Title

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ORIGINAL LABORATORY RESEARCH REPORT

A Spatial Memory Deficit in Male But Not Female Rats After Neonatal Diazepam Exposure: A New Model for Developmental Sedative Neurotoxicity

Gregory A. Chinn, MD, PhD, Marcus H. Cummins, BS, and Jeffrey W. Sall, PhD, MD

BACKGROUND: Developmental anesthetic neurotoxicity is well described in animal models for GABAergic, sedating drugs. Here we investigate the role of the benzodiazepine, diazepam on spatial and recognition memory of young adult rats after neonatal exposure.
METHODS: On postnatal day 7, male (n = 30) and female (n = 30) rats were exposed to diazepam (30 mg/kg intraperitoneally) or vehicle. On postnatal day 42, animals started a series of behavioral tests including Barnes maze (spatial memory), object recognition battery (recognition memory), and open field and elevated plus maze (anxiety). In a separate cohort, blood gases were obtained from diazepam-exposed animals and compared to isoflurane-exposed animals (1 MAC for 4 hours).
RESULTS: Male animals exposed to diazepam had impaired performance in the Barnes maze and were unable to differentiate the goal quadrant from chance (1-sample *t* test; $t_{diazepam/male}$ (14) = 1.49, $P = .158$). Female rats exposed to diazepam performed the same as the vehicle controls ($t_{diazepam/female}$ (12) = 3.4, $P = .005$, $t_{vehicle/female}$ (14) = 3.62, $P = .003$, $t_{vehicle/male}$ (13) = 4.76, $P < .001$). There were no statistical differences in either males or females in measures of recognition memory, anxiety, or locomotor activity in other behavioral tests. Physiologic measurements of arterial blood gases taken from animals under sedation with diazepam were much less aberrant than those exposed to the volatile anesthetic isoflurane by *t* test ($pH_{diazepam}$ [M = 7.56, standard deviation {SD} = 0.11] versus $pH_{Isoflurane}$ [M = 7.15, SD = 0.02], $t(10) = 8.93$, $P < .001$; $Paco_{2diazepam}$ [M = 32.8 mm Hg, SD = 10.1] versus $Paco_{2Isoflurane}$ [M = 91.8 mm Hg, SD = 5.8], $t(10) = 8.93$, $P < .001$).
CONCLUSIONS: The spatial memory results are consistent with volatile anesthetic suggesting a model in which development of the GABA system plays a critical role in determining susceptibility to behavioral deficits. (Anesth Analg 2023;XXX:00–00)

KEY POINTS

- **Question:** What is the effect of neonatal diazepam exposure on spatial memory in adulthood by sex in rats?
- **Findings:** Male rats are susceptible to a spatial memory deficit while female rats are spared.
- **Meaning:** Sex differences in susceptibility to early-life benzodiazepine exposure are the same as a volatile anesthetic exposure previously reported and suggest a common GABAergic mechanism between the 2 exposures.

GLOSSARY

ABG = arterial blood gas; **ANOVA** = analysis of variance; **ARRIVE** = Animal Research: Reporting of In Vivo Experiments; **F_{IO_2}** = fraction of inspired oxygen; **GABA** = gamma aminobutyric acid; **IP** = intraperitoneal; **M** = mean; **MAC** = minimum alveolar concentration; **ns** = not significant; **P** = postnatal; **SD** = standard deviation; **W** = sum of signed ranks

Early-life exposure to GABAergic drugs can cause deleterious effects on neurodevelopment in preclinical models.[1,2] There currently is a debate about the clinical significance in humans and the extent of a potential problem given the results of several human studies.[3–6] However, there are now

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over 20 years of consistent data in animals, as diverse as rodents to nonhuman primates, that exposure to commonly used anesthetic drugs can cause deficits in adulthood in learning and memory.[7–10] These data raise the possibility that exposure to GABAergic agents during sensitive windows in development may cause neurocognitive changes, even if they are difficult to detect clinically.

We have approached this phenomenon as a developmental toxicology model and have used it to study sex dimorphisms in brain development. In our studies of neonatal rats, we have found that there is a window of susceptibility that is dependent on age, sex, and GABA excitation.[11–16] Male rats are susceptible to a spatial memory deficit after isoflurane exposure on postnatal day 7(P7) while female rats with the same exposure are spared. However, earlier exposure of female rats at P4 show vulnerability to isoflurane, and later exposure of male rats at P15 shows a loss of susceptibility. This effect is androgen mediated as blocking testosterone in male rats will also protect males from toxicity.[13] Similarly, exogenous testosterone given to female rats will cause them to be susceptible like males at P7.[12]

This susceptibility is dictated by the development of the GABAergic system. In the mature mammalian brain, gamma aminobutyric acid (GABA) is understood to be an inhibitory neurotransmitter, but in early neurodevelopment, the effect of GABA can be excitatory. This is due to the transmembrane chloride gradient of the neuron which dictates the flow of chloride when the GABAa receptor is activated. In the mature state, the chloride gradient is low intracellularly, due to the activity and expression of a potassium-chloride cotransporter KCC2 (SLC12A5) which transports chloride out of the cell, and the net effect of GABAa activation is a negative presynaptic potential. In early development, there is a different potassium-chloride cotransporter that is dominantly expressed that brings potassium intracellularly, NKCC1 (SLC12A2). The net effect of high intracellular chloride is GABAa activation can cause an excitatory (depolarizing) presynaptic potential.[17] There is a sex-specific difference in GABA development[18] which corresponds to our observed differences in susceptibility to anesthetic neurotoxicity.[11,16] We have shown that the protein levels of these cotransporters are also androgen sensitive with higher levels of the immature cotransporter (NKCC1) in males compared to females.[11] NKCC1 protein is also higher in females treated with exogenous testosterone compared to vehicle-treated females.[12] Conversely, males treated with the androgen blocker flutamide had higher levels of the mature transporter KCC2 in the brain.[12,13] Finally, pharmacologically blocking the effect of NKCC1 on males at the time of exposure (with bumetanide) also resulted in protection from developing a spatial memory deficit.[13]

These studies have been informative in establishing a model for understanding sex differences and timing of developmental anesthetic neurotoxicity. However, they have at least 2 limitations. One is the specificity of isoflurane is very low. Like all volatile anesthetics, it is known to have many sites of action, in addition to GABA agonism.[19,20] Isoflurane as a volatile anesthetic also causes significant respiratory depression during the exposure which can lead to physiologic aberrations and even death. While we have gone to significant lengths to show that the deficit we see is not caused by hypercarbia or hypoxia, the inability to individually control respiration and oxygenation makes this a model with limited human comparison.[21]

To address these issues, we have studied a new model of GABAergic developmental toxicity, by using the benzodiazepine, diazepam. Diazepam's site of action is well characterized on the GABAa receptor.[22] Diazepam also has an added benefit in that it has less respiratory depression compared to other powerful sedatives such as volatile anesthetics, barbiturates, and opioids.[23] Finally, it is a clinically relevant medication, routinely given to young children for anxiolysis or for sedation. Here we test whether the GABAergic effects of diazepam on postnatal day 7 rats will cause a spatial memory deficit in females or males.

METHODS

Animals

Protocols were approved by the University of California, San Francisco Institutional Animal Care and Use Committee, and all experiments were conducted in accordance with ARRIVE guidelines. Sprague Dawley rats were ordered from Charles River (South San Francisco, CA) and arrived on P2. Animals arrived as a same-sex litter of 10 with a foster dam. Pups were weaned at P21 and group housed with littermates. Animals were exposed to a reverse light-dark cycle (8 AM–8 PM) to allow for behavior testing during the animals' natural active phase (dark phase). They were allowed food and water ad libitum. Cages contained a single red plastic tube for enrichment.

Diazepam Exposure

Diazepam (Cayman, Ann Arbor, MI) was diluted in an aqueous vehicle used by compounding pharmacies.[24] Animals were injected intraperitoneally with a dose of 30 mg/kg. This dose was chosen based on a small pilot study in which we found the length of sedation was around 6 hours, which was the amount of time we had previously exposed animals to isoflurane.[11–13] Others have modeled the pharmacokinetics of a single intraperitoneal (IP) injection at 30 mg/kg of diazepam and found a rapid uptake in both serum and cerebrospinal fluid in rats.[25] Because the animals

were all approximately 18 g, a 5.4 mg/mL stock was made fresh and 0.1 mL of the stock (or vehicle) was injected with a 28 g needle. The injection site was quickly covered with a small amount of Vetbond (3M, St. Paul, MN) to prevent leakage. Diazepam-injected animals were monitored for 5 hours with body temperatures recorded every 15 minutes and regulated with a heated pad (Thermo Haake, Waltham, MA; Supplemental Digital Content 1, Supplemental Figure 1, http://links.lww.com/AA/E427), exposed to room air. Vehicle-injected animals were placed in the monitoring chamber at the beginning of the exposure but were returned to their respective dams after 15 minutes to minimize the effects of maternal deprivation which could disproportionally affect the nonsedated animals and influence future behavior experiments.[26]

Arterial Blood Gas

For the physiologic studies of diazepam exposure compared to isoflurane, exposures were conducted on separate days using the same volatile anesthesia exposure chamber.[11,13] The carrier gas was humidified oxygen and air (F_{IO_2} 0.5). CO_2 absorber pellets were placed in the chamber (Litholyme; Allied Healthcare, St. Louis, MO). A Datex-Ohmeda gas analyzer was used to monitor isoflurane, CO_2, and oxygen. All animals were P7 male rats for these experiments. The isoflurane group was subjected to 1 MAC of isoflurane for 4 hours using a down-titrated protocol.[15] The diazepam group was exposed to 30 mg/kg IP injection with ABGs collected at 2 hours (by 4 hours, sedation had noticeably waned as the animals were moving significantly more, so collection at 2 hours was chosen to model the animal's point of deepest sedation). Terminal blood gases were obtained via cardiac puncture in the exposure chamber by use of glove box. Samples were run on ABL 90 machine (Radiometer, Copenhagen, Denmark).

Behavior

Barnes Maze. On postnatal day 41, animals were exposed to the Barnes maze apparatus and trained to identify the location of the escape box over the course of 4 consecutive days as previously described.[11,27] Cues were placed on the walls of the testing room. The apparatus was cleaned with 70% ethanol between each trial, which lasted 4 minutes or until the animals found the escape box. If the animals did not find the location of the goal within the allotted time, then the animal was gently directed to the target hole. Latency and movement were tracked by a camera (Basler aca1280; Basler Inc, Exton, PA) and tracking software (Ethovision XT 11.5; Noldus Information Technology, Inc, Leesburg, VA). After 1-week delay from the last day of training, a probe trial was conducted in which the escape box was removed, and the animals' movements were tracked for 90 seconds. The order of testing was randomized for the first trial, then kept consistent throughout all rounds of testing. The location of the escape box around the maze was pseudorandomized to give equal distribution of the goals around the maze to each experimental group. Observers were blinded during behavior testing and data analysis.

Elevated Plus Maze. This test was performed using standard protocol to assess anxiety.[28] Apparatus consists of a plus-shaped platform elevated off the ground. Two opposite arms of the maze bounded by walls and the other 2 arms are open. The animal is placed in the center and allowed to freely explore over the course of 5 minutes. Animal movement was tracked by camera and tracking software.

Open Field. Animals were placed in a box with a 61 cm square base and 50 cm wall height. Two identical testing boxes were used simultaneously with a single camera used to track movements. Animals were allowed to freely explore for 4 minutes. Time spent in the inner portion of the box was compared to the time spent around the outer perimeter.

Recognition Memory. Recognition memory tests were performed as previously described.[7,11] Using the same apparatus as the open field, animals were introduced to a pair of objects in the exposure for 4 minutes. The animals were removed for 2 minutes during which time the objects were rearranged or changed. The animals were reintroduced to the arena and tracked for 4 minutes. Two different recognition memory scenarios were tested. The Novel Object scenario has 2 identical objects for the exposure, and a new object replacing one of the familiar objects in the test phase. The Object Place scenario has 2 different objects in the exposure, and the replacement of one of the objects with an object that is identical to the other (familiar object). The novel object is this newly introduced object that is in a novel position.

Statistics

Prism 9 (GraphPad, San Diego, CA) was used for statistical analysis and graph making. A power analysis was conducted a priori based on the Barnes maze results from isoflurane-treated animals in previous experiments[13,27] using G*Power 3.1 software (University of Dusseldorf, Germany). The calculated effect size ranged from 0.7 to 0.9 with a difference in means ranging from 17 to 25 and standard deviation from 24 to 27. With an α of .05 and a power of 0.8, we calculated a sample size ranging from 10 to 19 animals per group for this specific behavior (1-sample *t* test). This is consistent with the range of prior behavior

cohort sizes we have used in the past[12,13,27] and we picked n = 15 per group as a manageable cohort size for feasibility of completing tasks, realizing that this was the best estimate to capture the effects of a new treatment.

Data were subjected to Shapiro-Wilk normality testing which determined whether a parametric or nonparametric hypothesis testing statistic was used. Blood gas mean values (pH, Pao_2, $Paco_2$) were compared between groups (isoflurane versus diazepam) by unpaired *t* test. A mixed-effects model was applied to the Barnes Maze learning data to compare the effects of day of training, sex, treatment, and their interactions. Two-way analysis of variance (ANOVA) was performed for the open field and elevated plus maze to test for an effect by sex, treatment, and interaction. Additionally, 1-sample *t* tests were performed for the probe trial of the Barnes maze to test if the proportion of time spent in the goal quadrant was greater than chance (25%). In the object recognition trials, *t* tests (or Wilcoxon signed rank) were performed for each treatment group comparing the time spent investigating the novel and familiar objects during the testing phase. The discrimination index (time investigating novel object – time investigating familiar object, divided by total time investigating both objects) was also calculated and a 1-sample *t* test (or Wilcoxon signed rank test) was performed to test whether the discrimination of the novel object was better than chance (theoretical value 0). Posthoc multicomparisons were made (Dunnett's multiple comparison using an α threshold of .05 for multiplicity adjusted *P* values) to compare diazepam/male to other groups in the open field and the elevated plus maze. Dunnett's multiple comparison was also used to compare the first day of training to the last day of training in the Barnes maze learning acquisition and animal weights to compare treatment versus vehicle separately for males and females per day (α of .05 for multiplicity-adjusted *P* values).

In the Barnes maze training, 3 animals never learned the position of the goal by day 4 of training, n = 1 from vehicle/male group and n = 2 from diazepam/female group. These were included in the acquisition phase analysis but eliminated from the probe trial. In the *Object Place* Recognition paradigm, a single diazepam/male animal was eliminated for not exploring either object (total exploration was less than 10 seconds).

RESULTS

The physiologic effects of intraperitoneal diazepam exposure are significantly different from isoflurane exposure. The mean arterial pH of animals anesthetized under 1 MAC of isoflurane for 4 hours was (M = 7.15, SD = 0.02) compared to (M = 7.56, SD = 0.11) in the diazepam exposure group (test *t*, $t(10) = 8.93$, $P < .001$) (Figure 1). This pH difference was driven in by the difference in respiratory drive and CO_2 retention (M = 91.8 mm Hg, SD = 5.8) for isoflurane versus (M = 32.8 mm Hg, SD = 10.1) for diazepam ($t(10) = 12.4$, $P < .001$). Fio_2 was adjusted to 50% for the isoflurane and diazepam groups and there was no statistical difference in Pao_2 ($M_{isoflurane}$ = 107 mm Hg, SD = 36.7 versus $M_{diazepam}$ = 113 mm Hg, SD = 26.4; $t(10) = 0.33$, $P = .750$).

To test the effects of early-life diazepam exposure on learning and behavior, a cohort of animals, male (n = 30) and female (n = 30) rats were exposed to diazepam (IP 30 mg/kg) or vehicle and monitored for 5 hours on P7. Animals lost righting reflex within 15 minutes of injection and while righting reflex returned within 2 to 5 hours, animals remained sedated with limited ambulation. However, respiratory drive was maintained, and they remained visibly pink. Temperature was tightly controlled and monitored (M = 36.8°C, SD = 0.19) (Supplemental Digital Content 1, Supplemental Figure 1A, http://links.lww.com/AA/E427).

After weaning at P21, animals started Barnes maze training on P41. In the learning phase, there was no statistical difference in latency to the escape box (goal) by sex (mixed-effects model [$F(1,56) = 1.30$, $P = .260$]) or diazepam ($F[1,56] = 0.06$, $P = .810$; Figure 2). There was a learning effect over the course of the training ($F[3168] = 25.24$, $P < .001$). Interestingly, there was also an interaction of effects of training and sex in the mixed-effects model ($F[3168] = 3.37$, $P = .020$). No other interactions were statistically significant (Supplemental Digital Content 2, Supplemental Table 1, http://links.lww.com/AA/E428). Similarly, a post hoc Dunnett's multiple comparison showed a difference in latency from day 1 to 4 in every group (vehicle/female $P < .001$, diazepam/female $P < .001$, vehicle/male $P = .010$ and diazepam/male $P = .033$). This is like previous isoflurane exposures where a deficit in learning the escape positions was not detected in treated animals.[11–13,27]

One week after the acquisition of the goal position, a 90-second probe trial was conducted by removing the escape box. In this trial, there was no statistical difference by 1-sample *t* test in the time spent within the goal quadrant for the diazepam/male compared to chance ($t(14) = 1.49$, $P = .158$). By contrast, the other groups including females exposed to diazepam were not affected and spent statistically more time at the goal compared to chance (vehicle/female $t(14) = 3.62$, $P = .003$, diazepam/female $t(12) = 3.4$, $P = .005$, vehicle/male $t(13) = 4.76$, $P < .001$; Figure 2B).

After the Barnes maze trials, these animals were subjected to recognition memory tests. In the *Novel Object* paradigm (Figure 3A–C), all the groups spent

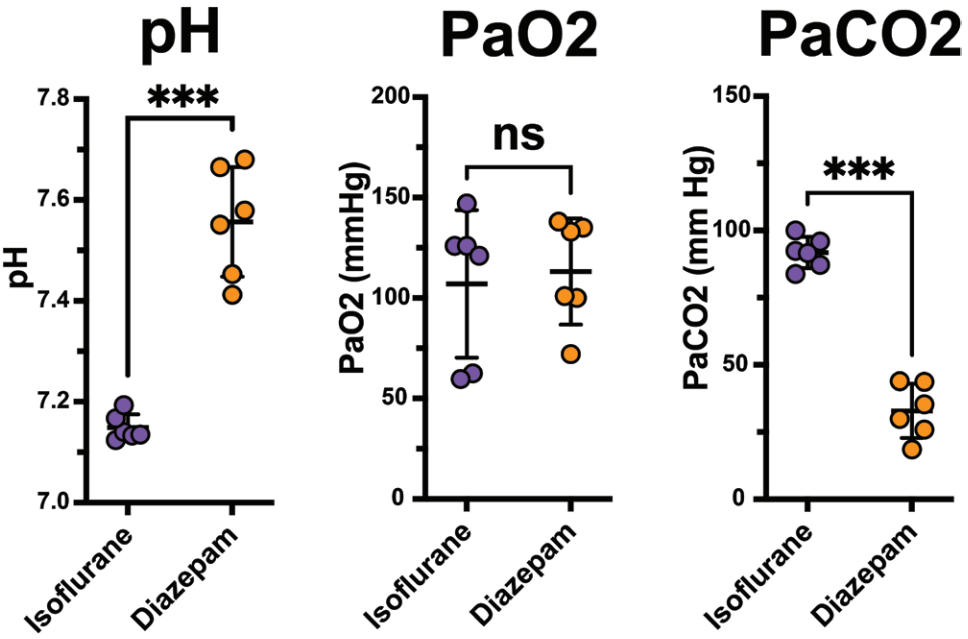

Figure 1. ABG values show a statistically significant difference in physiologic disturbance between sedation with isoflurane and diazepam in rats. ABGs obtained under exposure conditions (1 MAC of Isoflurane after 4 h, and diazepam injected intraperitoneally after 2 h; F_{IO_2} 0.5 for both exposures). The animals were significantly more acidotic in the isoflurane exposure compared to diazepam (mean 7.15 vs 7.56: *t* test, $t(10) = 8.93$, $P < .001$). This can be attributed to the clinically significant respiratory depression of isoflurane relative to diazepam as evidenced by the CO_2 retention in the isoflurane group (91.8 vs 32.8 mm Hg, $t(10) = 12.4$, $P < .001$). There was no statistical difference in Pa_{O_2} (107 vs 113 mm Hg, $t(10) = 0.33$ $P = .750$). Error bars = standard deviation. ***$P < .001$. ABG indicates arterial blood gas; F_{IO_2}, fraction of inspired oxygen; MAC, minimum alveolar concentration; ns, not significant.

more time with the novel object and performed above chance by discriminating above chance the novel object during the test, (discrimination index, Wilcoxon signed rank test-vehicle/female $W = 118$, $P < .001$, diazepam/female $W = 98$, $P < .001$, vehicle/male $W = 118$, $P = .003$, diazepam/male $W = 102$, $P = .002$.)

In a more challenging recognition task (Object Place recognition) (Figure 3D–F), all groups spent significantly more time with the novel object compared to the familiar except for the diazepam-treated males which did not reach significance by paired *t* test (vehicle/female $t(14) = 2.82$, $P = .014$, diazepam/female $t(14) = 3.12$, $P = .007$, vehicle/male $t(14) = 3.85$, $P = .002$, diazepam/male $t(13) = 2.08$, $P = .057$). However, analysis by discrimination index showed all groups performed better than chance including the diazepam/males (1-sample *t* test, vehicle/female $t(14) = 2.98$, $P = .010$, diazepam/female $t(14) = 3.47$, $P = .001$, vehicle/male $t(14) = 4.13$, $P = .004$, diazepam/male $t(13) = 2.26$, $P = .042$). These suggest that diazepam exposure in males may affect recognition memory, although the effect size is small.

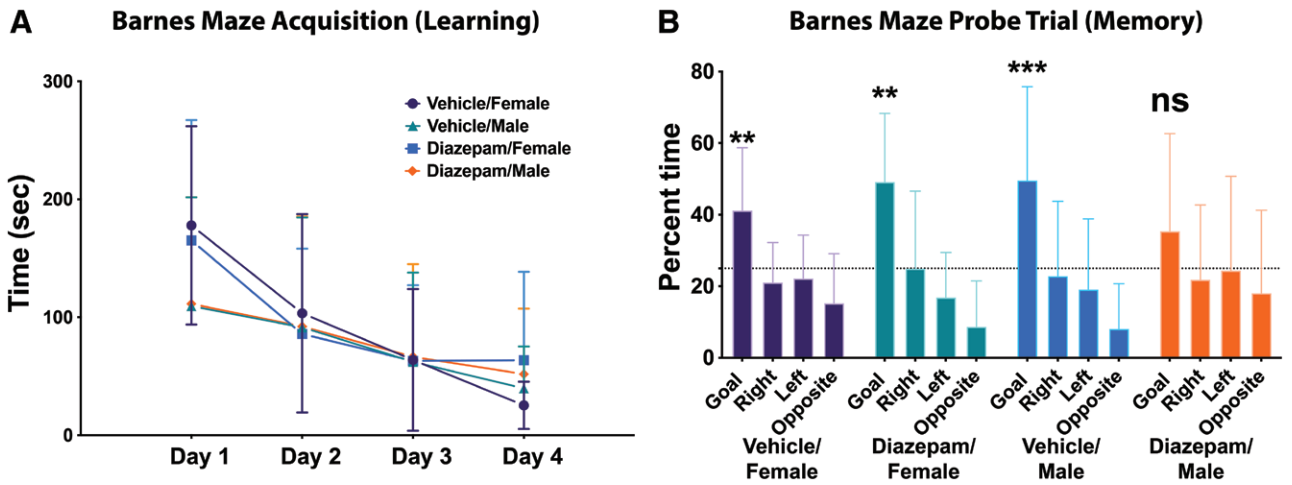

Figure 2. Spatial memory deficit in male rats but not female rats after diazepam exposure. A, Latency to escape in the Barnes maze was tracked over 4 consecutive days of training (n = 15 per group). There was no statistical difference by sex in a mixed-effects model ($F[1,56] = 1.30$, $P = .260$) or diazepam ($F[1,56] = 0.06$, $P = .810$). There was an effect of training ($F[3168] = 25.24$, $P < .001$). Post hoc Dunnett's multiple comparison showed a difference in latency from day 1 to 4 in every group (vehicle/female $P < .001$, diazepam/female $P < .001$, vehicle/male $P = .010$, and diazepam/male $P = .033$). B, The 90-s probe trial was performed 1 wk after the last day of learning. All groups except the diazepam/male group spent more time than chance in the goal quadrant (1-sample *t* test with theoretical mean 25%. vehicle/female $t(14) = 3.62$, $P = .003$, diazepam/female $t(12) = 3.4$, $P = .005$, vehicle/male $t(13) = 4.76$, $P < .001$ and diazepam/male $t(14) = 1.49$, $P = .158$). Error bars = standard deviation **$P < .010$, ***$P < .001$. ns indicates not significant.

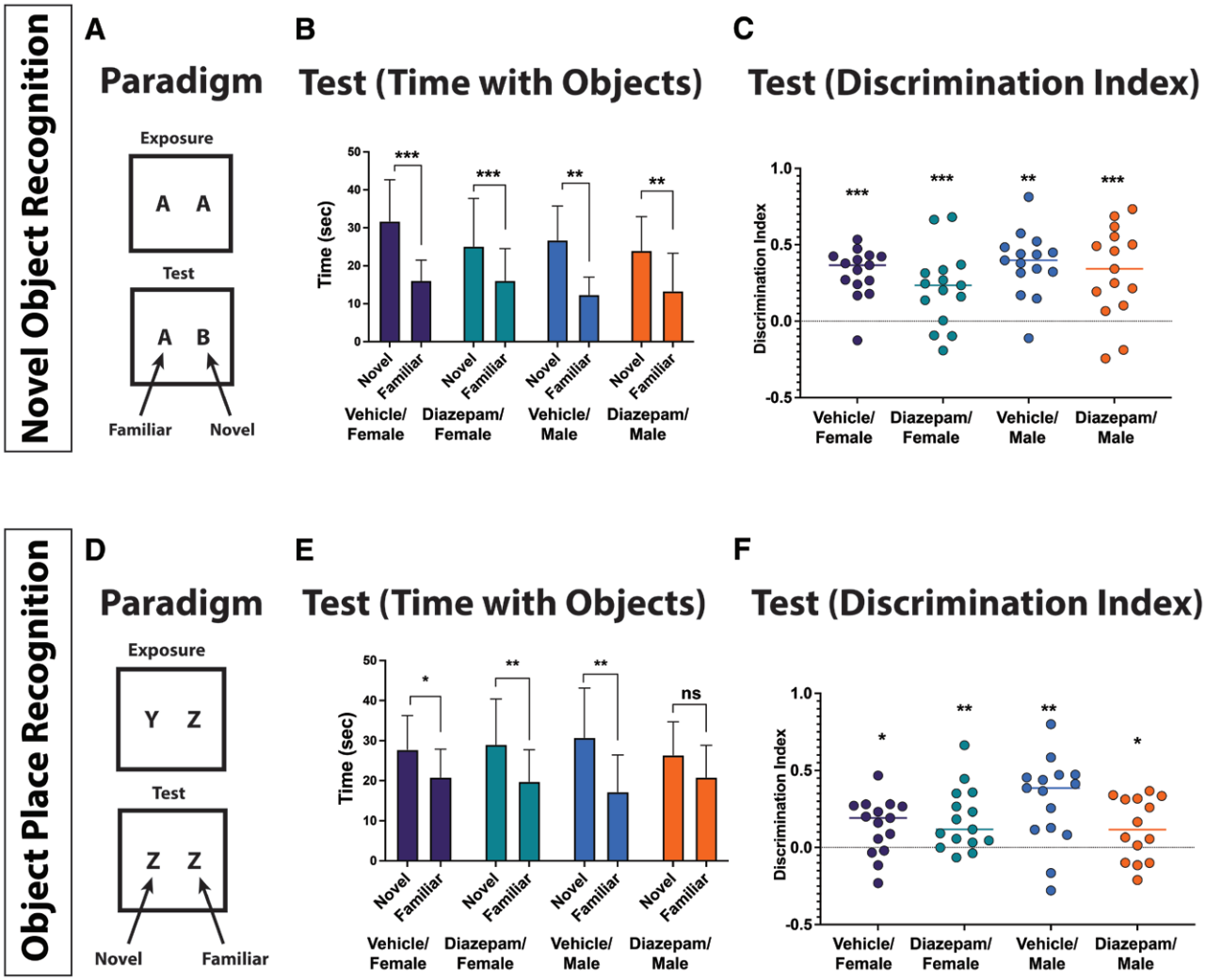

Figure 3. Object recognition may be affected in male rats treated with diazepam, but not females. A–C. Novel object recognition task was successfully accomplished in all treatment groups in both the comparison of the time spent with objects or the discrimination index; all spent significantly more time with the novel object over chance. D–F, Object Place recognition is a more challenging paradigm for animals to learn, and the diazepam/male group performed worse than the other groups as it did not spend significantly more time with the novel object when comparing times by *t* test, $t(13) = 2.08$, $P = .057$. This group did statistically spend more time than chance when measured by discrimination index, but *P* value was only slightly under .05 (1-sample *t* test, $t(13) = 2.26$, $P = .041$). Error bars = standard deviation. $*P < .050$, $**P < .010$, $***P < .001$.

Benzodiazepines can affect mood and anxiety if withdrawn or in the context of chronic use,[29,30] so we were interested in evaluating if there was an anxiety component to this model. In the open field test in which animals can freely explore an empty arena, there was no statistical difference in exploration between controls and treated animals in terms of the amount of time spent in the inner portion of the arena or the perimeter (Figure 4A–C). There was a statistically significant difference by sex, in the overall distance traveled ($F[1,56] = 13.83$, $P < .001$), but no statistical difference by treatment ($F[1,56] = 0.001$, $P = .97$) or interaction between sex and treatment ($F[1,56] = 0.52$, $P = .470$). Similarly, post hoc Sidak's multiple comparison showed no statistical difference between diazepam/males and vehicle/males ($t(56) = 0.54$, $P = .932$) but did find differences between both vehicle/females and diazepam/males ($t(56) = 3.14$, $P = .008$) and diazepam/females and diazepam/males ($t(56) = 2.65$, $P = .031$) in terms of total distance traveled.

In another test of anxiety, the elevated plus maze, animals showed no statistical difference in exploration between treated and untreated animals, and similarly preferred to spend most of the time in the protected limbs of the maze (Figure 4D–F). There was a statistically significant difference by sex in the total distance of exploration ($F[1,56] = 4.95$, $P = .030$), but it was not significant for treatment ($F[1,56] = 0.31$, $P = .579$) or the interaction of sex and treatment ($F[1,56] = 0.06$, $P = .081$). Post hoc Dunnett's multiple comparison showed no statistical difference in the distance

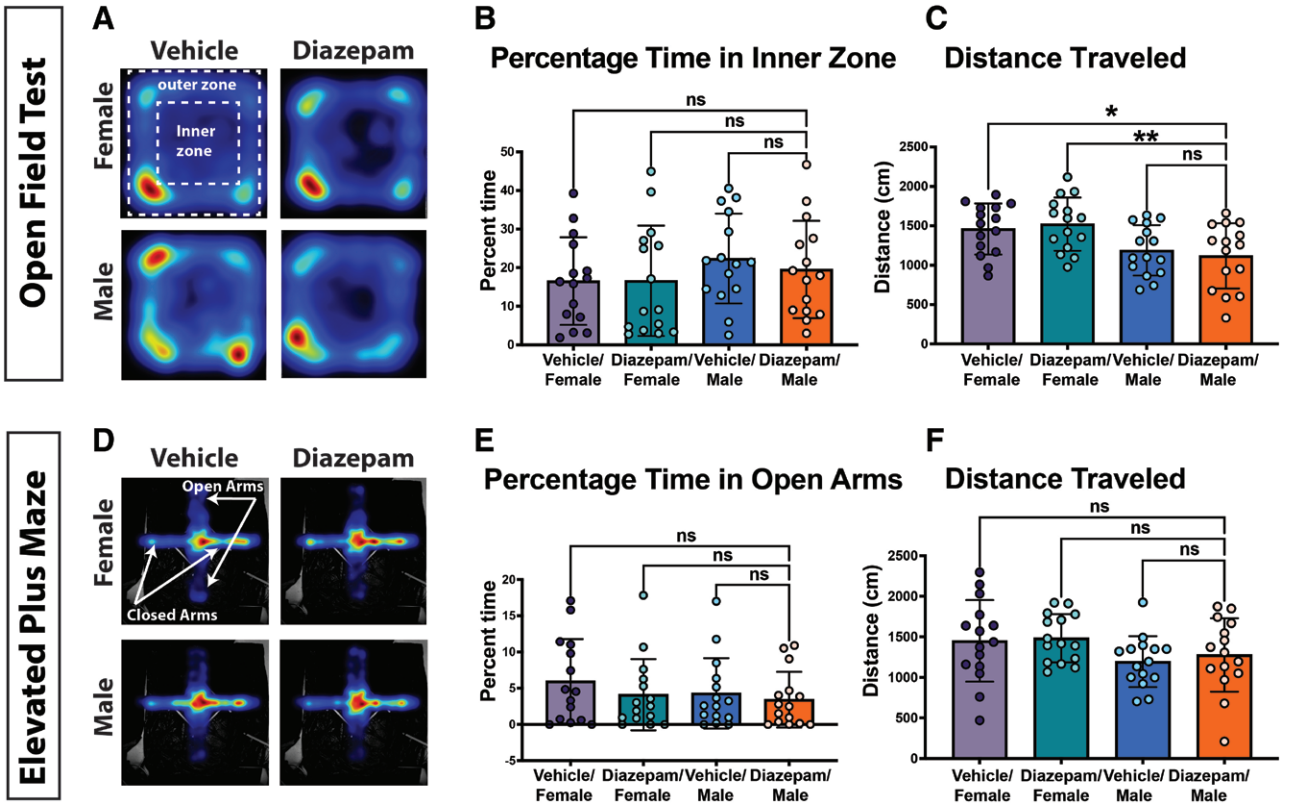

Figure 4. Measures of anxiety are not altered with early-life diazepam exposure. A, Open Field test averaged heat maps show the portion of time exploring the open field. B, There was no statistical difference by treatment of sex by 2-way ANOVA, or Dunn's multiple comparison of diazepam males to other treatment groups in the time spent in the inner zone. C, The total distance travel was significantly more by sex (F[1,56] = 13.83, P < .001) Sidak's multiple comparison also showed no statistical difference between males but did find differences between both female groups and the diazepam/male group. D, Average heat map of the elevated plus maze by group. E, Similar to the open field test, there was no statistical difference by sex or treatment by 2-way ANOVA of the time spent in the open arms. F, Similarly, there was also a statistically significant difference by sex in the exploration pattern with total distance traveled increased in females (F[1,56] = 4.953, P = .030), although Dunnett's multiple comparison showed no statistical difference from the diazepam/male group and other groups. Error bars = standard deviation. *P < .050, **P < .010. ANOVA indicates analysis of variance; ns, not significant.

explored between the diazepam/male group and other groups.

Finally, there were no statistically significant differences in animal weight between vehicle and diazepam exposure on P14, P20, P27, and P35 for males and females. However, on P148, there was a statistically significant difference by Dunnett's multiple comparison in both vehicle/males versus diazepam/males (q(280) = 7.83, P < .001) and vehicle/females versus diazepam/females (q(280) = 4.35, P = .012) between the control and treatment (Supplemental Digital Content 1, Supplemental Figure 1b, http://links.lww.com/AA/E427).

DISCUSSION

Diazepam, a stereotypic benzodiazepine, when administered in early life can cause a spatial memory deficit in early adulthood that is dependent on sex. This is a recapitulation of prior work using volatile anesthetic gas. While volatile anesthetics are potent GABAergic drugs, they also have many other targets which could account for this behavioral deficit. The results from these studies suggest that the mechanism of developmental anesthetic neurotoxicity with volatile anesthetics shares a similar mechanism with benzodiazepines, which have more specificity for the GABAergic system. This opens the door for more pathway-targeted experiments with this new model for studying this persistent problem.

Although the novel recognition behavior experiment did not completely recapitulate prior results with isoflurane, there were several similarities including all groups were able to discriminate the novel object.[11,13] The diazepam/male group in the more difficult recognition memory test, Object Place recognition, also performed worse than the other groups, reaching significance in the discrimination index, but not in time spent at novel object versus familiar object. In comparison, the other groups, including females exposed to diazepam have very statistically significant differences in discrimination index or time spent at novel with P < .001. In previous studies of early-life isoflurane exposure, males in the treatment group have a statistically significant deficit in this

domain. The fact that we did not see the same deficit with diazepam suggests that isoflurane may induce a more significant insult than diazepam within this memory domain.[11,13] Alternatively, these results could be reflective of non-GABAergic effects of isoflurane.

We have previously shown that the susceptibility to development of a spatial memory deficit corresponds to maturity of the potassium chloride cotransporters at the time of exposure. It is these transporters that determine the direction of the chloride gradient resulting in GABA agonism acting in an excitatory or inhibitory manner. Male rats at P7 have significantly higher levels of the immature potassium chloride cotransporter (NKCC1) compared to females of the same age and have lower levels of the mature transporter (KCC2).[13] We believe this accounts for the development of the spatial memory deficits in males but not females exposed to GABAergic agents on P7.

GABAergic agents are also potent regulators of the expression of potassium chloride cotransporters themselves. Exposure to volatile anesthetics increases expression of NKCC1 and decreases KCC2 in the brain within hours of exposure.[31–33] This functionally leads to developmentally altered GABA depolarization which may also have important consequences on normal neuronal development, including delaying dendrite and synapse maturation or GABA mediated neurogenesis[34,35] and may explain why short, repeated exposures are often more problematic than a single exposure.[36,37] Disruption of the development of the GABA system also has important implications for hyperexcitable neuronal states like epilepsy. Finally, the strong regulation of these transporters by sex steroids, the presumed basis for sex differences, has implications for developmental sex differences and potential treatments of males and females.[12,13,33,38,39]

While exogenous diazepam exposure could potentially influence adult anxiety levels, we did not observe statistically significant differences in treatment compared to control with either measure of anxiety-elevated plus maze or the open field test. Interestingly, these observational behaviors showed statistically significant differences in exploration by sex which others have observed as well.[40] Both treated and untreated males had less exploratory behaviors, relative to females. This is important to consider for studies that use a combination of male and female animals as matching group sizes and numbers is an important control for these measures.

Another benefit of this is the significant improvement in physiologic parameters compared to volatile anesthetic exposure. Given the limitations of volatile anesthetic delivery to small rodents in a controlled chamber, individual animals are not intubated, and ventilation is not controlled as in human anesthesia delivery. This results in respiratory depression which can cause significant hypercarbia and acidosis and can lead to death. It is common to have several mortalities in an exposure depending on the number of animals and length of exposure. By contrast, we did not have a single mortality during exposure in the diazepam cohorts. It is important to note that hypercarbia and hypoxia have been suggested to be the underlying cause of the observed deficits, but experiments on animals solely exposed to carbon dioxide found hypercarbia in isolation does not cause behavioral deficits.[21]

This study has several limitations. The studies reported are descriptive in nature describing a new model system for study of neonatal sedation and do not probe the mechanism of toxicity. While the pattern of sex differences in susceptibility are the same as we have previously reported in experiments using volatile anesthetic[11–13] and both drugs are putatively working through GABA agonism, we did not specifically test if our effect is due to this action. We also made an assumption in our ABG studies that the baseline blood gases of treated and untreated animals are the same. We were unable to do these studies because it would require a terminal blood draw by cardiac puncture in an unanesthetized animal which was beyond the limits of our approved protocol (although such data exist in the literature and one could assume that it would remain the same in untreated animals). We also cannot exclude a possibility that the slight pH increase (pH 7.56, respiratory alkalosis) during diazepam exposure may play a role in the development of the cognitive deficit, although the CO_2 is with normal limits (32.8 mm Hg). Finally, care should be taken with extrapolating results to humans as the dose and route of administration are optimized for a rodent model of GABA exposure, not clinical sedation.

This study establishes a rodent model to study early-life GABAergic neurotoxicity that is associated with less physiologic abnormalities than volatile anesthetic exposure. Given the widespread use of benzodiazepines in children for a variety of indications, from seizure disorder, to sedation, to anxiolysis, there remain questions regarding their long-term effects. While their "safety" profile with regards to respiratory depression is sometimes favored over opioids or intravenous anesthetics like (propofol), these studies and others raise the specter that they too may have deleterious clinical effects and deserve further study.

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DISCLOSURES

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