

Olfactory Repeated Discrimination Reversal in Rats: Effects of Chlordiazepoxide, Dizocilpine, and Morphine

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Effects of a benzodiazepine (chlordiazepoxide), an *N*-methyl-D-aspartate receptor antagonist (dizocilpine), and an opiate agonist (morphine) were studied with a procedure designed to assess effects of drugs and other manipulations on nonspatial learning in rats. In each session, rats were exposed to 2 different 2-choice odor-discrimination problems with food reinforcement for correct responses. One problem (performance discrimination) remained the same throughout the study. That is, 1 odor was always correct (S+) and the other was never correct (S-). For the other problem (reversal discrimination), stimuli changed every session. Six different odors were used to program the reversal discrimination; on any given session, S+ was a stimulus that had served as S- the last time it had appeared, S- was a stimulus that had been S+ on its last appearance. Thus, in each session, learning a discrimination reversal could be studied along with the performance of a comparable, but previously learned, discrimination. Chlordiazepoxide interfered with reversal learning at doses that had no effect on the performance discrimination. Morphine and dizocilpine also impaired reversal learning but only at doses that also affected performance of the well-learned performance discrimination.

Keywords: benzodiazepine, NMDA antagonist, learning, repeated acquisition, morphine

A critical problem associated with procedures used to study learning and memory in nonhuman subjects is the difficulty of distinguishing the effects of drugs or other variables that are selective to learning processes from effects on other aspects of performance (sensory-motor abilities, motivation, etc.). A technique that has been used effectively to make this distinction is the multiple-component, repeated acquisition/performance (RAP) procedure (Thompson & Moerschbaecher, 1979). The standard RAP procedure requires subjects to learn sequences of reinforced lever-press responses. In one stimulus context, the same sequence is reinforced in every experimental session (performance), whereas in a different context, a new response sequence is required in each session (acquisition). Thus, in a given session, the experimenter can observe the effects of a drug or other manipulation on learning a new sequence and compare this directly with the drug's effect on performance of the previously learned sequence. When drugs affect learning at doses that do not alter performance of a comparable behavioral sequence, drug actions that are specific to acquisition processes are often inferred, and an impressive literature on the pharmacology and toxicology of learning has been generated

with such procedures, primarily with monkeys and pigeons as subjects (Cohn & Paule, 1995; Eckerman & Bushnell, 1992).

One aspect of the RAP procedure that has limited its utility in neuroscience is that it is difficult to train the requisite behavioral chains in rodents. A RAP procedure developed in our laboratory for rats that permits relatively rapid acquisition used a variation of the Morris (1981) swim task (MST; Keith & Galizio, 1997). In one pool, rats learned a new platform position in each session (acquisition component), whereas in a different pool the platform was located in the same position in every session (performance component). Under these conditions, rats come to display rapid, within-session learning of the platform position, with acquisition often evident in a single trial. Through the use of this procedure, benzodiazepines (e.g., chlordiazepoxide) and an opiate agonist (morphine) consistently impaired spatial acquisition at doses that had no effect on swimming to the well-learned component (Galizio, Keith, Mansfield, & Pitts, 2003; Keith & Galizio, 1997; Keith, Pitts, Pezzuti, & Galizio, 2003). However, effects of *N*-methyl-D-aspartate (NMDA) antagonists such as dizocilpine (MK801) LY235959 have generally not been selective (Galizio et al., 2003; Keith & Galizio, 1997).

Of potential importance is that the MST involves spatial learning in contrast with the nonspatial operant procedures of the traditional RAP techniques. There is evidence that spatial and nonspatial learning may involve different brain regions; and thus, may differ in pharmacological sensitivity as well (Eichenbaum & Cohen, 2001; O'Keefe & Nadel, 1978). In order to determine the generality of the drug effects observed in the MST studies, a nonspatial procedure suitable for rats is needed. The main purpose of the present study was to develop such a procedure by using olfactory discrimination learning.

In contrast to the slow rates of visual discrimination learning shown by rats, rapid within-session olfactory discrimination can be

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readily demonstrated (Slotnick, Hanford, & Hodos, 2000). Simple methods of studying olfactory discriminations have been developed in which rats are trained to dig in cups containing a mixture of sand and spices or other odorants to obtain a buried food pellet (Birrel & Brown, 2000; Bunsey & Eichenbaum, 1996). In order to adapt these procedures for a RAP methodology, rats were exposed to two different odor pairs in each session. For both pairs, the stimulus cup associated with one odor contained a food pellet (S+) and the other odor did not (S-). One of the stimulus pairs was constructed such that in each session food was produced by digging in a stimulus that had served as S- in the last session it had been used, but not for digging in the other member of the pair, which had served as S+ in the last session it had been used. Thus, a form of discrimination reversal was required in each session. The other stimulus pair served as a control with one stimulus designated as S+ and the other S- in every session throughout the experiment. Rats were trained under these conditions until asymptotic performances were reached, after which the effects of a benzodiazepine agonist (chlordiazepoxide [CDP]), an opiate agonist, morphine, and a noncompetitive NMDA antagonist (dizocilpine [DZP]) were assessed. Thus, a second purpose of the study was to determine whether compounds affecting benzodiazepine, opiate, and NMDA receptor systems would affect nonspatial learning in the same way that they affect spatial learning.

Method

Subjects

Subjects were 10 male Holtzman Sprague-Dawley albino rats that were 120–180 days old at the start of the study. The rats were housed individually in hanging steel cages with a reversed 12-hr light-dark cycle. The subjects' diet was restricted to the sucrose pellets used as reinforcement and to standard rat chow pellets available for 1 hour each day (usually half an hour after they completed a testing session). The subjects had unlimited access to water in their home cage.

Apparatus

The apparatus was a modified operant chamber with interior dimensions 28 cm long × 26 cm wide × 30 cm high. The floor of the apparatus was constructed of stainless steel bars 0.5 cm in diameter and spaced 1.3 cm apart. The apparatus was modified for the experiment by removing a 5-cm section of the front wall permitting a stimulus presentation tray to be inserted. The plastic tray was 28 cm long × 12 cm wide × 4 cm high and contained two symmetrical circular holes 5 cm in diameter, 8 cm from the wall, and 5 cm from each other. Plastic cups (2 oz.) were placed in these holes for stimulus presentation. When the tray was completely inserted, the two cups (8 cm apart) were accessible to the rat. A small speaker adjacent to the operant box emitted constant white masking noise (70 dB).

Stimuli

Olfactory stimuli were generated by mixing household spices (celery, cinnamon, garlic, ginger, mustard, onion, paprika, and sage) or coffee with sterilized play sand. The cups were filled to approximately 1 cm below the rim with scented sand, and the cup designated as correct (S+) on a particular trial was baited by placing a sucrose pellet 1 cm below the surface of sand with tweezers. A pellet was inserted and removed from the stimulus cup that was designated as incorrect on a trial (S-) to ensure that neither displacement of the sand nor the scent of the pellet or tweezers would serve as potential cues. A ratio of 1 g of spice per 100 g of sand was

used because pilot research suggested that these spice levels were sufficient to mask the scent of the sucrose pellet. In these pilot studies, the rats of the present study as well as additional rats were exposed to multiple trials on which they could choose between two cups filled with sand with a specified concentration of one of the above spices, one baited with a sucrose pellet and the other not. No evidence of above-chance pellet detection was obtained at the 1-g/100-g concentrations with any of spices used in the present study.

Procedure

Pretraining. Initially, 45-mg sucrose pellets were placed in empty stimulus cups in the tray, which was inserted into the chamber until the pellets were consumed. In subsequent sessions, food pellets were buried progressively deeper in unscented sand until subjects were reliably consuming pellets buried to a depth of 1 cm.

Repeated reversal training. In each session of this phase, rats were exposed to a simultaneous odor discrimination with two different spices—one designated as the correct stimulus (S+) and the other as incorrect (S-) for that session. Each trial began with the insertion of the stimulus tray with one S+ and one S- cup. The S+ was in the left cup on half the trials (10), and it was in the right cup on the other half of the trials; the order was random with the constraint that S+ was not presented on either side more than twice in succession. A response was defined as the displacement of sand by a digging motion with the paws and/or penetration of sand with the snout. The trial continued until a correct response was made and the animal consumed the pellet or until 30 s had elapsed, whichever came first. During the intertrial interval (approximately 5 s), the experimenter recorded the response, removed the cups, and replaced them with the cups programmed for the next trial. Different stimulus cups were used for each trial to ensure that no carry over scents from previous trials could influence performance.

Stimuli were chosen to serve as S+ and S- from a pool of eight before each session. Across sessions, the order in which these stimuli appeared was randomized with the following constraints: Once an odor served as S+ on a session, it had to serve as S- before appearing again as S+; once a stimulus was used in a session, it was not used again until all other stimuli had been used at least once; and, finally, particular stimulus pairs were not used consecutively. Thus, in each session, a discrimination reversal could be studied because the rat learned to dig in a cup containing an odor that was S- the last time it appeared and not to dig in a cup containing an odor that was S+ the last time it appeared. Rats were tested under these conditions until they met a criterion of 90% correct or better on the final ten trials of each of five consecutive sessions.

Repeated reversal/performance phase. In this phase a second simultaneous discrimination was added to the repeated acquisition sequence each session, but this discrimination was invariant across sessions. That is, one of the stimuli was designated as S+ throughout the experiment, whereas the other was designated as S-. Thus, this discrimination (performance discrimination) did not have to be learned within each session. Stimuli for the performance discrimination were chosen arbitrarily for each rat. During this phase, the number of trials per session was increased to 24, with 16 reversal-learning and 8 performance trials. The performance trials were randomly interspersed between the reversal trials, but no more than two performance trials could appear consecutively. For analysis, the reversal trials were divided into four blocks of four trials and the performance trials were into four blocks of two trials. When criteria were met of 100% correct throughout the session for performance and 87.5% correct for the last two blocks of reversal trials for eight consecutive sessions, the drug-administration phase began. As the experiment progressed, 20 sessions were arbitrarily selected to test interrater reliability. In these sessions, one of two raters was blind as to which stimulus was correct or incorrect, and there was agreement with respect to response choices on 99.1% of the trials.

Drug phase. Sessions were conducted 5 days per week and injections were given on Tuesdays and Fridays. Five rats were studied with CDP, 5

with DZP, and 7 with morphine. Most rats were tested under more than one drug, and when this occurred, 2 weeks of baseline training without injections intervened between one drug study and the next. Chlordiazepoxide hydrochloride (Sigma), dizocilpine (MK-801) maleate (Tocris), and morphine sulfate (National Institute on Drug Abuse) were dissolved in 0.9% saline and administered in a volume of 1 ml/kg. CDP and morphine injections were given 15 min prior to the session. DZP was administered 30 min prior to the session. Doses were administered two to four times, in an ascending order initially and in a semirandom order thereafter.

Data analysis. For each subject, mean percentage correct was computed for each block and averaged for all determinations at each dose, including saline. As a second control measure, a baseline was determined by using the eight criterion sessions that preceded the drug administration phase. Means determined for each subject were entered into a Dose \times Component (reversal vs. performance) \times Block within-subjects analysis of variance (ANOVA). Post hoc comparisons were made with Tukey's honestly significant difference (HSD) test.

Results

The pretraining phase of the study required from 7 to 14 sessions. Considerably more sessions were required for rats to reach criterion for the repeated reversal phase (range = 15–39; $M = 23.9$). Finally, the rats required an additional 12 to 51 training sessions ($M = 26.5$) to reach criterion following the introduction of the performance discrimination in the repeated reversal/performance phase.

Figure 1 shows the effects of the various drugs on the performance (open triangles) and reversal (solid circles) discriminations, with mean percentage correct on the vertical axis and within-session trials blocks on the horizontal axis. The first two panels show percentage correct on baseline and saline control sessions for the three studies and reveal that rats were learning the reversals to high levels of accuracy within the session while maintaining virtually perfect accuracy on the well-learned performance discrimination. Reversal learning occurred rapidly under baseline conditions. In the initial trial block (first four trials), baseline and saline accuracies were slightly above chance for the reversal discrimination, ranging from 55% to 70% correct across the three studies. However, by the second trial block, the reversal discrimination had been learned with accuracies usually at 85% correct or better.

Low doses of CDP (1.0 and 3.0 mg/kg) did not affect accuracy in either condition. The 10.0-mg/kg dose, however, appeared to produce selective effects: This dose impaired reversal learning without affecting the performance discrimination. Reversal learning was also clearly impaired at 17.0 mg/kg, with only a small drop in accuracy on the performance discrimination. A Dose \times Component \times Block repeated measures ANOVA confirmed the dose-dependent impairment of accuracy with a significant main effect of CDP dose, $F(5, 20) = 12.10$, $p < .01$, and the reliability of the selective effects on reversal learning by a significant Dose \times Component interaction, $F(5, 20) = 5.64$, $p < .01$. Post hoc analysis (Tukey's HSD test) reveal that the effects of 10 and 17 mg/kg CDZ on reversal learning were significantly different from those in the saline and block (BL) conditions but that none of the doses produced a statistically significant impairment of the performance discrimination relative to either saline or BL.

Morphine also disrupted reversal learning in a dose-dependent fashion, but the doses that produced declines in reversal accuracies

also tended to disrupt performance accuracies as well. Reversal learning was somewhat impaired at the 5.6- and 10.0-mg/kg doses, but these were accompanied by small disruptions in performance. Both reversal and performance discriminations were substantially impaired at the 17.0-mg/kg dose. Consistent with such an interpretation, the main effect of morphine dose was statistically significant, $F(5, 30) = 41.88$, $p < .01$, but the Dose \times Component interaction term failed to reach significance ($p > .05$). Post hoc analysis indicated that only the 17.0-mg/kg dose produced effects that differed significantly from the saline and BL conditions ($p < .05$).

Low doses of DZP (0.03 mg/kg) did not affect accuracy appreciably in either component, and the 0.1-mg/kg dose produced only small impairments on both the performance and reversal discriminations. At 0.3-mg/kg DZP, accuracy in both discriminations declined to well below chance levels due to a lack of responding by most rats. The nonselective impairment produced by DZP was confirmed statistically with a Dose \times Component \times Block repeated measures ANOVA with a significant main effect of dose, $F(4, 16) = 11.86$, $p < .01$, and the absence of a significant Dose \times Component interaction ($p > .05$). Only the highest dose (0.3) produced effects that differed significantly from the saline and BL conditions ($p < .05$).

Discussion

The present study demonstrates the feasibility and utility of extending the RAP procedure to the analysis of olfactory discrimination learning in rats. In the repeated reversal phase of the experiment, all rats eventually showed rapid, within-session acquisition of a discrimination in which the S+ and S- were stimuli whose functions were reversed on each appearance. The high levels of accuracy maintained on the performance discrimination throughout the session made it possible to evaluate drug effects on reversal learning while simultaneously evaluating the performance of a previously learned problem.

Intermediate doses of CDP (10 mg/kg) interfered with reversal learning without affecting performance. These results replicated the effects of benzodiazepines on spatial learning by rats in the MST (Keith & Galizio, 1997; Keith et al., 2003) and extended those findings to a nonspatial learning problem. The selective disruption of learning by CDP in the present study was also consistent with findings of selective learning impairments produced by benzodiazepines by using repeated acquisition procedures in other species, including pigeons (e.g., Thompson, 1975) and monkeys (e.g., Auta, Winsauer, Faust, Lambert, & Moerschbaecher, 1997). Thus, the present study confirms the generality of benzodiazepine impairment of both spatial and nonspatial learning across species.

Morphine also impaired reversal learning but only at relatively high doses that also interfered with performance. At the 5.6- and 10.0-mg/kg doses, reversal accuracies did appear to be affected somewhat more substantially than were accuracies on the performance discrimination, but there was no dose that produced statistically significant selective effects. These results were consistent with previous studies of opiate effects using nonspatial repeated acquisition in monkeys (e.g., Moerschbaecher & Thompson, 1983), which have generally reported only nonselective effects of

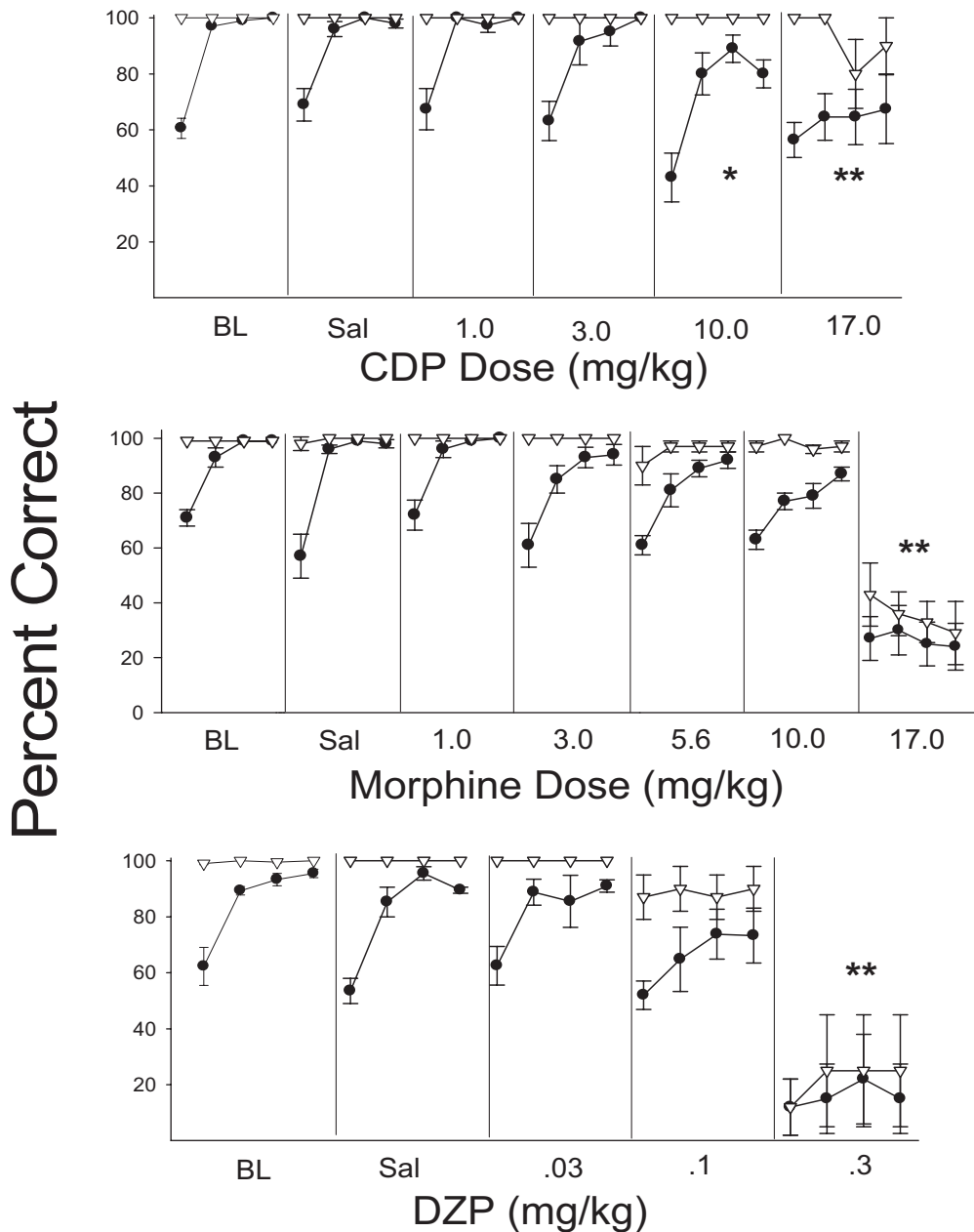


Figure 1. Mean percentage correct is plotted on repeated reversal learning (solid circles) and performance (open triangles) discriminations for the drugs tested (A: chlordiazepoxide [CDP]; B: morphine; C: dizocilpine [DZP], bottom). BL (baseline) presents means obtained on the eight criterion sessions that preceded the drug administration phase. Sal (saline) shows means obtained when saline was injected. Vertical bars indicate standard error of the mean. When error bars are not visible, they are smaller than the symbol used to represent the data point. A single asterisk indicates a significant difference (Tukey's honestly significant difference test: $p < .05$) in reversal learning, but not in performance, between the effects of that dose and both control conditions (BL and Sal). Two asterisks indicate that both reversal learning and performance in differed from controls ($p < .05$).

opiate drugs. It is interesting that the present findings with respect to morphine differed from the selective effects on spatial learning in the MST noted by Galizio et al. (2003). The present findings of nonselective effects of morphine in rats suggest that differences in morphine effects observed with the MST and nonspatial RAP procedures in previous studies are probably not simply the result of

the species used. A more likely possibility is that the opioid system may play a more critical role in spatial than in nonspatial learning. However, other explanations of these effects cannot be ruled out. For example, the MST involves different reinforcers than the repeated reversal procedure (escape from the pool vs. food). Further explorations of opiate effects on selected learning models are

necessary to determine the generality of spatial versus nonspatial differences with respect to opiate drugs.

Like morphine, NMDA receptor antagonist DZP also produced dose-dependent impairment of accuracy on both discriminations that were nonselective. Disruption of reversal learning did not reliably occur until doses that were high enough to impair performance were reached. These results were consistent with previous findings of nonselective effects of NMDA receptor antagonists on spatial learning by rats (Galizio et al., 2003; Keith & Galizio, 1997). However, studies of NMDA receptor antagonists on repeated acquisition of nonspatial problems in monkeys have generally yielded selective effects (e.g., France, Moerschbaecher & Woods, 1991). The basis for the differences in NMDA antagonist effects reported across procedures remains unclear. Our findings suggest that it is not simply the case that NMDA antagonists affect nonspatial and spatial learning differently.

Several strengths and limitations of the olfactory RAP procedure are worth noting. One disadvantage is that the procedure is quite labor intensive. However, automated olfactory apparatus are commercially available and could be used effectively with the procedure (e.g., Slotnick et al., 2000). Also, potential difficulties in interpretation of selective effects should be noted. Performance and acquisition components in RAP procedures differ in several respects other than the learning requirement. For example, acquisition or reversal is more difficult than the performance task and more likely to be associated with errors. Thus, even with RAP procedures, inferences about selective effects on learning processes are not straightforward. That said, the procedure does permit direct comparison of reversal learning with the performance of a comparable nonreversed discrimination in the same session and allows dose response functions to be determined for individual rats. These features represent improvements over the controls offered by alternative procedures. Another aspect of the procedure that should make it a valuable tool is that the baseline performances were developed fairly rapidly and were characterized by stable and high levels of discrimination accuracy. In contrast, repeated acquisition of response chains in rats often show high levels of between- and within-subjects variability and require more extensive training to achieve stable, accurate baseline levels (cf. Winsauer, Rodriguez, Cha, & Moerschbaecher, 1999). In addition to these practical concerns, the fact that discrimination learning in rats with olfactory stimuli occurs so rapidly, after just a few training trials, may make this a useful model of human learning. On balance, the repeated reversal procedure with olfactory stimuli has much to commend it as a technique to study the neurobiology of learning in rats.

References

Auta, J., Winsauer, P. J., Faust, W. B., Lambert, P., & Moerschbaecher, J. M. (1997). Effects of negative allosteric modulators of gamma-aminobutyric acid A receptors on complex behavioral processes in

monkeys. *Journal of Pharmacology and Experimental Therapeutics*, 280, 316–325.

- Birrel, J. M., & Brown, V. J. (2000). Medial frontal cortex mediates perceptual attentional set shifting in the rat. *Journal of Neuroscience*, 20, 4320–4324.
- Bunsey, M., & Eichenbaum, H. (1996, January 18). Conservation of hippocampal memory function in rats and humans. *Nature*, 379, 255–257.
- Cohn, J., & Paule, M. G. (1995). Repeated acquisition of response sequences: The analysis of behavior in transition. *Neuroscience and Biobehavioral Reviews*, 19, 397–406.
- Eckerman, D. A., & Bushnell, P. J. (1992). The toxicology of cognition: Attention, learning and memory. In H. Tilson & C. Mitchell (Eds.), *Neurotoxicology* (pp. 213–269). New York: Raven Press.
- Eichenbaum, H., & Cohen, N. J. (2001). *From conditioning to conscious recollection: Memory systems of the brain*. New York: Oxford University Press.
- France, C. P., Moerschbaecher, J. M., & Woods, J. H. (1991). MK-801 and related compounds in monkeys: Discriminative stimulus effects and effects on a conditional discrimination. *Journal of Pharmacology and Experimental Therapeutics*, 257, 727–734.
- Galizio, M., Keith, J. R., Mansfield, W., & Pitts, R. C. (2003). Repeated spatial acquisition: Effects of NMDA antagonists and morphine. *Experimental and Clinical Psychopharmacology*, 11, 79–90.
- Keith, J. R., & Galizio, M. (1997). Acquisition in the Morris swim task is impaired by a benzodiazepine but not an NMDA antagonist: A new procedure for distinguishing acquisition and performance effects. *Psychobiology*, 25, 217–228.
- Keith, J. R., Pitts, R. C., Pezzuti, T., & Galizio, M. (2003). GABA-A modulator effects on a multiple-component, repeated-acquisition test of spatial learning. *Behavioural Pharmacology*, 14, 67–76.
- Moerschbaecher, J. M., & Thompson, D. M. (1983). Differential effects of prototype opioid agonists on the acquisition and performance of conditional discriminations in monkeys. *Journal of Pharmacology and Experimental Therapeutics*, 226, 738–748.
- Morris, R. G. M. (1981). Spatial localization does not require the presence of local cues. *Learning and Motivation*, 12, 239–260.
- O'Keefe, J., & Nadel, L. (1978). *The hippocampus as a cognitive map*. Oxford, England: Clarendon Press.
- Slotnick, B. M., Hanford, L., & Hodos, W. (2000). Can rats acquire an olfactory learning set? *Journal of Experimental Psychology: Animal Behavior Processes*, 26, 399–415.
- Thompson, D. M. (1975). Repeated acquisition of response sequences: Stimulus control and drugs. *Journal of the Experimental Analysis of Behavior*, 23, 429–436.
- Thompson, D. M., & Moerschbaecher, J. M. (1979). An experimental analysis of the effects of d-amphetamine and cocaine on the acquisition and performance of response chains in monkeys. *Journal of the Experimental Analysis of Behavior*, 32, 433–444.
- Winsauer, P. J., Rodriguez, F. H., Cha, A. E., & Moerschbaecher, J. M. (1999). Full and partial 5-HT-1A receptor agonists disrupt learning and performance in rats. *Journal of Pharmacology and Experimental Therapeutics*, 288, 335–347.

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