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Isotretinoin (13-*cis*-retinoic acid) alters learning and memory, but not anxiety-like behavior, in the adult rat

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ABSTRACT

Isotretinoin (ISO, 13-*cis*-retinoic acid) is commonly prescribed as *Accutane* for the treatment of acne. ISO is a known teratogen and the physical side effects of the drug have been well documented. However, possible psychological risks associated with the drug have yet to be determined. Retinoid receptors are abundant in the striatum and hippocampus, brain structures involved in implicit and explicit memory processes, respectively. The current study examined whether ISO influenced implicit or explicit memory processes using a two-stage radial-arm maze (RAM) task. The two stages were identical, except for the method of presenting arm choices to the rats: one at a time (Stage 1) or in pairs (Stage 2). Male rats (n=12/group) were tested on both stages of the RAM during chronic oral treatment with ISO (0, 5, 10, or 15 mg/kg/day). Performance indicated that ISO impaired explicit memory in Stage 2, but retention tests one month after ISO exposure ended, indicated recovery from this explicit memory impairment and evidence of enhanced implicit memory in the 10 mg and 15 mg ISO rats. These data indicate extensive, enduring memory effects from oral ISO treatment at doses likely to produce serum levels within the range typically used to treat acne in humans.

Vitamin A and its derivatives, the retinoids, are essential for the development and maintenance of body tissues and central nervous system function (Lane and Bailey, 2005; Maden, 2007). Isotretinoin (ISO, 13-*cis*-retinoic acid) is a naturally-occurring retinoid that is also the active ingredient in the acne medication *Accutane* (Hoffman-LaRoche, Nutley, NJ). First synthesized in 1955, ISO has proven to be highly effective in treating dermatological diseases; however, prescription use of the drug has been rigidly controlled ever since its 1982 FDA approval, mainly because it has been associated with severe teratogenic effects, but also because it has been associated with a variety of physical side effects (e.g., dry/peeling skin, muscle pain, vomiting, headache, fatigue, intracranial hypertension, tremors or seizures, numbness or paralysis, and blurred or double vision) in users (Roche, 2002). Not surprisingly, because ISO is a derivative of vitamin A, its side effects are similar to the symptoms experienced with vitamin A toxicity, or hypervitaminosis A (O'Donnell, 2003, 2004). Due to the many side effects associated with ISO's use, it has never been approved as a first-line acne treatment, but only for cases of severe recalcitrant nodular acne, a severe form of acne generally considered to be resistant to standard treatments (e.g., oral antibiotics). However, in recent years, the use of ISO for less severe cases of acne has reportedly been on the rise with recent statistics showing that half of all prescriptions are written for patients not diagnosed with severe acne (Bremner and McCaffery, 2008). *Accutane* use more than tripled during the previous decade with nearly 2 million prescriptions being filled in the U.S. alone during the year 2000 (Roche, 2002).

While many physical side effects and risks have been associated with prenatal and adult exposure to ISO, possible psychological risks associated with adult use have yet to be fully determined. Reports of suicidal ideation, depression, personality changes, memory loss, violence, and aggression in patients taking ISO have raised concerns in recent years about serious psychological effects associated with the drug (O'Donnell, 2003). While studies investigating potential psychological risks of ISO have been equivocal (Bremner et al., 2005; Chia et al., 2005; Cohen et al., 2007; Ferguson et al., 2005; Hull and D'Arcy, 2003; Magin et al., 2005; Marqueling and Zane, 2005; O'Reilly et al., 2006), ISO's list of contraindications and warnings has continually increased over the years to now include the possibility of acute or chronic psychiatric disorders, including sadness, depression, irritability, increased aggression, loss of concentration, loss of appetite, and suicide-related behavior (Roche, 2002).

The distribution of retinoid receptors in the cortex, hippocampus, and dopamine (DA)-innervated areas such as the striatum (caudate/putamen), nucleus accumbens, and the olfactory tubercle (Krezel et al., 1999; Zetterstrom et al., 1994, 1999) suggests that several areas of the adult brain respond to retinoids, and the functional processes of these brain regions may be influenced by manipulation of retinoid availability (Lane and Bailey, 2005). Thus, a major focus of research investigating the role of retinoids in the central nervous system has

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been their interaction with DA systems (Samad et al., 1997; Krezel et al., 1998). These studies have generally supported the hypothesis that regulation of DA receptors by retinoid receptor activation influences the expression of DA in the central nervous system (Samad et al., 1997). For instance, Krezel et al. (1998) found that the expression of D_1 and D_2 receptors was reduced in the ventral striatum of adult retinoid receptor mutant mice. Functionally, these mice displayed impaired locomotion, decreased coordination, and a reduction in DA signaling in the mesolimbic system compared to normal mice. Response to cocaine, which normally increases locomotion by increasing DA signaling in the mesolimbic system, was also blunted. While these changes may be due to alterations that take place during embryonic development, research suggests that retinoids still play an important, although perhaps different, role in the mature brain (see Lane and Bailey, 2005 for review). Together, these findings highlight the involvement of retinoid receptors in the regulation of brain DA systems known to be involved in regulation of movement, reward/reinforcement, and implicit forms of learning and memory.

In recent years, retinoids have also been demonstrated to have an important role in the learning and memory systems of the hippocampus. In retinoid receptor knockout mice, a decrease in hippocampal long-term potentiation (LTP) and long-term depression (LTD), with a concomitant impairment in spatial learning and memory, has been demonstrated (Chiang et al., 1998). Furthermore, animals fed a vitamin A deficient (VAD) diet have been shown to have impaired hippocampal LTP/LTD, reduced size of CA1 neurons, a reduction in retinoid receptor mRNAs, and reduced neuronal protein RC3, a protein involved in the functional plasticity of hippocampal synapses during relational memory processing (Misner et al., 2001; Cocco et al., 2002; Etchamendy et al., 2003). Functionally, these VAD animals have been shown to be impaired in hippocampal-dependent learning and memory tasks (Cocco et al., 2002; Etchamendy et al., 2003). Further evidence of a role for retinoids in learning and memory processes comes from the demonstration that many of the VAD-induced impairments mentioned above are reversible when animals are returned to a vitamin A sufficient diet (Misner et al., 2001; Etchamendy et al., 2003). Given that both DA and acetylcholine (ACh) have been shown to play important roles in hippocampal LTP/LTD (Kusuki et al., 1997; Otmakhova and Lisman, 1998; Disterhoft and Oh, 2003) and have been shown to be impaired in VAD mice (Cocco et al., 2002) and retinoid receptor deficient mice (Krezel et al., 1998), these neurotransmitter systems have been implicated in these hippocampal impairments. While it is important to note that differences in the route of drug administration, species, and age, will almost certainly result in differences in RA metabolism and signaling mechanisms, the literature suggests that, regardless of these differences, alterations in retinoic acid influence hippocampal learning and memory systems.

A recent study by Etchamendy et al. (2001) is of particular relevance to understanding the role of retinoid receptors in learning and memory processes. This study used a two-stage radial-arm maze (RAM) paradigm that distinguished between the expression of implicit and explicit memory. During Stage 1 of this task, individual arms of the RAM were presented repeatedly to rats one by one (i.e., successive go/no-go discrimination), either baited or unbaited, until they learned to distinguish which arms contained food rewards. Successful discrimination was indicated by shorter latencies to enter baited than unbaited arms. Stage 2 of this task used the same go/no-go reward contingencies learned in Stage 1, but grouped the single arm presentations into adjacent pairs that would be presented concurrently. Animals were thus presented with an explicit choice between one baited (positive) arm and one unbaited (negative) arm.

The uniqueness of this two-stage paradigm is that it is thought to provide a test of both implicit and explicit learning and memory processes within the same testing apparatus (Marighetto et al., 1999, 2000). The first stage of this task assesses implicit memory, which facilitates particular routines that do not require relational comparisons, and is heavily reliant on striatal mechanisms. Clinical and behavioral evidence suggests that the striatum is centrally involved in the stimulus–response associations and procedural learning that leads to habit formation and the improved performance of routine behaviors (Jog et al., 1999; Packard and Knowlton, 2002; Poldrack and Packard, 2003; Squire, 1998; Teng et al., 2000). The second stage, which requires animals to make a relational/concurrent discrimination, tests explicit memory (Etchamendy et al., 2001; Touzani et al., 2003). Explicit memory is required for flexibility in comparing and contrasting items in memory as well as the capacity to support inferential use of memories in novel situations. This type of memory requires the hippocampus and neighboring parahippocampal and rhinal cortex structures (Cohen et al., 1997; Etchamendy et al., 2001; Squire, 1998).

Etchamendy et al. (2001) used this two-stage paradigm to evaluate a possible association between the cognitive impairments in aged mice and the down regulation of retinoid signaling (Etchamendy et al., 2001). Implicit memory (Stage 1) was unimpaired in aged mice, but an explicit memory deficit (Stage 2) was completely alleviated by administration of retinoic acid. Hippocampal levels of retinoid receptors, expression of specific target genes associated with these receptors, and hippocampal LTP were also restored to near-young adult levels after acute administration of retinoic acid. All of these facilitative effects of RA could be abolished by the co-administration of a retinoic acid receptor antagonist. These findings suggest that retinoic acid can alter hippocampal-dependent processes. In another test using this same paradigm, Marighetto et al. (2000) examined hippocampectomized rats and found that performance was impaired only in tasks that emphasized comparison of items (Stage 2) and not those that encouraged separate representations for individual items (Stage 1). This finding further supports the theory that explicit memory requires relational representations of past experiences and that these comparisons are hippocampal-dependent (Marighetto et al., 2000).

Utilizing this same paradigm, a major goal of the present study was to investigate whether adult exposure to ISO alters implicit or explicit learning and memory processes in the rat. Previous ISO studies have focused on two aspects of behavior: depression-like behaviors and learning/memory effects. Despite evidence (Crandall et al., 2004; Sakai et al., 2004) that 13-*cis*-RA suppresses hippocampal cell proliferation, neurogenesis, and survival (similar to findings in depressed patients), Ferguson et al. (2005) found that chronic 13-*cis*-RA exposure did not severely affect depression-like behaviors in rats. Three studies have specifically looked at learning and memory effects of 13-*cis*-RA in adults, but have found apparently discrepant results. Crandall et al. (2004) found that 13-*cis*-RA exposure reduced hippocampal neurogenesis and performance on the hippocampal-dependent radial-arm maze task. However, Ferguson and Berry (2007) found no evidence of learning and memory effects of 13-*cis*-RA, despite testing rats in three different spatial tasks (Morris water maze, 8-arm radial maze, and a dry land maze) which each used a different type of reinforcer (i.e., water escape, food reinforcement, or water reinforcement). In a third study, O'Donnell et al. (2003) found that, similar to Etchamendy et al.'s (2001) findings with aged mice, 13-*cis*-RA reversed the amnesic effects of dimethyl sulphoxide (DMSO) exposure using conditioned avoidance and Morris water maze tasks.

In the current study, animals were chronically exposed to ISO and subjected to four behavioral tests intended to more fully evaluate the effects of ISO on learning and memory in adult rats and rule out non-specific effects of the drug (e.g., motor activity or anxiety effects). First, animals were tested in both stages of the two-stage implicit/explicit memory task. Animals were then tested in an open field task, which is widely used to examine anxiety levels in animals as demonstrated by their activity levels and overall interaction with the environment (i.e., motor activity) (Goto et al., 1993). Third, animals were tested in an elevated plus maze. The elevated plus maze is commonly used to study anxiety (Hogg, 1996) based on the natural aversion of rodents to explore open spaces as well as the innate fear rodents have for

elevated places. These tests of anxiety-like behavior also served to clarify the basis of any effects observed in the learning and memory tasks (i.e., anxiety could function as a potential confounding factor in these tasks). Finally, in order to evaluate any potential long-term effects of ISO, all rats were administered a retention test in both the implicit and explicit stages of the RAM 30 days after discontinuation of the drug.

1. Methods

1.1. Subjects

Forty-eight male Long Evans rats (46 days old at the beginning of the study), bred in the Western Illinois University animal colony, were pair-housed in standard wire-mesh cages in same-treatment pairs. Rats were weighed daily throughout the study. Animals were fed an ad lib diet of standard chow pellets (LabDiet 5012, PMI Nutrition International, Brentwood, MO) and water until five days prior to the start of testing. On postnatal day 52 (PND 52) rats were placed on a restricted diet of 14 g/day standard rat chow, provided daily upon completion of the experimental task in order to maintain motivation for food reward during all subsequent behavioral testing (i.e., food restriction continued until all testing was completed). During feeding, the animals were placed in separate cages for 4 h to ensure that each rat had full access to the 14 g/day and that food intake could be monitored, after which they were returned to their home cages. Water was available ad lib, except during behavioral testing procedures (described below). The rats were housed in a temperature-controlled room under a 12-hour reverse light cycle with all experimental procedures occurring during the dark cycle. All procedures were conducted in accordance with the guidelines established by the Western Illinois University Institutional Animal Care and Use Committee and American Psychological Association ethical procedures.

1.2. Drug administration

Administration of either ISO or canola oil (vehicle control) via 18-gauge oral gavage needles (Kent Scientific, Torrington, CT) began five days prior to behavioral testing (PND 52). The rats were randomly assigned to one of four groups ($n=12$/group) according to the dose of ISO to be administered: 0, 5, 10, or 15 mg/kg/day. The dose range chosen for administration produces serum levels that correspond to humans taking the recommended doses and represent approximately 0–15× the human dose due to the differences in human and rat metabolism (Ferguson et al., 2006). The half-life in humans ranges from 6–36 h, while the half-life in rats is just over an hour (Chien et al., 1992, Ferguson et al., 2006).

Capsules of ISO (Accutane, Roche Pharmaceuticals, Nutley, NJ) were opened and prepared daily in canola oil at a volume of 3 mg/ml. In order to maintain the drug in solution, the drug vials were placed on a mechanical stirrer. Because ISO is light sensitive, all drug preparations were conducted in a darkened room and amber vials were used for drug storage. Drugs were administered once daily after testing procedures had been completed and prior to the rats' four-hour access to their daily food ration. Drug administration began on the last day of maze habituation (PND 52) and continued until testing was completed on PND 83. The experimenter performing the behavioral testing remained blind to the drug conditions and group assignments throughout testing.

1.2.1. Apparatus

The radial-arm maze was elevated 1.00 m from the floor and consisted of 12 wooden arms (73.75 cm long × 10.00 cm wide × 6.25 cm thick) with translucent Plexiglas walls on each side that stood 16.50 cm high and extended 36.80 cm down the length of the arm. At the end of each arm was a food well in which a Nestle® mini chocolate chip food reward could be placed. The arms extended from a circular arena that was 61.25 cm in diameter. Each arm was separated from the arena via a 25.00 cm guillotine-type Plexiglas door, which the experimenter used to control access to each arm. All doors were controlled via fishing line strung through a hole in the top of the door; the line then passed through a pulley hanging from the ceiling directly above the center of the maze. Each of the twelve lines was attached to its own lever on a panel in the corner of the room. The levers were used to individually control the opening and closing of each door.

The maze was located in the center of a dimly lit room and was surrounded by visual cues such as the experimenter and posters on the walls. In order to prevent odors from affecting performance, the maze was wiped with antibacterial spray between each animal's test session. During testing, white noise broadcasted from a stereo inside the room masked any outside noise.

1.2.2. Habituation

Beginning on PND 46, each rat was allowed to habituate to the RAM once a day for 7 consecutive days. During habituation, each rat was given free access to the center arena and to all six arms to be used during the experiment. Twelve chocolate chips were placed in the arena near the six arms that were to be used during the experiment. A session lasted until all of the chocolate chips had been eaten or until 5 min elapsed, whichever came first. Food rations were decreased by 1 g in rats that did not show motivation to eat the chocolate chips, as expressed by failure to eat half of the chips by Day 4 of habituation. Habituation ensured that all rats were familiarized to the maze and were motivated to eat all 12 chocolate chips. All rats successfully ate all twelve chips by the final day (Day 7) of habituation.

1.2.3. Discrimination tasks

Each rat was individually assigned to a set of six adjacent arms. Of the six arms, three were always baited and three were never baited. As depicted in Fig. 1, the relative locations of these arms were such that these six arms could later be grouped into three pairs of adjacent arms (A, B, and C) with opposing reward values (Stage 2 below). In order to control for possible side preferences, the reward contingencies were such that if A and B had the left arm baited, C would have the right arm

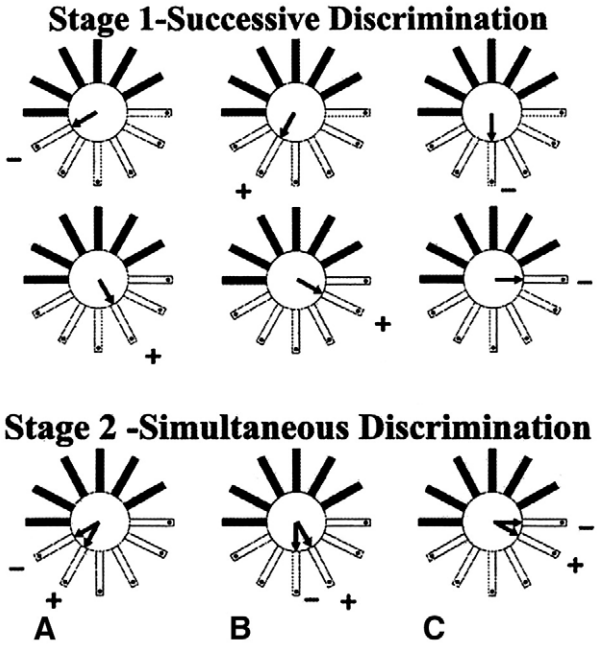

Fig. 1. Diagram of Stage 1 and Stage 2 of the radial-arm maze, error bars reflect standard error of the means (SEMs).

baited. The only parameter that changed from Stage 1 to Stage 2 was the manner in which the arms were presented to the rats: one at a time, or in pairs of two.

1.2.3.1. Stage 1—concurrent successive discrimination (go/no-go). After an animal completed habituation, it was evaluated on concurrent successive discrimination performance. This stage, conducted from PND 57 through PND 76 (20 sessions), assessed implicit memory based on stimulus–response relationships and the storage of those memories. In order for the animals to acquire the reward contingencies associated with each arm (baited or non-baited), each rat was tested in a series of trials in which they were confronted with only one of the six arms open at a time. For each of these go/no-go trials, the door to this one arm was opened for a maximum of 60 s. If the rat failed to enter the arm during this time, the door was closed and the trial ended. If the rat entered the arm and reached the food well within the allotted 60 s, the door was closed as soon as it returned to the center arena. In all cases, the trial ended as soon as the door closed; the rat was removed from the apparatus and placed in a nearby holding cage for 15 s between trials, which constituted the inter-trial interval (ITI). During this time, the experimenter quickly wiped down each arm with antibacterial spray and prepared the maze for the succeeding trial. Each daily session consisted of every arm being opened two times in a random fashion for a total of 12 trials per session. After 20 days on Stage 1, rats had achieved a response criterion of 2.0 for the latency discrimination ratio for two consecutive days (see below) and advanced to Stage 2.

As defined below, three behaviors were recorded for analysis of Stage 1 performance: latency discrimination ratio, arm speed, and latency to respond at the beginning of a trial. The ability of rats to distinguish between baited and non-baited arms was evaluated by accuracy in a latency discrimination ratio, the ratio between the median response latency to enter the never-baited arms and the median response latency to enter the always-baited arms. While a ratio was not calculated, "arm speed", or the time elapsed between the moment when all four paws were in the arm and the moment the food well was reached, was recorded for both the never-baited arms and the always-baited arms. Similar to "arm speed", "response latency", or the time elapsed from the beginning of the trial (opening of the door) to the moment all four paws were in an arm, was recorded for both the never-baited arms and the always-baited arms. When animals did not enter an arm completely on a given trial, a time of 60 s (maximum time allotted) was recorded for the response latency and arm speed measures. Response latencies and arm speed enabled evaluation of the animals' behaviors before and after a choice was made, respectively, and whether ISO-exposed or control group rats performed the task more efficiently. These measures were evaluated as a function of whether baited or non-baited arms were chosen to indicate whether ISO exposure affected the ability to distinguish between the reward contingencies.

1.2.3.2. Stage 2—concurrent simultaneous (2-choice) discrimination. On PND 77, rats advanced to Stage 2 (concurrent simultaneous discrimination) and were tested on this stage for five days. Each trial consisted of the rat being confronted with access to two adjacent arms with opposing reward contingencies they had previously learned to discriminate individually. Each daily session consisted of 12 consecutive trials made up of alternate presentations of each of the three pairs in a pseudorandom sequence. Presentation of a pair involved opening the doors to both arms and allowing the rat to make a choice as to which arm to enter. A "choice" was recorded when the rat had placed all four paws in one of the two arms. This choice triggered the closure of the door to the alternate arm. A "correct response" was recorded when the rat chose to enter the baited arm as opposed to the non-baited arm. The trial was completed when the rat returned to the center arena of the radial-arm maze after having made three choices. At this time, the rat was removed from the center arena and placed in a nearby holding cage for 15 s as the experimenter prepared the RAM for the next trial, constituting the ITI.

Similar to Stage 1, three behaviors were recorded for analysis of Stage 2 performance: percent correct, arm speed, and latency to respond at the beginning of a trial. "Percent correct" served as a measure of the accuracy with which animals chose a baited, rather than non-baited arm when forced to make a response on each of the five days on this stage. By focusing on the capacity of explicit memory to support inferential use of memories in novel situations, this measure allowed for evaluation of whether ISO exposure alters the ability to form complex, relational associations among items previously experienced separately. "Arm speed" was recorded to enable evaluation of the animals' behaviors after all four paws were in the arm and was considered an index of motivation as well as an indication that the animals were learning the task. Median "response latencies" were evaluated as a secondary measure of whether ISO exposure affects rats' ability to distinguish between reward contingencies. Presumably, if a rat had difficulty distinguishing between a baited and unbaited arm, it would take longer to choose an arm than a rat that could easily distinguish the reward contingencies.

1.3. Open field task

1.3.1. Apparatus

Open field testing took place in a gray wooden enclosure (48×48×8 in.) placed in the middle of a dimly lit room. The floor of the apparatus consisted of black 8″×8″ grids. During testing, white noise broadcasted from a stereo inside the closed room masked any outside noise.

1.3.2. Behavioral testing procedures

On PND 82, all 48 animals were tested once in the open field task to evaluate overall activity level. Each rat was placed in the center of the testing arena and timed for 5 min while two experimenters manually counted the number of grid crossings made by the animal during the testing period. A crossing was defined as placing all four paws into a neighboring square. Crossings of the grids along the perimeter of the open field were considered "Outside Crossings" while crossings that occurred throughout the rest of the open field were considered "Center Crossings". A ratio of Outside Crossings to Center Crossings was analyzed as an assessment of whether ISO affected anxiety. Animals higher in anxiety were expected to spend more time near the outside walls of the enclosure than in the center of the field when compared to animals lower in anxiety. The mean of the two experimenters' totals was used as each rat's score on the open field task for subsequent data analyses. Between each animal's test session, the apparatus was wiped down with antibacterial spray to remove odors in the box.

1.4. Elevated plus maze

1.4.1. Apparatus

The elevated plus maze was constructed of wood and consisted of two open arms (50 cm long×10 cm wide) arranged perpendicular to two enclosed arms (50 cm long×10 cm wide×40 cm tall). The maze was elevated 50 cm from the floor and placed in the middle of a dimly lit room. During testing, white noise broadcasted from a stereo inside the closed room masked any outside noise.

1.4.2. Behavioral testing procedures

On PND 83, all 48 animals were tested once in the elevated plus maze task. During this task, each rat was placed into the center of the maze, alternately facing an open or a closed arm (balanced across treatments), for 5 min while two experimenters manually counted the number of arm entries (an animal placing all four paws in an arm) and the amount of time spent in the open arms compared to closed arms.

The measurements recorded by both experimenters were averaged for each rat for use in subsequent data analyses. Between each animal's test session, the apparatus was wiped down with antibacterial spray to remove odors. A ratio of "Closed Arm Entries" to "Open Arm Entries" and "Percentage of Time in Open Arms" was analyzed in order to assess whether ISO affected anxiety. Animals higher in anxiety were expected to enter the closed arms more frequently and to spend more time in closed arms than in open arms when compared to animals lower in anxiety.

1.5. Radial-arm maze follow-up test

After the above tests were completed, all drug administration procedures were discontinued. After a 30-day drug-free period, rats were retested on Stages 1 and 2 of the RAM in order to investigate any long-term effects of ISO. Thus, on PND 114, all rats began a 2-day retention test on Stage 1 of the RAM discrimination task. During Stage 1 retesting, each arm was opened two times in a random fashion for a total of 12 trials per day. The Stage 1 retest procedures and feeding schedule were identical to those used previously. On PND 116, all rats began a 2-day retention test on Stage 2 of the RAM discrimination task. During Stage 2 retesting, each daily session consisted of 12 trials made up of alternate presentations of one of the three pairs of arms in a random sequence. These Stage 2 retest procedures and feeding schedule were also identical to those used previously.

1.6. Radial-arm maze: general statistical procedures

Analysis of variance models were used to analyze both RAM tasks. The independent variables were DRUG, BLOCK (Stage 1, described below), DAY (Stage 2), and REINFORCEMENT. "DRUG" was treated as a between subject variable with four levels (0, 5, 10, and 15 mg/kg/day of ISO). BLOCK and DAY were treated as within-subjects variables in order to determine how performance changed due to repetition of the task. "REINFORCEMENT" (whether an arm was baited or unbaited) was also a within-subjects variable and was used to ascertain whether the presence of a reward would enhance performance differently across treatment groups. Analyses of the three dependent measures for both Stage 1 and Stage 2 [accuracy (i.e., "latency discrimination ratio" in Stage 1; "percent correct" in Stage 2), response latency, and arm speed] included both main effects and interaction effects. For Stage 1, the latency discrimination ratio was calculated using the ratio $[(E-)-(E+)]/[(E-)+(E+)]$ for each 5-day block of 12 trials. $E-$ represents the median latency to enter the non-baited arms while $E+$ represents the median latency to enter the baited arms. This same formula was also used to analyze the 2 days of Stage 1 retest. For Stage 2, percent correct was calculated by dividing the number of correct responses by the number of trials per session (12 trials); this value was then multiplied by 100. Also, for each rat, the median arm speed and median response latency for the baited and non-baited arms for each daily session were recorded for analyses. Analyses were run using SPSS for Windows version 11.0. Fisher's PLSD test, with $\alpha=0.05$, was used for all pairwise comparisons.

2. Results

2.1. Body weights

A repeated measures ANOVA was conducted comparing body weights prior to the first day of drug administration (PND 55) and the final day of Stage 2 RAM testing (PND 84) to determine if food restriction or drug administration differentially influenced weight gain across drug groups. No significant differences were found [$F(3, 44)=1.32$, $p=0.28$].

2.2. Radial-arm maze: Stage 1

For each of the following analyses, data from Stage 1 were divided into four blocks with five sessions in each block (20 sessions total) in order to reduce session-by-session variability and to normalize the distributions. The value for each block represents the mean of the five daily sessions included in that block.

2.2.1. Latency discrimination ratio

Performance under each of the three doses of ISO was compared to Controls (see Table 1A). DRUG had no significant effect on the ability to learn the discrimination task [$F(3, 44)=2.33$, $p=0.10$]. A significant main effect of BLOCK [$F(3, 132)=20.91$, $p<0.01$] indicated that overall ability to discriminate improved significantly across all blocks of sessions for all groups. No DRUG×BLOCK interaction [$F(9, 132)=1.18$, $p=0.31$] was found.

2.2.2. Response latencies (RL)

There was no main effect of DRUG [$F(3, 44)=0.59$, $p=0.62$] on RL; however, as expected with learning of the task, a significant REINFORCEMENT×BLOCK interaction [$F(3, 42)=8.77$, $p<0.01$] revealed that RL on the baited arms decreased across blocks of

Table 1
Stage 1 radial-arm maze data across blocks and treatment groups

	Block 1	Block 2	Block 3	Block 4
A. Discrimination ratio Stage 1				
0 mg/kg	1.17(±0.15)	3.46(±0.99)	4.28(±0.71)	5.60(±1.26)
5 mg/kg	1.74(±0.22)	2.37(±0.42)	3.70(±0.37)	4.25(±0.61)
10 mg/kg	1.46(±0.35)	1.58(±0.15)	3.17(±0.89)	2.83(±0.48)
15 mg/kg	1.55(±0.24)	1.89(±0.44)	4.03(±0.92)	3.42(±0.62)
B. Response latency Stage 1 (unbaited/baited)				
0 mg/kg	8.87/9.16(±1.84/±1.68)	5.91/2.82(±1.06/±0.35)	8.30/2.55(±1.83/±0.20)	9.64/2.17(±2.45/±0.24)
5 mg/kg	9.77/7.69(±2.32/±1.88)	4.99/2.76(±0.68/±0.35)	7.06/2.18(±1.93/±0.37)	7.51/1.99(±1.68/±0.22)
10 mg/kg	8.04/9.24(±2.26/±2.75)	2.97/2.23(±0.32/±0.21)	6.38/2.88(±1.79/±0.98)	3.75/1.53(±0.63/±0.13)
15 mg/kg	10.93/10.96(±3.99/±4.70)	6.07/4.91(±2.14/±2.16)	8.91/3.64(±1.94/±0.99)	6.07/2.17(±1.16/±0.51)
C. Speed Stage 1 (unbaited/baited)				
0 mg/kg	6.20/4.91(±2.21/±1.61)	2.67/1.03(±1.10/±0.19)	2.63/1.00(±0.71/±0.00)	11.30/1.00(±4.18/±0.00)
5 mg/kg	7.08/5.03(±2.05/±1.53)	1.67/1.08(±0.24/±0.04)	3.92/1.00(±1.14/±0.01)	8.46/1.00(±2.60/±0.00)
10 mg/kg	6.18/6.76(±2.68/±2.49)	1.82/1.03(±0.27/±0.03)	7.42/2.50(±1.59/±1.06)	9.63/1.03(±2.22/±0.03)
15 mg/kg	8.63/9.74(±4.76/±4.89)	6.37/3.68(±2.24/±2.62)	6.75/2.14(±1.97/±0.66)	10.56/1.61(±3.06/±0.61)

Each panel represents data for each treatment group across blocks of trials (5 days/block). In Panel A, data represent mean discrimination ratio data and depict a similar progression of performance between treatment groups. Panel B, data represent mean latency to enter the arms (unbaited/baited). In panel C, data represent mean time to travel down the arms (unbaited/baited).

sessions; whereas, RL on the unbaited arms did not change reliably across blocks (see Table 1B). Tests of simple effects revealed that the effect of REINFORCEMENT on RL was not significant in BLOCK 1 ($p=0.86$); however, the effect was significant in BLOCKS 2, 3, and 4, (p's<0.01). No DRUG×BLOCK interaction [$F(9, 102.37)=0.67$, $p=0.73$] was found. A marginally significant DRUG×REINFORCEMENT interaction [$F(3, 44)=2.56$, $p=0.07$] was found, but no significant post hoc comparisons were found. The three-way interaction was not significant [$F<1$].

2.2.3. Arm speed

An analysis across all blocks found no significant main effect of DRUG [$F(3, 44)=0.89$, $p=0.46$] or interaction effects with DRUG on arm speed; however, a significant REINFORCEMENT×BLOCK interaction [$F(3, 42)=9.56$, $p<0.01$] indicated that speed on the baited arms decreased (i.e., rats reached the food well faster) across blocks, but speed on the unbaited arms did not change significantly across blocks (see Table 1B). Tests of simple effects revealed that the effect of reinforcement on arm speed was not significant in BLOCK 1 ($p=0.47$); however, the effect was significant in BLOCKS 2, 3, and 4, (p's<0.01).

2.3. Stage 2

2.3.1. Percent correct

As Fig. 2 depicts, a main effect of DRUG on percent correct [$F(3, 44)=3.54$, $p=0.02$] was found, with post hoc comparisons revealing that Controls performed significantly better than animals exposed to 10 mg/kg ($p=0.02$) and marginally better than animals exposed to 15 mg/kg ($p=0.08$). A main effect of DAY [$F(4, 41)=2.61$, $p=0.05$] occurred; however, post hoc tests revealed this was solely due to improved performance on DAY 4 of this task ($p<0.01$). No DRUG×DAY interaction [$F(12, 108.77)=0.39$, $p=0.96$] was found.

2.3.2. Response latencies

There was no main effect of DRUG on response latencies [$F(3, 44)=2.16$, $p=0.11$]. A main effect of DAY [$F(4, 41)=5.04$, $p<0.01$] indicated that mean response latencies were longer (animals were slower to respond) on DAY 2 ($M=8.00$ s, SEM=0.96) than on DAY 1 ($M=4.88$ s, SEM=0.90) and DAY 3 ($M=4.35$ s, SEM=0.54). DAY 4 ($M=5.24$ s, SEM=0.52) and DAY 5 ($M=5.86$ s, SEM=1.33) did not differ from any other days. No DRUG×DAY interaction [$F(12, 108.77)=1.16$, $p=0.32$] was found.

2.3.3. Arm speed

No main effect of DRUG [$F(3, 44)=1.17$, $p=0.33$] was found for arm speed. A significant main effect of DAY [$F(4, 41)=4.45$, $p<0.01$] occurred due to slower performance on DAY 2 of this task [mean arm speed (s): Day1=5.47, Day 2=9.80, Day 3=5.34, Day 4=5.10, Day 5=6.06], but no DRUG×DAY interaction [$F(12, 108.77)=0.422$, $p=0.95$] was found.

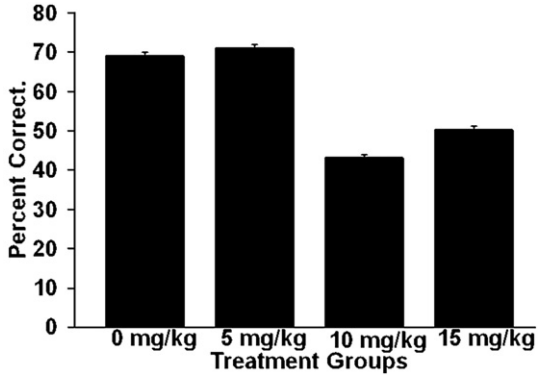

Fig. 2. Mean percent correct for Stage 2 for each DRUG group ($n=12$/group), error bars reflect standard error of the means (SEMs).

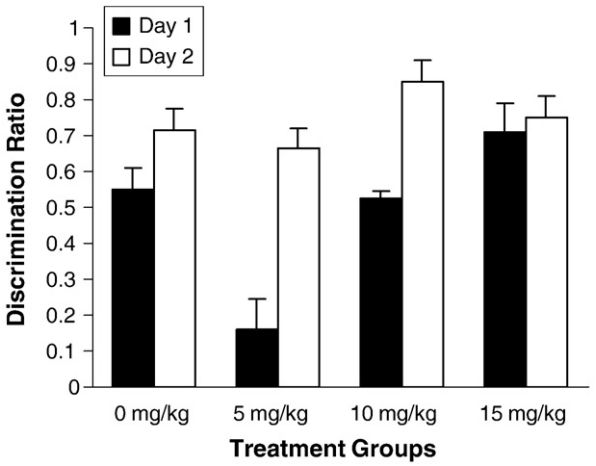

Fig. 3. Median latency discrimination ratios for Stage 1 retest ($n=12$/group) for each DRUG group on each DAY of testing, error bars reflect standard error of the means (SEMs).

2.4. Open field task

One-way ANOVAs were conducted to examine the effect of DRUG on open field performance. No significant effects of DRUG were found on the number of total crossings [$F(3, 44)=0.668$, $p=0.58$] or the ratio of outside crossings to center crossings [$F(3, 44)=1.11$, $p=0.36$]. Thus, exposure to ISO did not appear to affect overall activity level or anxiety.

2.5. Elevated plus maze

A one-way ANOVA was run on the elevated plus maze data to evaluate the effect of DRUG dose. There were no DRUG effects on the total number of arm entries [$F(3, 44)=0.25$, $p=0.86$], the ratio of open arm entries to closed arm entries [$F(3, 44)=1.00$, $p=0.41$], or the amount of time spent in the open arms [$F(3, 44)=0.26$, $p=0.85$]. These results, along with those from the open field task, indicate that ISO does not appear to alter activity or anxiety levels.

2.6. Radial-arm maze: retest Stage 1

2.6.1. Latency discrimination ratio

As depicted in Fig. 3, a DRUG×DAY interaction was found [$F(3, 44)=2.99$, $p<0.05$]. Post hoc tests indicated that animals exposed to 5 mg/kg were less able to discriminate between the baited and unbaited arms than those exposed to 15 mg/kg, 10 mg/kg, and Controls on Day 1 (all p's<0.05). None of the groups differed on Day 2 of the retest.

2.6.2. Response latencies

As depicted in Fig. 4, a significant DRUG×REINFORCEMENT interaction [$F(3, 44)=4.97$, $p=0.01$] indicated group differences in latency to enter unbaited arms [$F(3, 44)=4.64$, $p=0.01$] during the Stage 1 retest. Post hoc tests revealed that the Control and 5 mg/kg groups were faster to respond to the unbaited arms than animals that received either 10 mg/kg ($p=0.02$) or 15 mg/kg ($p=0.03$) doses of ISO.

2.6.3. Arm speed

There were no significant DRUG-related effects on arm speed. A REINFORCEMENT×DAY interaction [$F(1, 44)=20.76$, $p<0.01$] indicated that speed in the baited arms increased on the second day while speed in the unbaited arms decreased on the second day. These arm speed

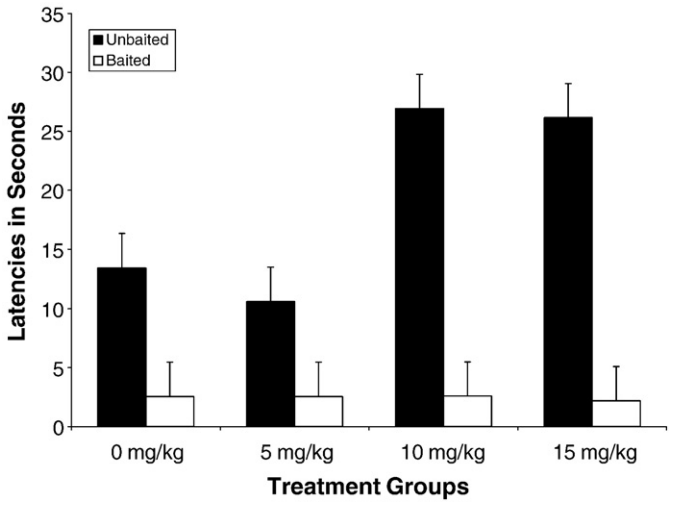

Fig. 4. Mean response latencies (in seconds) for Stage 1 retest for both unbaited and baited arms for each DRUG group (n=12/group), error bars reflect standard error of the means (SEMs).

findings suggest that the animals became reoriented to the task demands of this stage as retesting progressed.

2.7. Radial-arm maze: retest Stage 2

2.7.1. Percent correct

A main effect of DAY [$F(1, 44)=6.58$, $p=0.01$] showed that percent correct decreased between DAY 1 (Mean=87.15%, SEM=2.38%) and DAY 2 (Mean=80.03%, SEM=2.10%); but, there were no significant DRUG or DRUG×DAY effects on percent correct. Across both days, the percent correct for the 0 mg/kg group was 81.95%, SEM=4.53; the percent correct for the 5 mg/kg group was 78.48%, SEM=6.19; the percent correct for the 10 mg/kg group was 89.24%, SEM=2.35; the percent correct for the 15 mg/kg group was 84.73%, SEM=2.49. When comparing these percentages with the final day of Stage 2, these numbers reflect a 12.75% improvement for the 0 mg/kg group, a 7.28% improvement for the 5 mg/kg group, a 46.04% improvement for the 10 mg/kg group, and a 34.33% improvement for the 15 mg/kg group between Stage 2 and Stage 2 retest.

2.7.2. Response latencies

There were no significant DRUG or DRUG×DAY effects on response latencies; however, a main effect of DAY [$F(1, 44)=5.57$, $p=0.02$] indicated that response latencies increased between DAY 1 (M=6.32 s, SEM=0.89) and DAY 2 (M=9.13 s, SEM=1.35).

2.7.3. Arm speed

There were no significant DRUG or DRUG×DAY effects on arm speed; however, a main effect of DAY [$F(1, 41)=6.89$, $p=0.01$] on arm speed indicated that rats became increasingly slower across the two days of testing (DAY 1 M=5.72 s, SEM=0.90; DAY 2 M=9.30 s, SEM=1.48).

3. General discussion

Although ISO is known to severely disrupt brain development, few studies have examined the drug's effects on the adult brain. The present study sought to examine the behavioral effects of ISO exposure during adulthood by testing the performance of rats in tasks designed to measure implicit and explicit memory, reaction time, motivation, exploratory activity, and anxiety. These particular behaviors were chosen, in part, because retinoid receptors have been shown to be present in high densities in structures thought to control these behaviors (e.g., striatum and hippocampus). Moreover, previous studies have shown that altering retinoid activity reduced hippocampal cell proliferation and suppressed midbrain and striatal DA signaling (Crandall et al., 2004; Krezel et al., 1998). These earlier findings led us to predict that ISO would affect explicit memory (due to effects on hippocampal DA receptors), and implicit memory and activity levels (perhaps due to striatal effects). In the current study, explicit forms of memory were found to be impaired with concurrent ISO exposure. However, there was no evidence of ISO effects on implicit memory, motivation, reaction time (response latencies), activity, or anxiety.

The simultaneous, two-choice discrimination impairment found in Stage 2 of the radial-arm task was an anticipated finding based on previous studies demonstrating a role for retinoic acid (RA) receptors in hippocampal functioning (Crandall et al., 2004; Etchamendy et al., 2001, 2003; Misner et al., 2001). This second stage of the RAM task requires the animal to use previously-acquired information in a novel manner that necessitates the use of relational representations (here: the simultaneous presentation of two arms, each with previously-acquired valences, requiring an explicit choice). This second stage assessed explicit memory by requiring the animal to use information acquired in Stage 1 flexibly in a novel (2-choice) situation. The flexible use of information in this manner is, arguably, a form of "explicit memory" and has been shown to depend on the functional integrity of the hippocampus and related temporal lobe structures (Marighetto et al., 1999; Marighetto et al., 2000; Eichenbaum et al., 1992).

A plausible explanation for the explicit memory deficit in Stage 2 is the decline in adult hippocampal neurogenesis and cell proliferation shown to be induced by ISO exposure (Crandall et al., 2004). Several studies have demonstrated that manipulation of RA levels (either depletion or supplementation) suppresses hippocampal neurogenesis (Crandall et al., 2004; Misner et al., 2001). Given that neurogenesis has been correlated with learning/memory performance (Gould et al., 1999), one would expect to see impairment of hippocampal-dependent tasks following ISO exposure. Another piece of supporting evidence comes from studies of hippocampal LTP/LTD. Although observed with RA deficiency instead of acute ISO treatment, the fact that manipulation of RA has been shown to impair hippocampal LTP/LTD suggests that ISO may interfere with RA's normal role supporting synaptic effectiveness (see Misner et al., 2001), and therefore interfere with hippocampal LTP/LTD processes involved in learning and memory. Lastly, multiple neurotransmitter systems thought to support hippocampal function (e.g., DA, ACh, GABA, glutamate) have been shown to be dependent on RA availability and RA receptor integrity (for review, see Lane and Bailey, 2005). Thus, adult exposure to ISO can be expected to affect any or all of these neurochemical systems.

Although the results from Stage 1 of the RAM task indicated that ISO did not significantly interfere with the initial learning of the baited/unbaited arm discrimination, the response latency and discrimination ratio data from the 30-day retention test ("Stage 1 retest") implied that animals given either of the two highest doses of ISO (10 mg/kg or 15 mg/kg) showed enhanced retention of the earlier learning. The response latency data were particularly notable: while no ISO-related effects on response latencies were seen during the initial Stage 1 testing, at Stage 1 retest both the Control and 5 mg/kg groups were faster to respond on the unbaited (incorrect) arms than animals exposed to 10 or 15 mg/kg ISO. Thus, the Control and 5 mg/kg groups were less proficient at discriminating "unbaited" from "baited" arms. Essentially, the opposite pattern of effects occurred for Stage 2 of the task. The treatment effects seen in the initial Stage 2 testing were no longer present in the Stage 2 retention test (Stage 2 retest), suggesting that there were no long-term effects on explicit memory processes in rats previously exposed to ISO. Notably, during the Stage 2 retest, performance in the 10 mg/kg and 15 mg/kg groups had risen to the levels of Controls, thus suggesting that the Stage 2 deficit was

perhaps more of a performance/retrieval deficit than storage problem. Taken together, the radial-arm maze retention tests show that there were no apparent long-term negative effects of ISO thirty days after discontinuation of the drug.

While the underlying mechanism remains unclear, it seems that ISO improved the long-term retention of the implicit/procedural discrimination task. The dose-dependent facilitation of long-term implicit memory observed with the 30-day retention test suggests that even though the drug did not significantly affect performance in the short term, the cessation of treatment resulted in enhanced long-term accessibility to this information. This finding was certainly unexpected, and should be replicated. One potential explanation is that if the hippocampal, explicit memory system was unable to effectively solve the simultaneous 2-choice task, then the basal ganglia system would continue to strengthen its control over behavior, and thus, reinforce the already well-established tendency to approach the task as a go/no-go (S-R) situation (i.e., "over-learning" of Stage 1 may occur). That is, the difficulty these rats had using the individual arm information to perform the choice/relational Stage 2 task may have been partly due to a tendency to continue responding in an implicit, S-R mode (i.e., making go/no-go responses to individual arms) instead of using the individual arm information to make relational (choice) representations. Thus, the five sessions of Stage 2 testing may have functioned as continued S-R training for these animals. Support for this interpretation comes from several studies indicating that the basal ganglia and hippocampal memory systems often compete or interfere with one another. Furthermore, when one of these systems is unable to provide an adequate solution in a situation involving novel information or task demands, the other system tends to dominate control of behavior (Packard and Knowlton, 2002; Sherry and Schacter, 1987). Regardless of the mechanism behind this effect, it was an unexpected finding that requires replication before further inferences can be drawn.

Another question raised by the retest results, especially in light of the discussion in the previous paragraph, is: why wasn't there an ISO effect on the retest of Stage 2? The impairment observed in Stage 2 when animals were undergoing treatment with ISO was no longer observed when they were retested after thirty days without the drug. This finding suggests that the drug effects observed earlier were differences in performance/expression of learning that occurred due to the ISO exposure. In other words, once the drug was removed and animals were retested they were apparently then able to express learning that they did not display when initially tested. Alternatively, the lack of differences in the Stage 2 retest could result from differences in forgetting between groups across the thirty day retention interval. However, it is notable that the overall performance on Day 1 of the Stage 2 retest was actually better (87%) than the final day of the initial Stage 2 test (53%). More specifically, the 0 mg/kg group showed a 12.75% improvement; the 5 mg/kg group, a 7.28% improvement; the 10 mg/kg group a 46.04% improvement; and the 15 mg/kg group, a 34.33% improvement between Stage 2 and Stage 2 retest. A case could be made that the cessation of oral gavage treatments prior to the retest may have played a part in the overall (across groups) improvement in performance; however, such an explanation does not elucidate why groups given the two highest doses of ISO showed such a dramatic improvement between Stage 2 and Stage 2 retest.

The measures of activity and anxiety (open field and plus maze) were included in this study to investigate potential alterations in exploratory activity or anxiety associated with ISO exposure. The lack of differences in arm speed and response latencies (reaction times) in the RAM indicates that ISO-treated rats were not impaired with regard to motor abilities, motivation, or perceptual processes. Although it is possible that these behaviors were unaffected due to limitations in our model (e.g., length of drug exposure, drug doses chosen, or task sensitivity) these explanations seem unlikely because the length of exposure and drug doses were sufficient to reveal significant learning and memory effects. Moreover, by the time animals were tested in the open field and plus maze tasks they had been exposed to the ISO much longer (35+ days) than when initially tested in the RAM. With regard to the drug doses, those used in the present study (5, 10, and 15 mg/kg) were overlapping in range with those used in the Ferguson et al. (2005) study (7.5 and 22.5 mg/kg) in which they based their doses on research indicating that 7.5 mg/kg produces serum levels of ISO comparable to those of human *Accutane* users (Ferguson et al., 2005, 2006). Notably, similar to the present study, Ferguson et al. (2005), also found little evidence for ISO-induced effects using the open field task.

Overall the findings from this study indicate that adult exposure to ISO impairs explicit/relational memory, while possibly enhancing long-term retrieval of implicit/procedural memory. Learning and memory effects due to RA system alterations appear to be very robust in the literature. Manipulations of this system through genetic knockouts, pharmacological means, or aging all produce learning and memory effects (Chiang et al., 1998; Crandall et al., 2004; Misner et al., 2001; Etchamendy et al., 2001; however, also see Ferguson and Berry, 2007). However, the direction of these learning and memory effects seems to depend on the animal's current level of cognitive facility. For instance, in animals with impaired memory, whether due to aging, experimentally-induced amnesia, or vitamin A deficiency, supplementation with ISO tends to *improve* performance. Similarly, ISO has also been demonstrated to have a neuroprotective effect in Alzheimer's disease models (Ono et al., 2004; Sahin et al., 2005). However, when healthy, adult animals are exposed to ISO it tends to cause learning and memory *impairments*, as seen in the present study and in Crandall et al. (2004). Thus, it may be appropriate to hypothesize that ISO produces a classic inverted-U dose curve with regard to cognitive effects. Further research will be needed to corroborate this potential effect and to try to delineate the specific CNS levels of ISO needed to optimize cognitive functioning in order to more fully understand the dual micronutrient/neurotoxicant role of RA and its precursor vitamin A.

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