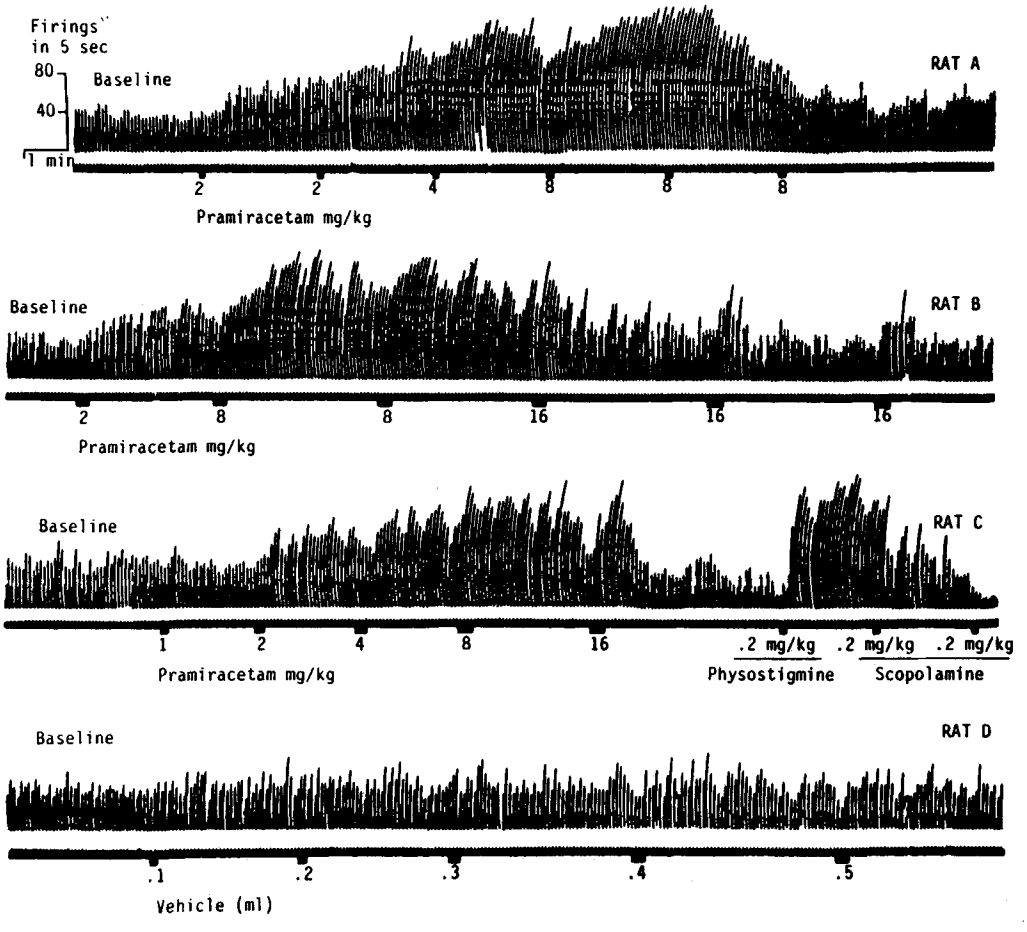

Figure 3. Effect of cumulative intravenous doses of pramiracetam on firing rates of single neurons in the ventral pallidum of three different rats. Record of rat C also shows that once the therapeutic window has been closed by a high cumulative dose of pramiracetam, the neuron is still capable of normal response to physostigmine and scopolamine. Record of rat D shows that cumulative doses of vehicle are without effect. Each reset of pen occurred after 5-sec recording periods of neuron firing.

1–13-Hz band for the frontal cortex), although in figure 2 the findings are expressed as mean percentage change from the placebo control group at the 90-min posttreatment reading.

As is well known, and as we have recently again demonstrated[3], electroencephalographic arousal in the hippocampus is associated with an increase in voltage output in the 4–8-Hz frequency band. As figure 2 shows, pramiracetam produced increased voltage output from the hippocampus in the 4–8-Hz frequency band, indicative of hippocampal arousal. This effect was largest at 20 mg/kg of the drug. Remarkably, both higher and lower doses produced smaller effects and at either extreme of dosage the arousal action of pramiracetam virtually disappeared. Thus, here again we encountered a therapeutic window.

The above action was paralleled by similar effects on the frontal cortical EEG. Cortical arousal is associated with decreased voltage output in the 1–13-Hz frequency band[3]. As figure 2 shows, pramiracetam had this kind of effect on the EEG of the frontal cortex. This arousal action was again largest at 20 mg/kg of the drug. And, once again, at both the highest and lowest doses tested, the arousal action was lost, demonstrating the operation of a therapeutic window in the frontal cortical EEG.

Single neuron therapeutic window. In our single cell studies we used microelectrodes placed extracellularly on neurons in the ventral pallidum of anesthetized (ketamine HCl) young adult male Long-Evans rats. The basic method employed has been described in detail elsewhere[9]. In brief, the microelectrodes were made of glass and were saline filled. Neuron firing rates were recorded by a polygraph as accumulated pen movement over 5-sec intervals. These records were DC amplifier generated. Pramiracetam was dissolved in saline and administered via the femoral vein. Dosing was cumulative, the individual dose administrations being spaced generally 3–4 min apart, the amount of time required for the effect of each dose increment to attain a stabilized peak action. Prior to the first drug increment in each test, a 5–10 min long period of baseline firing rate was recorded. As another control, several tests were run with appropriate volumes of saline administered as separate increments over the entire experimental period (minimum of 22 min long).

The findings are summarized in figure 3, which presents the findings from four different rats. In rats A, B, and C, different size increments of pramiracetam were used (to explore the influence of increment size on the result). In spite of that, the general pattern of the effects on firing rate was similar in all three rats. Thus, incremental doses of pramiracetam caused firing rates to increase monotonically up to total doses of 16, 10, and 15 mg/kg in rats A, B, and C, respectively. Additional doses caused firing rates to decline in each case to about the original baseline level, which shows the operation of a therapeutic window even at the level of the single neuron.

Furthermore, figure 3 shows the results of two separate control procedures. First, near the end of the test involving rat C, a small dose of physostigmine caused firing to increase greatly once again, and then two small doses of scopolamine caused firing almost to stop. This control operation demonstrates that the neuron remains viable and capable of normal pharmacologic response after the therapeutic window has been closed by pramiracetam. Second, the test of rat D shows simply that repeated doses of saline have no effect on firing rate over the duration of the test.

A final control test is presented in figure 4, which shows the effect of incremental doses of physostigmine on neuron firing rates in two different areas of the brain, one of these areas (ventral pallidum) being the same area studied in the cases presented in figure 3. Remarkably, no therapeutic window was ever seen under physostigmine event though incremental dosing was carried out to lethal levels. Clearly, not all drugs exhibit a therapeutic window. Moreover, this control test shows that the therapeutic window phenomena evidenced by pramiracetam can hardly be attributed to mere toxicity, and also not to a cholinergic agonist action of pramiracetam.

Discussion. Remaining to be discussed is the question of what mechanisms underlie the therapeutic window? Of course, we really don't know. But we can rule out some possibilities. Certainly it is not toxicity, as the physostigmine result (fig. 4) makes clear, as does also the fact that pramiracetam is essentially free of behavioral toxicity[1,2]. We can also state with certainty that the closing of the window is not caused by a hypercholinergic state produced by pramiracetam. The results presented in both figures 3 and 4 rule out this notion. Originally this notion had some plausibility because, although pramiracetam does not bind to muscarinic receptors, it does increase high-affinity choline uptake[2]. But now this possibility can be ruled out. Lastly, we can rule out mechanisms peripheral to the brain as important to the effect. We know this because we have been able to show the therapeutic window of pramiracetam on hippocampal CA-1 pyramidal cell firing rates even when very small doses of the drug are injected directly into the lateral ventricles of the brain[10].

Therefore, we currently believe the following. The therapeutic window of pramiracetam arises from mechanisms within the brain. It is not peculiar to pramiracetam (although not all drugs

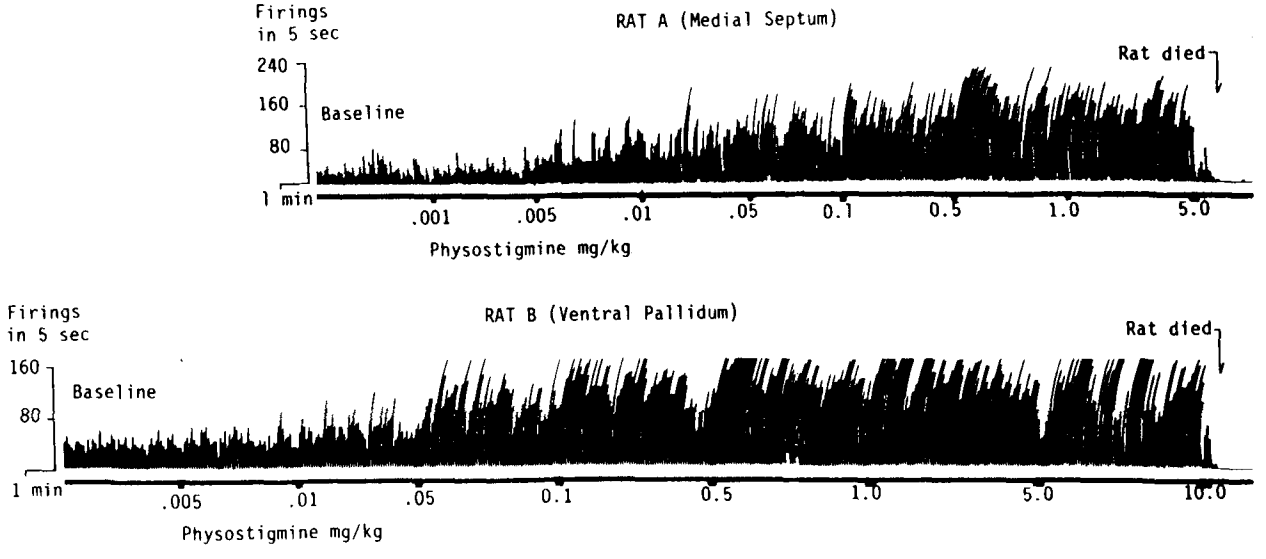

Figure 4. Effect of cumulative intravenous doses of physostigmine on firing rates of single neurons in the medial septum (rat A) and ventral pallidum (rat B). No therapeutic window was evidenced even though dose level increased to point where rats died. Other details as in figure 3.

show a therapeutic window) because we have observed the same phenomenon with other drugs of this class[10]. It remains for future work to clarify the exact mechanisms involved; however, it appears to involve a negative feedback system of some kind – at least that is our current guess. The clinician should be made aware of the therapeutic window evidenced by pramiracetam and other drugs of its class, because they may encounter situations where better therapeutic effects can be achieved only by reducing dosage.

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Nerve-mediated action of forskolin on guinea pig ileal mucosa

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Summary. The effects of forskolin on myenteric neuronal activity and mucosal function were examined in guinea pig ileum. Forskolin increased the excitability of myenteric neurons, and increased mucosal chloride secretion by stimulating enteric neurons as well as by acting directly on enterocytes.
Key words. Forskolin; intestinal secretion; myenteric neurons, neural stimulation; mucosal transport.

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii,* stimulates adenylate cyclase by activating the catalytic subunit[2,3]. Forskolin has been reported to inhibit sodium absorption and to convert chloride absorption to secretion in the rat descending colon[4]. These effects are similar to the changes in ion transport that have been observed in both ileum and colon for other substances known to alter intracellular cyclic-AMP levels[5,6].

The small intestinal mucosa is innervated by nerve processes that originate in intrinsic ganglia of the submucosal and myenteric plexuses and in extrinsic ganglia[7]. Recent studies in this laboratory have shown that stimulation of enteric nerves evokes chloride secretion in isolated flat sheets of guinea pig ileum[8,9]. This response is mediated, in part, by acetylcholine that is released from enteric cholinergic neurons[8]. Because enteric neurons modulate mucosal function, exogenous substances added to the solutions bathing in vitro tissues could have dual actions, and alter transport function either by acting directly on transporting cells or paracrine cells or by stimulating enteric neurons that innervate enterocytes.

The aim of this study was to compare the action of forskolin on enteric neurons and on the intestinal epithelium of the guinea pig. The results suggest that forskolin stimulates chloride secretion by direct action on enterocytes as well as by activation of the innervation of the enterocytes.

Methods. Non-albino guinea pigs of either sex weighing 315–525 g were stunned by a blow to the head and exsanguinated. The terminal 10 cm of ileum was discarded and a 10 cm segment of the remaining ileum was removed.

Conventional methods, which are described in detail elsewhere, were used to record intracellular electrical activity in myenteric ganglion cells in vitro[10]. Forskolin was applied to the neurons by addition to the superfusion solution (Krebs solution). Ethanol alone was added to the superfusion solution as a control to rule out possible actions of the vehicle in which forskolin was dissolved.

Alterations in neuronal excitability were determined by counting the number of action potentials evoked by intracellular injection of rectangular depolarizing pulses of constant current and duration before, during the presence and after washout of forskolin (fig. 1). A statistically-significant increase in the mean number of spikes evoked per current pulse was interpreted as reflecting an increase in neuronal excitability.

For the mucosal function studies, segments of ileum were stripped of both circular and longitudinal muscles and were mounted as flat sheets in flux chambers. This dissection procedure removed the myenteric ganglia and left intact the submucosal ganglia. The chambers were equipped for measuring transmural electrical potential differences and short-circuit currents (Isc). Warmed and oxygenated solutions of identical composition bathed mucosal and serosal surfaces of the tissues. Under these conditions changes in Isc reflected alterations in active ion transport. Forskolin or ethanol carrier was added to the serosal bathing fluids. Electrical field stimulation was used to assess the effectiveness of neuronal blockade by tetrodotoxin (TTX). Alanine or carbachol was added at the termination of the experiment to determine tissue responsiveness to absorptive or secretory stimuli, respectively.

Results and discussion. In order to determine whether forskolin altered enteric neuronal activity, 0.5–1.0 μM forskolin was added to the superfusion fluid bathing myenteric neurons. Application of forskolin enhanced the excitability of the AH/Type 2 neurons (fig. 1). This was the case for all of 19 cells that were tested in preparations from 14 guinea pigs with one to three trials per cell. The augmented excitability was accompanied by depolarization, increased input resistance, reduction of amplitude and duration of postspike hyperpolarization and by spontaneous spike discharge (fig. 2). Washing with drug-free solution reversed these effects; however, reversal required 5–15 min of washing when the time of exposure to forskolin was 30 sec to 1 min.

Forskolin mimicked the changes in neuronal behavior that oc-