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# Role of Acetylcholine in Negative Patterning in Turtles (*Chrysemys picta*)

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Turtles were run on a negative patterning task involving 2 positive elements, a key with white stripes on a black background, and a solid red key, and a compound stimulus combining the 2 elements, white stripes on a red background. Injections of scopolamine, methylscopolamine, or saline were started at the same time that the compound stimulus was introduced, after the animals had been autoshaped to press the key for each of the elements. Scopolamine disrupted the learning of negative patterning, but methylscopolamine had no effect. In contrast, learning of a simple discrimination between the elements was not affected by scopolamine. These results show that muscarinic cholinergic receptors are involved in the learning of negative patterning in turtles.

*Keywords:* autoshaping, basal forebrain, negative patterning, scopolamine, turtles

The basal forebrain of turtles, like that of mammals, contains cholinergic cells that project to the cerebral cortex (Ouimet, Patrick, & Ebner, 1985; Powers & Reiner, 1993; Schuss & Powers, 1998). The homologue of the dorsal cortex in mammals is uncertain (see Aboitiz, Morales, & Montiel, 2003; Powers, 2003), but it has been shown to be involved in learning and memory (e.g., Blau & Powers, 1989; Grisham & Powers, 1989; Petrillo, Ritter, & Powers, 1994; Reiner & Powers, 1983).

In the negative patterning task, three stimuli are presented: two elements are each reinforced (A+, B+), and a compound stimulus (AB–) consisting of a combination of the two elements is unreinforced (Pavlov, 1927; Rudy & Sutherland, 1989; Woodbury, 1943). This task is theoretically interesting because, to solve it, animals must learn not to respond to a stimulus that is made up of two elements that are reinforced. Learning theories that are based on the accumulation of associative strength to individual elements cannot explain the learning of negative patterning, in which animals learn to respond to each element but learn not to respond to the compound composed of those elements. One type of theory to explain such learning is configural theory (Rudy & Sutherland, 1989; Sutherland & Rudy, 1989), which postulates that two forms of learning exist, learning to respond to elements (elemental association) and to more complex conjunctions of stimuli such as are found in compound stimuli (configural association). Elemental associations are the basis of learning in situations in which a given stimulus has a fixed relationship between its presentation and reinforcement, such as a discrimination between A+ and B–, whereas configural associations are the basis of learning when different responses are required to different combinations or relationships between stimuli. Rudy and Sutherland further hypothesized that the configural association system was mediated by the hippocampus, but that elemental associations could be learned without hippocampal involvement. This hypothesis has been supported in some studies (Alvarado & Rudy, 1995; Hata, Kumai, & Okaichi, 2007; Richmond, Nichols, Deacon, & Rawlins, 1997; Rudy & Sutherland, 1989; Whishaw & Tomie, 1989) but not in all (Bussey et al., 2000; Davidson, McKernan, & Jarrard, 1993; Moreira & Bueno, 2003; Papadimitriou & Wynne, 1999).

Butt and coworkers (Butt & Hodge, 1997; Butt, Noble, Rogers, & Rea, 2002) proposed that the basal forebrain cholinergic system mediates configural learning but not elemental learning, and they supported this notion with data showing that lesions of the basal forebrain cholinergic cells made by 192 IgG-saporin lesions, which destroy cholinergic cells but leave other cells intact, impair negative patterning acquisition in rats but not elemental discrimination (Butt et al., 2002). These authors also postulated a role for attention in the configural learning deficits seen after lesions of the cholinergic system: they suggested that learning about the compound requires attention to both elements simultaneously, and if animals were impaired in their ability to attend, they would show configural learning impairments.

Recently we demonstrated that blockade of nitric oxide disrupted the learning of negative patterning in turtles (Yeh & Powers, 2005). In that study we hypothesized that the cholinergic system in turtles would be involved in the learning of the negative patterning problem because nitric oxide cells are found in close proximity to cholinergic cells in the basal forebrain of turtles (Bruning, Wiese, & Mayer, 1994). As described above, acquisition of negative patterning has been shown to be impaired in rats after lesions of the cholinergic basal forebrain (Butt & Hodge, 1997; Butt et al., 2002). In addition, impairment on negative patterning has been found in rats after blockade of muscarinic cholinergic receptors with scopolamine (P. M. Moran, 1992; Richmond et al., 1997). The scopolamine findings are somewhat problematic, however, because in one case (P. M. Moran, 1992), only retention was blocked, not acquisition, and in the other (Richmond et al., 1997), which tested only retention; blockade of peripheral muscarinic

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receptors with methylscopolamine had the same effect as scopolamine. In the present study we expected that blocking acetylcholine with scopolamine would disrupt the acquisition of negative patterning performance, as the nitric oxide synthase blocker L-NAME had in our previous study (Yeh & Powers, 2005). Scopolamine has been shown in a previous study to impair memory for a maze in turtles (Petrillo et al., 1994).

We investigated the effects of scopolamine, methylscopolamine, and saline injections on the learning of a negative patterning task. Methylscopolamine was used as a control for peripheral effects because it does not cross the blood–brain barrier. The negative patterning task was identical to that used by Yeh and Powers (2005). The elemental stimuli, both reinforced, were a red key and a black key with white vertical stripes. The compound stimulus, which was unreinforced, was a red key with white vertical stripes on it. Autoshaping, an appetitive classical conditioning paradigm, was used to train the turtles.

In addition, a control experiment using a simple go/no go discrimination between the elements in the compound, a red key and a black key with white vertical stripes, was run, to ascertain the effect of scopolamine on such a task. This task too was identical to the simple discrimination used by Yeh and Powers (2005).

## Method

### *Subjects*

The subjects were 27 painted turtles (*Chrysemys picta*) obtained from Seltrut Inc. (Port Ritchey, FL). The turtles were housed individually in tanks containing water with a platform on which they could bask. The room was kept at a constant temperature of 30 °C, and on a 14:10-hr light–dark cycle. The experiment was run during the light part of the cycle. Prior to the experiment, the turtles were fed beef baby food in their home cage.

### *Apparatus*

The apparatus was an enclosure of watertight black Plexiglas, measuring 21.2 × 23.0 cm in length and width, and 20.5 cm in height. Centrally located 18.8 cm above the floor, on the front wall of the chamber, was a houselight. Below the houselight, side-by-side, were the food magazine located 8.7 cm from the right wall and 7.0 cm from the floor and the response key (3.0 cm in diameter), 9.0 cm from the left wall and 7.0 cm from the floor. The food magazine had a translucent plastic disk, which measured 2.2 cm in diameter with an opening of 1.0 cm in the center. Food reward, beef baby food (Beech-Nut Nutritional Comp., Canajoharie, NY), was delivered through an aperture in the center of the disk, by a rubber tube attached to a syringe. The syringe was mounted on a Davis liquid pump (Model LR 131 Davis Scientific Instruments, Studio Center, CA). The response key was a transparent plastic disk, which was illuminated from behind, and stimuli could be presented to the turtle inside the chamber via rear-mounted projectors (Industrial Electronic Engineers, Inc., Van Nuys, CA). Depressions of the response key were relayed to a computer that controlled and recorded responses in the experimental chamber. The stimuli were a solid red key, a key with three vertical 2 mm wide white lines, separated from one another by 3 mm, on a black background, or a key with the same three vertical lines on a red background (the compound). Water was added to the chamber to the depth of 4 cm because turtles eat under water.

### *Procedure: Negative Patterning Experiment*

The turtles were pretrained to eat from the food magazine. They were then autoshaped using two stimuli, both reinforced. The stimuli were the solid red key and the key with a black background and white vertical stripes. They were given 20 trials per day, 10 of each stimulus, in random order, with a variable intertrial interval of 90 s. There was no response contingency. The light behind the response key was turned on for 15 s, and at its offset, food was delivered (0.2 ml baby beef per reinforcement) and the magazine light was illuminated for 15 s. The number of responses during the stimulus on each trial was recorded, and from those numbers, the probability of response per day (number of trials with a response, divided by number of trials, for each condition) could be calculated. This training continued for 18 days.

On Day 19, the compound stimulus, which was white vertical stripes on a red key, was introduced, and the turtles began to receive drug injections. In this stage, 40 trials were given per day, 10 reinforced trials with a red key, 10 reinforced trials with white stripes on a black key, and 20 unreinforced trials with the compound. The unreinforced key remained on for 15 s and was followed by the intertrial interval, which continued to be 90 s. The turtles were run for 24 days on this procedure.

Eight turtles were given scopolamine injections (6.4 mg/kg in 1.0 ml saline/kg), 4 were given methylscopolamine (6.8 mg/kg in 1.0 ml saline/kg), and 3 were given saline (1.0 ml/kg). The injections were given intraperitoneally (in front of the rear leg) 30 min before the animals were run in the experiment. We had previously used this injection amount and interval between injection and testing successfully (Petrillo et al., 1994). These amounts of scopolamine, which are larger than those typically used in mammals, have been shown not to have any effects on general activity in turtles (Petrillo et al., 1994) but to disrupt memory for a cross-shaped maze.

### *Procedure: Elemental Discrimination Experiment*

The turtles were assigned randomly to two groups, saline ($n$ = 6) and scopolamine ($n$ = 6). They were magazine trained as described above and then autoshaped on a discrimination between the red key and the black key with white stripes on it. For all turtles, the red key was designated as the positive stimulus. Daily injections of scopolamine, at the same dose as was used in the negative patterning experiment, or saline, began at the outset of discrimination training. There were 20 trials per day, 10 reinforced and 10 nonreinforced in random order. The turtles were fed 2 ml of their daily ration in their home cage 30 min after completion of the experimental session each day. All other details of the experiment were identical to the negative patterning experiment.

## Results

### *Negative Patterning*

The data on negative patterning were combined into 3-day blocks. The performance of the group given methylscopolamine,

which does not cross the blood–brain barrier, was not different from that of the controls given saline for either probability or number of responses (for group differences, all $ps > .05$), and the results of the two control groups, methylscopolamine and saline, were combined. In addition, there was no difference between the response to red and stripes throughout the experiment, for either probability or number of responses (all $ps > .05$); therefore the responses to these elemental stimuli were combined.

*Probability of response.* Figure 1A shows the probability of response in 3-day blocks with the elements combined. As can be seen, both groups learned to respond to the elements in the first six blocks. There was no difference between the groups in this phase of the experiment, which is to be expected because they were not receiving injections during this phase of the experiment. A significant effect of block was found for this phase, $F(5, 65) = 23.58$, $p < .001$, $\eta_p^2 = .64$.

In Blocks 7 through 14, when scopolamine, methylscopolamine, or saline was introduced, the effect of scopolamine appeared to be to reduce the responding to the elements and to increase the responding to the compound. Analysis of variance of the results from the compound training phase of the experiment showed a significant main effect for stimulus, $F(1, 13) = 59.65$, $p < .001$, $\eta_p^2 = .82$; a significant interaction of stimulus by block, $F(7, 91) = 10.88$, $p < .001$, $\eta_p^2 = .46$; a significant interaction between group and stimuli, $F(1, 13) = 14.66$, $p = .002$, $\eta_p^2 = .53$; and a significant Group × Stimulus × Block interaction, $F(7, 91) = 2.82$, $p = .011$, $\eta_p^2 = .18$; the last two confirming the effect of scopolamine on performance. Although comparison of the groups on response to the elements or the compound across blocks separately failed to show any significant group differences, comparison of the groups on the last block of trials showed a significant interaction between stimulus and group, $F(1, 13) = 15.47$, $p = .002$, $\eta_p^2 = .54$. Further $t$ tests showed that the groups differed on the elements in this block, $t(13) = 2.3$, $p = .04$, but not on the compound, $t(13) = 1.2$, $p > .05$.

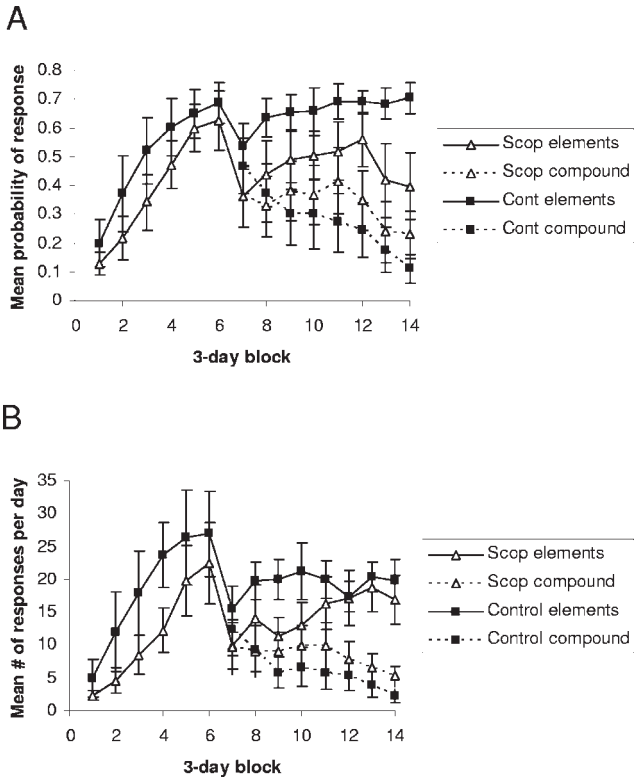


*Figure 1.* Results from experiment on the effects of scopolamine on negative patterning, plotted in 3-day blocks. Data from animals given saline and methylscopolamine are combined as the control group. Scopolamine, methylscopolamine, and saline injections were introduced at the beginning of Block 7. (A): The mean probability of response (±*SEM*) is plotted as a function of 3-day block. Scop elements and scop compound refer to the performance of the scopolamine group on the elements (red and stripes) and the compound stimulus, respectively. Cont elements and cont compound refer to the performance of the control group on the elements and the compound stimulus, respectively. (B): The mean number of responses per day response (±*SEM*) is plotted as a function of 3-day block.

*Number of responses.* Figure 1B shows the mean number of responses per day for each 3-day block, with responses to the elements combined. Number data for the three turtles injected with saline were accidentally lost for the first six blocks, the blocks before scopolamine and the compound were introduced; therefore the control data for those blocks represent only the methylscopolamine group ($n = 4$).

As can be seen in Figure 1B, mean number of responses increased for both groups over the first six blocks, before the introduction of the drug injections. For number of responses, the only significant effect in the training phase of the experiment was that of block, $F(5, 50) = 13.67$, $p < .001$, $\eta_p^2 = .58$. In the compound training phase of the experiment, the responses of both groups dropped when the injections were introduced, but the responses for the scopolamine group were lower on the elements and showed less differentiation than those of the control group. Significant effects were found for stimulus, $F(1, 13) = 50.92$, $p < .001$, $\eta_p^2 = .80$; the interaction of stimulus by block, $F(7, 91) = 8.32$, $p < .001$, $\eta_p^2 = .39$; and the interaction of stimulus by group, $F(1, 13) = 6.14$, $p = .03$, $\eta_p^2 = .32$. Analyses of simple effects in these data, as were done for the probability data, were unable to demonstrate the source of the significant interaction.

## *Simple Go/No Go Discrimination*

Figure 2A shows the performance of the two groups in the scopolamine experiment in terms of probability of response. As can be seen, there was a tendency for the saline group to respond more than the scopolamine group in this experiment, but there was a great deal of variability in the performance. In particular, one control turtle and three scopolamine turtles never learned to hit the key. Figure 2B shows the results with these turtles dropped from the analysis. Here the similarity in responding between the two groups can be seen. Repeated-measures analyses of variances (ANOVAs) of the data in Figure 2A showed that there was a significant effect of stimulus, $F(1, 10) = 7.68$, $p = .02$, $\eta_p^2 = .43$, but no main effect for groups (saline vs. scopolamine), $F < 1$, $\eta_p^2 = .07$; no other main effects or interactions were significant, and no partial eta-squared for an interaction with group was above .08. For number of responses, the results including all the subjects were similar: the main effect for stimulus approached significance, $F(1, 10) = 4.22$, $p = .067$, $\eta_p^2 = .30$; but the main effect for groups was not significant, $F(1, 10) = 1.45$, $p > .05$, $\eta_p^2 = .13$; nor were

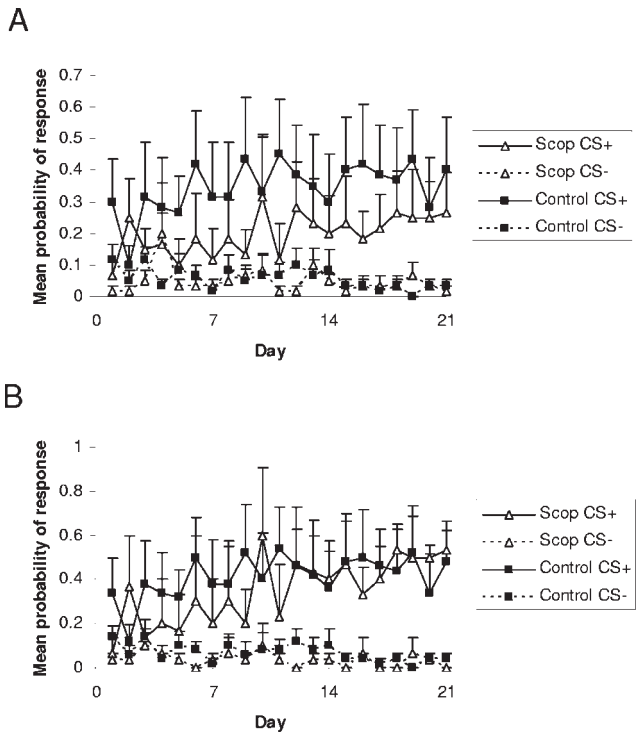


*Figure 2.* Results from experiment on the effects of scopolamine on a simple discrimination between red (CS+) and stripes (CS–). The mean probability of response (±*SEM*) is plotted as a function of day. Scop CS+ and Scop CS– refer to the performance of the scopolamine group on the positive and negative stimuli, respectively. Control CS+ and Control CS– refer to the performance of the control group on the two stimuli. (A): Data from all cases. (B): Data from selected cases (see text for explanation).

any other main effects or interactions, and no partial eta-squared for an interaction with group was above .15. When the data from Figure 2B were analyzed, however, the findings showed a significant main effect for stimulus, $F(1, 6) = 9.76$, $p = .02$, $\eta_p^2 = .62$; and a significant interaction between stimulus and day, $F(20, 120) = 1.81$, $p = .03$, $\eta_p^2 = .23$; illustrating that learning did take place. Again there was no effect of groups and no interaction of groups with any stimulus or day (all effect sizes $<.13$). For number of responses on these selected cases, nothing was significant, and all effect sizes involving group were less than .11.

## Discussion

Thus the performance of turtles given scopolamine on negative patterning was impaired relative to those given saline or methylscopolamine. The fact that methylscopolamine had no effect indicates that the effect of scopolamine was not due to peripheral effects because methylscopolamine does not cross the blood–brain barrier. In contrast, scopolamine had no effect on the learning of a simple discrimination between the elements of red and stripes, demonstrating that this elemental discrimination does not require the cholinergic system.

Performance on the elemental discrimination was unaffected by scopolamine. Inclusion or omission of turtles that did not hit the key made no difference in the results: In either case no group difference was found. These findings are consistent with the data from another study in our laboratory (Naimoli, Libby, & Powers, 2009) showing no deficit on an instrumental horizontal–vertical discrimination in turtles given scopolamine.

In this study, as in our previous study of negative patterning (Yeh & Powers, 2005), significant effects were found more frequently with the probability of response measure than with the number of responses measure. Number of responses is more variable, and therefore it is more difficult to obtain reliable differences between groups.

The scopolamine group in this experiment showed a deficit because they responded less to the elements than the control group did. This deficit appears to be a consequence of the nonreinforcement of the compound stimuli. Both groups showed a drop in response when the compound was introduced, but the scopolamine group never recovered responding to the elements, and therefore their performance on the discrimination was impaired. This deficit is similar to the deficit found after blockade of nitric oxide in turtles (Yeh & Powers, 2005). Reanalysis of the data in that study showed that the groups differed significantly on the elements at the end of training, not on the compound. Thus, as might be expected if blockade of nitric oxide and blockade of acetylcholine were both affecting the same system, both manipulations produced a similar pattern of results.

Previous work in our laboratory has shown that both scopolamine and lesions of the cholinergic basal forebrain and dorsal cortex disrupt maze retention (Petrillo et al., 1994), and the disruptions shown in these cases were similar. These findings provided evidence that scopolamine exerts its effects on maze retention by blocking acetylcholine in the telencephalon, specifically in the dorsal cortex. We hypothesize that the effects seen here are also due to blockade of cholinergic synapses in the dorsal cortex. Cholinergic cells are also found in the medial septum in turtles (Powers & Reiner, 1993), however, and these cells probably project to the reptilian homologue of the hippocampus, the medial cortex. Thus the effect seen in this study may be due to the cholinergic blockade of the medial septum—medial cortex projection, or some other cholinergic system, not the basal forebrain-dorsal cortex projection. Richmond et al. (1997) found that scopolamine had the same effect on negative patterning in hippocampal lesioned rats as it did on control rats, suggesting that the scopolamine effect is on a different system, perhaps the basal forebrain cholinergic system.

In mammals, basal forebrain or hippocampal cholinergic lesions made with 192 IgG-saporin did not disrupt spatial learning (Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995; Torres et al., 1994). Lesions of both the cholinergic basal forebrain and the medial cortex, the homologue of the hippocampus, disrupt spatial learning in turtles (Petrillo et al., 1994; Rodriguez et al., 2002), but specific cholinergic lesions made with 192 IgG-saporin lesions have not been studied. Thus we do not know whether the effects seen with medial cortex lesions, basal forebrain lesions, or dorsal cortex lesions are due specifically to loss of cholinergic inputs. Scopolamine may impair both spatial learning and negative patterning because it blocks cholinergic receptors in both the basal forebrain and medial septum-hippocampal system.

As mentioned earlier, L-NAME, a nitric oxide synthase inhibitor, which blocks nitric oxide, also impaired negative patterning in turtles (Yeh & Powers, 2005). This impairment is consistent with

the presumed role of acetylcholine in this task, as nitric oxide-positive neurons are also found in the basal forebrain of turtles (Bruning et al., 1994). In mammals nitric oxide in the basal forebrain modulates acetylcholine release (Prast & Philippu, 1992; Vazquez, Lydic, & Baghdoyan, 2002).

Studies in mammals have demonstrated the importance of acetylcholine in negative patterning. In rats, quisqualic acid or 192 IgG-saporin lesions of the nucleus basalis magnocellularis in the basal forebrain, the latter of which destroy only cholinergic cells, impaired negative patterning (Butt & Hodge, 1998; Butt et al., 2002). In a microdialysis study, acetylcholine release was increased during the execution of a negative patterning task in rats (Hata et al., 2007). Turtles are descended from the stem amniotes that gave rise to both reptiles and mammals (e.g., Rieppel, 1999). Thus, the finding that a cholinergic receptor blocker impairs negative patterning learning in turtles suggests that the role of acetylcholine in negative patterning learning has been inherited from the reptilian ancestors of mammals.

Two studies of the effect of scopolamine on negative patterning in rats have yielded disappointing results. P. M. Moran (1992) found a deficit on retention but not acquisition of negative patterning with a dose of 0.6 mg/kg. No effect was found with a dose of 0.1 mg/kg. Richmond et al. (1997), in a markedly different task (auditory stimuli and two choice discrimination), found that both scopolamine and methylscopolamine, in doses from 0.025 to 0.1 mg/kg, impaired rats' retention of negative patterning, suggesting that the effects were peripheral, not central. These authors studied only retention. Thus the two studies that found effects of scopolamine on negative patterning in rats found it on retention of negative patterning, not acquisition. We did not study retention of negative patterning in this experiment, but Yeh and Powers (2005) found no effect of nitric oxide blockade on retention of negative patterning.

The results of this study can be understood in terms of a distinction between simple, or elemental, association learning and configural association learning (Rudy & Sutherland, 1989). Blocking muscarinic receptors impaired configural association learning but not simple associative learning in this experiment. According to configural learning theory, configural associations are required when the stimuli do not have a one-to-one relationship with the responses to be made. Thus the animal must learn the relationship between several stimuli and the response contingencies in effect. In negative patterning, such response contingencies are present, in that the elements are sometimes reinforced (when presented alone) and sometimes unreinforced (when presented in a configuration). Elemental association learning is sufficient when the stimuli, or elements, are always associated with reinforcement or nonreinforcement. The data presented here support the idea that muscarinic receptors are not necessary for elemental association learning in turtles.

Although Rudy and Sutherland (1989) proposed a dissociation between simple and configural association learning after lesions of the hippocampus, subsequent research has shown that mammals do not always show deficits on negative patterning after hippocampal lesions (Davidson et al., 1993; Moreira & Bueno, 2003; Papadimitriou & Wynne, 1999) or on other tasks that tap configural association learning (e.g., Gallagher & Holland, 1992; Whishaw & Tomie, 1991). Rudy and Sutherland (1995) revised their theory to attribute configural association learning to an interaction between the hippocampus and cortex. Although much less work has been done on the effects of basal forebrain cholinergic lesions on negative patterning, these lesions have been found in two studies to impair negative patterning (Butt & Hodge, 1998; Butt et al., 2002). Butt and colleagues interpreted their findings in terms of configural learning theory, but they pointed out that much of the data on the function of basal forebrain cholinergic area suggests that this area is involved in attention not learning (Chen, Baxter, & Rodefer, 2004; Chiba, Bucci, Holland, & Gallagher, 1995; McGaughy, Dalley, Morrison, Everitt, & Robbins, 2002; McGaughy, Kaiser, & Sarter, 1996; Muir, Everitt, & Robbins, 1994; Turchi & Sarter, 1997). They extended the configural learning idea to attention by pointing out that attention to two stimuli simultaneously is required when animals learn a negative patterning problem. If the basal forebrain cholinergic system is involved in such attention, then negative patterning would be impaired by lesion of the basal forebrain or blockade of cholinergic receptors.

Data from our laboratory suggest that the cholinergic system in turtles is involved in selective attention. Lesions of the dorsal cortex impair long-term habituation of head withdrawal to a looming stimulus in turtles (A. Moran, Wojcik, Cangiane, & Powers, 1998). Thus without the dorsal cortex, animals are impaired in learning to ignore stimuli that have no consequence. Because the dorsal cortex is the target of a projection from the cholinergic basal forebrain (Bruce & Butler, 1984; Ouimet et al., 1985; Schuss & Powers, 1998), this finding also suggested an attentional deficit after disruptions of the basal forebrain cholinergic system. The present finding of a deficit in negative patterning after cholinergic blockade can be understood as a deficit in attention, if turtles given scopolamine have difficulty simultaneously attending to both stimuli when they are presented in compound.

The spatial learning deficit seen with scopolamine and cholinergic system lesions (Petrillo et al., 1994) can also be understood in attentional terms. Animals that are not able to focus their attention on the relevant stimuli are likely to have deficits in finding their way in an environment with multiple stimuli that appear differently from different vantage points in a maze.

Thus, we have shown that scopolamine impairs negative patterning acquisition in turtles but does not affect learning of a simple elemental discrimination. Future research will investigate the effects of scopolamine and lesions of the basal forebrain cholinergic area and dorsal cortex on attentional processes.

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