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Pregnenolone sulfate enhances post-training memory processes when injected in very low doses into limbic system structures: The amygdala is by far the most sensitive

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ABSTRACT **Immediate post-training, stereotactically guided, intraparenchymal administration of pregnenolone sulfate (PS) into the amygdala, septum, mammillary bodies, or caudate nucleus and of PS, dehydroepiandrosterone sulfate, and corticosterone into the hippocampus was performed in mice that had been weakly trained in a foot-shock active avoidance paradigm. Intrahippocampal injection of PS resulted in memory enhancement (ME) at a lower dose than was found with dehydroepiandrosterone sulfate and corticosterone. Intraamygdally administered PS was approximately 10^4 times more potent on a molar basis in producing ME than when PS was injected into the hippocampus and approximately 10^5 times more potent than when injected into the septum or mammillary bodies. ME did not occur on injection of PS into the caudate nucleus over the range of doses tested in the other brain structures. The finding that fewer than 150 molecules of PS significantly enhanced post-training memory processes when injected into the amygdala establishes PS as the most potent memory enhancer yet reported and the amygdala as the most sensitive brain region for ME by any substance yet tested.**

Steroids play multifactorial roles in physiology. They are pleiotropic facilitators of coordinative processes that enable neural, endocrine, immune, and metabolic systems, separately and together, to cycle freely through their operational modes in solving problems of survival and reproduction and in achieving rebalancing when malfunctioning occurs (1–8).

The biosynthesis of steroid hormones usually begins with the formation from cholesterol of pregnenolone, from which the sex steroids, glucocorticoids, and mineralocorticoids eventually derive. In seeking to identify manipulable rate-limiting processes in age-related or disease-related deterioration of nervous system function, we focused initially on dehydroepiandrosterone (DHEA) and its sulfate (DHEAS). The latter, chiefly adrenally derived in extracerebral tissues (1) and largely locally produced in cerebral tissues (8), can serve as precursors for both androgenic and estrogenic steroids. Blood levels of DHEA and DHEAS decrease progressively with age in both sexes (9). Low concentrations of DHEA and DHEAS reduced neuronal death, decreased astrocytic proliferation, and promoted postmitotic differentiated states in both neurons and glia when added to brain cell cultures (10, 11) and enhanced memory for foot-shock active avoidance training (FAAT) in weakly trained mice (10, 12, 13). Pregnenolone, pregnenolone sulfate (PS), androstenedione, testosterone, dihydrotestosterone, and aldosterone also produced memory enhancement (ME), while estrone, estradiol, progesterone, and 16β-bromoepiandrosterone did not. By using intracerebroventricular administration, dose–response curves with pregnenolone, PS, DHEA, and DHEAS showed PS to be most potent, with significant ME occurring at 3.5×10^{-15} mol per mouse (14).

Substances administered intracerebroventricularly penetrate to several brain regions. Prior to attempting to define mechanisms of actions of PS, it was necessary to determine whether or not regional differences exist in sensitivity to its action so as to help identify the neural circuitry most importantly involved. Memory-active substances frequently have differing effects when injected into structures of the forebrain limbic system—e.g., mammillary bodies, septum, amygdala, and hippocampus (e.g., see refs. 15 and 16; however, see ref. 17, for an exception). In the present experiments, tests of retention of FAAT were made in mice after post-training injection of PS into the above structures. In addition, injections were made into the caudate nucleus, a part of the basal ganglia, as a "control" region in the sense that it is not considered to play a specific role in retention of learning of conditioned fear responses, although injection of several substances into it have modulatory effects (e.g., see refs. 15 and 18).

All test substances were administered after training so that they could not affect acquisition. Retention was tested 1 week later so that retention of test performance would not be directly affected by administration of the steroids. Lack of differences in escape latencies between vehicle controls and mice receiving any of the doses of steroids tested indicates that ME in mice receiving the steroids was not attributable to proactive motor facilitatory effects of the steroids. Since the substances tested could not directly affect performance during either training or testing, we interpret the changes in retention test performance as being the result of changes in memory processing occurring shortly after training.

MATERIALS AND METHODS

Test Animals and Surgical Procedures. After at least 2 weeks in the laboratory, CD-1 male mice obtained from Charles River Breeding Laboratories at 6 weeks of age were caged individually 24–48 h prior to training and remained singly housed until retention was tested 1 week later. Animal rooms were on a 12-h light/12-h dark cycle with light going on at 0600 h. Mice were assigned randomly to groups of 15 or more and were trained and tested between the hours of 0800 and 1400. Surgical procedures for preparing mice for injections of test solutions bilaterally into the hippocampus, amygdala, and caudate and unilaterally into mammillary bodies and septum have been described (12–17). In brief, mice were anesthetized with methoxyflurane and placed in a stereotaxic

Abbreviations: FAAT, foot-shock active avoidance training; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; PS, pregnenolone sulfate; ME, memory enhancement, memory-enhancing, or memory enhancer.

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instrument, and a hole was drilled through the skull over each injection site after deflecting the scalp. Mice were trained 48 h after surgery. Immediately after training, the test solution was injected over a period of 60 s into the target structure through 30-gauge blunt stainless steel hypodermic tubing (Small Parts, Miami) attached to a 10-μl syringe with PE-10 tubing and driven by a Sage Instruments (Boston) syringe pump (model 341A). The method of injection resulted in reliable administration into desired target structures as determined by locating in frozen brain structures the site to which the tip of the injection tubing had penetrated. The site of injection was confirmed histologically by using a mouse brain stereotaxic atlas (19).

Apparatus and Training and Testing Procedures. The T-maze used consisted of a black plastic alley with a start box at one end and two goal boxes at the other. The start box was separated from the alley by a plastic door that prevented movement down the alley until training began. An electrifiable stainless steel rod floor ran throughout the maze to deliver scrambled foot shock (12–17).

Mice were not permitted to explore the maze prior to training. Training began when a mouse was placed in the start box. The door was raised and a buzzer sounded simultaneously; 5 s later foot shock was applied. The goal box entered on the first trial was designated "incorrect" and the foot shock was continued until the mouse entered the other goal box, which in all subsequent trials was designated as "correct" for the particular mouse. Four training trials were given with an intertrial interval of 30 s, a warning buzzer of 55 dB, and foot-shock intensity of 0.30 mA. Vehicle or steroid solutions were administered within 2 min after training. One week later, T-maze training was resumed until each mouse made five avoidance responses in six consecutive training trials. Retention was measured by the number of trials required for each mouse to meet this criterion; the fewer trials required, the greater the retention of learning. The mice in this study were weakly trained so that ME effects could be detected.

Test Solutions. PS was obtained from Steraloids (Wilton, NH); DHEAS was from Syntex (Palo Alto, CA); corticosterone was from Sigma. DHEAS and corticosterone were dissolved in saline. PS (1 mg) was dissolved in 100 μl of absolute ethanol and diluted slowly with 10 ml of distilled water. Subsequent 1:10 serial dilutions were made with physiological saline by micro or bulk dilution. The maximal content of ethanol ever present in a test solution was 0.05%. Injections of saline containing the same amounts of ethanol as the steroid-containing test solutions invariably gave results statistically indistinguishable from those obtained with saline alone (see Table 1).

When we found that PS improved retention at remarkably low doses upon injection into the amygdala, we decided to test solutions made and coded in another laboratory, breaking the code only after performance of the tests. O. H. Lowry and M. E. Pufateri (Department of Pharmacology, Washington University School of Medicine) kindly cooperated in this effort, employing calibrated micropipettes and volumetric flasks to make dilutions. PS (1 mg) was dissolved in 100 μl of absolute ethanol at 50°C and dilution was made to a concentration of 10^{-7} g/ml with deionized water. Serial dilutions were made successively by taking 97.7 μl of solution and diluting with saline to 100 ml to give the required concentrations. The solution at 10^{-19} g/μl was prepared by diluting 5 ml of the solution at 10^{-18} g/μl to 50 ml with saline. Vehicle controls were supplied that contained the same concentrations of ethanol as in the PS solutions at 10^{-18} and 10^{-19} g/μl.

Statistical Treatment. Significance of the overall effect of treatment was determined by a one-way analysis of variance (ANOVA) for each steroid dose–response study. Dunnett's *t* test was used to make multiple comparisons between mean trials to criterion for drug treatment groups and the control group (20, 21).

RESULTS

ANOVA showed there to be significant overall ME effects for intrahippocampal injection of PS [$F(7,112) = 8.36$; $P < 0.001$], DHEAS [$F(4.70) = 6.98$; $P < 0.001$], and corticosterone [$F(4.70) = 8.37$; $P < 0.001$] (Fig. 1). By far the most potent action was exerted by PS. Quantities between 10^{-16} g or 2.4×10^{-18} mol per mouse and 10^{-12} g or 2.4×10^{-14} mol per mouse gave mean trials to criterion that were significantly lower than the vehicle controls ($P < 0.01$). Dilutions made serially with gas-tight Hamilton syringes (model 1710N) and those made by bulk dilution of amounts measured with volumetric pipettes and diluted in large volumes to achieve the dose of 2.4×10^{-18} mol per mouse in the home laboratory gave closely similar mean trials to criterion with groups of 15 mice each: 6.59 ± 0.28 for the former and 6.57 ± 0.32 for the latter, both with significantly fewer trials to criterion ($P < 0.01$) than the vehicle controls (9.20 ± 0.35). Multiplying mol of PS per mouse by Avogadro's number, 6.02×10^{23} molecules per mol, we calculated that 1.45×10^{6} molecules of PS were sufficient to produce significant ME on intrahippocampal injection. DHEAS and corticosterone showed significant ME only at much higher concentrations and over smaller concentration ranges than PS (Fig. 1). Clearly, PS was more potent than the other steroids tested and its effects extended over a greater range of concentrations.

When PS was tested in the amygdala, mammillary bodies, septum, and caudate nucleus (Fig. 1), one way ANOVAs showed significant ME effects for amygdala [$F(8,126) = 10.31$; $P < 0.001$], septum [$F(9,208) = 9.31$; $P < 0.001$], and mammillary bodies [$F(9.140) = 844$; $P < 0.001$] but not for the caudate [$F(4.70) = 1.05$; $P > 0.1$]. Two-way ANOVA showed that the dose–response curves for mammillary bodies and septal injection did not differ significantly from each other but that both were significantly different from the curves obtained for hippocampus and amygdala.

The retention test scores on intraamygdalar injection of very dilute solutions of PS prepared in Lowry's laboratory were closely similar to those found with similar solutions prepared in the home laboratory (Table 1), giving means for trials to criterion within 1 SEM of each other. Between 15 and 145 molecules of PS produced ME. Although we were initially skeptical of the ME shown with such extremely low doses, the closely similar results obtained with solutions prepared in two laboratories by different dilution procedures and six replications in the home laboratory gave us confidence in the validity of the results.

DISCUSSION

The finding that fewer than 150 molecules of PS can enhance post-training memory processes when injected into the amygdala in mice establishes PS as the most potent ME yet reported. The ME observed after injection of PS into hippocampus, septum, and mammillary bodies probably is attributable to direct effects of PS in these structures and not to diffusion among them or from them into the amygdala. If diffusion were occurring, then injection of PS into the caudate nucleus should have improved retention, since it is adjacent to the septum and both septum and caudate are approximately equidistant from the amygdala.

Almost all MEs show U-shaped dose–response curves over appropriately chosen dose regions. Typically, with progressive increases in dose, ME increases from the control level to a maximum, and with further increases in dose, ME decreases until performance becomes similar to that of the controls (22). The decrements of ME observed at higher doses may be attributable to incoordinations induced in management of intracellular free Ca^{2+}, leading to disorganization of orderly release of reaction cascades (23, 24). The U-shaped dose–

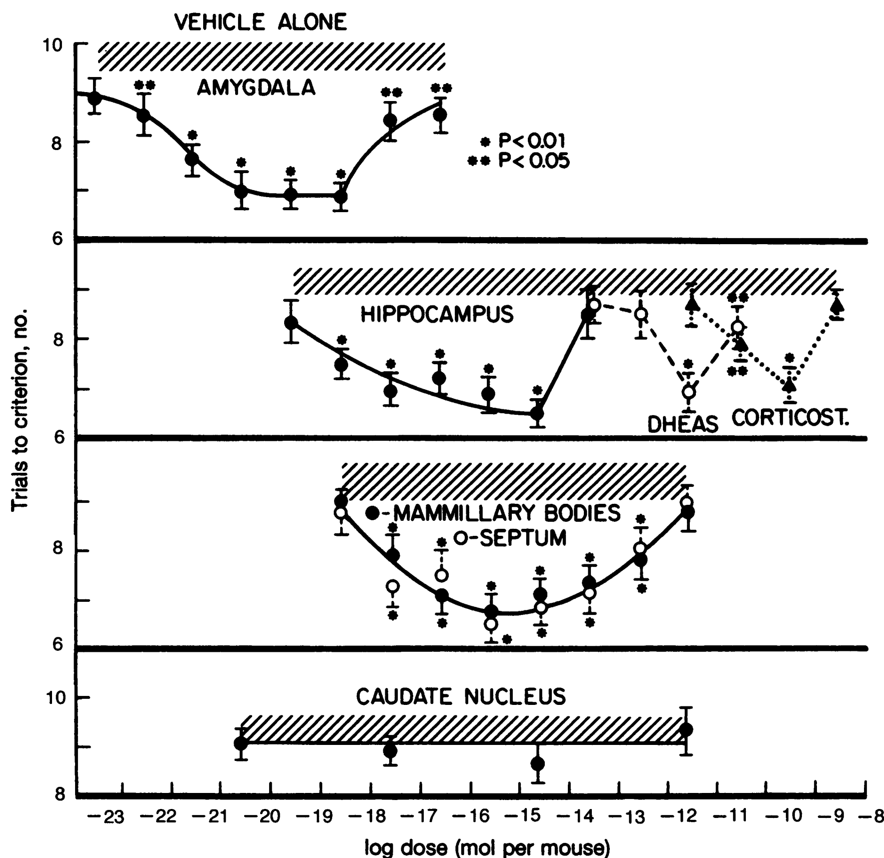

FIG. 1. Effects of post-training intraparenchymal injection of steroids on retention of FAAT in male mice. The mean and SEM for trials to criterion are shown for 15 animals at each dose indicated. The means differing from vehicle alone at $P < 0.01$ (*) or at $P < 0.05$ (**) based on Dunnett's t tests are indicated. The shaded areas are the mean ± SEM for trials to criterion for vehicle controls.

response curve described above usually covers a 2- to 5-fold dose range. However, in the case of injection of PS into limbic system structures, the curves extended over the much greater dose ranges of four to six orders of magnitude (Fig. 1). The latter differentiates the PS effects from those of the usual MEs (e.g., excitatory neurotransmitter agonists) and suggests that the dose–response curve for PS may be a composite of at least two effects. At the upper dose range PS may act like other MEs, and at the lower range, PS may act differently. A binding protein with sufficiently high affinity for PS to qualify as a receptor for it has not yet been identified. However, the existence of such an entity has not been ruled out (e.g., see ref. 25). The left-hand portion of the dose–response curve for the amygdala (Fig. 1) is not inconsistent with such a possibility. A diligent search for a receptor with extremely high affinity for PS is warranted, especially in the amygdala. If such a receptor were found and characterized, the elucidation of the molecular mechanism of the action of PS would be facilitated greatly.

When injected into the amygdala, PS was approximately 10^4 times more potent on a molar basis in producing ME than when injected into the hippocampus and approximately 10^5 times more potent than when injected into the septum or mammillary bodies. There is much evidence supporting the hypothesis that processing of sensory data in the amygdala

Table 1. ME effects in weakly trained male mice of intraamygdalar post-training injection of extremely small quantities of PS

Site of solution preparation	No. of mice	PS dose per mouse g	mol	No. of molecules	Trials to criterion, no.	P value for comparison with vehicle controls
Lowry laboratory	20	10^{-18}	2.4×10^{-21}	1450	7.10 ± 0.19	<0.01
	20	10^{-19}	2.4×10^{-22}	145	7.45 ± 0.20	<0.01
Flood laboratory	15	10^{-18}	2.4×10^{-21}	1450	7.00 ± 0.34	<0.01
	15	10^{-19}	2.4×10^{-22}	145	7.60 ± 0.32	<0.01
	15	10^{-20}	2.4×10^{-23}	15	8.53 ± 0.41	<0.05

Data for trials to criterion are expressed as the mean ± SEM. Mol were calculated by dividing the weight in grams by the molecular weight of the sodium salt of PS, 418.6. The numbers of molecules were calculated by multiplying mol by 6.02×10^{23} molecules per mol, Avogadro's number. The vehicles containing the amounts of alcohol present in the higher and lower dose solutions prepared by the Lowry laboratory gave trials to criterion of 9.50 ± 0.27 and 9.50 ± 0.17, respectively, in groups of 20 mice each. The vehicle containing the maximal amount of alcohol (0.05%) at the highest concentration of PS employed in Fig. 1 gave a value of 9.80 ± 0.29 and saline alone gave a value of 9.27 ± 0.29 in groups of 15 mice each. In no instance were the results obtained with vehicle controls significantly different from those obtained with saline controls.

assigns emotional significance to it and, when the stimuli are aversive, elicits behavioral, autonomic, and humoral responses typical of what is commonly known as fear (unconditioned) (26, 27). The amygdala is possibly the central station where unconditioned (e.g., foot shock) and conditioned stimuli (e.g., buzzer sound) meet, as in this study. When the two stimuli are experienced simultaneously or the conditioned stimulus is experienced first, fear-conditioned learning takes place, the sounding of the buzzer alone eventually eliciting the fear response, which in our paradigm is the running of the alley of the T-maze to the correct goal box sufficiently rapidly to avoid receiving foot shock. The latter response is more complex than a simple conditioned increase of heart rate, for example, and minimally requires important participation of septohippocampal and cerebellar structures.

Extensive lesion, pharmacological, and electrophysiological studies establish the central nucleus of the amygdala to be the critical mediator of fear learning. Sensory information of various modalities enters the amygdala through its basal and lateral nuclei that communicate bidirectionally with the central nucleus (26, 27). From the latter emanate outputs to various neural pathways that trigger unconditioned fear responses and within it occur the plastic changes that associate the fear responses with nonaversive stimuli that result in conditioned fear responses. However, the site of long-term memory storage may be elsewhere, e.g., the cortex (26). It is in the amygdala that the mechanism for the notable ME effect of PS may profitably be sought. It remains to be determined whether or not the sensitivity to PS reported herein for the amygdala can be generalized to types of learning that are centered in other brain regions (26).

We support a cGMP hypothesis of action of PS and other MEs on facilitation of memory processing and indicate below why PS may be particularly effective in this regard (see ref. 28). In the following we reiterate briefly some aspects of this hypothesis. The time-sequence coordination of the membrane-ionic events is such that there are pulsatile localized increases in cytosolic free Ca^{2+} that reflect accurately the amounts and durations of depolarizations to which membranes are subjected. The patterns of such Ca^{2+} transients may have great informational content and may determine how membrane-effective experiences are encoded by neurons (29). The free Ca^{2+} either directly or via its interaction with Ca^{2+} binding proteins, of which calmodulin, parvalbumin, troponin C, S-100, and calbindin are examples, releases cascades of many intracellular processes, including activation of genes. Concurrently, chemical modifications (e.g., phosphorylation or dephosphorylation) of the Ca^{2+} binding proteins and allosteric effects exerted by noncovalent binding of substances to them alter their affinities for Ca^{2+} (30). Relatedly, changes occur in activities of enzymes involved in metabolism of the cyclic nucleotides, cAMP and cGMP, changing their turnover rates, relative amounts, and intracellular distributions. There occur consequential changes in activities of protein kinases and phosphatases that act on specific substrate proteins. Phosphorylation controls the activity of many enzymes and the conformational states of nonenzymatic proteins and, therefore, a multiplicity of cellular processes (31).

Among the changes occurring when free cytosolic Ca^{2+} becomes elevated is an increase in Ca^{2+}–calmodulin, which activates many cascade-initiating targets. Many substances are known to interact allosterically with calmodulin to regulate activation of calmodulin-dependent targets (29, 32, 33). Among the targets activated by Ca^{2+}–calmodulin are those that lead to increases in cGMP (see ref. 28). Many substances with overall excitatory effects in the nervous system, among which are the known MEs, appear to have cGMP as messenger for their action (34). By direct action on presynaptic terminals, cGMP can produce long-term enhancement of transmitter release (35) and, therefore, probably long-term increases in efficacy of information transmittal in activated nerve circuits.

It may be that in weakly trained animals, such as used in the present study, the post-training "window of opportunity" for MEs to exert their action is the time during which the effects of initially suboptimally increased levels of Ca^{2+}–calmodulin can be driven to higher levels by increasing cytosolic free Ca^{2+} or guanyl cyclase activity may be increased directly. In the latter regard, it is particularly interesting not only that PS is a negative modulator of the inhibitory γ-aminobutyric acid receptor complex and a positive modulator of the excitatory *N*-methyl-D-aspartate receptor complex, thereby raising excitability of the system as a whole (36) but also that PS greatly amplifies the increase in cytosolic free Ca^{2+} that occurs during stimulation of *N*-methyl-D-aspartate receptors (37–39). PS also may be a direct activator of soluble guanylate cyclase (40). Thus, PS alone of the known MEs, by enhancement at multiple sites of pathways leading from neural excitation to increased metabolism of cGMP, could exert a remarkable synergistic amplification of neural transmission at much lower concentrations than would be expected from action of a given amount of PS on any one of the above systems alone. This might help explain the powerful effects of PS on retention of FAAT upon intracerebroventricular or intrahippocampal injection. However, the striking results upon intraamygdalar injection encourage us to look for possible additional amplificatory mechanisms, which currently are inapparent.

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