Gene by Environment Interaction Leads to Sensitivity to the Anti-Aggressive Effect of Δ^9-Tetrahydrocannabinol

By

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Gene by Environment Interaction Leads to Sensitivity to the Anti-Aggressive Effect of Δ^9-Tetrahydrocannabinol

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Abstract

Pathological reactive aggression is a neurodevelopmental disorder characterized by exaggerated violent and explosive responses that are disproportionate to provocation, and a propensity for cannabis abuse. The susceptibility to pathological aggression is shaped by several genes. In particular, substantial evidence has highlighted a major role of the gene which encodes monoamine oxidase A (MAOA), the primary catabolic enzyme for monoamine neurotransmitters. In fact, one of the strongest predictors of male pathological aggression is the gene by environment (GxE) interaction between the low transcriptional activity polymorphisms of MAOA (MAOA-uVNTR) and early life stress such as childhood neglect or abuse. Given that reactive aggressive individuals tend to consume cannabis, it was hypothesized that this GxE interaction leads to neurobiological changes that confer a sensitivity to the effects of cannabis consumption. In line with this idea, numerous previous studies have concluded that cannabinoid receptor 1 (CB1), the receptor that is activated by the major psychoactive ingredient in cannabis, Δ^9-tetrahydrocannabinol (THC), is responsible for the anti-aggressive effects of cannabis consumption. In particular, it has been shown that these effects are mediated by CB1 localized on glutamatergic neurons. Cannabinoid receptor 2 (CB2) has been shown to a lesser extent to reduce aggression.

To uncover the connection between cannabis consumption and pathological reactive aggression, our lab recently developed the first mouse model of this GxE interaction, based on subjecting a transgenic MAOA hypomorphic mouse model ($MAOA^{Neo}$) to early-life stress (ES). Unlike their wild-type and non-stressed controls, $MAOA^{Neo}$ male pups subjected to ES from postnatal day (PND) 1 through 7 developed a marked increase in aggression from the onset of adolescence at PND28, throughout adulthood. This developmental trajectory strikingly mimics the ontogeny of pathological reactive aggression in humans; making this the most relevant and translatable model available.

To test whether the GxE interaction leads to neurobiological changes in CB1 and CB2, ES-MAOA^{Neo} males were euthanized, as well as their non-stressed (NS) and wild type (WT) littermates, then their hypothalamus, amygdala, and midbrain was isolated. These regions compose the major aggression circuit in the brain. ES-MAOA^{Neo} mice develop elevated hypothalamic CB1 in response to early life stress compared to ES-WT littermates. Additionally, MAOA^{Neo} mice develop increased amygdalar CB1, though this effect is independent of ES conditions. Neither midbrain CB1 nor CB2 in any analyzed region change in response to either genetic or condition factor. Based on these findings, it was hypothesized that ES-MAOA^{Neo} mice would be selectively sensitive to the anti-aggressive effects of ultra-low-dose (0.03 mg/kg) THC. Indeed, the ES-MAOA^{Neo} mice fought for a smaller duration at both juvenile and adult stages, and this dose does not induce locomotor- or anxiety-related effects. However, ultra-low-dose THC did not show an improvement in risk assessment, suggesting that the effect of THC is to selectively reduce aggression, not to rescue the pathological aggressive behavioral phenotype. Despite being unable to determine if these effects are mediated by CB1, an inhibitor of glutamate release (riluzole) strongly reduced aggression in both fighting bouts and duration.

Together, these results suggest that ES-MAOA^{Neo} interaction confers a sensitivity to the anti-aggressive effects of THC. Based on these findings, it is proposed that CB1 is posited to be a biomarker that may predict pathological aggression in humans. Future studies should be done to determine if ultra-low-dose THC treatments activate CB1 on glutamatergic or GABAergic neurons, if the upregulation event in hypothalamic CB1 occurs on glutamatergic or GABAergic neurons, and should also examine CB1 changes in other pertinent brain regions.

Dedicated to my family for their support and love

Acknowledgements

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1 INTRODUCTION

1.1 PATHOLOGICAL AGGRESSION OVERVIEW, BACKGROUND, AND HALLMARKS

1.1.1 What is aggression and when is it considered pathological?

Aggression is the intent or execution of physical harm toward another and it is a standard behavior that is common among all animals and humans. It can serve numerous purposes, but in mammals, it is most often executed in the securing of food, water, shelter (or territory), and sexual opportunities. As humans, the necessities of life are commonly associated with money and 'property'; it is learned from an early age that there is a competition for these resources and that social cues can be recognized and utilized to prevent the loss of these necessities. Through schooling or parenting, the proper response to any particular cue is learned and a preference in response is often generated. In other words, rules are instilled within us at an early age, and as a juvenile, we become more independent, we start testing the limits of what social behavior is acceptable, in an attempt to learn 'risk versus reward'. This process of learning that which is socially or morally acceptable occurs in early in life and manifests itself as impulsivity and misconduct. It is unsurprising, then, that a stressful, or unsupervised early life environment greatly increases the likelihood of juvenile misconduct [1]. Aggressive behaviors may be expressed as 'proactive' or 'reactive. Proactive aggression is premeditated and goal-oriented as in armed robbery. Conversely, reactive aggression occurs in response to an external stimulus such as social provocation. When the aggressive response is disproportionate to the external stimuli, this is considered pathological aggression.

Pathological aggression is characterized by frequent outbursts of overtly violent, criminal behavior. The relationship between aggression and drug abuse is particularly intriguing, given that aggressive patients exhibit a significantly higher rate of risk for drug abuse and/or dependence.

The individual risk factors for both cannabis consumption and reactive aggression overlap [2], and as discussed later, a strong association between cannabis consumption and reactive aggression has been found [3].

Our society does allow for an individual to commit acts of aggression against other people in few, specific instances, such as war and self-defense. In other instances, however, it is illegal. As a result, the impulses that drive and inhibit aggression are influenced by the risk of receiving penalty by parents in childhood or by the laws of our society as an adult. Though not all perpetrators of violent crimes are pathologically aggressive, statistics of violent crimes are often highly useful in tracking pathological aggression. The socioeconomic burden of pathological aggression is extremely significant, and the costs of court fees, state-sponsored healthcare, and emergency services have been estimated at $590 billion in the U.S [4].

In the last two decades, one particular enzyme has become highly implicated in the pathogenesis of aggression, monoamine oxidase A (MAOA). Low transcriptional activity of the MAOA gene confers a vulnerability to impulsive aggression, especially to those that contain the 2- and 3- repeat of the upstream variable number tandem repeat (MAOA-uVNTR) [5]. More recent lines of research have uncovered that early life stress such as childhood neglect and abuse strongly predicts conduct disorder, violent crime conviction, and proclivity toward violence in a sample of prisoners [6]. This work has since become the basis of a number of studies examining the gene by environment (GxE) interaction that produces pathological aggression [6]. Other predictors, like early life stresses, Brunner's syndrome, antisocial personality, and substance abuse have been studied extensively [7-12]. From these multiple lines of research, most agree that pathological aggression is a learned behavior. Experiencing neglect and abuse at early stages disrupts this learning process by showing that aggressive behavior is acceptable. One study found that early

observation of violence prior to age ten strongly predicts juvenile violent behavior [13]. Despite the substantial work that has been done, the GxE interaction predictor of pathological aggression is the strongest of predictors of pathological aggression.

1.1.2 Critical brain regions: cortical control vs aggressive impulse

For the last two decades, aggression research has largely focused on the dual, opposing natures that allow for the expression, or conversely the suppression of aggressive manifestations [14-18]. The foundation of these natures appears to lie in brain circuitry. As will be discussed, pathologically aggressive individuals consistently exhibit neurobiological changes within the regions described next, and these changes are displayed in their behaviors: impaired risk assessment, hostile attribution bias, and impulsivity.

In the mammalian brain, the region most directly responsible for the execution of aggression is the periaqueductal gray (PAG), a cluster of midbrain neurons surrounding the cerebral aqueduct, with direct downstream projections onto key autonomic effectors. Early studies found that extrinsic electrical stimulation of this region will immediately result in defensive postures and rage vocalizations [19]. The PAG receives glutamatergic input from the anterior hypothalamus [19], which integrates the signals from the amygdala, prefrontal cortex (PFC), and the nucleus accumbens (NAcc); regions that are critical for emotional modulation. Numerous studies have identified that deficient PFC signaling, also known as hypofrontality, is highly associated with pathological aggression and impulsivity [20, 21]. The PFC determines the presence of threat, right from wrong, and it organizes planning. Typically, reactive aggressive individuals have impaired ability to recognize angry facial expressions [22, 23], suggesting an inability of the PFC to properly evaluate threat. It is largely accepted that the pathological aggression risk factors likely result in major signaling changes within these circuits, though the specific neurotransmitter that is

responsible is highly debated [24]. When threatening cues are recognized, the aggression circuit is initiated and this ultimately is interpreted as an aggressive impulse, though, not all aggressive impulses are executed. The brain regions that generate aggressive drives operate in concert with the (PFC) [16, 25, 26]; a region that acts as a mediator, and when the impulse is inappropriate or disproportionate, may oppose the execution of aggressive impulses [14, 15, 17].

1.2 PATHOLOGICAL MECHANISMS

For such a significant societal problem, there are surprisingly few pharmacological treatments for aggression. As discussed above, dopamine receptor antagonists are effective, but these drugs are often accompanied by a host of side effects, including sedation and extrapyramidal symptoms, which often lead to poor patient compliance [27, 28] which underscores a critical need to elucidate the underlying neurobiological mechanisms of pathological aggression in order to identify novel drug targets and improve therapy.

1.2.1 Cannabinoid receptor signaling

As mentioned before, pathological reactive aggression has been associated with juvenile cannabis consumption [3]. One study has shown that reactive aggressive adolescents tend to be rejected by peers, and later adopt cannabis consumption, likely as a mechanism to self-medicate [2]. Psychoactive cannabinoids have been found to activate or antagonize cannabinoid 1 receptor (CB1), a GPCR expressed in several brain regions that regulate anxiety-related behaviors such as the PFC, amygdala, and the PAG [29]. CB1 is coupled to Gαi, so when CB1 is active (typically after postsynaptic neuron depolarization), there is a decrease in adenylyl cyclase activity, and initiation of the mitogen-activated protein (MAP) kinase pathway. Ultimately, reductions in calcium influx lead to fewer fusions of neurotransmitter vesicles with the cell membrane. Depending on the neurotransmitter, glutamate or gamma-amino butyric acid (GABA), CB1

activation leads to depolarization-induced suppression of excitation (DSE) or inhibition (DSI), respectively. DSE results in fewer postsynaptic depolarizations while DSI increases it. These two effects are recognized as the contributions of CB1 to behavior [30].

Polymorphisms of CNR1 typically result in a vulnerability to delirium, addiction, and impulsivity [31]. Polymorphisms of CNR2 do exist, though they have been associated with peripheral health issues [32]; not associated with aggression.

1.2.1.1 Known locations of cannabinoid receptor 1

Cannabinoids have been found to have numerous biological functions. They are located presynaptically, where they control neurotransmitter release. It is unsurprising, then, that CB1 receptors are widely distributed throughout the brain. Brain regions that express CB1 include the hippocampus, hypothalamus, amygdala, PFC and other cortical regions, PAG, stria terminalis, and the thalamus [29].

1.2.1.2 Contribution of CB1 activity to aggressive responses

Extensive studies have been done to uncover the role of CB1 in anxiety-related behaviors, however, the results from these studies may have implications for aggression studies, because the brain regions that are responsible for both of these constructs overlap. The contributions of DSE and DSI to anxiety-related behaviors have been uncovered by initial CB1 studies. CB1 agonist treatment typically results in anxiolytic responses, with exceptions [33]. This suggests that exogenous CB1 agonists bind to CB1 on glutamatergic neurons in anxiety-generating brain regions (amygdala) or GABAergic neurons in anxiety-modulating areas (PFC). Treatments with CB1 antagonists tend to increase anxiety-related behavior [34]. Further, CB1 KO mice exhibit anxiogenic responses in the light/dark box (LDB) paradigm and the elevated plus maze (EPM) [35]. The LDB and EPM tests are well-validated paradigms in which the rodent subject is given

an approach task. Each test has an illuminated region, and a dark, enclosed region. These tests utilize the exploratory nature of the rodent and the averseness of rodents to light or open spaces. Overall, the results suggest that CB1 KO mice lose fine control of the neurotransmission in anxiety- and aggression-mediating regions.

To address anxiety response variability in terms of subject vulnerability, Tambaro et al. increased the expression of CB1 in mouse PFC by chronically administering a CB1 antagonist/inverse agonist AM251 [36]. After a 3 day washout period, these mice were administered a dose of a CB1 agonist (CP55,940) and were tested in the EPM. CP55,940 treated mice that were pretreated with AM251 spent less time in the open arms of the EPM. This suggests that increased expression of CB1 may predispose an individual to an anxiogenic response. Although, this conclusion is based on several assumptions. Firstly, AM251 pretreatment only increased the expression of CB1 in the PFC. It also decreased the expression of CB1 in the hippocampus. Presumably, these changes occur to oppose the enhanced amygdalar activity that tends to accompany CB1 antagonism. It is possible that, instead of relative CB1 expression, CB1 sensitivity is greatly enhanced, and this allows for the CB1 agonist dose-curve to rapidly shift left. Overall, this experiment supports that vulnerabilities to the side effects of cannabinoids may be conferred by changes in CB1 receptor expression or sensitivity in different populations. Further, these changes in CB1 expression may confer vulnerabilities to specific side effects of cannabis consumption.

As described earlier, cannabinoid-mediated DSE and DSI are facilitated by CB1 on different neuronal subpopulations. The next step in cannabinoid studies is to determine the role of these neuronal subpopulations in the behavioral effects of cannabis. Rey et al. administered either a low or high dose CB1 agonist to two separate genotypes of mice [37]. These types included knockout of CB1 on glutamatergic and GABAergic. They found that GABA-CB1 KO mice were resistant

to the anxiogenic effect of a high dose CB1 agonist, suggesting that DSI contributes significantly to the generation of anxiety-like behavior. In keeping with this, glutamatergic CB1 KO mice did not show reduced anxiety when treated with a CB1 agonist in low doses. Overall, this experiment depicts CB1 as a modulator (DSE) or facilitator (DSI) of anxiety-related behavior depending on the neuronal subpopulation it is expressed on. Further, the direction (DSE-anxiolytic and DSI-anxiogenic) suggests that the overall mediation of anxiety by CB1 is occurring in a brain region such as the amygdala. A similar experiment showed that glutamatergic CB1 KO mice spent a greater percentage of time fighting intruder conspecifics, while GABAergic CB1 KO did not [38]. This suggests that initiation of DSE is the likely mechanism that reduces anxiety and reduces aggression upon acute treatment of low dose cannabis.

1.2.1.3 CB1 content is known to change in response to psychiatric illness

CB1 content is known to change under several circumstances, including maternal deprivation [39]. Depression induces PFC CB1 expression, as suicide victims have higher levels of CB1 [40]. Both acute and chronic alcohol abuse upregulate cerebral CB1 [41]. Together, this suggests that numerous environmental factors may lead to upregulation of CB1 and are likely to contribute to pathological aggression.

1.2.1.4 Brief discussion on cannabinoid receptor 2

Cannabinoids also bind to cannabinoid 2 receptor (CB2), a GPCR which is also coupled to Gαi. It is expressed mainly in immune cells, where it regulates cytokine release. CB2 is expressed in the brain, though it is much more widely expressed in glial cells than neurons [42]. As a result, CB2 has not been thoroughly examined for its contribution to anxiety and aggression. In 2006, Ashton et al. postulated that CB2 may be expressed in neurons because in a brain-specific

immunohistochemical study CB2 did not co-localize with GFAP, a marker for astrocytes [43]. This presented the possibility that CB2 might be responsible for the side effects of marijuana use. CB2 has also been examined for its role in aggression, though to a much lesser extent. CB2 KO male mice have been shown to fight male conspecifics for a greater duration in both the social interaction and resident-intruder test [44]. In the same study, CB2 was found to regulate the expression of both MAOA in the dorsal Raphe nuclei. The mechanism by which CB2 may contribute to the control of aggression is yet unknown, though there are recent studies that suggest it may be related to its role in reducing cytokine release.

1.2.2 Does Δ^9-tetrahydrocannabinol potentiate or reduce aggression?

Previous studies that examined the aggression-modifying effects of Δ^9-tetrahydrocannabinol (THC), the major psychoactive ingredient in marijuana, have shown conflicting results. Early studies associated juvenile marijuana use with aggression [45]. Though higher doses of THC have shown increases in aggression [46], a majority of studies published since then suggest that THC reduces aggression [47]. The discrepancy between effects of THC may be caused by the numerous receptors that THC can activate. To control for this potential confound, one study utilized a selective CB1 agonist to determine the role of CB1 in aggression and showed that selective activation of CB1 reduces aggression [48].

This apparent conflict leads to the two major hypotheses regarding the association of THC and aggression. The causal hypothesis posits that marijuana use leads to subsequent increases in aggression, though this hypothesis has little support. Rather, the majority of cannabinoid studies conclude that consumers of cannabis use marijuana to alleviate the negative emotional states that may lead to externalized aggression, be it anxiety, frustration, stress, or aggressive impulses [49].

1.2.3 THC may have biphasic effects on aggression, anxiety, or related behaviors

THC has numerous pharmacological effects, including anxiety reduction, euphoria induction, and increased appetite. When taken in large doses, THC can precipitate anxiety, psychosis, panic, decreased motor control, and impaired learning [50]. Theoretically, any change in anxiety level could unpredictably influence the outward expression of aggression. Therefore, the possibility of anxiolysis and locomotion must be considered in THC pharmacological studies.

1.3 PRECLINICAL MODELS OF PATHOLOGICAL AGGRESSION

1.3.1 Historical rodent models of aggression

There are several known methods of inducing aggression in rodents, though it is most common to socially isolate the test rodent before the aggression test [51]. Male rodents are territorial in nature; they seek out their own territory and defend it from rival males. Frustration- and anticipation-induced aggression models have also been utilized for the purpose of studying aggression, that is, the test rodent tends to be more aggressive after omission of an expected reward or if it has had limited access to an opponent. Other studies that examine rodent female aggression typically utilize the maternal aggression model, by introducing an unfamiliar female conspecific access to her nest [52].

1.3.2 GxE factors

1.3.2.1 Gene by environment (GxE) factors strongly predict aggression in both rodent models and humans

As previously discussed, Caspi et al. found that in individuals that had a low translationally active polymorphism of MAOA, early life stress such as neglect and abuse strongly predicted violent behavior [6]. Considering that previous studies do not utilize a proper rodent model that accounts for the strong GxE risk factors, a study must be done to uncover how these risk factors interaction

to generate changes in cannabinoid receptor levels, and suggest the mechanism by which THC provides an anti-aggressive effect in a model of pathological aggression.

1.3.3 The ES-MAOA^{Neo} model

1.3.3.1 Behavioral characteristics of the ES-MAOA^{Neo} model of male rodent pathological aggression

Our lab previously generated a mouse model that mimicked this MAOA-uVNTR and early life stress GxE interaction [53]. The MAOA gene of these mice contains a neomycin resistance cassette in intron 12 which produces mainly MAOA copies with truncated C-terminals (non-functional copies) and a small portion of functional MAOA protein that is identical to WT mouse MAOA. When male test mice of this MAOA^{Neo} genotype experience daily maternal separation and saline injection (ES) for the first seven postnatal days (PND), they exhibit much higher levels of aggression compared to WT littermates or non-stressed litters (data not shown) in the resident-intruder aggression test at both PND 28 and 70. These ES-MAOA^{Neo} males also exhibit repetitive behavior in the marble burying test, reduced locomotion in the open field test, and antisocial behaviors in the social interaction test compared to unstressed and WT controls (data not shown).

1.3.3.2 Neurochemical characteristics of the ES-MAOA^{Neo} model

As discussed previously, male MAOA^{Neo} mice exhibit higher levels of 5HT in the PFC, but dopamine and norepinephrine are unchanged. An analysis of receptor expression has not yet been done, and it is unknown if neurotransmitter levels change in MAOA^{Neo} mice in response to early life stress conditions.

1.4 GOAL OF RESEARCH

1.4.1 Gaps in research

Though most studies conclude aggression is reduced by THC and that activation of CB1 reduces the manifestations of aggression, it is still not known why *pathologically* aggressive juveniles use marijuana. Despite the immense amount of work that has been done in this field several key questions are still unanswered: For pathologically aggressive individuals,

1. Does THC reduce or increase violent outbursts?
2. Do the GxE factors that lead to pathological aggression produce neurobiological changes?

Based on this background, the following thesis has been formed: ES-MAOA^{Neo} factors confer a sensitivity to the anti-aggressive effects of ultra-low-dose THC.

2 METHODS

2.1 EXPERIMENTAL DESIGN

2.1.1 Neurochemical analysis: Mouse Model, Age, Selected brain regions

For this experiment, male rodents (both MAOA^{Neo} and wild type [WT]) were either exposed to the early life stress [ES] condition or daily handling [NS] condition for the first week. Upon reaching PND 70, the mice were euthanized and the brains were promptly dissected for receptor analysis.

2.1.2 Behavioral analysis: Mouse model, age, selected behaviors

For the juvenile behavioral THC experiments, both ES-MAOA^{Neo} and ES-WT male mice were genotyped upon weaning on PND 21. They were subjected to the following behavioral paradigms: on PND 23, Tube dominance test followed by social isolation; on PND 28 and 31, the mice were subjected to a counter-balanced within-subject resident-intruder aggression paradigm in which they were treated with 0.03 mg/kg THC in 5% kolliphor-EL, 5% ethanol or vehicle; on PND 35, the mice were treated with 0.03 or 0.3 mg/kg THC or vehicle, and then were subjected to the open field locomotion test, immediately followed by the elevated plus maze paradigm.

For the juvenile behavioral THC/AM251 experiments, both ES-MAOA^{Neo} and ES-WT male mice were genotyped upon weaning on PND 21. They were subjected to the following behavioral paradigms: on PND 23, Tube dominance test followed by social isolation; on PND 28, the test mice were injected with the 5% kolliphor-EL, 5% ethanol vehicle and subjected to the resident-intruder aggression paradigm; on PND 31, the mice were treated with 0.03 mg/kg THC with or without 1.0 mg/kg AM251 in 5% kolliphor-EL, 5% ethanol vehicle, and then were subjected to another resident-intruder aggression.

For the adult behavioral THC experiments, both ES-MAOA^{Neo} and ES-WT male mice were genotyped upon weaning on PND 21 and were socially isolated on PND 70. On PND 84, the mice

were subjected to a between-subject resident-intruder aggression paradigm in which they were treated with 0.03 mg/kg THC in 5% kolliphor-EL, 5% ethanol or vehicle. On PND 90, the mice were treated with 0.03 or 0.3 mg/kg THC or vehicle, and then were subjected to the open field locomotion test, immediately followed by the elevated plus maze paradigm

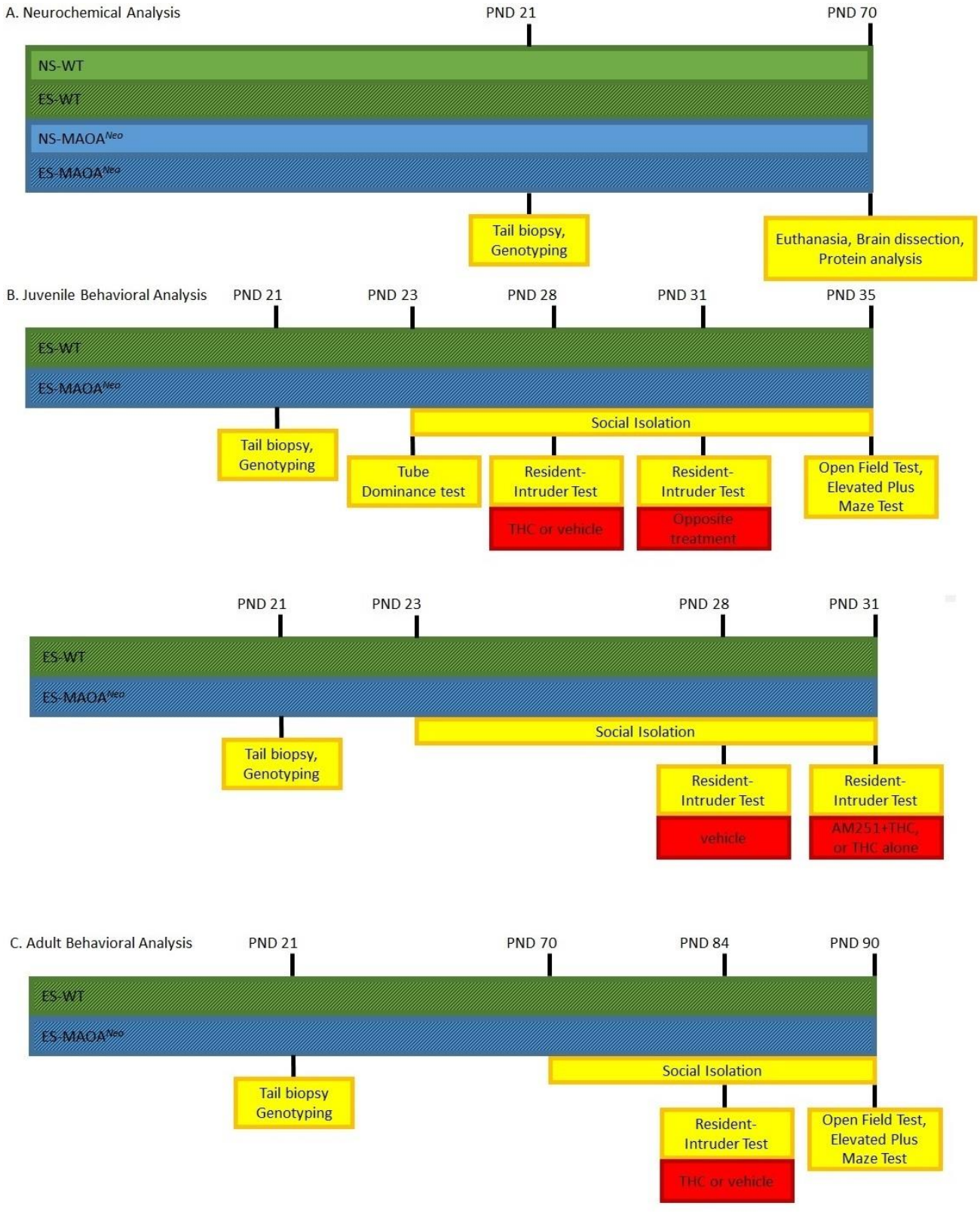


Figure 2.1 Experimental Design. (A) Neurochemical analysis of test mice, (B) juvenile behavioral analysis of test mice, and (C) adult behavioral analysis.

2.2 METHODS

2.2.1 Drugs

THC is the psychoactive phytocannabinoids in cannabis that is a nonselective agonist of CB1 and CB2. Stock (in ethanol) drug was purchased from Sigma-Aldrich (1972-08-3). AM251 is an antagonist/inverse agonist of CB1, and it was purchased in powder form from Cayman chemical (183232-66-8). Though the precise mechanism of riluzole is unclear, it has been shown to reduce glutamate release *in vivo*. Riluzole was also purchased from Cayman chemical (850608-87-6).

2.2.2 Animals

All activities with live animals, including behavioral testing, was done in accordance with the protocol that was approved by the University of Kansas Institutional Animal Care and Use Committee.

2.2.2.1 Animal husbandry

Male 129S6 mice were used for these studies. The juveniles weighed between 10-15 g, and the adult mice weighed between 25-35 g, though the MAOA^{Neo} mice typically weigh much less than their WT littermates (data not shown). Animals were group-housed in cages with food [2018] and water available ad libitum, except during periods of social isolation, in which they were isolated in their own private cage with access to food and water available ad libitum. In both cases, the room was maintained at 22°C, on a 12-h light/dark cycle (lights on 08:00 hours). Light and sound were maintained at 10 lx and 70 dB for all behavioral tests unless otherwise indicated. Experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Animal Use Committees of the University of Kansas.

2.2.2.2 Breeding

For the generation of the test mice used in this study, it was crucial that the ES-WT mice and ES-MAOA^{Neo} mice were littermates. Therefore, the breeding cages setup for this set of experiments consisted of a WT male paired with a heterozygous MAOA^{Neo} female. The male offspring from this pair were either homozygous WT or MAOA^{Neo}. Females were excluded from the study and used for future breeding pairs.

2.2.2.3 Genotyping

Tail biopsies were performed upon weaning of mice on PND 21. DNA was extracted from the tails via the HOTSHOT method. To properly determine the genotype of the mice, a PCR was run using the GoTaq Green master mix (M712) with the appropriate negative controls. The products underwent electrophoresis on a 4% agarose in a buffer of 40mM Tris, 20mM acetic acid, and 1mM EDTA containing 0.0001% ethidium bromide. The resulting bands were imaged on a BIO-RAD EZ gel documentation imager. Male mice were assigned to the MAOA^{Neo} genotype if DNA band amplified at approximately 500 base pairs or to the WT genotype if amplified at approximately 300 base pairs.

2.2.2.4 Early life stress [ES]

In order to simulate early life neglect and abuse, test mice were subjected to a daily saline injection and maternal separation for 30 minutes on PND 1 and 2-4 hours daily between PND 2 and 7. All animals within the litter received the same manipulation. During separation, the pups were placed into a new cage in a special, temperature-controlled room. Non-stressed [NS] controls were also removed from their cages (5 minutes) to account for handling stress.

2.2.2.5 Habituation

Prior to behavioral testing, all test animals were habituated to the testing room in which the behavioral paradigm took place for a period of 30 minutes. This minimized the error associated with the environmental visual, odorous, and audial cues during the testing phase.

2.2.2.6 Dissections

Brains of test mice were dissected immediately after induction of anesthesia by isoflurane inhalation and rapid decapitation via guillotine. The following brain regions were isolated for analysis: hypothalamus, amygdala, and midbrain. Additional regions were isolated and stored for future analysis: prefrontal cortex, striatum, and hippocampus.

2.2.3 Neurochemistry

2.2.3.1 Protein Isolation and quantification

CB1 and CB2 receptor quantification were measured via western blot. Following brain extraction, regions of interest were dissected using a stereotaxic atlas and immediately frozen and stored at -80°C. Samples were homogenized twice in ice-cold buffer (230 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, 50 mM NaF, 10 mM sodium pyrophosphate containing protease/phosphatase inhibitor cocktail, pH 7.4) using a hand homogenizer, followed by centrifugation at 1500 rpm for 5 minutes at -20°C. The supernatants from these homogenizations and centrifugations were combined and then were ultra-centrifuged at 13000 rpm for 15 minutes at 4°C. The pellet formed from this process contained the target protein, and it was resuspended in ice-cold homogenization buffer, aliquoted, and then stored at -80°. Protein concentration was estimated via Bradford assay.

2.2.3.2 SDS-PAGE

Isolated protein samples were diluted with 4x Laemmli Sample Buffer (Bio-Rad #1610747) with 5% β-mercaptoethanol and then denatured via boiling at 95°C. 20 mg protein was added to each well of an AnykD Criterion TGX Precast Midi Protein Gel, 26 well, 15 µL (Bio-Rad #5671125).

2.2.3.3 Immunoblot

After gel electrophoresis, the protein is transferred to a nitrocellulose membrane via the Criterion blotter. The membranes are then incubated with the following antibodies: Cannabinoid receptor 1 (GeneTex #GTX100219), Cannabinoid receptor 2 (GeneTex #GTX23561), anti-rabbit HRP (GeneTex #GTX221666-01), anti-goat HRP, and anti-mouse HRP. The incubated membranes were then imaged via the Bio-Rad EZ gel documentation system.

2.2.4 Behavioral paradigms

2.2.4.1 Drug treatment

For the resident-intruder aggression paradigm, ultra-low-dose (0.03 mg/kg) THC or vehicle was administered 20 minutes before habituation and testing. For the AM251 tests, 1.0 mg/kg was administered 10 minutes before THC injection. For the open field and elevated plus maze, ultra-low-dose or low-dose (0.3 mg/kg) THC or vehicle was administered 35 minutes before the open field/elevated plus maze testing. For the riluzole aggression tests, 1.0 mg/kg riluzole was administered immediately before a 10 minute habituation.

2.2.4.2 Tube Dominance Test

To assess the generation of dominance between the two groups, ES-MAOA^{Neo} and ES-WT from the same litter are paired for this test on PND 23. The Tube Dominance Test takes place within a 12-inch transparent plastic tube at 75°F and 400 lux. The test mice enter into either side of a 12-inch transparent plastic tube, they meet at the center of the tube, and the more dominant mouse

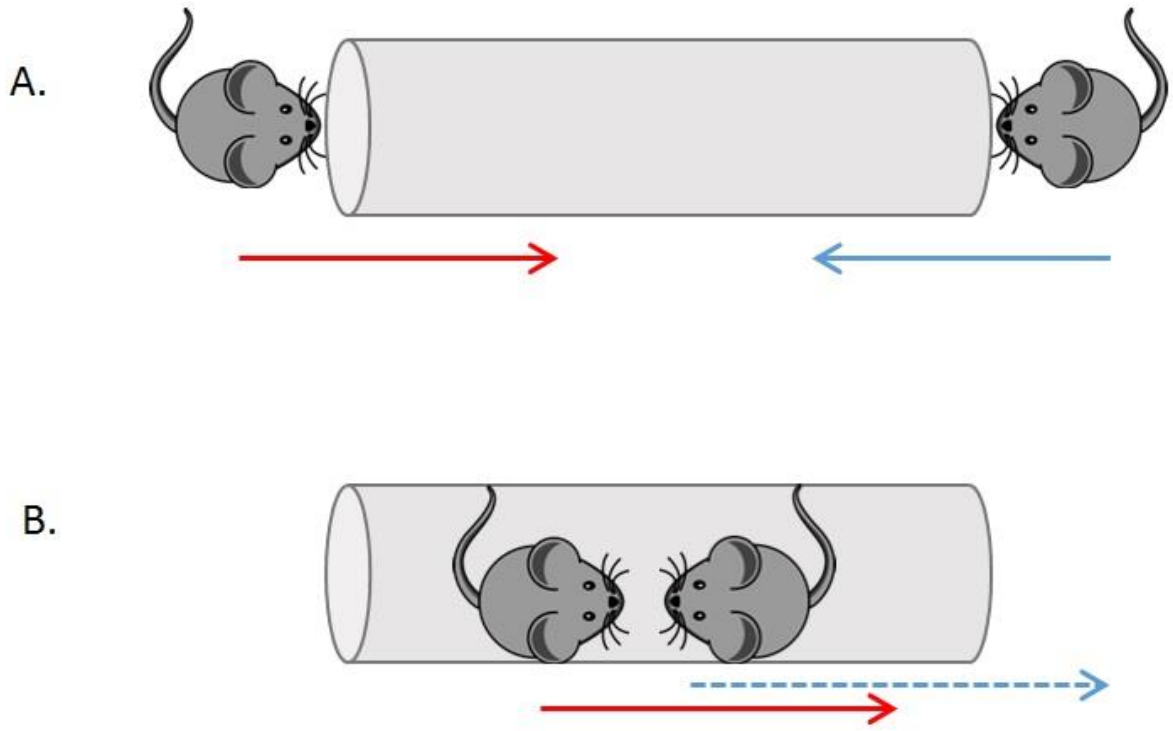


Figure 2.2 Diagram of the Tube Dominance Test. (A) Both test mice enter the tube, but (B) the subservient mouse is forced to move backward to exit the tube.

forces the subservient mouse to back out of the tube. Pairs that did not meet or interact in the center of the tube were excluded from the dominance analysis. Results for this test were analyzed via the sign test.

2.2.4.3 Rat Risk Assessment

The purpose of this test is to assess rodent risk assessment between the various animal groups: NS-MAOA^{Neo}, NS-WT, ES-MAOA^{Neo}, and ES-WT. The Rat Risk Assessment takes place within the T-maze, at 75°F and 3 lux, and consists of two phases: training and testing. For the training phase, the test mouse is placed within an enclosed 'starting' chamber, on one branch of the T-maze for habituation. After 5 minutes, the door is opened and the test mouse may freely explore the rest of the T-maze for 5 minutes duration. The testing phase takes place the next day and is identical to

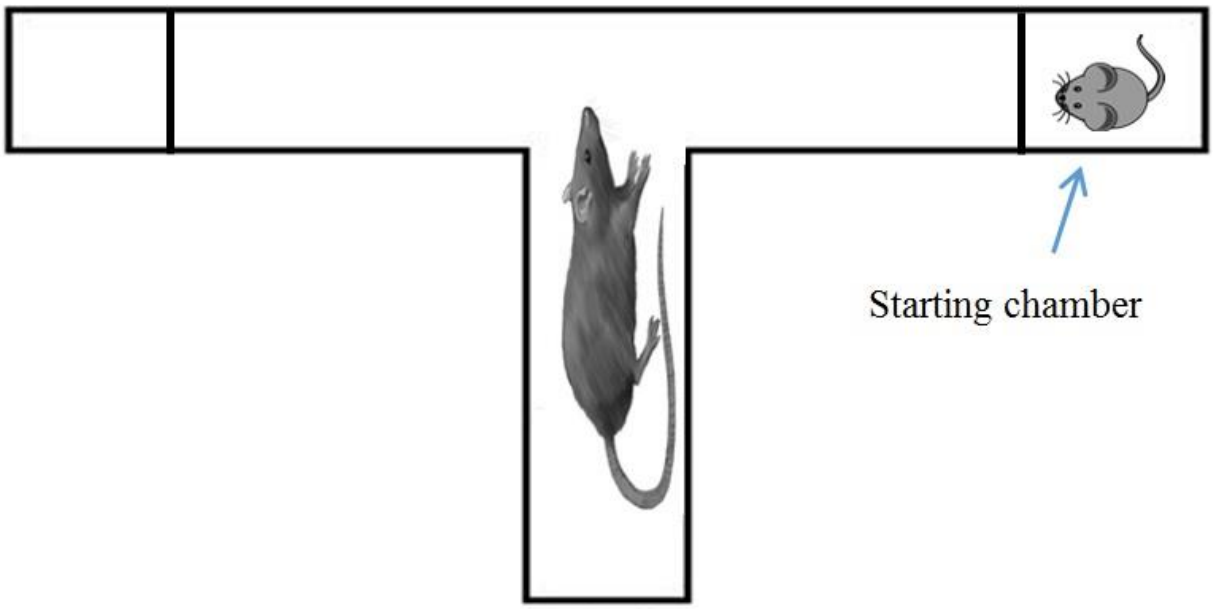


Figure 2.3 Diagram of the Rat Risk Assessment Test. All test mice habituate within the starting chamber for 5 minutes, and then are released to explore the maze with the anesthetized rat.

the training phase, except for the presence of an anesthetized rat within the long arm of the T-maze. Time spent sniffing and climbing the rat was analyzed.

2.2.4.4 Open Field Test

The purpose of the open field test is to assess the general movement and activity levels of rodents. In this specific experiment, we tested whether ultra-low-dose (0.03 mg/kg THC) or low dose (0.3 mg/kg THC) reduced the activity levels of ES-MAOA^{Neo} or ES-WT mice. The paradigm takes place within a box, 40 cm x 40 cm x 40 cm, at 10 lux (in the center) and 75°F. The test mouse was placed into the box 35 minutes after drug treatment, for 10 minutes duration. Total movement,

meandering, and center time was measured via Ethovision, and then analyzed.

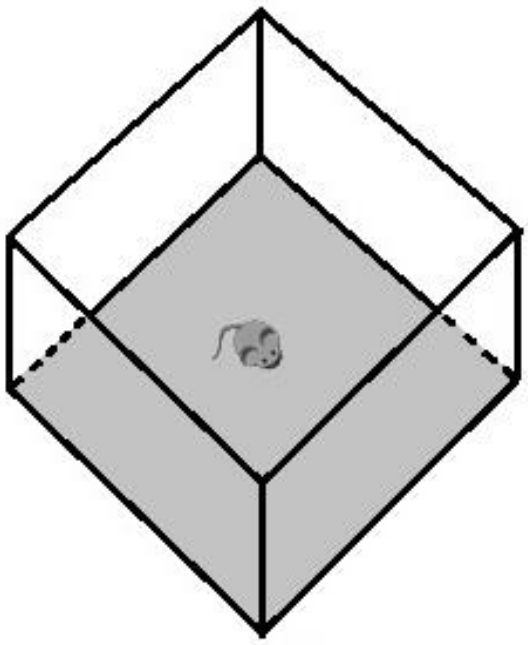


Figure 2.4 Diagram of the Open Field Test. The test mouse is placed in the center of the box and is allowed to freely explore the chamber.

2.2.4.5 Elevated Plus Maze Test

The purpose of the elevated plus maze test is to assess the risk assessment and anxiety-related behavior of rodents. In this experiment, we tested whether ultra-low-dose (0.03 mg/kg THC) or low dose (0.3 mg/kg THC) reduced either of these parameters in ES-MAOA^{Neo} or ES-WT mice. The elevated plus maze contains two open arms and two closed arms. The lighting in the open arms is 2 lux and the temperature is 75°F. The test mouse is placed in the center of the maze, facing an open arm, and is allowed to freely explore the maze for 10 minutes. Time spent in arms

and frequency and latency of entering, as well as frequency of head dips, were analyzed.

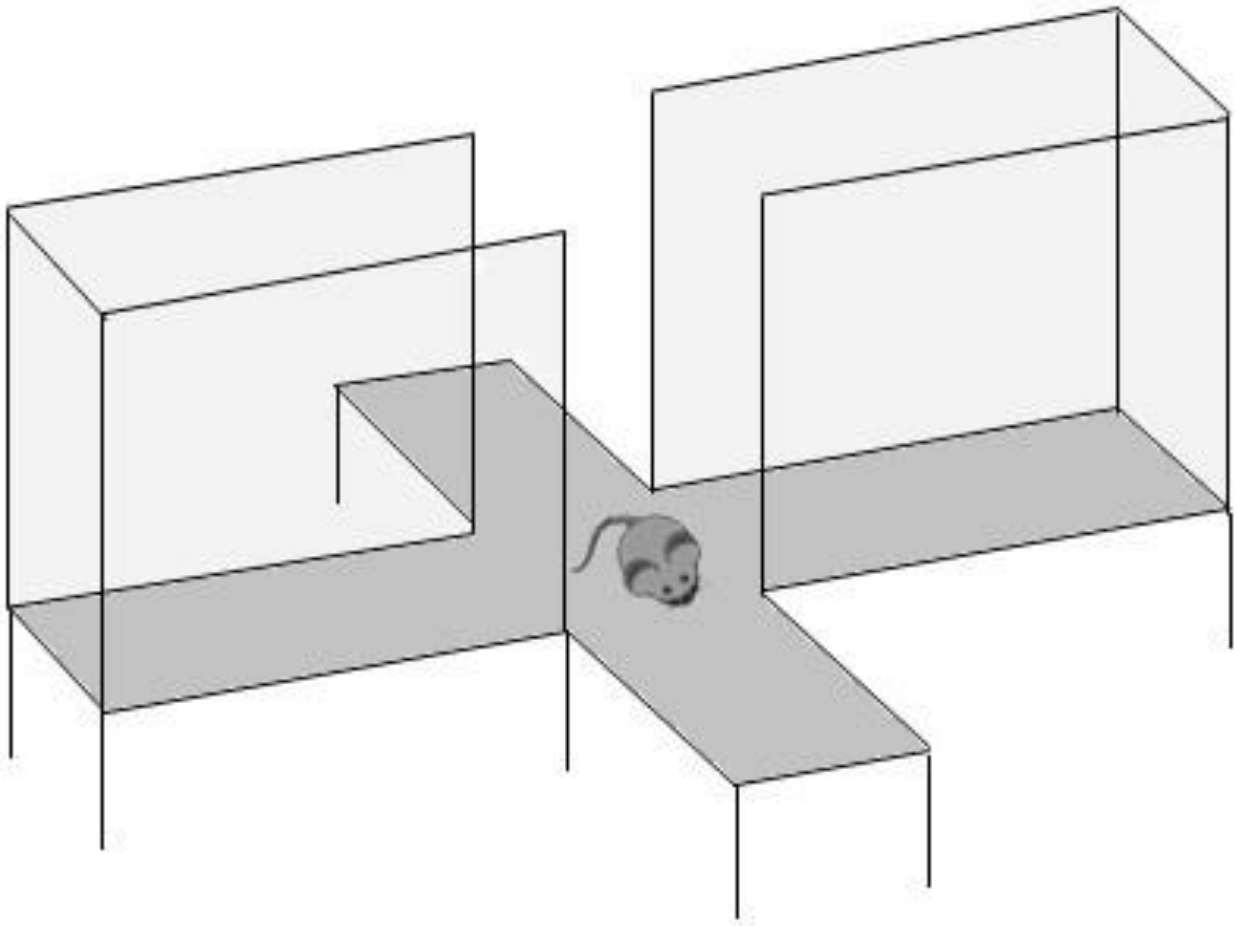


Figure 2.5 Diagram of the Elevated Plus Maze test. The test mouse is placed directly in the center of the maze, while facing the open arm, and is allowed to freely explore the maze.

2.2.4.6 Resident-Intruder Rodent Aggression Paradigm

As the name implies, this paradigm measures aggression by allowing a test mouse to freely express aggressive responses to a home cage intruder (a natural stimulus). The test occurs at 3 lux, 75°F, for 10 minutes. The intruder is placed in the cage, opposite the test mouse. In the first experiment, we tested whether low dose THC (0.03 mg/kg, IP) increased or reduced aggression in ES-MAOA^{Neo}, using ES-WT and vehicle treated controls. For these tests, the frequency, duration, and latency of attacking was measured and analyzed via 2-way ANOVA, with Tukey's procedure as a

post-hoc tool. However, the riluzole experiment utilizes a two-tailed Student's t-test for data analysis.

2.3 STATISTICAL ANALYSIS

In this study, groups were formed from two variables (ES/NS conditions and MAOA^{Neo}/WT genotypes, or MAOA^{Neo}/WT genotypes and drug/vehicle treatment). Therefore, 2-way analysis of variance was done for a majority of data, except where noted. *Post-hoc* analysis was performed when appropriate via Tukey's procedure.

3 RESULTS

3.1 NEUROCHEMICAL ANALYSIS

To investigate how cannabinoid receptor content changes in response to the interaction between early life stress and low transcriptional MAOA genotype, we exposed the both MAOA^{Neo} and WT test mice to either ES or NS conditions, then isolated three brain regions for western blot analysis.

3.1.1 ES-MAOA^{Neo} interaction upregulates hypothalamic CB1 in mice

The hypothalamus is a crucial region that regulates aggressive behaviors; activity in this region has been shown to directly stimulate fighting in animals. To test whether the ES-MAOA^{Neo} interaction changes cannabinoid changes, CB1 and CB2 receptor content was measured via immunoblotting. There was a highly significant interaction in hypothalamic CB1 (Fig. 3.1 A) ($F(1,27)= 16.60$; $p<0.001$), though there was no main effect of genotype ($F(1,27)= 0.6755$; NS) or ES condition ($F(1,27)= 2.860$; NS). *Post-hoc* analysis revealed that ES-MAOA^{Neo} contains higher levels of CB1 compared to NS-MAOA^{Neo} ($p<0.01$) or ES-WT mice ($p<0.01$). There was neither

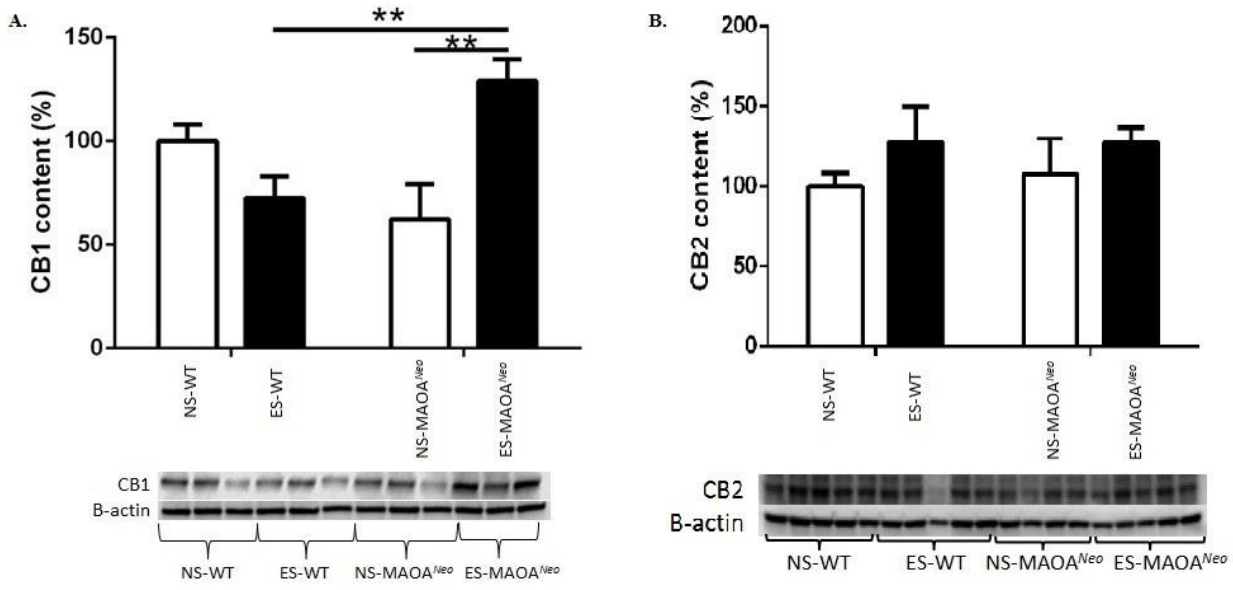


Figure 3.1 ES-MAOA^{Neo} interaction upregulates hypothalamic CB1. The expression of (A) CB1 and (B) CB2 content are shown. Data were normalized to the internal loading control, β-actin, and to the NS-WT group. Representative blot shown. Data are shown as mean ± standard error of the mean, n=3, **p<0.01.

an interaction in CB2 content (Fig. 3.1 B) ($F(1,29)= 0.6231$; NS) nor main effect of genotype ($F(1,29)= 3.494$; NS) or ES condition ($F(1,29)= 0.5194$; NS).

3.1.2 Amygdalar CB1 is upregulated in MAOA^{Neo} mice

The amygdala is well-known for its role in externalized and aversive behavior. Cannabinoids have been recently explored for their biphasic effects on anxiety and fear by working in this area. Therefore, it was hypothesized that there is an upregulation of cannabinoid receptors in ES-MAOA^{Neo} mice. However, there was neither an interaction in CB1 content (Fig. 3.2 A) ($F(1,26)= 0.01308$; NS) nor main effect of ES condition ($F(1,26)= 0.1352$; NS). Conversely, there was an increase in CB1 in MAOA^{Neo} mice compared to WT mice ($F(1,26)= 4.963$; $p<0.05$). There was neither an interaction in CB2 content (Fig. 3.2 B) ($F(1,26)= 0.05353$; NS) nor main effect of genotype ($F(1,26)= 0.03820$; NS) or ES condition ($F(1,26)= 0.1675$; NS).

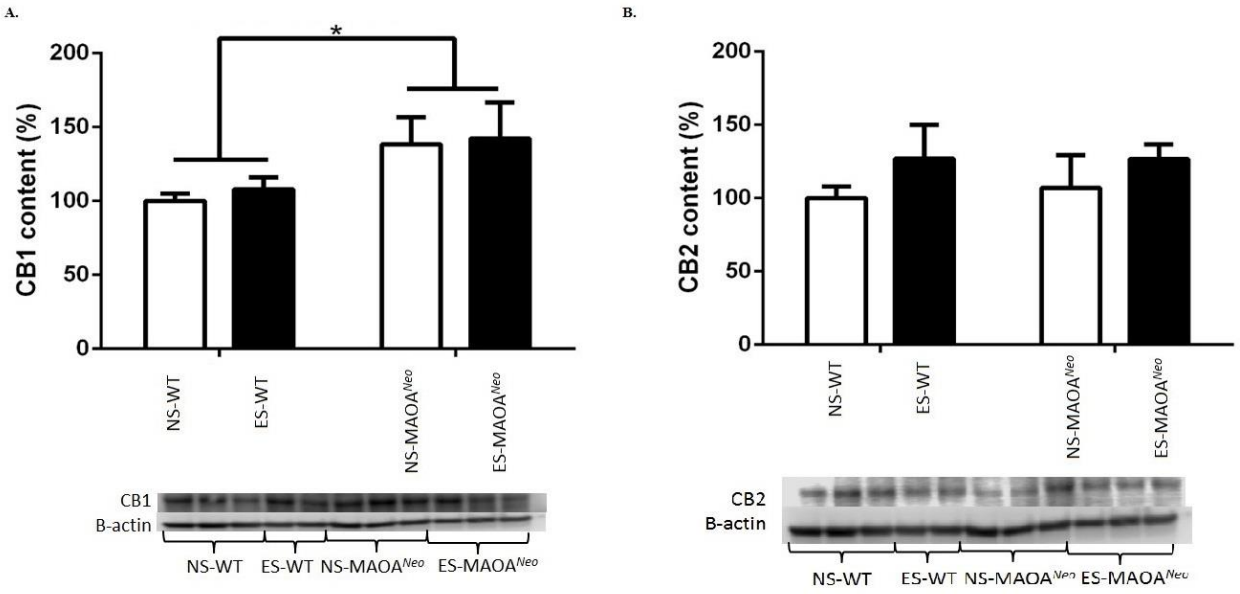


Figure 3.2 Amygdalar CB1 is elevated in ES-MAOA^{Neo} mice. The expression of (A) CB1 content and (B) CB2 content are shown. Data were normalized to the internal loading control, β-actin and NS-WT group. Representative blot shown. Data are shown as mean ± standard error of the mean, n=3, *p<0.05.

3.1.3 Midbrain CB1 content does not change in response to ES-MAOA^{Neo} interaction

At a dose of 1 mg/kg, THC is known to induce conditioned place preference. It is thought that this effect is mediated by activation of CB1 within the ventral tegmental area, the neurons that project dopamine in the mesolimbic system. An upregulation of CB1 within the midbrain of pathologically aggressive individuals would explain their association with cannabis consumption. Therefore, it was hypothesized that midbrain CB1 in ES-MAOA^{Neo} mice would be upregulated. Surprisingly, there was neither an interaction in CB1 receptor content (Fig. 3.3) ($F(1,12)= 0.6543$; NS) nor main effect of genotype ($F(1,12)= 0.6353$; NS) or ES condition $F(1,12)= 0.0005248$; NS).

3.2 BEHAVIORAL ANALYSIS

The neurobiological findings suggest that the ES-MAOA^{Neo} interaction leads to neurobiological changes within the aggression circuit that may predispose these animals to THC sensitivity. The next step in this line of research was to determine the role of THC in the expression of aggression,

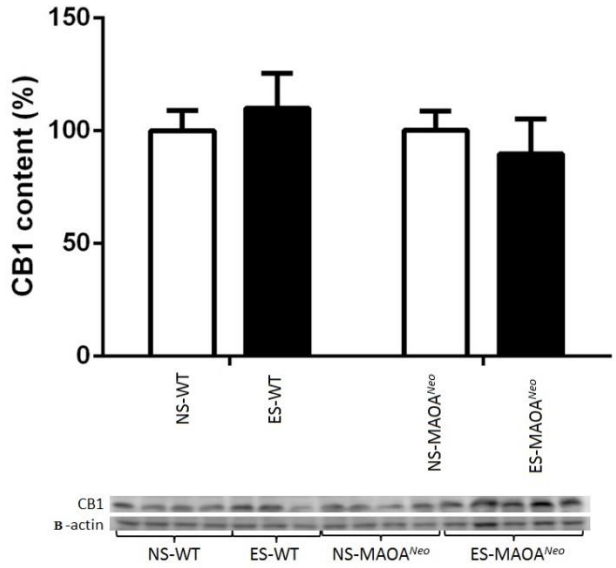


Figure 3.3 ES-MAOA^{Neo} interaction does not induce changes in midbrain CB1. Data were normalized to the internal loading control, β-actin and NS-WT group. Representative blot shown. Data are shown as mean ± standard error of the mean, n=2.

the potential role of CB1, and if general changes in locomotion or anxiety-related behaviors could account any change in aggression manifestation. Given that our laboratory's previous aggression research with the ES-MAOA^{Neo} model was done in adulthood, and that the clinical literature suggests that cannabis consumption and impulsive aggression greatly overlap in adolescent years, it was critical to verify the effects of THC at both juvenile and adult life stages.

3.2.1 ES-MAOA^{Neo} mice are not more dominant than ES-WT littermates

To determine whether ES-MAOA^{Neo} mice develop aggressive tendencies as a result of social hierarchical interactions with ES-WT littermates, the relative dominance was tested by matching juvenile male ES-MAOA^{Neo} mice with their male ES-WT littermates. Although there is a clear trend of ES-MAOA^{Neo} dominance over ES-WT, the result is not significant (Fig. 3.4) (Sign test: test statistic = 2, critical value = 1; NS).

3.2.2 Ultra-low-dose THC reduces aggression in ES-MAOA^{Neo} mice

The early neurobiological finding that CB1 is upregulated in the hypothalamus of ES-MAOA^{Neo} mice suggests that these mice are highly sensitive to the effects of CB1 agonists. Additionally, a key aim of this study is to determine the role of acute cannabis consumption in pathologically

aggressive individuals. Therefore, it was critical to identify whether ultra-low-dose THC (0.03 mg/kg, IP) would increase or decrease the manifestations of aggression in ES-MAOA^{Neo} mice. In the juvenile resident-intruder test, male ES-MAOA^{Neo} test mice showed greatly increased fighting bouts compared to ES-WT controls (Fig. 3.5 A) ($F(1,21)= 13.33$; $p<0.01$), though there was neither an interaction ($F(1,21)= 0.8404$; NS) nor effect of THC treatment ($F(1,21)= 3.806$; NS). Conversely, there was an interaction in fighting duration (Fig. 3.5 A) ($F(1,20)= 4.382$; $p<0.05$), and as expected, ES-MAOA^{Neo} mice fought for a longer duration than ES-WT mice ($F(1,20)= 17.01$; $p<0.001$). However, ultra-low-dose THC reduced fighting duration in both genotype groups ($F(1,20)= 5.528$; $p<0.05$). *Post-hoc* analysis revealed that ultra-low-dose THC-treated ES-MAOA^{Neo} mice fought for smaller duration than vehicle-treated ES-MAOA^{Neo} mice ($p<0.05$), but there is no difference in fighting duration between the vehicle and ultra-low-dose THC ES-WT mice, despite the main effect of THC across both genotypes.

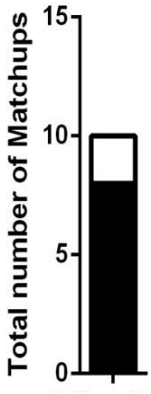


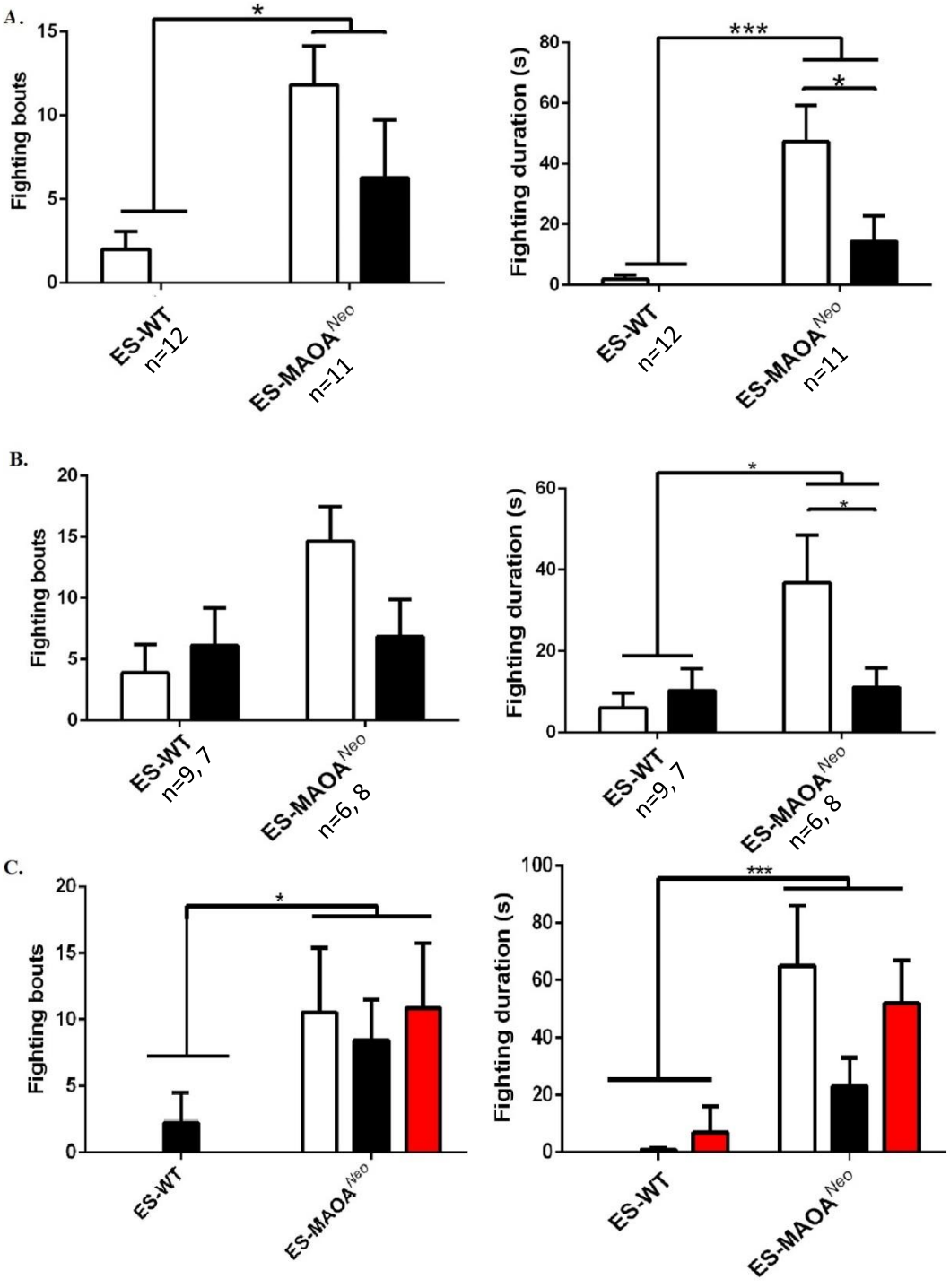
Figure 3.4 The Tube Dominance Test. Matched pairs are made between ES-MAOA^{Neo} and ES-WT littermates. Black represents trials won by ES-MAOA^{Neo}, while white represents trials won by ES-WT.

In the adult resident-intruder tests, male ES-MAOA^{Neo} test mice show neither an interaction in fighting bouts (Fig. 3.5 B) ($F(1,26)= 3.143$; NS) nor main effect of genotype ($F(1,26)= 4.126$; NS) or THC treatment ($F(1,26)= 0.9551$; NS). However, there is a significant interaction in fighting duration (Fig. 3.5 B) ($F(1,26)= 5.661$; $p<0.05$), and also a significant increase in fighting duration of ES-MAOA^{Neo} mice compared to ES-WT mice regardless of THC treatment ($F(1,26)= 6.312$; $p<0.05$), but no main effect of THC treatment ($F(1,26)= 2.888$; NS). *Post-hoc* analysis revealed that vehicle treated ES-MAOA^{Neo} mice fought for a greater duration than ES-WT mice ($p<0.05$), and that ultra-low-dose THC-treated ES-MAOA^{Neo} mice fought for a smaller duration compared to vehicle treated ES-MAOA^{Neo} mice ($p<0.05$).

As discussed, in order to evaluate the role of CB1 in the reduction of aggression by THC, a CB1 antagonist (AM251, 1.0 mg/kg, IP) or vehicle was administered in combination with ultra-low-dose THC to another cohort, and then they were subjected to another resident-intruder aggression test. There was neither an interaction in fighting bouts (Fig. 3.5 C) ($F(2,38)= 0.1289$; NS), nor a main effect of drug treatment ($F(2,38)= 0.0004637$; NS), however, the ES-MAOA^{Neo} mice fought more often than ES-WT mice ($F(1,38)= 5.185$; $p<0.05$). There was neither an interaction in fighting duration (Fig. 3.5 C) ($F(2,52)= 1.073$; NS), nor the main effect of drug treatment ($F(2,52)= 1.136$; NS), however there was a significant increase in the fighting duration of ES-MAOA^{Neo} mice compared to ES-WT mice ($F(1,52)= 13.46$; $p<0.001$). It is important to note that the n value is low for this experiment (the ES-MAOA^{Neo} THC-treated group and THC/AM251 combination group both had n=4), given the number of groups, and that this experiment should be repeated in the future with an appropriate n value per group. This, together with the other experiments that show THC reduces aggression, suggests that CB1 solely mediates the anti-aggressive effect of THC, or it works in concert with other cannabinoid receptors to some degree.

THC is hypothesized to reduce aggression via activation of CB1 on glutamatergic neurons within the aggression circuit. The resulting effect, DSE, reduces glutamate release and reduces activation of downstream neurons. To illustrate the role of glutamate release in aggression, ES-MAOA^{Neo} mice were treated with riluzole (an inhibitor of glutamate release). There was a significant reduction in fighting bouts following riluzole treatments (Fig. 3.9) ($t= 3.548$, $df= 13$; $p<0.01$). Additionally, there was a significant reduction in fighting duration following riluzole treatment compared to vehicle treatment (Fig. 3.9) ($t= 5.083$, $df= 13$; $p<0.001$).

Figure 3.5 Ultra-low-dose THC reduces aggression in ES-MAOA^{Neo} mice. Each line shows the frequency and duration of fighting in the resident intruder paradigm after treatment with 0.03 mg/kg THC (black), 0.03 mg/kg THC in combination with 1.0 mg/kg AM251 (red), or vehicle. (A) Juvenile ultra-low-dose THC test, (B) adult ultra-low-dose THC test, and (C) juvenile ultra-low-dose THC (n=7) and combination AM251/ultra-low-dose THC (n=5) test. Data are shown as mean ± standard error of the mean. *p<0.05, ***p<0.001.



3.2.3 ES-MAOA^{Neo} mice exhibit impaired risk assessment

To show whether or not the ES-MAOA^{Neo} interaction reduces risk assessment, we submitted the test mice of NS and ES conditions, and also MAOA^{Neo} and WT genotype, to the rat risk assessment test. There was neither an interaction in rat sniffing duration (Fig. 3.6 A) ($F(1,33)= 0.2921$; NS) nor a main effect of genotype ($F(1,33)= 3.335$; NS) or condition ($F(1,33)= 1.426$; NS). However, there was an interaction in rat climbing (Fig. 3.6 A) ($F(1,29)= 5.282$; $p<0.05$) and an increase in climbing as a result of both MAOA^{Neo} genotype ($F(1,29)= 6.040$; $p<0.05$) and ES conditions ($F(1,29)= 9.459$; $p<0.01$). *Post-hoc* analysis revealed that ES-MAOA^{Neo} test mice climbed the rat more than NS-MAOA^{Neo} mice ($p<0.01$), ES-WT mice ($p<0.05$), or NS-WT mice ($p<0.01$). There was neither an interaction in fecal boli count (Fig. 3.6 A) ($F(1,32)= 0.9929$; NS) nor main effect of ES condition ($F(1,32)= 0.5019$; NS), however, MAOA^{Neo} test mice defecated fewer times than WT test mice($F(1,32)= 4.228$; $p<0.05$). This suggests that WT mice were either more aware of the threat that the predator posed, or were perhaps more afraid.

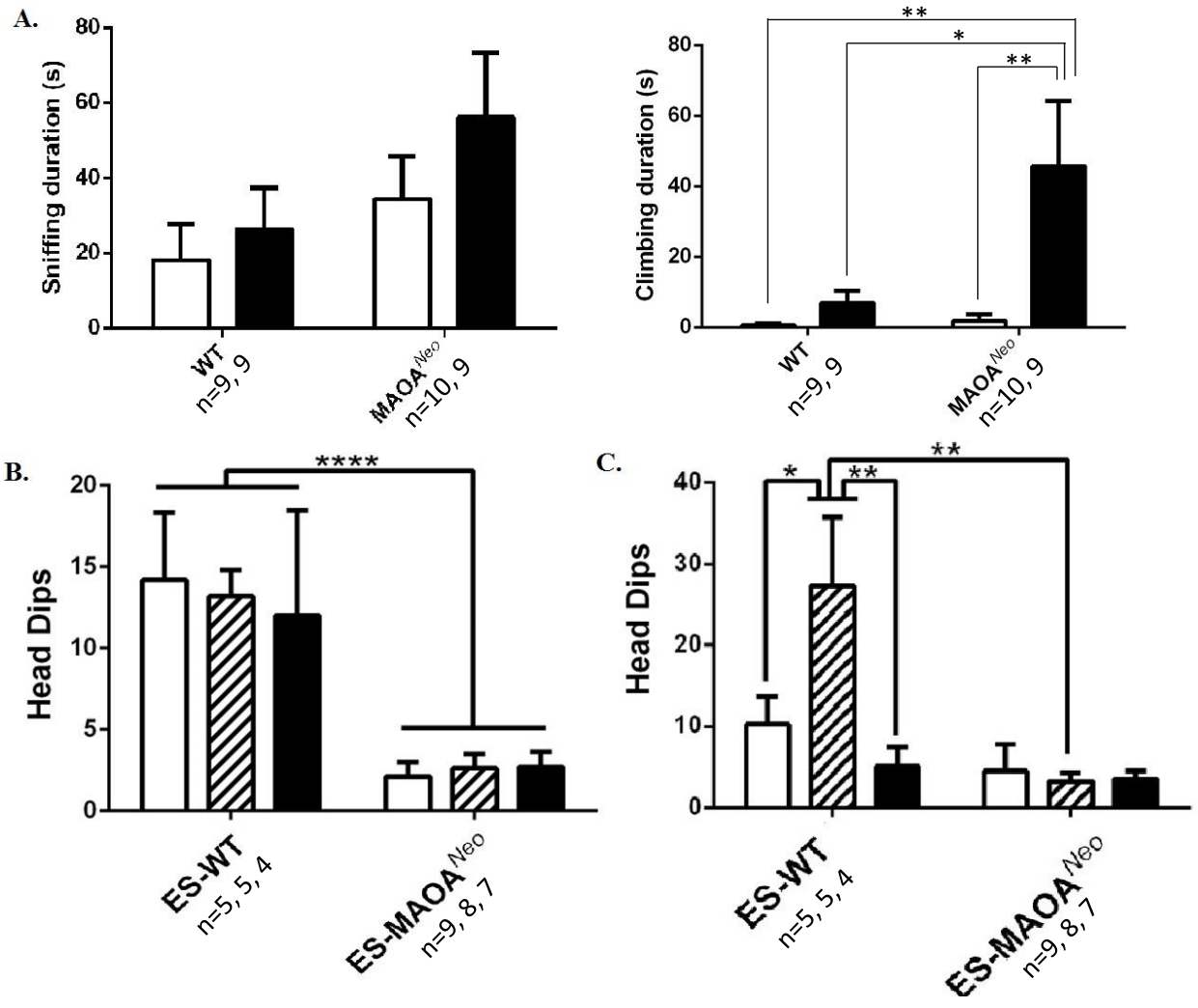


Figure 3.6 ES-MAOA^{Neo} mice exhibit impaired risk assessment, unrecovered by ultra-low-dose THC. (A) Sniffing and climbing duration as counted during the rat risk assessment test, black representing ES condition. (B) Head dips counted during the juvenile elevated plus maze test and (C) head dips counted during the adult elevated plus maze test. Striped represents ultra-low-dose THC treatment (0.03 mg/kg, IP) and black represents low dose THC (0.3 mg/kg, IP). Data are shown as mean ± standard error of the mean. *p<0.05, **p<0.01, ****p<0.0001.

Considering that ultra-low-dose THC reduces aggression in ES-MAOA^{Neo} mice, it is hypothesized that the reduction in aggression is conferred by an increase in risk assessment. Rodent head dips in the elevated plus maze are a measure of risk assessment, so this parameter was examined. Juvenile ES-MAOA^{Neo} mice did not undergo as many head dips as ES-WT test mice regardless of THC treatment (Fig. 3.6 B) ($F(1,32)= 29.04$; $p<0.0001$). However, there was neither an interaction in head dips ($F(F2,32)= 0.1694$; NS), nor a main effect of THC ($F(2,32)= 0.09037$; NS).

In the adult elevated plus maze test, there was an interaction in head dips (Fig. 3.6 C) ($F(2,21)= 4.368$; $p<0.01$), but also there was a reduction of head dips in ES-MAOA^{Neo} mice compared to ES-WT controls ($F(1,21)= 9.611$; $p<0.01$). *Post-hoc* analysis revealed that ultra-low-dose THC increased head dips in ES-WT mice compared to low-dose THC-treated ES-WT mice ($p<0.05$) and ultra-low-dose THC-treated ES-MAOA^{Neo} mice ($p<0.01$).

3.2.4 Ultra-low-dose THC does not induce locomotor effects

Another possible explanation for the reduction in aggression by ultra-low-dose THC is that this dose produces a reduction in general locomotion. While this is highly unlikely to occur at this dose in the ES-WT mice, this possibility was important to test for, especially considering that other experiments posit ES-MAOA^{Neo} mice are sensitive to the effects of ultra-low-dose THC. To test for this possibility, juvenile and adult test mice received ultra-low-dose THC (0.03 mg/kg, IP), low dose THC (0.3 mg/kg, IP), or vehicle, and were subjected to an open field test. In the juvenile open field test, there was neither an interaction in total movement (Fig. 3.7 A) ($F(2,29)= 1.342$; NS) nor main effect of genotype ($F(1,29)= 2.746$; NS) or THC treatment ($F(2,29)= 1.168$; NS). The ES-MAOA^{Neo} mice meandered more than ES-WT littermates (Fig. 3.7 A) ($F(1,29)= 8.631$; $p<0.01$), independent of THC treatment ($F(2,29)= 0.7236$; NS). There was no interaction in juvenile meandering ($F(2,29)= 0.2565$; NS).

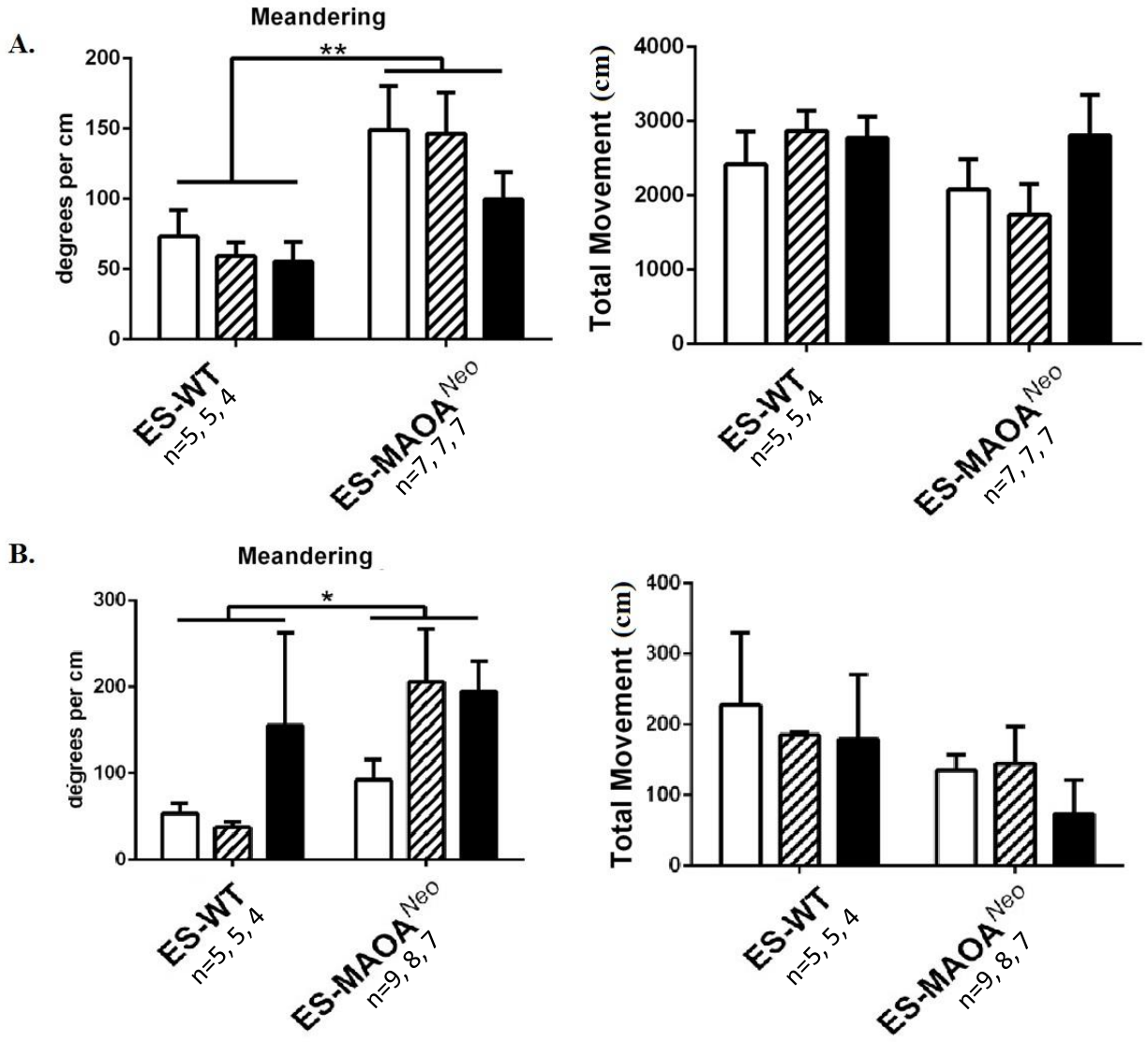


Figure 3.7 Ultra-low-dose THC does not induce locomotor effects. (A) Juvenile meandering and total distance moved in the open field test. (B) Adult meandering and total distance moved in the open field test. Striped represents 0.03 mg/kg THC, black represents 0.3 mg/kg THC. Data are shown as mean ± standard error of the mean. *p<0.05, **p<0.01.

In the adult open field test, there was neither an interaction in total movement (Fig. 3.7 B) ($F(2,19)=0.1766$; NS) nor main effect of genotype ($F(1,19)=2.715$; NS) or THC ($F(2,19)=0.4282$; NS), upon treating ES-MAOA^{Neo} or ES-WT mice with low or ultra-low-dose THC. There was neither an interaction in meandering (Fig. 3.7 B) ($F(2,20)=1.333$; NS) nor a main effect of THC

treatment ($F(2,20)=2.380$; NS), but ES-MAOA^{Neo} meandered to a greater extent ($F(1,20)=4.726$; $p<0.05$).

3.2.5 Ultra-low-dose THC does not influence anxiety-related behavior

It is also possible that THC-induced reduction of aggression occurs because of an increase in anxiety-related behavior. The argument could be made that an anxious mouse or a mouse in fear would be less likely to engage in aggressive behavior. To test for this, juvenile and adult test mice received ultra-low-dose THC (0.03 mg/kg, IP), low dose THC (0.3 mg/kg, IP), or vehicle, and were subjected to an open field and elevated plus maze test. Though the open field test is a well-accepted paradigm that measures changes in locomotion, center entries and duration may be considered a measure of anxiety-related behavior. In other words, a reduction in thigmotaxis in the open field test may be interpreted as a reduction in anxiety-related behavior. In the juvenile open field test, there was neither an interaction in center entries (Fig. 3.8 A) ($F(2,29)= 0.4168$; NS) nor a main effect of THC treatment ($F(2,29)= 1.772$; NS) or genotype ($F(1,29)= 2.467$; NS). There was a reduction in center duration in ES-MAOA^{Neo} mice (Fig. 3.8 A) ($F(1,29)= 6.955$; $p<0.05$), though there was neither an interaction ($F(2,29)= 0.6703$; NS) nor THC treatment effect ($F(2,29)= 0.3019$; NS). There was neither an interaction in fecal boli count (Fig. 3.8 A) ($F(2,29)= 0.1088$; NS) nor an effect of THC treatment ($F(2,29)= 0.4975$; NS) or genotype ($F(1,29)= 0.6396$; NS).

In the juvenile elevated plus maze test, there was neither an interaction in center duration (Fig. 3.8 A) ($F(2,32)= 0.7681$; NS), nor a main effect of genotype ($F(1,32)= 0.5451$; NS) or THC treatment ($F(2,32)= 0.6609$; NS). There was neither an interaction in closed arm duration (Fig. 3.8 A) ($F(2,32)= 0.1825$; NS) nor a main effect of genotype ($F(1,32)= 0.1877$; NS) or THC treatment ($F(2,32)= 0.6555$; NS). There was neither a significant interaction in fecal boli count (Fig. 3.8 A) ($F(2,32)= 0.8315$; NS) nor main effect in genotype ($F(1,32)= 3.976$; NS) or drug treatment

(F(2,32)= 1.443; NS). There was neither an interaction in open arm duration (Fig. 3.8 A) (F(2,32)= 0.06731; NS) nor a main effect of genotype (F(1,32)= 3.847; NS) or THC treatment (F(,2,32)= 0.4238; NS). There was neither an interaction in open arm entries (Fig. 3.8 A) (F(2,29)= 0.4481; NS) nor a main effect of genotype (F(1,29)= 2.983; NS) or THC treatment (F(2,29)= 0.3456; NS). Together, this suggests that ultra-low or low dose THC does not induce anxiety-related behavior in juvenile mice of either genotype.

In the adult open field test, ES-MAOA^{Neo} test mice showed a reduction in center time compared to ES-WT littermates (Fig. 3.8 B) (F(1,20)= 13.17; $p<0.01$). There was also a significant interaction in center time (F(2,20)= 6.106; $p<0.01$), *post-hoc* analysis revealed that ultra-low-dose THC reduced center duration in ES-MAOA^{Neo} test mice more than ES-WT littermates ($p<0.01$).

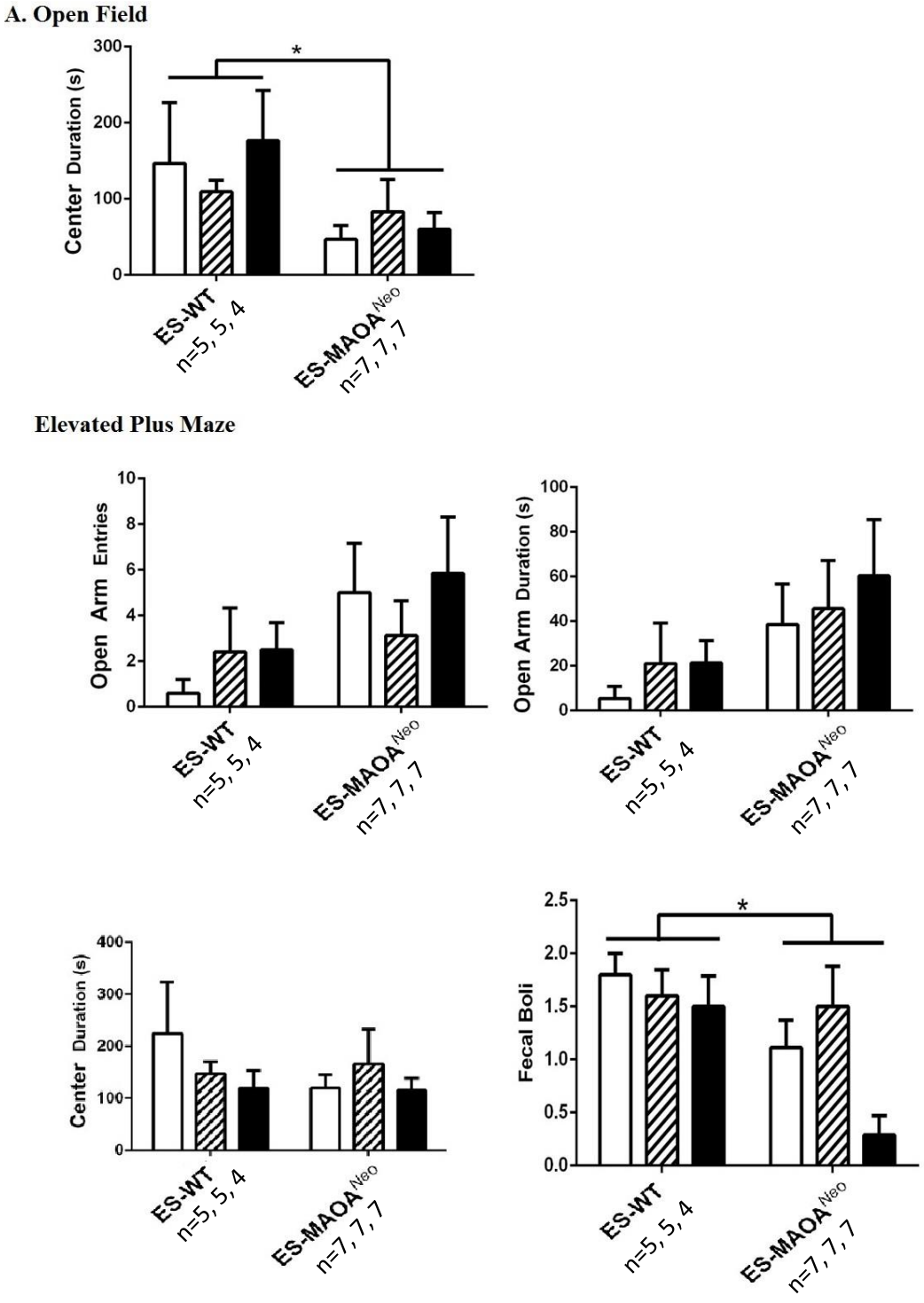
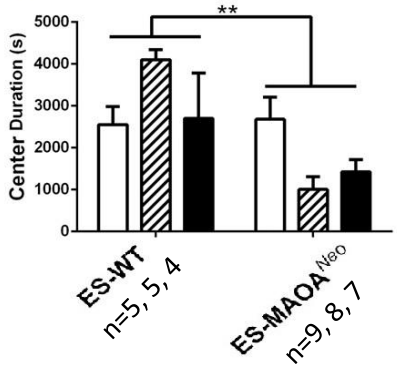
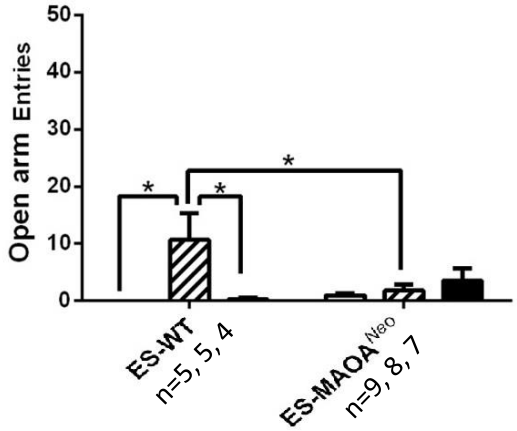


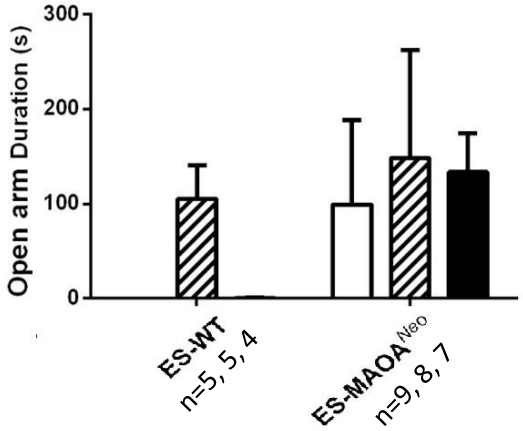
Figure 3.8 Ultra-low-dose THC does not reduce anxiety-related behaviors in ES-MAOA^{Neo} mice. Center duration for the open field test, and open arm entries, duration, center duration, and fecal boli for (A) juvenile test mice and (B) adult test mice. Striped represents 0.03 mg/kg THC, black represents 0.3 mg/kg THC. Data are shown as mean ± standard error of the mean. *p<0.05, **p<0.01. Figure continued on next page.

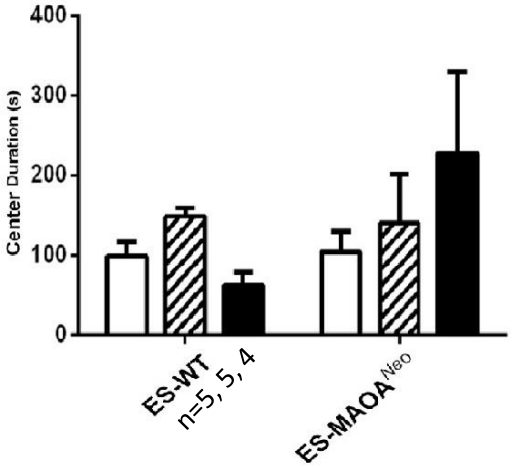
B. Open Field

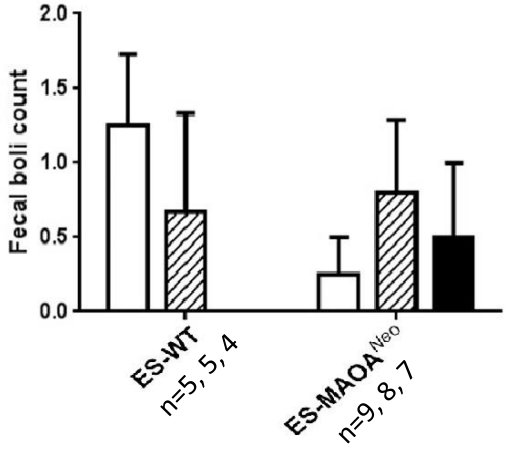


Elevated Plus Maze









In the adult elevated plus maze test, there was neither an interaction in fecal boli count (Fig. 3.8 B) ($F(2,21)= 1.139$; NS) nor a main effect of genotype ($F(1,21)= 0.7541$; NS) or THC treatment ($F(2,21)= 0.5591$; NS). ES-MAOA^{Neo} mice enter the center less than ES-WT mice (Fig. 3.8 B)

(F(1,21)= 14.96; p<0.001), though there is no effect of THC treatment (F(2,21)= 0.2841; NS) nor an interaction (F(2,21)= 0.7745; NS). There was an interaction in open arm entries (Fig. 3.8 B) (F(2,21)= 5.393; p<0.05), *post-hoc* analysis revealed that ultra-low-dose THC-treated ES-WT mice entered the open arm more often than untreated ES-WT (p<0.05), low dose THC-treated ES-WT mice (p<0.05), and ultra-low-dose treated ES-MAOA^{Neo} mice (p<0.05). ES-MAOA^{Neo} mice entered the closed arm less often than ES-WT (Fig. 3.8 B) (F(1,21)=15.77; p<0.001), though there was neither interaction (F(2,21)= 0.1182; NS) nor effect of THC treatment (F(2,21)= 1.064; NS). There was neither an interaction in center duration (Fig. 3.8 B) (F(2,21)= 1.455; NS) nor main effect of genotype (F(1,21)= 1.169; NS) or THC treatment (F(2,21)= 0.3337; NS). Despite the differences in open arm entries, there was neither an interaction in open arm duration (Fig. 3.8 B) (F(2,21)= 0.2369; NS) nor main effect of genotype (F(1,21)= 2.315; NS) or THC treatment (F(2,21)= 0.5874; NS). ES-WT mice had greater closed arm duration than ES-MAOA^{Neo} mice (Fig. 3.8 B) (F(1,21)= 5.284; p<0.05), though there was neither an interaction (F(2,21)= 0.6037; NS) nor main effect of THC treatment (F(2,21)= 1.435; NS).

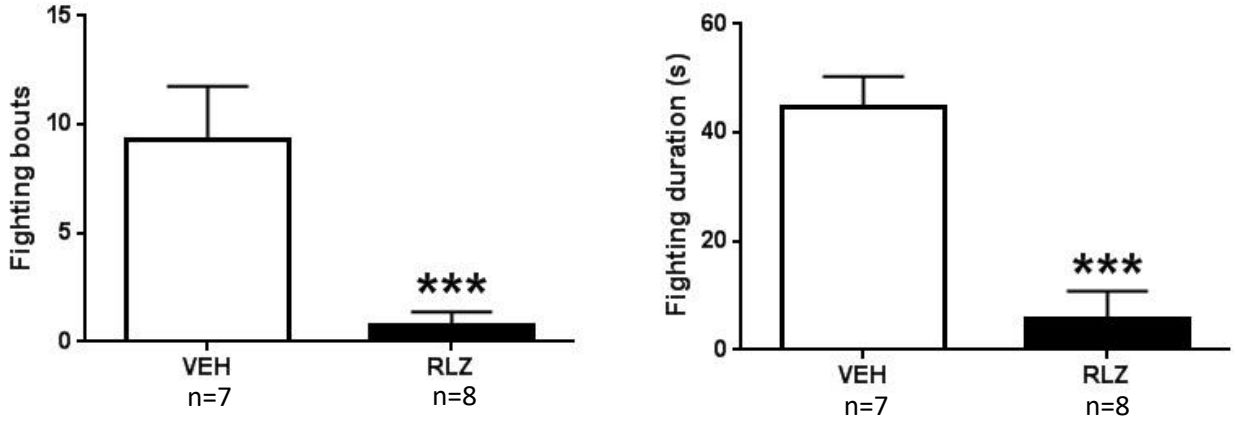


Figure 3.9 Riluzole reduces aggression in ES-MAOA^{Neo} mice. Fighting bouts and duration shown from the resident-intruder test in PND 70 mice. Data are shown as mean ± standard error of the mean. ***p<0.001.

4 DISCUSSION

4.1 INTERPRETATION

The results from the current study clearly show that the ES-MAOA^{Neo} interaction, and the ES condition and MAOA^{Neo} genotype factors alone, produce neurobiological changes within the aggression circuitry. These upregulation events include hypothalamic CB1 increases in response to the ES-MAOA^{Neo} interaction, amygdalar CB1 increases in response to MAOA^{Neo} genotype. The anterior hypothalamus projects glutamatergic signals to the dorsolateral PAG, the key region responsible for the execution of aggression [54-56]. Activation of CB1 within that region may trigger DSE, and reduce glutamatergic activity there. Ultimately, these changes explain the enhanced sensitivity to the anti-aggressive effects of THC of male ES-MAOA^{Neo} mice. Though it is unknown how this effect would be experienced by humans, the hypothalamus engages numerous biological systems that ready an organism for an aggressive encounter [57, 58], and may reduce the aggressive impulses experienced by pathologically aggressive individuals.

It is thought that activation of CB1 receptors on GABAergic interneurons in the midbrain disinhibit the mesolimbic DA system [59]. At a dose of 1 mg/kg, THC induces a shift in conditioned place preference [60]. Further, both MAOA^{Neo} genotype or ES condition are risk factors for cannabis abuse. Surprisingly, midbrain CB1 does not respond to either risk factor. It is possible, however, that changes in receptor expression at mesocorticolimbic terminals, such as the PFC, the ventral striatum, the hippocampus, or another unexamined region may be responsible for the vulnerability to cannabis abuse.

In this study, ultra-low-dose THC reduced aggression in the ES-MAOA^{Neo} group. Despite previous studies showing that THC reduces aggression [61], their rodent models do not reflect the clinical pathology. Translationally speaking, this ES-MAOA^{Neo} group represents pathologically

reactive aggressive individuals, and thus, this study is highly clinically relevant. Ultra-low-dose THC did not induce changes in total movement or meandering in the open field test, nor changes in open arm frequency or duration in the elevated plus maze, suggesting that the reduction in aggression is not due to THC-induced locomotor effects or changes in anxiety-related behaviors. When ultra-low-dose THC was co-administered with AM251, a CB1-receptor antagonist, no significant difference between the ES-MAOA^{Neo} treatments could be found. Overall, this supports that the anti-aggressive effect of THC in this model is indicative of a reduction in aggression circuitry activation. To illustrate this point, blocking glutamate release with riluzole treatment similarly produced an anti-aggressive effect in this ES-MAOA^{Neo} model.

Numerous other studies have shown that activation of CB1 in rodent models leads to an anti-aggressive effect. Early CB1 KO studies show that these mice exhibit out-of-context threat, and greater fighting durations [48]. More recent selective studies show that glutamatergic CB1 KO mice have higher aggression levels compared to GABAergic CB1 KO [38], suggesting that the anti-aggressive effect is mediated by CB1 on glutamatergic neurons, likely by eliminating DSE from the aggression circuit.

4.2 THE ROLE OF CB1 IN PATHOLOGICAL AGGRESSION

Previous studies have consistently found that acute CB1 activation by exogenous agonists tends to produce anti-aggressive effects in most rodent models of aggression [48]. However, these studies only establish the role of CB1 in the aggressive responses and do not truly explain how CB1 activation contributes to *pathological* aggression and cannabis abuse comorbidity. Total CB1 KO rodent models produce effects not limited to the aggression circuitry. For example, the numerous CB1 KO models, including the glutamatergic- and GABAergic-specific CB1 KO, impair DSE (and DSI) as a homeostatic mechanism and induces a state of hyperglutamatergia which has been

shown in other studies to enhance excitability (and perhaps aggression) due to enhanced glutamatergic connections between frontocortical and accumbal regions.

The ES-MAOA^{Neo} interaction is a highly relevant model of male pathological aggression which translates very well to those numerous patients that have the MAOA polymorphism that experienced early life neglect or abuse. Additionally, this study uniquely combines a neurobiological and behavioral approach that allows for the conclusion of the proper function of CB1.

4.2.1 CB1 upregulation: Is it a compensatory or causal mechanism?

Despite the ES-MAOA^{Neo} interaction reliably producing aggression in mice, this model shows higher levels of CB1 within the amygdala and the hypothalamus which are key regions in the execution of aggressive responses. The argument could be made that this model of pathological aggression seemingly contradicts the pattern that lower levels of CB1 produce aggression because the aggressive male ES-MAOA^{Neo} mice contain higher levels of CB1 in the hypothalamus. However, we propose that this upregulation event is a homeostatic event meant to counter a state of hyperglutamatergia within the aggression circuit.

4.2.2 Does THC reduce aggression via CB1 on glutamatergic or GABAergic neurons?

As discussed, CB1 can exist on either a glutamatergic or a GABAergic neuron. One weakness of the current study is that it does not identify the subpopulation of neurons in which CB1 is upregulated in a particular region. Though this would significantly complicate the experimental design, it may yield results that cannot be identified with the current design. For example, the midbrain contains both glutamatergic neurons within the PAG and GABAergic interneurons in the VTA. Even if there is an upregulation in one of these subpopulations, if there is a similar decrease in another subpopulation within the region, then it is possible that no change is found. Despite the

current study finding no difference in CB1, there may be differences in sub-region or neuronal subpopulation. Other studies have found that glutamatergic neuron-specific CB1 KO express intensified threatening and aggressive behavior [38], while GABAergic neuron-specific CB1 KO did not express any difference.

4.2.2.1 CB2

Considering the newly discovered role of CB2 in anxiety and aggression as reported by other groups [62-64], it was initially hypothesized that the ES-MAOA^{Neo} interaction would lead to upregulations in CB2. Despite the lack of upregulation in this model, it is still possible that CB2 contributes to the anti-aggressive effects of THC. When AM251 was co-administered with ultra-low-dose THC for the resident-intruder test, we did not see any difference in aggression between ES-MAOA^{Neo} mice that were AM251 treated and vehicle or THC alone. It is possible that, given the 2-way ANOVA design, a greater sample size is required before an effect is visible. Given that other groups have seen aggressive behaviors in CB2 KO mice, it would be unsurprising if AM251 is unable to abolish the anti-aggressive effects of ultra-low-dose THC in this model.

4.2.3 CB1 as a biomarker

Though several psychosocial metrics can be used to predict pathological aggression [65], there are surprisingly few biological markers available to verify the development of this disorder. Indeed, identification of proper biomarkers would improve opportunities for diagnosis and therapeutic intervention. The current study supports that pathologically aggressive individuals may express higher hypothalamic CB1 in adulthood. This upregulation of CB1 indicates functional consequences in response to GxE factors that modern healthcare may utilize in the identification of pathologically aggressive individuals.

4.2.4 Hypothalamus-specific CB1 activation may be further examined as an anti-aggressive therapeutic target

Advancing our understanding of aberrant aggression pathways will allow us to enhance the efficacy and selectivity of treatments, especially for adolescents; the most vulnerable and largest demographic of pathologically aggressive individuals [66]. Neuroleptics can induce elevated prolactin; a detrimental effect for juveniles. Another common anti-aggressive treatment, fluoxetine, may take several weeks to work and can even further raise their risk of suicide [67]. In contrast, cannabinoid drugs may themselves exert various beneficial effects, including anti-aggressive treatment, if at doses low enough to avoid adverse psychiatric effects.

4.3 FUTURE DIRECTIONS

4.3.1 Could aberrant stress signaling cascades increase pathological aggression?

Marijuana consumption is often accompanied by intense personal stress [68]. Indeed, cannabis consumption typically results in activation of the HPA axis [69]. In light of this connection, several groups have examined this previously missing aspect of cannabis research. As discussed, CB1 has been shown to be a critical mediator of amygdalar activity and aggression [35]. Patel et al. designed an experiment to determine the relationship between amygdalar activity and restraint stress with and without a CB1 agonist [70]. Using Fos as a marker, they showed that while neither 30 minutes of restraint stress nor a CB1 agonist (THC or CP55940) increased amygdalar activity, together they produced a robust increase.

4.4 CONCLUSION

The current study utilizes a highly relevant ES-MAOA^{Neo} mouse model to examine the relationship between pathological aggression and cannabis consumption comorbidity. The experimental design integrated both neurobiological and behavioral approaches and found that the ES-

MAOA^{Neo} interactions upregulate hypothalamic CB1 and may predispose the ES-MAOA^{Neo} mice to a sensitivity to the anti-aggressive effects of THC. The ES-MAOA^{Neo} mice fought for a smaller duration when treated with ultra-low-dose THC; a dose that does not induce locomotor effects or anxiety-related behavior.

Together, these studies suggest that the GxE interactions that lead to pathological aggression in humans leads to neurobiological changes in cannabinoid receptor expression that confer sensitivity to the anti-aggressive effects of marijuana, and may be responsible for the initiation of juvenile cannabis consumption.

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