

Effect of CB1 Receptor Antagonist, AM251, on Plasma Corticosterone levels
and Anxious Behavior in Sprague-Dawley Rats

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1. Abstract

Anxiety disorders are the most common mental illness in the United States. Debilitating symptoms relating to anxiety disorders affect over 40 million American adults, representing over 18 percent of the United States population (ADAA). Research suggests that methods of acute cortisol elevation may serve as therapy for some anxiety disorders (Wisloska-Stanek et al., 2012). Intraperitoneal administration of AM251, a CB1 antagonist, has been shown to elevate plasma corticosterone levels in rodents, as well as elevate behavioral depression in mice (Ginsberg et al., 2010 and Steiner et al., 2007). These findings suggest that, in addition to clinical treatment of depression, CB1 receptor modulating drugs may also have a clinical use in the treatment of anxiety disorders. Few studies have investigated intracranial routes of administration, however those that do suggest that AM251 may not be effective alone in initiating HPA hyperactivity, but instead prevent HPA suppression elicited by CB1 agonist administration. These findings develop a level of uncertainty regarding the mechanism by which CB1 mediated suppression of the HPA axis works (Evanson et al., 2010). The aim of the current experiment was to investigate the role of cannabinoid receptor signaling in the regulation of the HPA axis through the use of the CB1 antagonist AM251 to selectively block the function of retrograde CB1 signaling in the hypothalamic PVN. This molecule possesses an inverse agonist relationship with the CB1 receptor making it an ideal antagonist for experimentation (Steiner et al., 2007). Intracranial injections of two different concentrations of AM251 were administered unilaterally to the hypothalamic PVN in Sprague-Dawley rats, and compared to a control group through plasma corticosterone testing and elevated plus-maze testing. It has been demonstrated that CB1 signaling is significantly involved in the fast feedback response to HPA activation via glucocorticoid signaling (Evanson et al., 2010). This signaling pathway results in suppression and excitation of the HPA axis with regard to CB1 agonists and antagonists, respectively (Ginsberg et al., 2010). Therefore, the hypothesis was that administration of the antagonist AM251 would increase plasma corticosterone levels in a dose-dependent relationship, such that

administration of a lower concentration of AM251 would increase time spent in open arms of an elevated plus maze and decrease other observable anxious behaviors compared to the control. Likewise, administration of a higher concentration of AM251 would increase time spent in enclosed arms of an elevated plus maze and increase other observable anxious behaviors compared to control. Other anxious behaviors to be noted included dips, entries (4 limbs), intermediate entries (2 limbs), and peeks. Results suggest no significant effect of drug concentration on plasma corticosterone levels, although marginal trends were observed for plasma corticosterone between groups. Results additionally suggest no significant effect of AM251 administration on anxious behavior, although high levels of behavioral anxiety were observed from all groups. This study presents several novelties. No study has investigated a dose-response relationship of intracranial AM251 administration to the hypothalamic PVN. Additionally, no study has examined intracranial AM251 administration to the PVN with regard to a behavioral test for anxiety. This study was the first examine both, and how a possible dose-response relationship correlates with anxious behavior.

2. Introduction

2.1 Stress and the HPA axis

The hypothalamo-pituitary-adreno (HPA) axis is understood to be sensitive to both physical and psychological stressors (Lovallo, 2005). Activation of parvocellular neurosecretory cells in the paraventricular nucleus (PVN) of the hypothalamus by a perceived stressor result in series of hormone secretions, which have been shown to have detrimental effects, both physiologically and psychologically (Riebe and Wotjak, 2011). Activation of the PVN results in a release of corticotrophin-releasing hormone (CRH) and argentine vasopressin (AVP), which then results in the activation of the anterior pituitary gland. Signaling in the anterior pituitary causes secretion of Adrenocorticotrophic hormone (ACTH) which then proceeds to initiate a hormonal response in the adrenal cortex, resulting in secretion of the glucocorticoid cortisol into general circulation with widespread consequences (Riebe and Wotjak, 2011).

Acute elevation of cortisol levels in humans and corticosterone in rodents accompanies sympathetic activation, promoting changes in cardiovascular function, which results in an increased awareness as a response to a stressor, known as the fight or flight response. However, chronic elevation of cortisol levels can have negative effects, such as cardiovascular complications, neuronal cell death, and depressive symptoms consistent with common affective disorders (Lovallo, 2005). Results from a recent study investigating the effects of variable systemic corticosterone levels on HPA regulation and behavior in rats suggest that lower doses of corticosterone enhanced the animal's ability to cope with different stressors through sympathetic activation. On the other hand, higher doses decreased the animal's ability to cope with environmental stressors and initiated symptoms of anxiety, as indicated by a longer duration of time spent in enclosed arms compared to open arms during plus maze testing (Catalani et al., 2011). These results suggest a significant relationship between HPA hormonal secretion and the

ability to manage stressors. Furthermore, these results also indicate a potential utility for cortisol modulating methods as a means to treat depressive symptoms and anxiety.

2.2 Endocannabinoids and Glucocorticoids

Cortisol secretion as a result of HPA activation, as well as other glucocorticoids are not only involved in sympathetic activation as a response to stressors, but are responsible for deactivation of the HPA axis through a fast feedback mechanism of glucocorticoid and endocannabinoid signaling (Ginsberg et al., 2010). Results from a study by Ginsberg and colleagues, comparing both agonism and antagonism of glucocorticoid receptors (GR's) involved in fast feedback suppression of the HPA axis further support this concept. Intraperitoneal (IP) administration of GR antagonist RU486, and cannabinoid receptor type 1 (CB1) antagonist AM251 (IP) in rats were shown to increase ACTH levels, whereas GR agonist RU28362 was shown to decrease ACTH secretion suggesting suppression of HPA activity. Another study by Evanson and colleagues (2010), investigating the relationship between intra-paraventricular nucleus (PVN) AM251 administration and GR fast feedback in rats via the glucocorticoid dexamethasone, convey similar findings. Results suggest that dexamethasone-mediated HPA suppression was reversed by administration of AM-251, although AM-251 administration alone had little effect. Furthermore, as a result of acute corticosterone secretion, a rapid increase in 2-arachydonyl glycerol (2-AG) levels, an endogenous cannabinoid, was observed through the use of restraint stress testing. This suggests that CB1 signaling is necessary for glucocorticoid mediated inhibition of the HPA axis to take place, as well as CB1 receptors being involved in the glucocorticoid fast feedback pathway, but leads to uncertainty regarding the mechanism by which CB1 mediated suppression works (Evanson et al., 2010).

CB1 receptors are primarily found in limbic brain regions such as the hippocampus and Amygdala, as well as the PVN, pituitary, and adrenal glands. The role of these structures in the regulation of emotion and stress suggests a significant role for the CB1 receptor in the regulation of these hormonal based

processes (Roche et al., 2007). In addition to excitation of the HPA axis via CB1 antagonism, the administration of CB1 agonists have been shown to inhibit HPA activity resulting in a decrease of cortisol secretion and downregulation of glucocorticoid receptors (Riebe et al., 2011). This further suggests significance for CB1 signaling in HPA regulation, as well as a possible role for CB1 modulation in the treatment of emotional disorders.

2.3 Characteristics of CB1 antagonist AM251

It has been demonstrated that CB1 signaling via the antagonist (or inverse agonist) AM251 can block glucocorticoid fast feedback of the HPA axis (Evanson et al., 2010). AM251 may also induce depressive behaviors through the same pathway (Steiner et al., 2007). Steiner and colleagues utilized Rimonibant (SR141716), a CB1 antagonist similar in structure to AM251, commonly used to treat eating disorders in humans, to mimic the effects of the genetic deactivation of CB1 receptors in mice. Both CB1 deactivation and pharmacological blockade of CB1 signaling induced depressive behaviors indicated by struggling during forced swim testing (FST), as well as increased blood corticosterone levels within an hour after SR141716 administration. However, after the administration of desipramine, a clinical antidepressant, no change in behavior was observed in CB1 deactivated mice compared to the rimonibant group, as indicated by FST. These results suggest a possible significance for selective CB1 antagonists in the treatment of depressive symptoms (Steiner et al., 2007).

AM251 has also been demonstrated to elevate blood corticosterone levels in rodents (Ginsberg et al., 2010). However recent evidence suggests that the effectiveness of AM251 to block fast feedback inhibition of the HPA axis, resulting in HPA hyperactivity, is a factor of both gender and time of administration (Atkinson et al., 2010). Results suggest that AM251 administration during the diurnal trough (9AM) and peak (5PM) prove more effective in elevating blood corticosterone levels than any other time of day. This effect is also sexually dimorphic, in that blood corticosterone levels in male rats

were significantly greater than females in response to acute AM251 administration (Atkinson et al., 2010). It has been suggested that methods of corticosterone modulation in rats may serve as therapy for anxiety disorders (Wisloska-Stanek et al., 2012). However, a specific relationship between CB1 modulation by AM251 administration and its effect on behavioral anxiety has not been thoroughly investigated.

AM251, as well as other CB1 antagonists may also play a significant role in metabolic activity and caloric intake (Chambers et al., 2004). Results from a study comparing AM251 and CB1 agonists, anandamide and methanandamide, on caloric intake indicate a significant reduction in caloric intake due to AM251 administration over a 6 day trial. Results from anandamide testing did not increase caloric intake as much as was expected, however methanandamide initiated a significant increase in caloric intake. These results suggest another possible role for AM251 in the clinical treatment of eating disorders (Chambers et al., 2004).

2.4 Validity of Elevated Plus-Maze testing

Elevated plus-maze testing is one of the most widely utilized anxiety tests in behavioral animal research (Hogg, 1996). The maze consists of two opposing enclosed arms and two non-enclosed opposing arms, where the animal is placed in the middle of the maze and left to freely explore. Final data are quantified as a record of the number of times the animal moves all four limbs into a given arm of the maze, as well as the duration of time spent in that arm. If the animal spends the majority of the time in closed arms of the maze, it is said that the animal is more anxious. Conversely, if more time is spent exploring the open arms of the maze relative to closed arms, the animal is less anxious. This observational record has been demonstrated to be a valid indicator of the anxiolytic effect of a given pharmaceutical as well as the natural characteristics of the strain of animal being tested (Hogg, 1996).

Elevated plus-maze testing has also been demonstrated to result in HPA activation (Appenrodt et al., 1999). Appenrodt and colleagues examined plasma ACTH levels in rats after exposure to an elevated plus-maze. Results suggest exposure to the maze significantly increased plasma ACTH levels, providing clear evidence of HPA activation (Appenrodt et al., 1999). These findings propose that the elevated plus-maze may serve as a suitable behavioral test for HPA experimentation.

3. Method

3.1 Animals

Adult male Sprague-Dawley rats (n=18) were utilized. Animals were housed separately and kept in a temperature and humidity controlled environment. Animals were allowed to adapt to their new surroundings 1 week prior to experimentation by daily handling to limit the possibility of confounding stressors. Animals were given *ad libitum* access to water and standard rat feed. Permission for experimental protocols and animal use was obtained through the Allegheny College Animal Research Committee (ARC).

3.2 Drugs

AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxamide; Sigma Aldrich) was suspended in vehicle (2%dimethyl sulfoxide and 98% physiological saline) and administered intra-PVN at a doses of 30 and 60 pmol (0.5 μ l). A Ketamine/Xylazine cocktail served as an anesthetic and was administered intraperitoneally (70mg-6mg/kg) prior to surgery. Euthosol solution was utilized for sacrifice and was administered intraperitoneally (1.0ml) prior to perfusion. Post surgery, Terramycin was administered orally in peanut butter as a prophylactic.

3.3 Cannula Surgeries

Rats were weighed then anesthetized with ketamine/xylazine cocktail (IP: 70mg-6mg/kg). After confirmation that the animal was anesthetized, the animal's head was shaved and it was placed in the stereotax. A midline incision was made and periosteum was scraped to lateral sides of the skull. After aligning cannula with PVN stereotaxic coordinates, one main hole was drilled in the skull for cannula placement, as well as additional holes for jewelers screws. The cannula tip was then inserted into the brain and advanced to required depth, then fixed to the skull using cranial cement. After cement hardened, the stereotaxic arm was raised, a cap and stylus was placed in the cannula opening, and the incision was sutured using square knot sutures. The animal was then returned to an individual cage with food, water, and antibiotics (Terramycin). Each animal was then allowed a 1 week recovery period.

3.4 Elevated plus maze

Cap and Stylus was removed and each animal was individually administered its respective dose through a micro-syringe. Thirty minutes after injection, all animals were examined through a plus maze to test anxiety (43.5 in. X 43.5 in.). Anxiety was assessed using the amount of time spent in the open arms of the maze. Specifically, more time in, or entries into open arms of the maze indicated that the animal was less anxious compared to more time in the enclosed arms of the maze. The apparatus consisted of four arms, each at a 90 degree angle from the other. Two of the arms were enclosed with high walls without ceilings, positioned 180 degrees apart from each other. Crossing the two enclosed arms were the open arms. Rats were removed from their cages and taken individually to the testing room. During each trial, each rat was placed in the center of the maze facing one of the open arms, and released to explore freely for 10 minutes. The amount of time spent in each arm and the number of times it entered each arm were recorded. Entry into an arm was defined by all four limbs crossing over into the arm. Other behavioral observations were also recorded including dips (nose lowered below the

floor of the maze), peeks (nose poke into any arm of the maze), and intermediate entries and exits (2 limbs crossing into an arm of the maze).

3.5 Perfusion and Histology

After administration of Euthosol solution (IP, 1.0ml), a mediolateral-oriented incision was made through the lining of the peritoneal cavity. A cut was then made below the sternum, through the diaphragm, to expose the rib cage. Cuts were then made through the lateral ribcage to expose the chest cavity, followed by the pinning of the sternum to the lower jaw with a towel clamp. After clearing any fatty tissue around the heart blood samples were taken from an incision made to the right atrium, followed by perfusion of formal saline into the left ventricle. After 3-5 minutes or clear evidence of rigidity, perfusate flow was stopped and the animal decapitated. The skull was removed with rongeurs and brains postfixed in formal saline for histological examination. Cannula placement was then identified under a microscope by buffered thionin nissl stain.

3.6 Enzyme Immunoassay

Blood samples were taken directly from the right atrium after sacrifice and before perfusion. Samples were collected into chilled tubes (100 μ L) and stored at -28°C. Samples were thawed and centrifuged after which plasma corticosterone was measured using an ELISA corticosterone immunoassay kit for rats (Enzo life sciences).

3.7 Statistics

Dose-response of AM251 concentration was examined by regression analysis and one way between subjects ANOVA. Main effects of experimental group on open time, closed time, total entries, closed entries, open entries, nose dips, nose peeks, and intermediate entries and exits were examined

by one way between subjects ANOVA. Interaction between closed entries and open entries was examined by one way within subjects ANOVA. All statistical data was processed by SPSS (IBM).

4. Results

4.1 Cannula Placement

Criteria for correct cannula placement included identification of the cannula track within .5mm proximity of the PVN without severe damage to the structure. The hit rate for microsyringe injections to the PVN was 13/18(72%). Only animals with properly placed cannula were included in the analyses of data in this study.

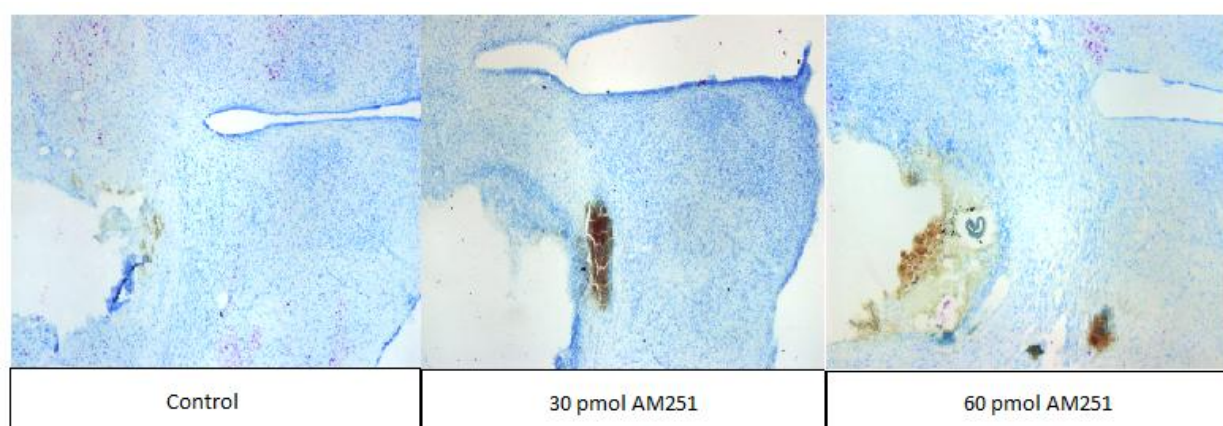


Figure 4.1: Images from histological identification of cannula placement. This is a depiction of a successful cannula placement from each experimental group. Cannula tracts were identified with buffered thionin nissl stain. All images are oriented in the same direction; dorsal (left side) ventral (right side) .For additional images see Appendix A.

4.2 ELISA immunoassay

After analysis of blood samples, ELISA assay results revealed that AM251 did not have any significant effect on plasma corticosterone levels. $F(2,10)=0.704$, $p=0.517$, $\eta^2=.123$. Although insignificant, there was an observed trend from differences in marginal means acquired from post tests

such that plasma corticosterone decreased with an increasing concentration of AM251 ($p > 0.05$ for all comparisons). For additional figures on corticosterone measures, refer to Appendix B.

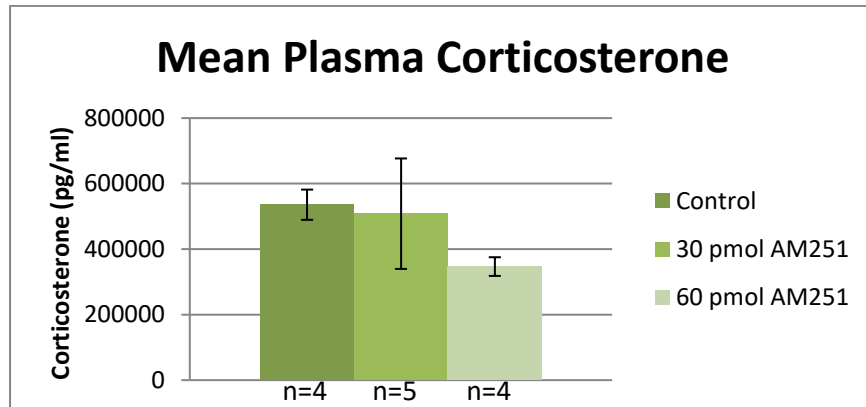


Figure 2: Mean Plasma Corticosterone by group. This is a depiction of all corticosterone values from ELISA assay, analyzed based on a standard curve of predetermined corticosterone concentrations. Plasma corticosterone values did decrease with an increasing concentration of AM251 but with inconsistent variability, depicted by error bars representing standard error. Corticosterone values are notated in pg/ml.

4.3 Plus Maze testing

Results from behavioral testing suggest that AM251 did not have any significant effect on time spent in open arms, $F(2,10)=2.333$, $p=0.147$, $\eta^2=0.318$, as well as observed time spent in closed arms, $F(2,10)=2.333$, $p=0.147$, $\eta^2=0.318$. Concentration of AM251 did not have a significant effect on open entries, $F(2,10)=1.646$, $p=0.241$, $\eta^2=0.248$, closed entries, $F(2,10)=0.425$, $p=0.665$, $\eta^2=0.078$, total entries, $F(2,10)=0.608$, $p=0.563$, $\eta^2=0.108$, nose dips, $F(2,10)=1.216$, $p=0.337$, $\eta^2=.196$, nose peeks, $F(2,10)=0.146$, $p=0.866$, $\eta^2=0.028$, or intermediate entries and exits, $F(2,10)=0.229$, $p=0.799$, $\eta^2=0.044$. There was a significant difference between open entries ($M=1.54$, $SD=2.145$) and closed entries ($M=4.15$, $SD=4.964$) for all subjects, $F(1,10)=6.617$, $p=0.028$, $\eta^2=0.398$. (For additional figures refer to Appendix C)

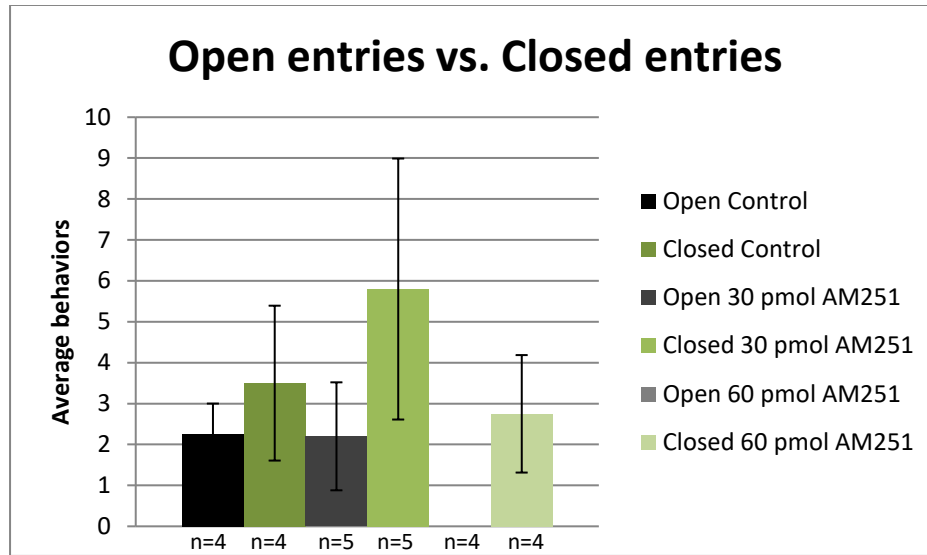


Figure 3: Mean closed and open entries. This is a depiction of average open entries by group side by side with average closed entries by group. Closed entries observed were significantly greater for all experimental groups compared to open entries for all experimental groups.

5. Discussion

This study serves to demonstrate an importance for CB1 signaling in the regulation of systems of stress response. AM251 did not have a significant effect on the concentration of circulating plasma corticosterone; results which have been previously demonstrated through similar studies (Evanson et al., 2010). However, an insignificant trend in marginal means suggests that increasing the concentration of AM251 elicited a decrease in plasma corticosterone ($p > 0.05$). These findings are novel from other research suggesting the blockade of fast feedback by AM251 administered to the PVN (Evanson et al., 2010). It seems that administration of the CB1 antagonist to the PVN decreases HPA activity, by promoting fast feedback of the HPA axis.

The hypothalamus has been demonstrated to have far reaching efferent projections to other limbic structures (McLaughlin et al., 2007). The function of these structures in the stress response has additionally been demonstrated to be altered by CB1 receptor modulation (Riebe and Wotjak, 2011).

Given that the role and physical nature of the endocannabinoid system in the management of stress and emotion is not yet fully understood, possible systemic effects of CB1 antagonism in the PVN may serve to provide an explanation to findings from the current study. It is likely that due to connections between the hypothalamus and other stress related structures, the blockade of CB1 signaling in the PVN may affect signaling in other structures through similar pathways.

Due to experimental limits, micro-syringe injections could have potentially been more precise. With the tip of the micro-syringe measuring roughly 0.1 mm in diameter, diffusion of AM251 to other local hypothalamic and non-hypothalamic areas may have been a possible confound, additionally serving as an explanation to our findings. It is likely that the drug diffused within an area roughly measuring 1 mm in diameter, complicating localization of the drug specifically to the PVN. With a diffusion area this large, the drug may have affected other local brain areas, possibly resulting in the systemic suppression of the HPA axis. Little research has been devoted to specific effects of CB1 antagonism in other regions of the hypothalamus.

It has previously been demonstrated that effects of CB1 blockade by AM251 in the HPA axis varies depending on the time of day the drug is administered (Atkinson et al., 2010). Given not all subjects in the current study were tested at the same time of day, diurnal effects of AM251 on fluctuating corticosterone levels may provide an explanation for the variability in plasma corticosterone levels observed. However, studies conducted by Atkinson and colleagues (2010) suggest that AM251 administration elevated HPA activity. These findings are not consistent with the decrease in plasma corticosterone observed in the current study. Systemic effects of CB1 antagonism in both the hypothalamus and other stress related limbic structures in combination with diurnal fluctuation of circulating plasma corticosterone levels, therefore remain reasonable explanations for the findings from the current study.

Data from behavioral testing was largely insignificant although several trends in marginal means suggest behavioral alteration possibly due to the administration of AM251. More time was spent in closed arms and less time was spent in open arms of the maze with an increasing concentration of AM251, negatively correlating with an increase in plasma corticosterone. Although subjects treated with 60 pmol AM251 made no open entries and spent all 10 minutes in closed arms of the maze, closed entries from previous closed arms were observed. This suggests that animals treated with 60 pmol AM251 were not incapacitated by treatment with the highest drug concentration.

More total entries and explorative behaviors were observed from subjects treated with 30 pmol AM251 than any other group. Conversely, subjects treated with 60 pmol AM251 showed less entries and explorative behavior than both control subjects and subjects treated with 30 pmol AM251. This may possibly be due to doubling the concentration of the drug. These findings are insignificant ($p > 0.05$), and great variability was observed from all groups. However, marginal trends suggest a possible dose-response effect of drug concentration on anxious behavior; such that, at lower concentrations AM251 suppresses anxious behavior, while at higher concentrations AM251 induced anxious behavior.

A significant difference between open and closed entries within subjects was observed, indicative of anxious behavior throughout all subjects. These findings may be due to experimental stress. Animals were handled prior to surgery but were inconsistently handled post surgery. Likewise, prior to each behavioral test, animals were handled for micro-syringe injections and for transportation to the testing room. Each of these procedures may act as an environmental stressor, possibly inducing anxious behavior as well as HPA activation. Additionally, it has been demonstrated that elevated plus maze exposure alone may act as an environmental stressor, ultimately elevating HPA axis activity (Appenrodt et al., 1999)

Tests examining differences between open and closed time were not necessary due to the how entries were defined in the current study. Animals were recorded as either being in open or closed arms because intermediate time was not defined nor accounted for. By the time the two hind limbs of the subject exited an arm of the maze, the front two limbs had already entered another arm of the maze. Similarly, if the animal entered, but then backed out of an arm, the behavior was either recorded as a nose peek or intermediate entry. This provided for all open and closed time to add up to all 10 minutes, eliminating the need for analysis of variance within subjects for these two variables.

Findings from this study support the hypothesis that administration of a lower concentration of AM251 would decrease anxious behavior and that a higher concentration of AM251 would increase anxious behavior, although findings were insignificant. Results fail to support the hypothesis that administration of AM251 would increase plasma corticosterone, nevertheless suggesting an importance for CB1 receptor activity in the mediation of the HPA axis response to stress. Although no significant behavioral or endocrine responses were elicited by administration of AM251, similar studies targeting effects of CB1 modulation in other connecting limbic and hypothalamic structures could further shed light on systemic cannabinoid interactions as well as behavioral implications. Previous research suggests that CB1 receptors are expressed in areas such as the lateral hypothalamus, and bed nucleus of the stria terminalis (Evanson et al., 2010), as well as regions of the hippocampus, and basolateral Amygdala (Riebe and Wotjak, 2011). These brain areas share connections to and from the PVN and remain candidates for investigation of possible systemic connections relating to HPA axis responses to stress.

In conclusion, we have demonstrated that HPA axis activity as well as anxious behavior may be capable of being altered by CB1 modulation. Although local administration of AM251 to the PVN did not significantly suppress HPA axis activity, research has shown various CB1 agonists in combination with CB1 antagonists to be effective modulators (Evanson et al., 2010). In the hypothalamus as well as other

limbic structures, the presence of CB1 receptors suggests a role in the regulation of hormonal and emotional processes. Systemic processes such as limbic control of emotion and HPA axis control of hormonal stress responses, as well as regulation of these processes may potentially be modulated through alteration of CB1 activity.

6. Acknowledgments

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Appendix A

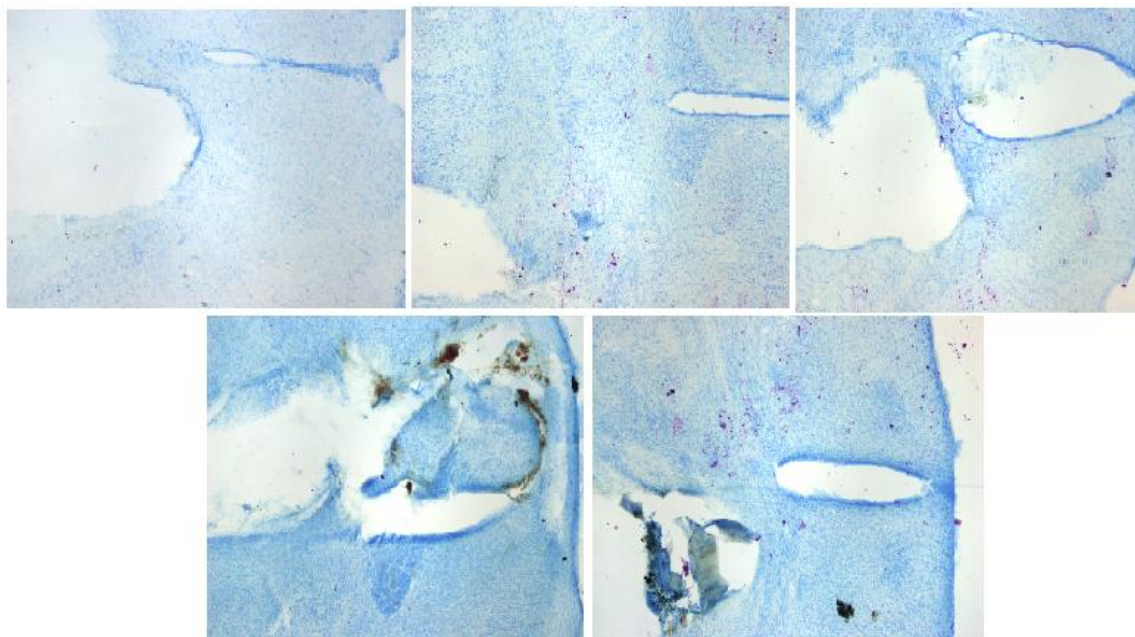


Figure A1: Additional histology images from control subjects. Cannula tracts were identified with buffered thionin nissl stain. All images are oriented in the same direction; ventral (right side) and dorsal (left side). Not all images depict successful cannula placement.

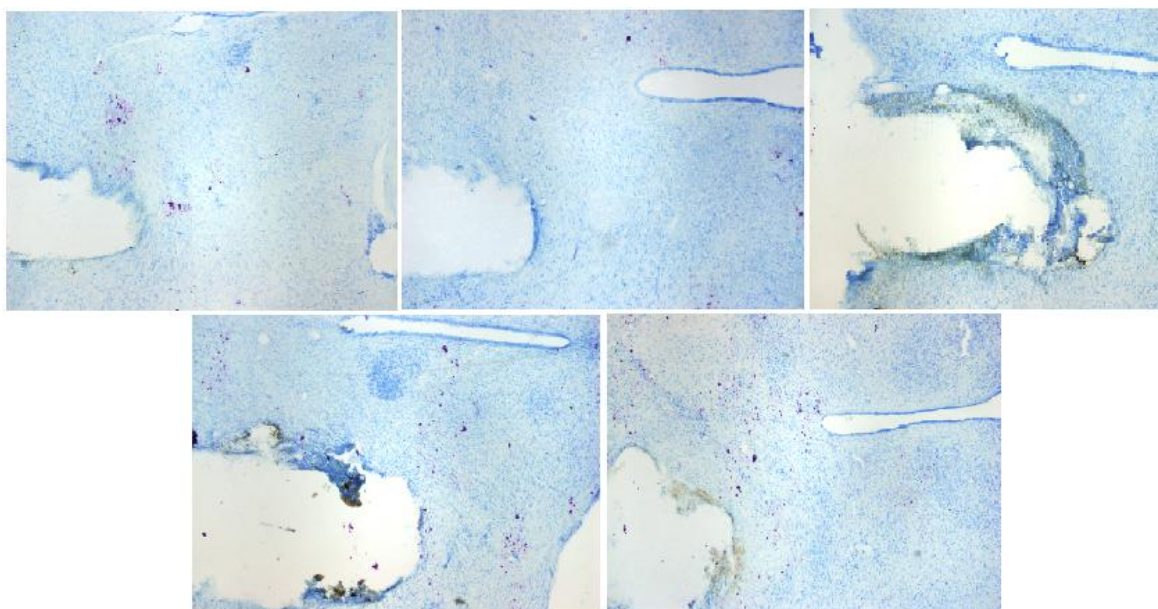


Figure A2: Additional histology images from subjects treated with 30 pmol AM251. Cannula tracts were identified with buffered thionin nissl stain. All images are oriented in the same direction; ventral (right side) and dorsal (left side). Not all images depict successful cannula placement.

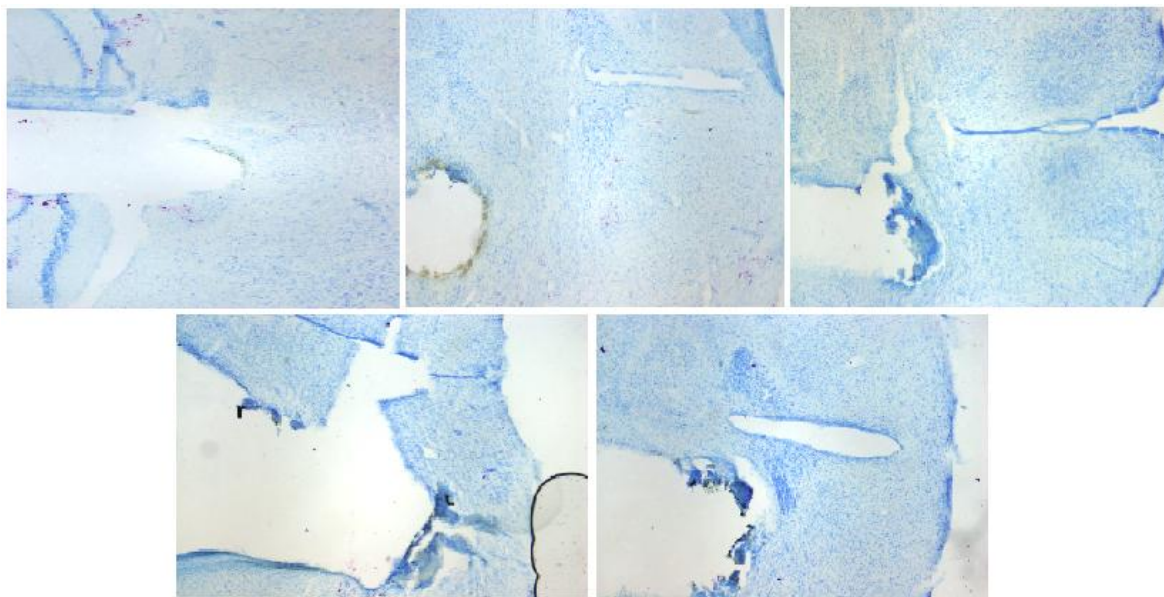


Figure A3: Additional histology images from subjects treated with 60 pmol AM251. Cannula tracts were identified with buffered thionin nissl stain. All images are oriented in the same direction; ventral (right side) and dorsal (left side). Not all images depict successful cannula placement.

Appendix B

Regression Summary**% Bound vs. Log Conc**

Count	5
Num. Missing	0
R	.990
R Squared	.980
Adjusted R Squared	.973
RMS Residual	3.766

ANOVA Table**% Bound vs. Log Conc**

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Regression	1	2073.138	2073.138	146.191	.0012
Residual	3	42.543	14.181		
Total	4	2115.681			

Regression Coefficients**% Bound vs. Log Conc**

	Coefficient	Std. Error	Std. Coeff.	t-Value	P-Value
Intercept	102.128	5.225	102.128	19.547	.0003
Log Conc	-20.599	1.704	-.990	-12.091	.0012

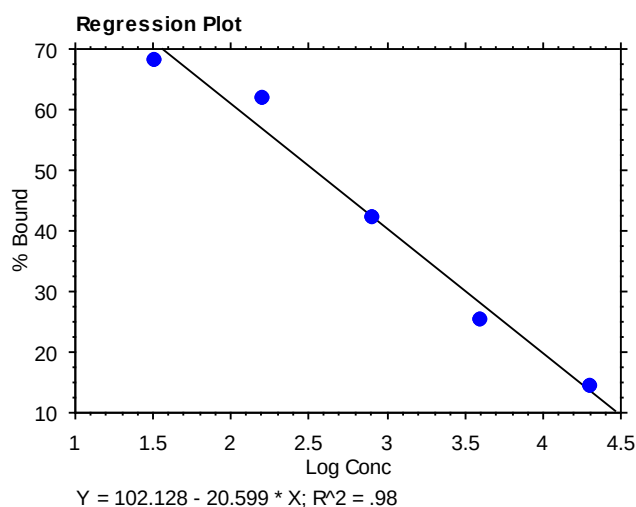


Figure B1: Elisa assay standard curve. This is a depiction of the standard curve used to determine unknown plasma corticosterone values from all samples. The log of all standard corticosterone values was analyzed with linear regression to determine an equation from which experimental corticosterone values were calculated and analyzed.

Table B1: ANOVA summary table for Corticosterone concentration (pg/ml).

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	4.256E10	.704	.517	.123
error (within groups)	10	6.042E10			

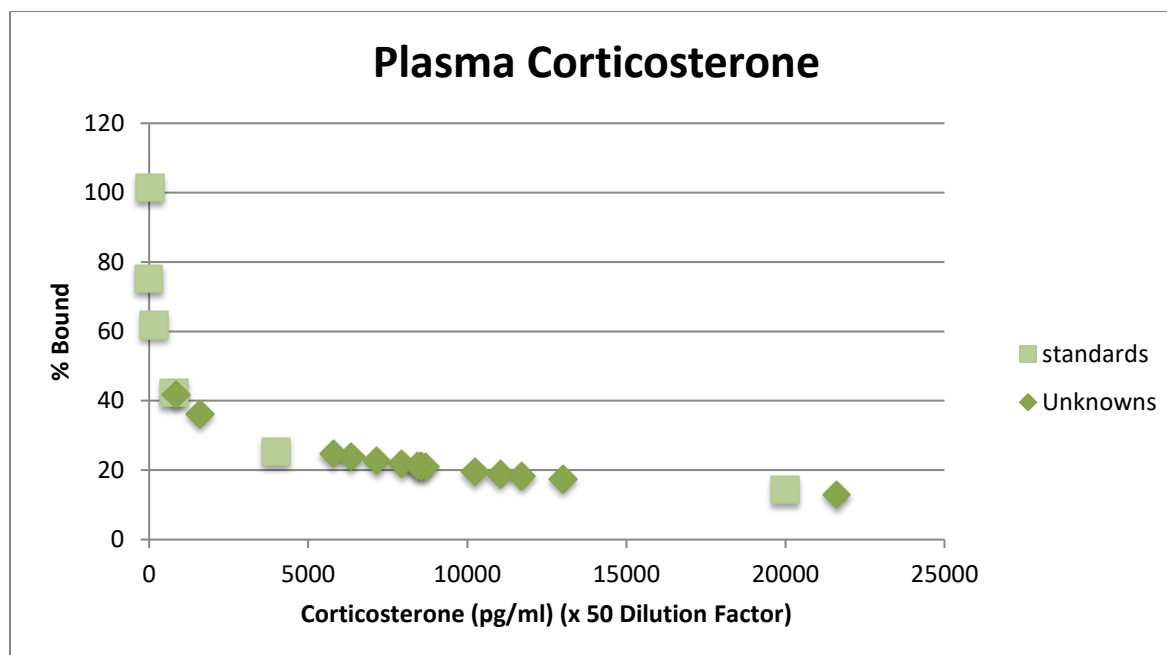


Figure B2: Plasma corticosterone concentration vs percent bound before x50 dilution factor. This is a depiction of the standard curve plotted alongside calculated corticosterone unknowns. Standard values were predetermined from the ELISA kit. Percent bound was calculated from optical density of dilute samples determined after reading the ELISA plate. Unknown corticosterone values were then calculated from the standard curve and multiplied by a dilution factor of X50 to determine actual corticosterone values per sample.

Appendix C

Table C1: ANOVA summary table for Open time

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	990.779	2.333	.147	.318
error (within groups)	10	424.675			

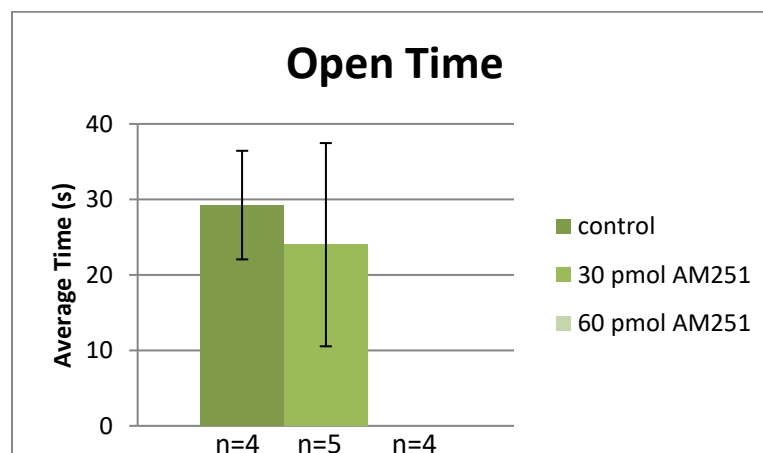


Figure C1: Average time in open arms by group. This is a depiction of mean time spent in open arms of the elevated plus maze. There was an overall decrease in observed time in open arms with an increasing concentration of AM251, although insignificant and with inconsistent variability. All subjects treated with 60 pmol AM251 did not spend any time in open arms of the maze.

Table C2: ANOVA summary table for Closed time

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	990.779	2.333	.147	.318
error (within groups)	10	424.675			

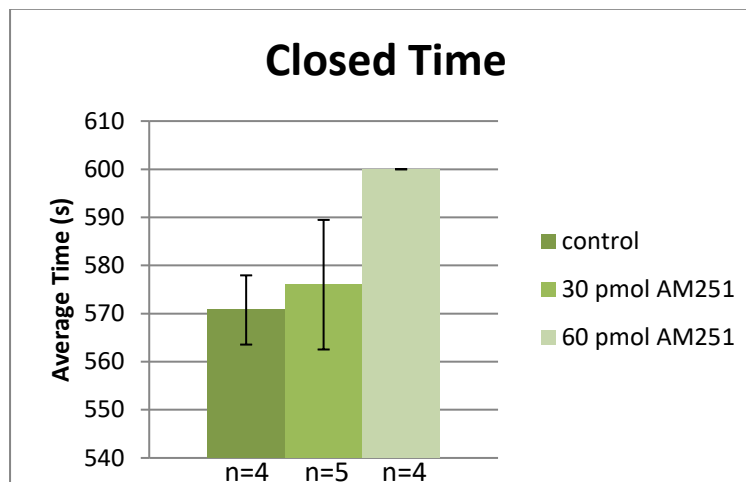


Figure C2: Average time in closed arms by group. This is a depiction of mean time spent in closed arms of the elevated plus maze. There was an overall increase in observed time in closed arms with an increasing concentration of AM251, although insignificant and with inconsistent variability. All subjects treated with 60 pmol AM251 spent all tested time in closed arms of the maze.

Table C3: ANOVA summary table for Open entries

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	6.840	1.646	.241	.248
error (within groups)	10	4.155			

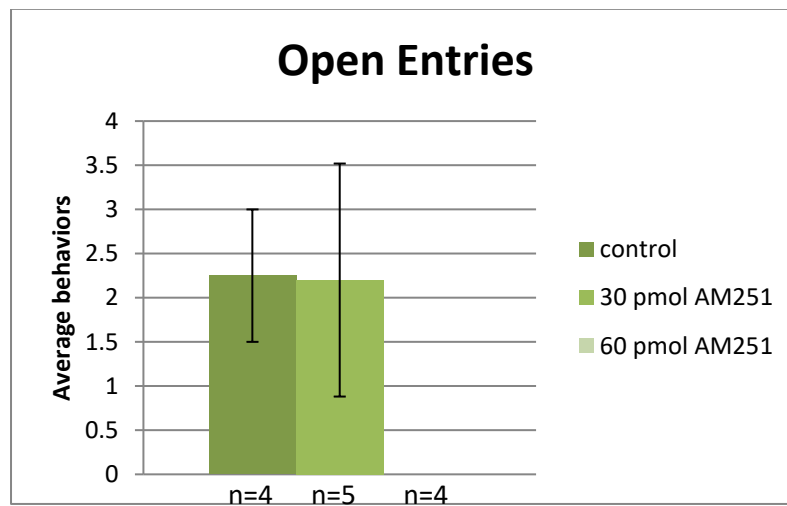


Figure C3: Average open arm entries by group. This is a depiction of mean entries into open arms of the elevated plus maze. There was an overall decrease in observed time in open arms with an increasing concentration of AM251, although insignificant and with inconsistent variability. All subjects treated with 60 pmol AM251 did not make any entries into open arms of the maze.

Table C4: ANOVA summary table for Closed entries

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	11.571	.425	.665	.078
error (within groups)	10	27.255			

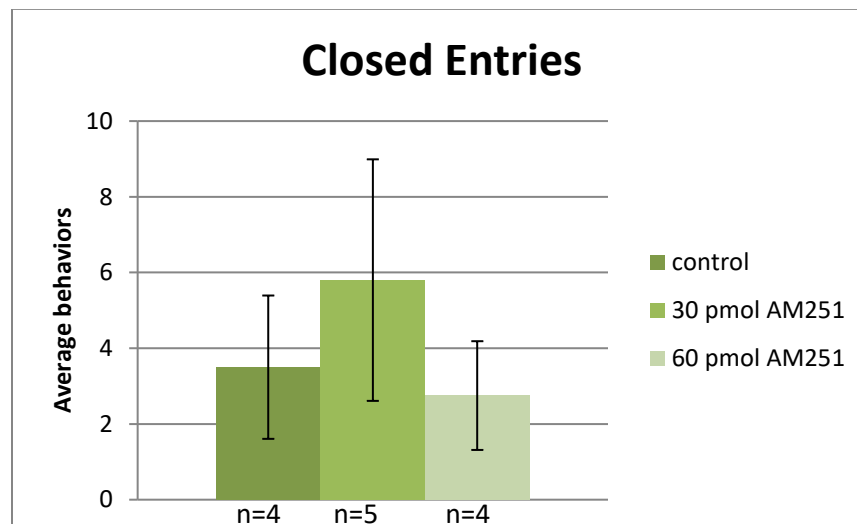


Figure C4: Average closed arm entries by group. This is a depiction of mean entries into closed arms of the elevated plus maze. There was no consistent effect of drug concentration on number of entries into closed arms of the maze.

Table C5: ANOVA summary table for Total entries

Source	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>	η_p^2
Group	2	30.635	.608	.563	.108
error (within groups)	10	50.350			

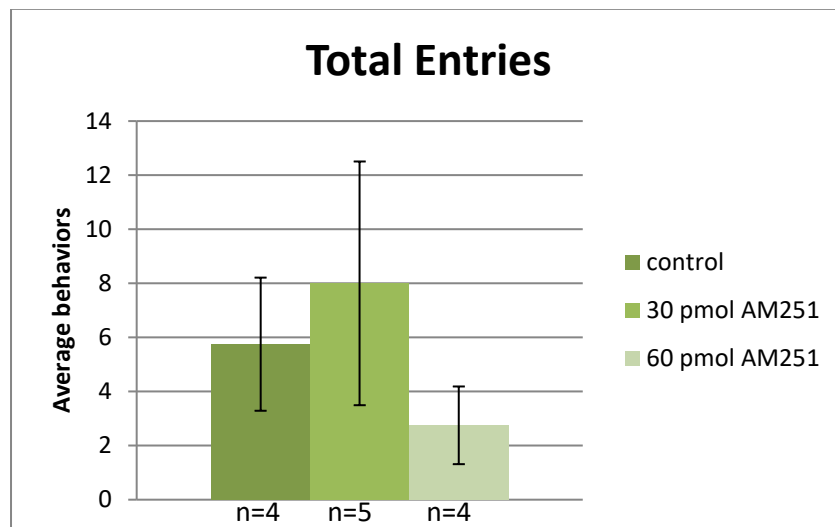


Figure C5: Average total arm entries by group. This is a depiction of mean entries into all arms of the elevated plus maze. There was no consistent effect of drug concentration on number of entries into all arms of the maze. No subjects were incapacitated by any treatment.

Table C6: ANOVA summary table for Nose dips

Source	<i>df</i>	MS	<i>F</i>	<i>P</i>	η_p^2
Group	2	11.379	1.216	.337	.196
error (within groups)	10	9.355			

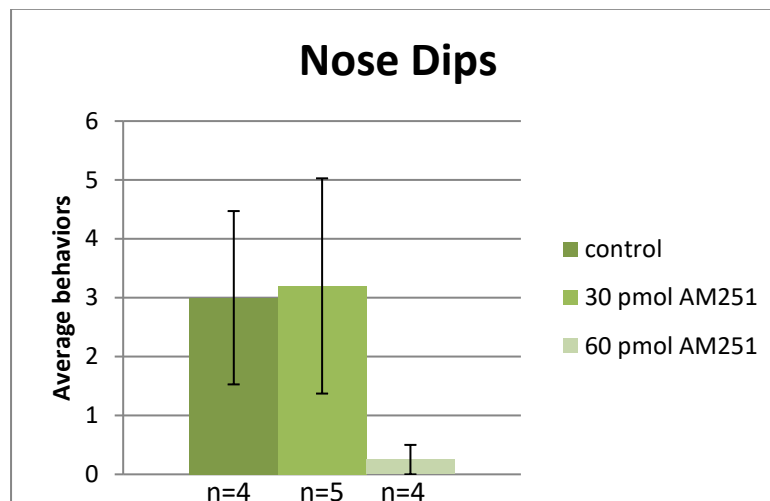


Figure C6: Average nose dips by group. This is a depiction of mean nose dips below the floor of the elevated plus maze. Although there was no significant effect of drug treatment on number of nose dips, subjects treated with 60 pmol AM251 did exhibit far fewer behaviors compared to other experimental groups.

Table C7: ANOVA summary table for Nose peeks

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	4.938	.146	.866	.028
error (within groups)	10	33.720			

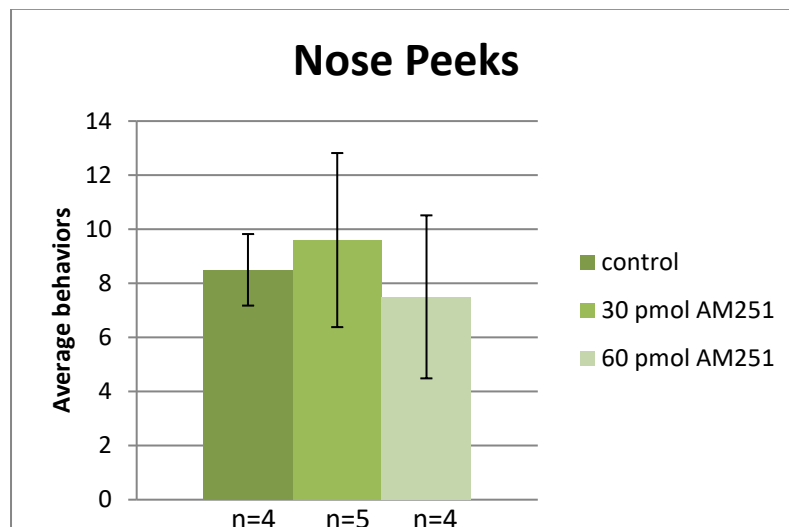


Figure C7: Average nose peeks by group. This is a depiction of mean nose peeks out of all arms of the elevated plus maze. There was no significant effect of drug treatment on number of nose peeks.

Table C8: ANOVA summary table for Intermediate entries/exits

Source	<i>df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Group	2	6.687	.229	.799	.044
error (within groups)	10	29.155			

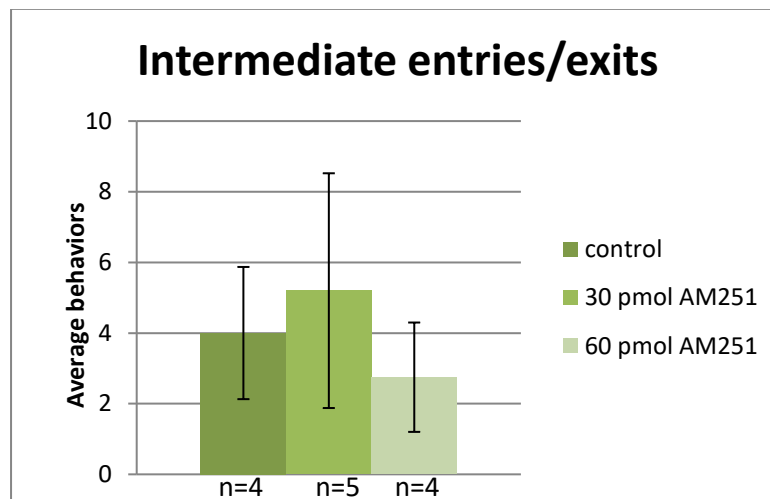


Figure C8: Average intermediate entries and exits by group. This is a depiction of mean intermediate entries and exits into all arms of the elevated plus maze. There was no consistent effect of drug concentration on number of intermediate entries and exits.

Table C9: ANOVA summary table for Open entries and Closed entries within subjects

Source	<i>Df</i>	MS	<i>F</i>	<i>p</i>	η_p^2
Between Subjects	2	15.317			
Within Subjects					
Entries	1	41.257	6.617	.028	.398
error (Ss x Entries)	10	6.235			