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Muscarinic Antagonists Are Anxiogenic in Rats Tested in the Black–White Box

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SMYTHE, J. W., D. MURPHY, S. BHATNAGAR, C. TIMOTHY AND B. COSTALL. *Muscarinic antagonists are anxiogenic in rats tested in the black-white box.* PHARMACOL BIOCHEM BEHAV **54**(1) 57–63, 1996. – Central cholinergic (ACh) projections have been shown to modulate stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and are integral to the expression of electrophysiological correlates of arousal, namely hippocampal theta rhythm. The degree to which these actions of ACh are behaviorally relevant has received comparatively less attention, and we sought to investigate if manipulations of ACh systems might also affect behaviors related to stress and arousal. We chose to examine indices of anxiety as revealed by changes in behavior elicited by the black-white box test, a relatively novel and recently validated model of rodent anxiety. Groups of rats were injected with either scopolamine hydrobromide (SCOP; 0, 0.05, and 0.10 mg/kg IP) or the peripherally acting scopolamine methyl bromide (methyl-SCOP; 0, 0.05, and 0.10 mg/kg IP) to compare and contrast the effects of central and peripheral ACh blockade on measures of anxiety. SCOP pretreatment significantly lowered latencies for rats to escape from the white to black compartment, while methyl-SCOP elevated latencies to reenter the white chamber from the black. Both drugs increased the amount of time rats spent in the black compartment and also suppressed exploration as revealed by decreased episodes of intercompartmental locomotion. Neither drug deleteriously affected locomotor activity, however; in fact, SCOP significantly increased locomotion in the white chamber. In the absence of motor disturbances to account for any group differences, we contend that both central and peripheral ACh blockade may affect measures of anxiety, perhaps by directly or indirectly affecting HPA activity. Central ACh systems may underlie sensory filtering whereby irrelevant stimuli are excluded from sensory processing. Antagonism of ACh transmission may render an animal incapable of correctly processing sensory information leading to hyperresponsiveness, which can manifest itself as enhanced anxiety and fear.

Acetylcholine Stress Hypothalamic-pituitary-adrenal axis Scopolamine Theta Arousal Anxiety Black-white box Peripheral Central

A LARGE body of evidence supports the notion that central acetylcholine (ACh) systems are involved in various behaviors, but especially those related to cognitive function. Indeed, the literature abounds with examples where cholinergic blockade impairs learning and memory performance, while conversely, cholinergic facilitation has been shown to enhance such performance (17,19,25,27). The contention that ACh is an essential component of cognition can also be derived from clinical data, especially in regards to age-related disorders such as Alzheimer's disease, where there is a direct association between the atrophy of basal forebrain ACh neurons and reductions in general cognitive abilities (6). Other researchers prefer to characterize ACh as part of a general activating or arousing system that necessarily affects learning and memory because of the obvious relationship that exists between any type of cognitive performance and the degree of attentiveness the animal displays (23,34). These ideas are not mutually exclusive, and are probably both valid to a point, although we tend to favor the latter view as somewhat more circumspect, and based on solid empirical support.

Particularly compelling support for ACh as a central arousing system can be found in numerous electrophysiological studies on the hippocampal formation (cornu ammonis (CA1–4), dentate gyrus, and subiculum). Field activity recordings made from the stratum oriens of CA1 and stratum moleculare of the dentate gyrus reveal two principal waveforms: theta, and large irregular activity (LIA) [reviewed in (7,8)]. Type 1 theta is a rhythmic, sinusoidal wave in the range of 3–12 Hz that is invariably present during motor behaviors, but absent in immobile, inattentive rats engaged in automatic be-

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haviors such as grooming and chewing (35). During these automatic behaviors, the predominant waveform exhibited is LIA, which is characterized as a broad range of low-frequency activity, with no peak frequency. An immobile rat rarely produces any theta activity, but can be induced to generate theta if presented with an arousing or fear-provoking stimulus (7). This fear/arousal-related theta is similar to Type 1 and has been designated Type 2. Type 2 theta can be completely abolished or prevented by prior treatment with cholinergic antagonists; alternatively, intrahippocampal or intraseptal injections of the ACh agonist carbachol elicit long trains of theta, regardless of the accompanying behaviors. Moreover, a number of anxiolytic agents significantly attenuate hippocampal theta activity (37), which is not to say this action underlies their efficacy as antianxiety agents, but it does suggest that theta may signify some degree of arousal related to internal aversive states.

Stressful stimuli produce a thoroughly characterized activation of the hypothalamic-pituitary-adrenal (HPA) axis, culminating in the enhanced synthesis and secretion of adrenocorticotrophin (ACTH) and corticosterone (CORT) into the systemic circulation (26). The HPA is, in part, controlled by a negative-feedback mechanism in the form of brain and pituitary CORT receptors that produce an inhibitory signal on further HPA activity. One key site for this inhibitory regulation lies within the hippocampal formation (15,18). In a preliminary study (5), we reported that intrahippocampal administration of the cholinergic antagonist scopolamine (SCOP) 20 min prior to restraint stress resulted in the marked hypersecretion of both ACTH and CORT, although basal hormone concentrations were unaffected and both hormones exhibited rapid recovery of baseline levels. Stress on its own is also known to elevate ACh release in the hippocampus proper, and interestingly, the circadian rhythm exhibited by ACTH and CORT is mirrored by a similar rhythm in ACh release (28,29). Furthermore, disruptions of the HPA axis produced by adrenalectomy significantly increase septal stimulation intensity required to elicit hippocampal theta activity (1). Thus, there would appear to be an intimate interplay between endocrine stress hormones and central ACh systems that supports the contention that ACh subserves aspects of arousal. The complexity of this endocrine–ACh relationship is not restricted to laboratory research with rats; Alzheimer's patients with accompanying degeneration of forebrain ACh cells profoundly hypersecrete cortisol in response to the dexamethasone suppression test (DST) (20).

On the basis of these electrophysiological and hormonal stress studies, we believe that central ACh systems mediate attention to aversive stimuli. Inhibition of central ACh neurotransmission seems to increase physiological indices of stress, while simultaneously abolishing arousal-related theta activity. At first glance these data appear to be contradictory, but may be parsimoniously explained by suggesting that ACh activity may be essential for an animal to accurately monitor and assess the degree of threat contained within any difficult or dangerous environment and initiate feedback control over the response. In the absence of functional ACh transmission, the animal overestimates the magnitude of threat and displays exaggerated endocrine stress responses. The occurrence of ACh-based theta activity reflects the central processing underlying normal risk estimation and internal behavioral control. Indeed, Bland (7) has suggested that ACh theta may subserve sensory processing related to subsequent motor behavior by signalling the intensity and speed of required locomotion in response to environmental cues. Thus, Type 2 ACh theta serves as an alerting system that primes an animal for escape or confrontation behaviors as dictated by its circumstances, and provides sensory feedback to the animal about the success of its motor output. Intraseptal infusions of procaine HCl, which temporarily suppress septohippocampal ACh neuronal activity and block theta activity, apparently increase subjective indices of anxiety (as revealed by increased freezing and gnawing behaviors), which supports this notion that theta is an essential component in normal risk assessment (24).

One logical extension of these ideas is that the arousal associated with stress physiology may also manifest itself as increased measures of anxiety. However, behavioral data to support this idea more directly are limited, although SCOP has been shown to augment indices of anxiety as measured in mice tested on the elevated plus maze (32), and chronic SCOP administration prevents the habituation of mice to specific aspects of fear/anxiety as revealed by the black–white box test (3,14). Moreover, ACh is known to affect the rate of fear conditioning, probably because of its ability to increase attention to the cue signalling the onset of the impending unconditioned stimulus (36). This group demonstrated that Pavlovian conditioning of fear (as measured by freezing behavior) was facilitated by postunconditioned stimulus–conditioned stimulus, SCOP administration. Hess and Blozovski (21) showed that intrahippocampal injections of atropine or SCOP elevated distress vocalizations and open field defecation, suggestive of augmented fear, in juvenile rats. Use of the black–white box has demonstrated it to be a valid model of rodent fear/anxiety and, we felt, could be usefully exploited to examine the behavioral sequelae to peripheral SCOP treatment, which also affects hippocampal theta activity and endocrine responses to acute stress (5,7). The following study was undertaken to assess how fear/anxiety as measured by the black–white box test would be affected by SCOP pretreatment in rats, given that this regimen also affects classical measures of stress induction. We hypothesized that central ACh neuronal activity is essential for accurate risk or threat assessment, and that, if these activities are compromised, an animal would exhibit hyper-reactivity to a stressful environment that should manifest itself in the form of heightened anxiety/fear in the black–white box. We compared both SCOP and methyl-SCOP (which does not significantly penetrate the blood–brain-barrier) to ascertain whether any observed effects were centrally or peripherally mediated. Some of these data have been presented in abstract form (33).

METHOD

Subjects

Adult male Lister Hooded rats, weighing between 350 and 450 g, served as experimental subjects. These were obtained from the breeding unit of the University of Bradford and maintained on site until testing. Rats were housed in groups of five in clear polycarbonate cages, with wood chip bedding material. Cage maintenance was undertaken twice weekly, but never on the day of testing. Food (standard rat chow) and tap water were provided ad lib. The housing room was climate controlled with 60% humidity and temperatures of approximately 22°C. Rats were housed under normal light cycle (on at 0800 and off at 2000 h). Testing was conducted during the lights-on cycle, and always starting at 1200 h. This ensured that testing was done under low basal ACTH and CORT conditions, to minimize individual variation in basal HPA activity.

Black-White Box Apparatus

The black-white test was similar to that previously employed in our laboratory (14), but built slightly larger to accommodate the increase in size of rats over mice. The overall dimensions of the box were 25 × 15 × 15 in (length, width, height). The bottom of the box was dissected by lines creating a grid appearance consisting of 5″ squares. The box was further divided into two chambers, the black (10 × 15 × 15 in) and the white (15 × 15 × 15 in) by a barrier possessing a doorway through which rats could traverse. The black compartment was illuminated with red lights, while the white compartment was intensely illuminated by bright white lights. A video camera, connected to a VHS recorder and monitor, was used to record each rats activities in the box. The entire black-white box was completely circumscribed by a heavy black curtain to minimize any possibility of distraction created by experimenter movement or ambient room lights.

Testing Procedure

On the day of testing, the rats were brought to the testing room and left for a 3-h period to acclimatize to the novel surroundings. They had continual access to food and water during this period and remained with their housing companions. Each rat was injected with a single dose of SCOP (0.0, 0.05, or 0.10 mg/kg/ml; n = 8–12/group), methyl-SCOP (0.0, 0.05, or 0.10 mg/kg/ml; n = 8–12/group) 20 min prior to testing by an investigator using coded drug bottles. Drug doses are expressed as the salt and prepared in distilled water. Individual rats were placed into the middle of the white compartment at the start of the trial and left for 10 min. The group order was counterbalanced according to a Latin square design. At the conclusion of the 10-min test period the rats were returned to their cages and another animal was placed into the box. In between rats the box was cleaned out with a 70% alcohol solution.

Behavioral Measures

The videotapes made on the day of testing were later scored by an investigator who was blind to the pretreatment regime. Each animal was scored for the following measures: a) time to escape from the white compartment to the black; b) time to reenter the white compartment from the black; c) total time in the black compartment; d) activity (squares crossed per unit time) in the white compartment; e) activity (squares crossed per unit time) in the black compartment; and f) number of crossings between the black and white chambers. To control for changes in activity levels due to differences in time distribution (i.e., rats spending more time in the black chamber invariably cross more squares), measures of activity are expressed as squares crossed per 10 s.

Drugs

(−)Scopolamine HBr (SCOP) and (−)scopolamine methyl bromide (methyl-SCOP) at the above concentrations were used. These were purchased from the Sigma Chemical Co. (Dorset, England) and prepared fresh each day in distilled water.

Statistical Analysis

All dependent measures were assessed using univariate analyses of variance (ANOVA). Each drug was analyzed independently from the other. Post hoc tests were performed using Student's t-test, applying a Bonferroni correction procedure to maintain the pair-wise comparison alpha level at 0.05 and minimize Type 1 errors (9).

RESULTS

Time to Escape White Chamber

Overall escape latencies were significantly altered by SCOP pretreatment $F(2, 25) = 3.53$, $p < 0.05$. Group comparisons revealed that SCOP at 0.1 mg/kg elicited more than a 60% reduction in time to exit the white chamber compared to VEH ($p < 0.01$). These data and those for the methyl-SCOP groups are depicted in Fig. 1. ANOVA on the methyl-SCOP data showed no overall main effect, $F(2, 25) = 0.70$, NS. Thus, central but not peripheral ACh blockade reduced escape time at the 0.10 mg/kg dose.

Time to Reenter White Chamber

ANOVA revealed no main effect of SCOP dose on this measure, $F(2, 25) = 1.26$, NS, although there was a tendency for SCOP-treated rats to reenter the white compartment relatively more quickly than VEH controls. Methyl-SCOP pretreatment exerted a powerful effect on time to reenter the white chamber, $F(2, 25) = 25.67$, $p < 0.001$, however. Latencies of the groups administered 0.05 and 0.10 mg/kg doses of methyl-SCOP were six and eight times higher than the VEH group ($ps < 0.001$). Means and SEMs are shown in Fig. 2.

Time Spent in Black Chamber

Both SCOP and methyl-SCOP treatment significantly affected the rats' time distribution, as demonstrated by separate

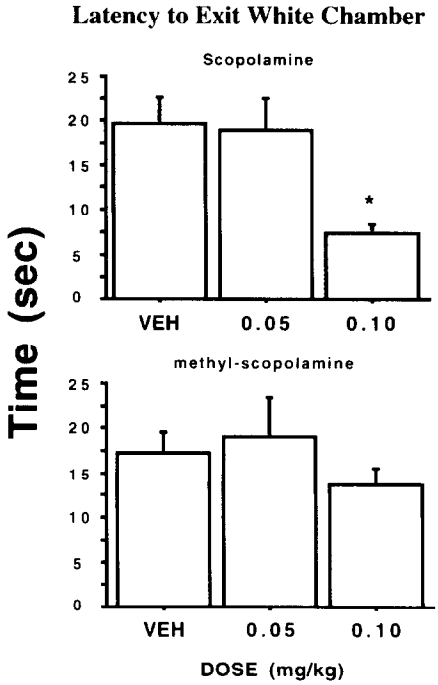

FIG. 1. Latencies to exit from the white chamber to the black chamber following initial placement in the black-white box. SCOP-treated rats were quicker to move to the black compartment, while methyl-SCOP-treated rats exhibited normal escape latencies. Values shown are means ± SEM. *Significantly different from VEH control group ($p < 0.05$).

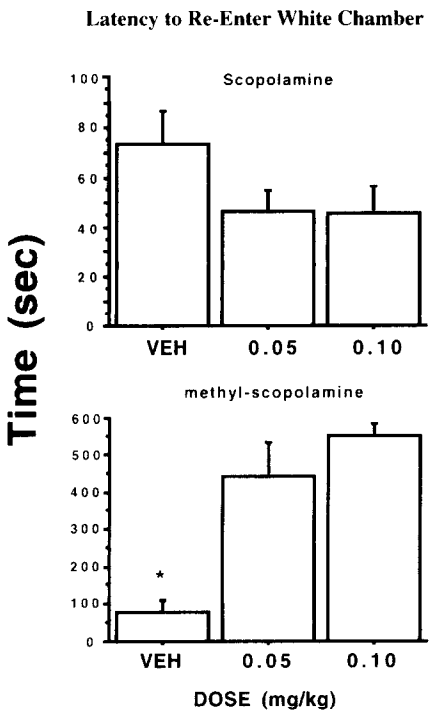

FIG. 2. Mean latencies before rats returned to explore the white chamber following their initial entry into the black compartment. SCOP-treated rats tended to return more quickly than control animals, but this was not significant. Methyl-SCOP-treated rats demonstrated extreme reluctance to reenter the white chamber. Values shown are means ± SEM. *Significantly different from drug-treated rats ($p < 0.05$).

ANOVAs, with $F(2, 25) = 10.77$, $p < 0.001$, and $F(2, 25) = 23.15$, $p < 0.0001$, respectively. All drug-treated rats exhibited higher amounts of time spent in the black compartment compared to their respective controls, and these data are shown in Fig. 3.

Number of Intercompartmental Crossings

Separate ANOVAs revealed significant effects of both SCOP, $F(2, 25) = 23.24$, $p < 0.001$, and methyl-SCOP, $F(2, 25) = 26.18$, $p < 0.001$. As shown in Fig. 4, all drug-treated rats exhibited suppressed exploration involving movements between the two chambers ($ps < 0.01$). Interestingly, the effect was somewhat more pronounced in the methyl-SCOP-treated rats, as though central ACh blockade supersedes that of peripheral ACh antagonism.

Locomotor Activity in Black and White Chambers

As shown graphically in Fig. 5, neither SCOP nor methyl-SCOP affected locomotor activity as measured in the black compartment. The ANOVAs were $F(2, 25) = 1.92$, NS, and $F(2, 25) = 2.45$, NS, respectively.

ANOVA did reveal a significant effect of SCOP on activity measured in the white chamber, however, with $F(2, 25) = 5.13$, $p < 0.01$. As shown in Fig. 5, SCOP significantly elevated locomotion compared to VEH controls ($ps < 0.05$). While methyl-SCOP also tended to elevate locomotor scores in the white chamber, this failed to reach statistical significance, $F(2, 20) = 2.7$, NS. The degrees of freedom are altered due to the exclusion of selected rats who spent too little time in the white chamber to permit meaningful analysis of time-dependent locomotor activity, because dividing activity counts by very low time values tended to grossly inflate activity rate values. One or two animals from each drug dose were excluded.

DISCUSSION

A substantial body of evidence supports the notion that central cholinergic projections are anatomically and functionally organized to mediate arousal and attention, and the present results from the black-white box confirm this. Levels of fear/anxiety were exacerbated by the cholinergic antagonist SCOP, as implicitly revealed by the increased time these rats spent in the black compartment compared to the white, and their initial rapid escape from the white to black chamber at the start of testing. Thus, the SCOP-treated rats preferred the dark area of the box, and showed diminished interest in their natural tendency to explore a novel environment. Conversely, methyl-SCOP-treated rats, with cholinergic disruption restricted to peripheral ACh systems, were also affected but in a qualitatively different manner. For instance, SCOP-treated rats were quick to exit the white chamber when initially placed into the box, while methyl-SCOP-treated rats displayed normal escape latencies. However, methyl-SCOP-treated rats were obviously reluctant to reenter the white compartment, while the SCOP pretreatment tended to facilitate scores on this measure. Regardless of these discrepancies, it is clear that peripheral ACh blockade can also influence fear and anxiety, albeit differently than central ACh blockade; thus, both pe-

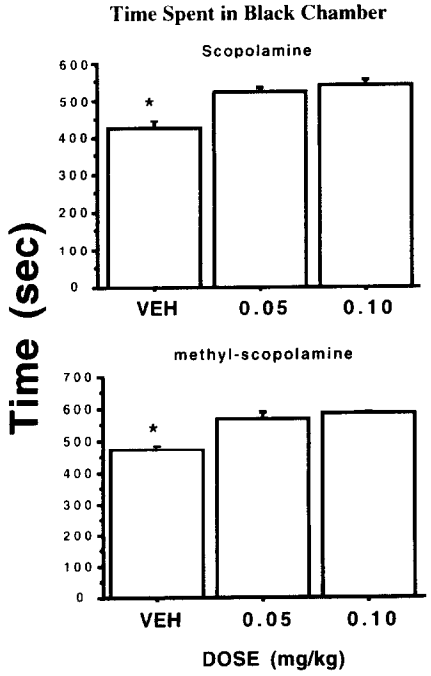

FIG. 3. Amount of time spent in the black chamber for the 600-s test trial. Both SCOP and methyl-SCOP administration significantly increased the duration of time spent in the black (safe) compartment. Values shown are means ± SEM. *Significantly different from drug-treated rats ($p < 0.05$).

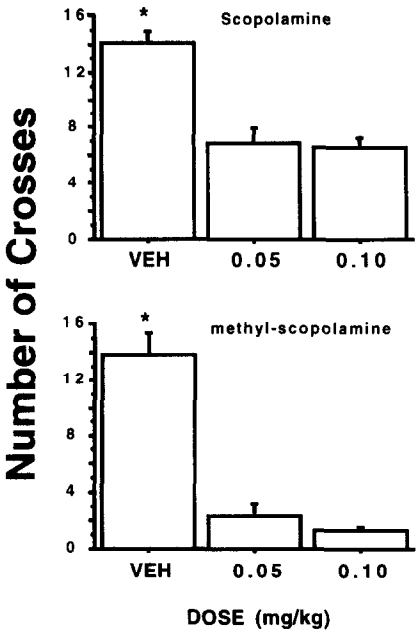

FIG. 4. Total number of intercompartmental crossings occurring in the 600-s test trial. Both SCOP- and methyl-SCOP-treated rats exhibited significantly fewer forays between the chambers, preferring to remain in the black chamber. Values shown are means ± SEM. *Significantly different from drug-treated groups ($p < 0.05$).

ripheral and central ACh systems somehow modulate these responses. By antagonizing ACh synaptic activity we observe that the intensely lit, open white compartment becomes significantly more aversive than the dark black chamber, and this is reflected by the alterations in time distribution across the two chambers. Furthermore, these differences are not due to changes in the underlying motor activity of these animals, because we did not observe any changes in locomotion once we corrected for the time distribution. In fact, other researchers have reported that SCOP tends to elevate locomotor activity (21,34), an effect we observed in SCOP-treated rats while in the white chambre. Thus, in the exclusion of gross motor deficits as a source of our black-white box test differences, we are left to conclude that ACh is an important regulator of fear/anxiety and that both peripheral and central mechanisms are involved.

In an earlier study, we reported that intrahippocampal infusions of SCOP potently enhanced stress-induced ACTH and CORT secretion (5). We posited that the septohippocampal cholinergic projections that are also responsible for providing the afferent drive for theta generation must be involved in some aspect of HPA inhibition as well. Activation of septohippocampal cholinergic projections may, in part, explain how the hippocampus exerts negative feedback control over HPA function. Consider the hippocampus as a comparator, monitoring the degree of HPA activity and matching it with the requirements of the animal given a particularly stressful encounter. Any response to an aversive situation depends on initially attending to the relevant cues, internally monitoring the physiological response, and precisely titrating that response with the needs of the animal. Cholinergic transmission probably subserves the selective attention to those cues, perhaps by filtering out irrelevant stimuli. By interfering with ACh transmission, the animal is incapable of monitoring or inhibiting the magnitude of its response because it is hypersensitive to all environmental stimuli including those that would normally be filtered out. This then manifests itself as either exaggerated endocrine stress responses, or as elevations in measures of behavioral anxiety as reported here. This sort of interplay may explain the relevance of ACh theta, elicited by arousing stimuli, and why ACh blockade at the level of the hippocampus may increase fear and HPA responsivity to stress. Induction of stress, produced by placing the rats in a novel, apparently threatening environment, leads to anxiety, which is increased by prior SCOP injections, perhaps due to its ability to increase HPA axis activity. Presumably, this means that any amount of stress may be a major contributory factor in the development of anxiety, or at least, that stress responses and anxiety may be coexpressed. The augmented anxiety exhibited by methyl-SCOP-treated rats may also be due to activation of HPA systems via a direct autonomic mechanism, although we have no empirical data to support this idea at this time.

Research with a somewhat different orientation has shown

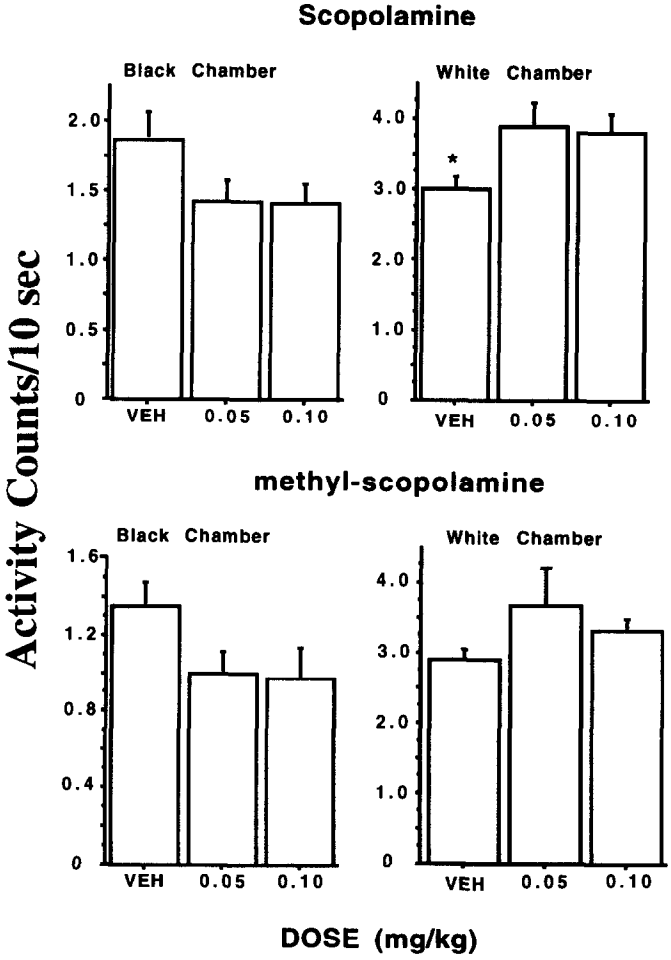

FIG. 5. Activity scores for all groups in each of the black and white compartments. Measures are expressed as activity counts per 10-s time period to determine rate of activity and correct for the unequal time distribution between the differently coloured chambers. Means ± SEM are depicted. *Significantly different from corresponding drug groups ($p < 0.05$).

that latent inhibition, where associational learning is delayed in rats preexposed, but not reinforced by the stimulus used as a CS, is impaired by hippocampal lesions (30,31). These data corroborate the contention that the hippocampus and related structures are important regulators of the salience of environmental stimuli, and together with our present data, imply that ACh innervation of the hippocampus is one component of such a mechanism. Further research into this area might prove useful, especially if the studies employ intrahippocampal drug administration rather than gross lesioning techniques.

Previous work from our laboratory has confirmed the construct validity of the black–white box as a model of anxiety (14). A number of anxiolytic drugs (benzodiazepines, buspirone, alcohol and opiates) suppress the inhibition of exploration induced by the highly illuminated, white field, while anxiogenic agents (such as yohimbine and FG7142) potentiate behavioral suppression (3,12,13). Recent studies have demonstrated that 5-HT_3 antagonists exert anxiolytic effects on a number of tasks (3,10,11). Interestingly, these same receptor subtypes mediate 5-HT-induced suppression of ACh release from entorhinal cortex slices (2). Thus blockade of 5-HT_3 receptors should elevate synaptic concentrations of ACh and may, therefore, act by facilitating ACh transmission. Such an idea would correspond with the notion above that ACh blockade may contribute to anxiogenesis, and suggest that the anxiolytic response elicited by 5-HT_3 antagonists may well derive from their augmentation of ACh release. They do prevent the impairment of habituation produced by SCOP in the black-white box in mice (3) which supports this notion.

There is general consensus that ACh function is integral to learning and memory (17,19), but how it may be involved has been the subject of some debate. It is possible that ACh facilitates learning and memory by enhancing attentional or arousal mechanisms (23). But it is also evident that disruptions of ACh may increase the overall degree of fearfulness exhibited by an animal. Clearly, studies in which ACh blockade has produced learning deficits must be cautiously interpreted because any overt impairments may originate from elevations of fear rather than from some interference with cognitive processing. We are unaware of human studies reporting anxiogenic effects following ACh blockade, however. Cholinomimetics do increase basal HPA activity in normal subjects and those suffering from affective disorders, effects that appear to be reliant on central muscarinic receptors [reviewed in (22)]. It is also worth noting that high plasma concentrations of CORT suppress the formation of long-term potentiation (LTP), one mechanism purportedly underlying learning (4,16). Cholinergic blockade might reduce learning by increasing CORT and thereby reduce synaptic plasticity as measured by LTP rather than directly altering cognitive processing.

In conclusion, ACh antagonism with SCOP or methyl-SCOP increases fear-associated behaviors as measured by the black-white box. This effect was dependent on central and peripheral mechanisms, although these sites of action can be qualitatively distinguished. Cholinergic transmission may be essential for an animal to mount an appropriate response to unfamiliar, fear-provoking environments. In the absence of normal ACh function, animals may be hyperreactive to cues related to the aversive task. Thus, ACh has a twofold involvement in fear-related behaviors: 1) to assist or modulate attention/arousal to aversive stimuli while simultaneously filtering out irrelevant signals; and 2) to initiate feedback control of physiological and behavioral responses to those aversive stimuli.

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