THE ROLE OF CONTEXT PREFERENCE AND AGE ON SINGLE TRIAL NICOTINE CONDITIONED PLACE PREFERENCE, AND THE ROLE OF DOSING CONTEXT ON MAPK ACTIVATION IN THE VENTRAL STRIATUM

by

Gina Fernandez

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of
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Psychology

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The Role of Context Preference and Age on Single Trial Nicotine Conditioned Place Preference, and the Role of Dosing Context on MAPK Activation in the Ventral Striatum

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

by

Gina Fernandez
Master of Arts
George Mason University, 2011

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DEDICATION

This dissertation is dedicated to my parents, my loving sisters and their cute little puppies.

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I would like to thank my lab mates for their invaluable support, especially Dr. Daniel Ehlinger and Dr. Jennifer Sontag. This project was the developed with the help of Dr. Hadley Bergstrom, to whom I owe a debt of gratitude. I would also like to thank my committee members, Dr. Karl Fryxell and Dr. Craig McDonald for their guidance and support. Finally, I would like to thank my advisor, Dr. Robert Smith, for his role in guiding my graduate career.

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ABSTRACT

THE ROLE OF CONTEXT PREFERENCE AND AGE ON SINGLE TRIAL NICOTINE CONDITIONED PLACE PREFERENCE, AND THE ROLE OF DOSING CONTEXT ON MAPK ACTIVATION IN THE VENTRAL STRIATUM

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George Mason University, 2014

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Adolescents are prone to risk taking behaviors that often lead to experimentation with drugs, such as nicotine. This behavioral profile coincides with a sensitization of the mesocorticolimbic dopamine pathway that receives input from the developing prefrontal cortex. Drug-seeking behaviors are also maintained through reinforced drug- cue associations, and can be tested using conditioned place preference (CPP). CPP is a behavioral measure of drug reward, in which a drug- cue relationship is established through associative learning. Previous work in our lab has demonstrated that adolescent Sprague Dawley rats, but not adults, form single trial nicotine CPP. Developmental differences in the establishment of a nicotine- context association could be due to differences in the cellular mechanisms underlying synaptic plasticity. One molecular substrate critical for the formation of long-term memory, as well as drug related neural plasticity is the mitogen activated protein kinase (MAPK) pathway. We conducted a

series of experiments to examine the relationship between age, nicotine exposure and MAPK activity on reward related behavior. Adult (∼ P70) and adolescent (P28) Sprague Dawley rats were trained for single trial nicotine CPP (0.5 mg/kg), and their initial CPP chamber preference was correlated with the strength of the nicotine-cue relationship. Adolescent rats with relatively higher dark CPP chamber preference (HDP) on day 1 of testing formed single trial nicotine CPP, an effect not seen in HDP adults. Adults with relatively lower dark CPP chamber preference (LDP) on day 1 of testing formed a significant aversion to the white chamber after single trial CPP, an effect not seen in HDP adults. Single trial nicotine CPP was attenuated in HDP adolescents pre-exposed to an MEK inhibitor (SL327; 50 mg/kg) during conditioning. There was no effect of SL327 in LDP adolescents. In a final experiment, adult and adolescent Sprague Dawley rats were exposed to a CPP conditioning session or a single nicotine versus saline injection in the homecage. Brains were processed for immunohistochemistry to visualize phosphorylated MAPK in the nucleus accumbens (NAc) and basolateral amygdala (BLA). In the adolescent NAc shell, there was a trend for increased amounts of pMAPK labeled cells following a nicotine injection in the CPP chamber versus the homecage, an effect not seen in the adult group. There was also a significant increase in the number of pMAPK labeled cells in adults following a saline injection in the CPP versus homecage context, an effect not seen in the adolescent group. In the NAc core, there was a significant increase in pMAPK labeled cells in both age groups after dosing in the CPP versus homecage environment, regardless of drug treatment. In the NAc shell and core, there were no differences in the amount of pMAPK labeled cells between saline or nicotine in

the CPP chamber in either age group, suggesting that pMAPK labeling was not specific to nicotine but may be related to injection stress. There were no effects of drug treatment, dosing context or age on pMAPK labeled cells in the BLA. Our results suggest that context preference can modulate the strength of a drug cue relationship across age groups, and during adolescence, this modulation involves MAPK activity. NAc shell and core MAPK activity may also modulate the response to an arousing stimulus based on context.

INTRODUCTION

Nicotine is one of the most heavily abused addictive substances in the United States, and oftentimes adolescents develop patterns of drug abuse that begin with cigarette use (Breslau et al., 1996; 2001; USDHHS, 2010). As many adult cigarette users began smoking during adolescence, it appears that tobacco use at an early age may be risky in terms of addiction liability (Nelson et al., 2006). Adolescents undergo a series of developmental neural changes that prime their susceptibility for compulsive drug seeking (Berheim et al., 2013; Spear, 2000; Bava & Tapert, 2010). For example, they exhibit a sensitized ventral striatal reward pathway, which increases their perception of the positive affect associated with nicotine (Bava & Tapert, 2010). Previous work from our lab found that adolescent rats establish a learned preference for context (conditioned place preference, or CPP) after only a single nicotine- context pairing, an effect not found in adults (Brielmaier et al., 2007). These results suggest that adolescents form stronger associative memories between nicotine and environmental cues compared to adults.

The formation of long term associative memories, such as the ones involved in CPP, requires activity of molecular signaling cascades that ultimately result in new gene expression and long-term synaptic changes. One of these pathways, the mitogen activated protein kinase (MAPK) cascade, is involved in the establishment of synaptic plasticity associated with long-term memory formation (Adams et al., 2000). Downstream targets

of MAPK phosphorylate transcription factors, such as CREB, which are known to be involved with memory formation in various brain regions (English & Sweatt, 1996; 1997). In general, MAPK works to integrate signals from neural cell receptors to intracellular signaling mechanisms (Sweatt, 2001).

MAPK is involved in a variety of associative learning paradigms, such as fear conditioning (Atkins et al., 1998; Schafe et al., 2000). For instance, deficits in fear extinction observed in adolescent rats are correlated with decreased phosphorylated MAPK (pMAPK) levels in the infralimbic cortex (Kim et al., 2010). Inhibiting MAPK attenuates contextual fear conditioning in mice (Raybuck & Gould, 2007) and cued fear conditioning in rats (Schafe et al., 2000). Disrupting MAPK signaling also leads to deficits in other behavioral learning paradigm, such as Morris water maze performance (Blum et al., 1999; Selcher et al., 1999), indicating the importance of MAPK signaling in behavioral learning models.

MAPK signaling has also been implicated in drug-mediated neural signaling and associative learning (Girault, 2007). pMAPK increases within the striatum in response to cocaine exposure (Valjent et al., 2000), as well as MDMA and tetrahydrocannabinol (Valjent et al., 2004). Nicotine also activates pMAPK in the cortex, nucleus accumbens (NAc) shell, central nucleus of the amygdala (CeA), and the basal nucleus of the stria terminalis (Valjent et al., 2004). Dopamine- 1 (D1) receptors are known to modulate the rewarding effects of drugs, and D1 antagonism blocks pMAPK in response to cocaine exposure. (Valjent et al., 2000). Additionally, systemic injections of SL327, a MAP kinase kinase (MEK) inhibitor, blocked cocaine-induced locomotion (Valjent et al.,

2000). MEK phosphorylates MAPK, and by blocking the activation of MAPK, Valjent found that MAPK signaling is involved addiction related behaviors. Although seemingly ubiquitous, MAPK activity has not been found to modulate all forms of drug related synaptic plasticity, such as ethanol (Groblweski, 2011) or morphine CPP dependence models (Mouledous, 2007).

CPP is a behavioral paradigm that measures the relationship between a stimulus, such as a rewarding drug, and its associated contexts. Most drugs of abuse that support self- administration also induce CPP (Bardo & Bevins, 2000; Tzschentke, 2007). One experimental design that takes into account an animals' preference for a particular context is a biased CPP procedure. The animals' preferred environment is determined during their initial exposure to the CPP apparatus, and drug pairings only occur in the non- preferred chamber (Calcagnetti, 1993; Le Foll & Goldberg, 2009). As mentioned previously, work in our lab found evidence of single trial nicotine CPP induction in early adolescent (P28) but not adult animals (P77) using a biased CPP paradigm (Brielmaier et al., 2007). This age effect is also seen in longer nicotine CPP protocols and across dosing regimens (Le Foll & Goldberg, 2005; Belluzi et al., 2004; Vastola et al., 2002).

Single trial nicotine CPP during adolescence is enhanced by previous exposure to a single stress inducing event and this effect is modulated by corticotropin- releasing factor type 1 receptors (Brielmaier et al., 2012). Brielmaier's results suggest that previous experiences can facilitate the establishment of stronger drug- cue associations, especially during an early developmental time period. In adults, single trial nicotine CPP requires D1 activation within the NAc shell, while inactivation of either D1 or D2 receptors in the

NAc has no effect on single trial nicotine CPP induction (Spina et al., 2006). These results speak to the role of the NAc shell, above that of the NAc core, during the acquisition of nicotine related reward behavior (Lecca et al., 2006; Sellings et al., 2008; D'Souza & Markou, 2014).

As in other behavioral learning paradigms, MAPK signaling has been implicated in some CPP models. Cocaine induced CPP activates pMAPK in the NAc core, a subregion of the accumbens related to reward (Miller & Marshall, 2005). Infusions of U0126, a MEK inhibitor, into the NAc, blocks CPP (Miller & Marshall, 2005). Cocaine CPP also causes increases of pMAPK in the basolateral amygdala (BLA) (Wells et al., 2013). Infusions of U0126 into the BLA, during reconsolidation, also blocks cocaine CPP reinstatement (Wells et al., 2013). Additionally, there are increased levels of pMAPK in the CeA after morphine induced CPP, and infusions of U012 into the CeA abolished place preference behavior (Li et al., 2011). A majority of cells with active pMAPK express NMDA receptors, and the NMDAr antagonist MK-801 suppressed MAPK activation, suggesting a calcium-regulated activation of pMAPK (Li et al., 2011). Nicotine CPP is also dependent on downstream targets of pMAPK, such as CREB and cFOS in the NAc core, prefrontal cortex and ventral tegmental area (VTA) (Pascual et al., 2009), as well as NAc shell (Brunzell et al., 2009). To date, studies that investigate the relationship between MAPK signaling and CPP have focused on adult murine models. Since adolescents are form drug related associative memories more readily than adults, it is important to examine the role of MAPK in memory formation within younger cohorts.

We conducted a series of experiments to test the relationship between age (adolescent versus adults), nicotine conditioning in a single trial CPP protocol and pMAPK labeling. First, we conducted single trial nicotine CPP experiment in adolescent rats, and by selecting for relative CPP dark chamber preference expressed during the first day of testing, we could also induce single trial nicotine CPP in adults. We then ran a set of experiments to classify chamber preference-related behavior with either novel object recognition or elevated plus maze using correlational analyses.

Given the role of MAPK in reward related learning and the attenuation of associative memories following MAPK inhibition, we tested whether inhibition of MAPK, via a systemic injection of SL327, impairs single trial nicotine CPP.

A final set of experiments examined differences in pMAPK expression within the NAc (shell and core) and BLA following a single CPP conditioning trial in both adult and adolescent rats using p44/42 MAPK immunohistochemistry. We studied pMAPK expression in the NAc and BLA because they are critical substrates required for the acquisition of nicotine CPP (Pascual et al., 2009; Hashemizadeh et al., 2014; Spina et al., 2006). To control for nicotine induced pMAPK activation resulting from exposure to novelty in the CPP chamber, another set of experiments examined adolescent and adult rats that were administered a single injection of either nicotine or saline in the homecage setting.

METHODS

Animals

Adolescent (P28) and adult (~P70) male Sprague Dawley rats, obtained from Harlan Laboratories (Indianapolis, IN), arrived one week before testing and were subject to individual handling by the animal care technician. Animals were group housed, and given access to food and water ad libitum. CPP and homecage injections occurred during the animal's light cycle.

Materials

Nicotine hydrogen tartrate salt (Sigma) and 0.9% saline were used for CPP conditioning and homecage injections. Nicotine was administered at a dose of 0.5 mg/kg and pH balanced to 7.4. SL327 (Abcam), an MEK inhibitor, was suspended in 15% DMSO at a concentration of 2.5 mg/ml and administered at 20 ml/kg to achieve a dose of 50 mg/kg. Vehicle solution consisted of 15% DMSO in saline, administered at a dose of 20 ml/kg. Animals were anesthetized with an 80 mg Ketamine HCL/12 mg Xylazine HCL mixture (Sigma Aldrich). Saline, nicotine and ketamine/xylazine were administered at an injection volume of 1 ml/kg body weight. Injections were administered using a $26^{1/2}$ gauge needle.

CPP conditioning took place in a two-chambered conditioned place preference insert (Med Associates, VT) located in a dimly lit room (4-6 lux within the white CPP chamber). The conditioning apparatus consisted of two Plexiglas chambers measuring

21x42x30 cm each. One chamber has black walls with a stainless steel rod floor and black tray paper lining, and the adjacent chamber consisted of white walls with a stainless steel mesh floor and white tray paper lining. There was a black removable guillotine door that separated the two chambers. A camera mounted above the inserts recorded each trial.

Novel object recognition (NOR) was tested within a Plexiglas activity chamber (61 x 61 x 14 cm) constructed in house that was enclosed on all sides and open to recording software from above. The objects used for this task were water bottles of different sizes filled with different colored sand; red, spherical paper weights; and soap dispensers filled with colored sand. These objects had previously been used to successfully test for NOR (unpublished observations).

The elevated plus maze (EPM; Kinder Scientific, CA) consisted of 4 Plexiglas arms measuring 11 x 51 cm. Two arms were enclosed on either side (11 x 51 x 18 cm) and all arms were elevated 86 cm off the ground. The arms were arranged in a 't' configuration, with the open arms facing each other. A camera was mounted over the apparatus and lighting was minimal (4 lux at the arm ends).

CPP Procedure

We used a "biased" place conditioning procedure in which animals were tested for their innate chamber preference and conditioned with nicotine in their non-preferred chamber (Brielmaier 2007; 2008; 2012). Testing consisted of three phases over 4 days: pretest, conditioning, and posttest. On the pretest, adolescent (P28) or adult (~P70) animals were placed in individual hanging wire cages and habituated to the testing room for 20 minutes. Animals were placed in the conditioning apparatus for 15 min with the

guillotine door removed to allow free access between both chambers. Placement into either chamber was counterbalanced.

Conditioning sessions occurred over the following 2 days. Animals were moved to the testing room, and over alternating days, animals randomly assigned to the nicotine group were be injected with 0.5 mg/kg nicotine subcutaneously (s.c.) and immediately placed in their initially non-preferred side or 0.9% saline and placed in their initially preferred chamber. Animals randomly assigned to the saline groups were injected with saline (s.c.) each day before placement in the respective compartment. Conditioning sessions lasted 15 minutes, and the order of sessions (nicotine or saline first) was counterbalanced within the groups.

On the posttest day, a 15-minute, drug-free choice test was conducted to determine preference shifts following conditioning. Conditions were identical to the pretest, with the guillotine door of the apparatus removed to allow animals free access to both chambers. CPP induction was determined via difference scores, which compared the time spent in the white, drug paired chamber on the posttest day versus pretest day. Figure 1 illustrates the single trial nicotine CPP protocol and table 1 denotes the number of animals used in the protocol.

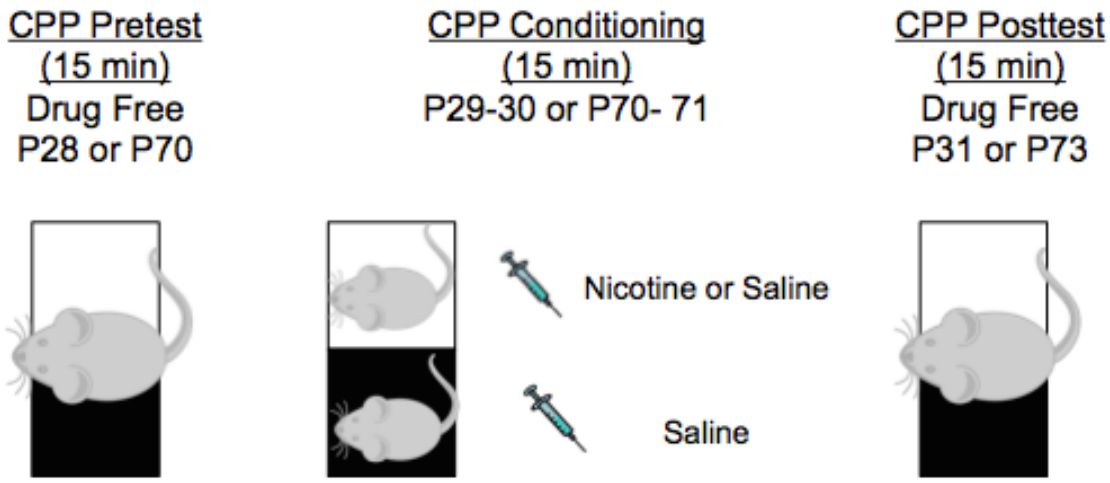


Figure 1: CPP Conditioning Protocol

Innate Behavior (NOR and EPM) and CPP Comparison

In order to define the relative dark CPP chamber preference animals exhibit during day 1 of CPP testing, we compared time spent in the white chamber on day 1 with behavior from novel object preference tests (NOR) and elevated plus maze (EPM). NOR examines aspects of novelty seeking and memory: rodents tend to approach novel objects, and in a NOR test, a rodent maintains the memory of a familiar object used during training, and when a novel object is presented, it preferentially spends time with it (Silvers et al., 2007). To look at the effect of memory and novelty seeking on CPP induction, we used a one trial NOR test (Silvers et al., 2007). At P26, animals underwent a six-minute habituation period in an empty activity chamber and immediately returned to their homecage. Twenty-four hours following the first habituation session, animals were exposed to the activity chamber for three training and one testing session, each of which were six minutes long, separated by one-hour intervals. Session 1 consisted of habituation to the empty activity chamber. During session 2, two identical objects were placed inside

of the chamber, towards the rear corners. The animals were unable to move or climb the objects. Session 3 was a replication of session 2, during which the same configuration of objects used in session 2 was presented. During the test session, one training object was replaced with a novel object. The novel object and corner presentation was counterbalanced. Figure 2 illustrates the NOR protocol. Data for the latency to approach the novel object, total time with the novel object, latency to the training object and total training object time were hand scored by a blind observer. The following day, animals (P28) were exposed to single trial nicotine CPP as described above. CPP pretest time in the white chamber and difference score results were correlated with NOR data to explore the relationship between novelty related memory and nicotine induced CPP.

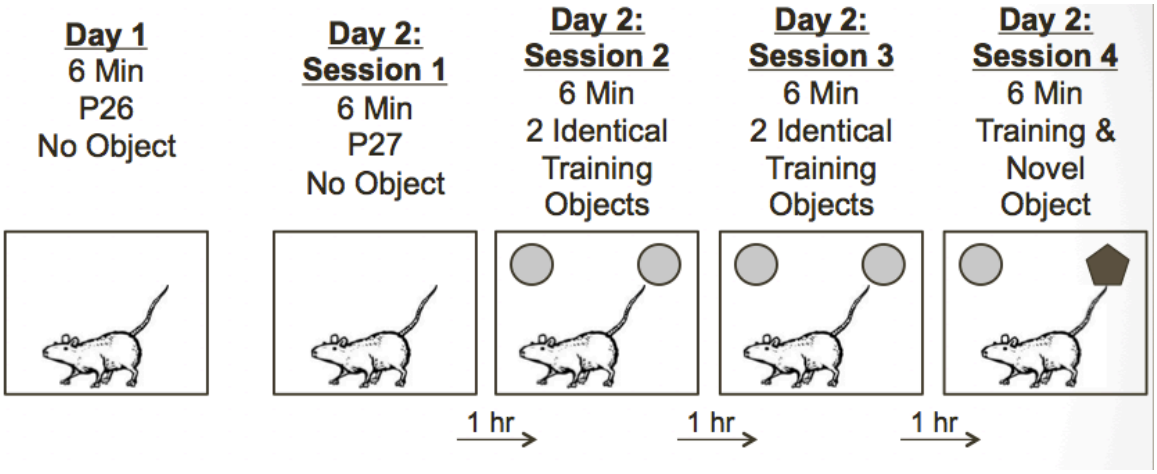


Figure 2: NOR Protocol

To examine at the effect of anxiety on single trial nicotine CPP, we also looked at EPM behavior in adolescence. Sprague Dawley rats (P28) were acclimated to the testing

room in individual hanging wire cages for 15 minutes. A rat was then placed in the center of the EPM maze and allowed to freely explore open and closed arms for a total of 5 minutes (File, 2004). After all animals were tested, they were returned to their group housing for an hour before beginning a single trial nicotine CPP protocol as described above. EPM data was hand scored for open arm entries, closed arm entries, total time in open arms and total time in closed arm. Arm time and arm entry was operationally defined as all four paws in the respective arm. EPM data was then correlated with CPP pretest time in the white chamber and CPP difference scores to investigate the influence of anxiety-like behavior on CPP induction.

MEK Inhibitor and Single Trial Nicotine CPP

To examine the role of MAPK signaling in single trial nicotine CPP, we ran a similar study to the CPP protocol previously described, but exposed adolescent male rats to SL327, an MEK inhibitor, prior to conditioning. On CPP conditioning days 2 and 3 (P29 and 30), 30 minutes prior to either saline or nicotine conditioning, adolescent animals were injected with 50 mg/kg SL327 (intraperitoneal), or vehicle, administered at a volume of 20 ml/kg. Animals were placed back in their hanging wire cages for 30 minutes, after which conditioning proceeded as previously described. Figure 3 illustrates this protocol.

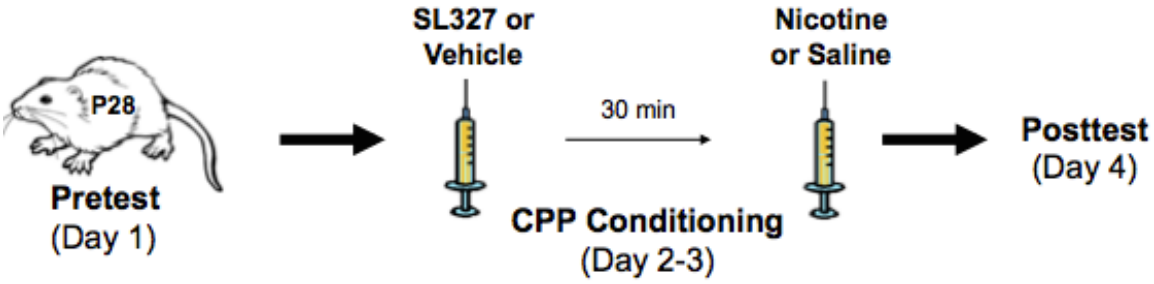


Figure 3: SL327 Dosing Procedure

pMAPK protocol for CPP Conditioning Context

To explore the effects of a single nicotine injection on synaptic activity, measured via pMAPK labeling, we ran a set of experiments in which adult and adolescent male brains were analyzed for p44/42 MAPK immunohistochemistry following drug injection. The first experiment examined the effects of age and drug on pMAPK counts after CPP conditioning. The CPP conditioning protocol is identical to pretest day 1 and conditioning session 1 from the CPP protocol. On day 1, adolescent (P28) and adult (~P70) animals were placed in individual hanging wire cages and allowed to habituate to the CPP testing room for 20 minutes. Animals were then placed in the conditioning apparatus for 15 min with the guillotine door removed to allow free access between both chambers. Animals were returned to their individual cages following the pretest. On day 2, animals were returned to the testing room and habituated for 20 minutes. Animals were then injected with 0.5 mg/kg nicotine (free base, s.c) or 0.9% saline (s.c) and confined to the non- preferred, white chamber for 15 minutes. After conditioning, animals were placed back in their individual hanging wire cages for 15 minutes, after which they were

anesthetized and prepped for histological analysis. Figure 4 illustrates the CPP conditioning procedure for histology.

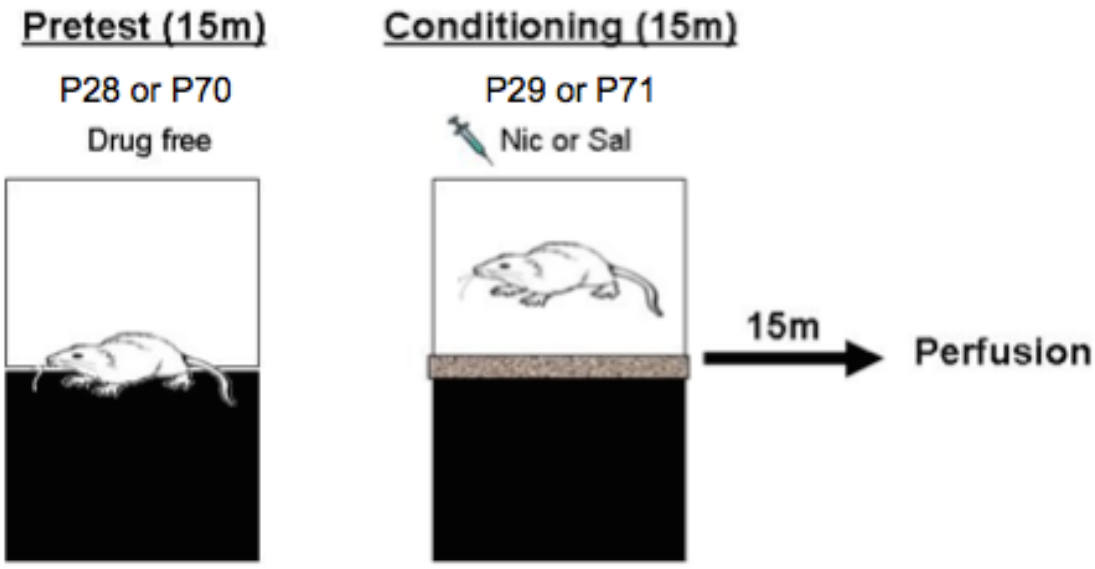


Figure 4: pMAPK protocol for CPP conditioning sessions

pMAPK protocol for HomeCage Injections

The second set of immunohistochemistry experiments controlled for novelty effects on pMAPK labeled cells. Adolescent (P29) and adult (∼P70) rats were injected with 0.5 mg/kg nicotine (free base, s.c) or 0.9% saline (s.c) in their homecage environment. Animals were placed back in their homecage, and thirty minutes following injections, animals were anesthetized and prepped for histological analysis. Figure 5 illustrates the homecage control procedure.

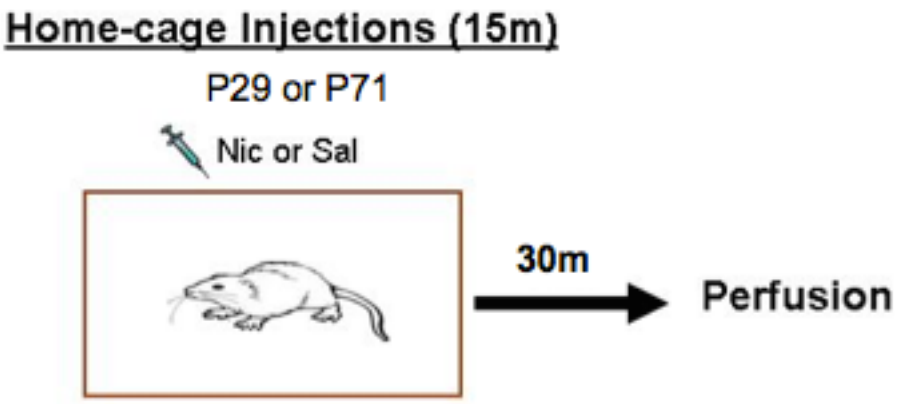


Figure 5: pMAPK protocol for home cage injections

Immunohistochemistry

Brains were perfused with 4% paraformaldehyde (Sigma Aldrich), sectioned at 40 µm (Leica VT100 Vibratome) and placed in 1x phosphate buffered saline (PBS; Fisher) overnight in a 3° C refrigerator. Tissue slices were washed in PBS 5 times and blocked in a .02% Triton (Sigma) and 1% bovine serum albumin (Fisher) solution for an hour on an orbital shaker plate. Sections were then incubated in rabbit polyclonal phospho-p44/p42 MAPK primary antibody (Thr202/Tyr204, 1:250 dilution, Cell Signaling) overnight at room temperature on an orbital shaker plate. The following day, slices were washed in PBS 5 times, and incubated with biotinylated goat anti-rabbit IgG (1:200 dilution, Vector Laboratories) for 2 hours at room temperature. Slices were then washed in PBS 3 times, and incubated at room temperature in avidin- biotin HRP complex (ABC Elite, Vector Laboratories) for an hour. Slices were washed 3 times in PBS and immunopositive neurons were visualized using an SG peroxidase kit (Vector Laboratories) for 10 minutes at room temperature. This protocol was adapted from Bergstrom et al., 2011. Table 1

denotes the number of animals used for histology, and figure 6 and 7 provide representative immune-labeled slices.

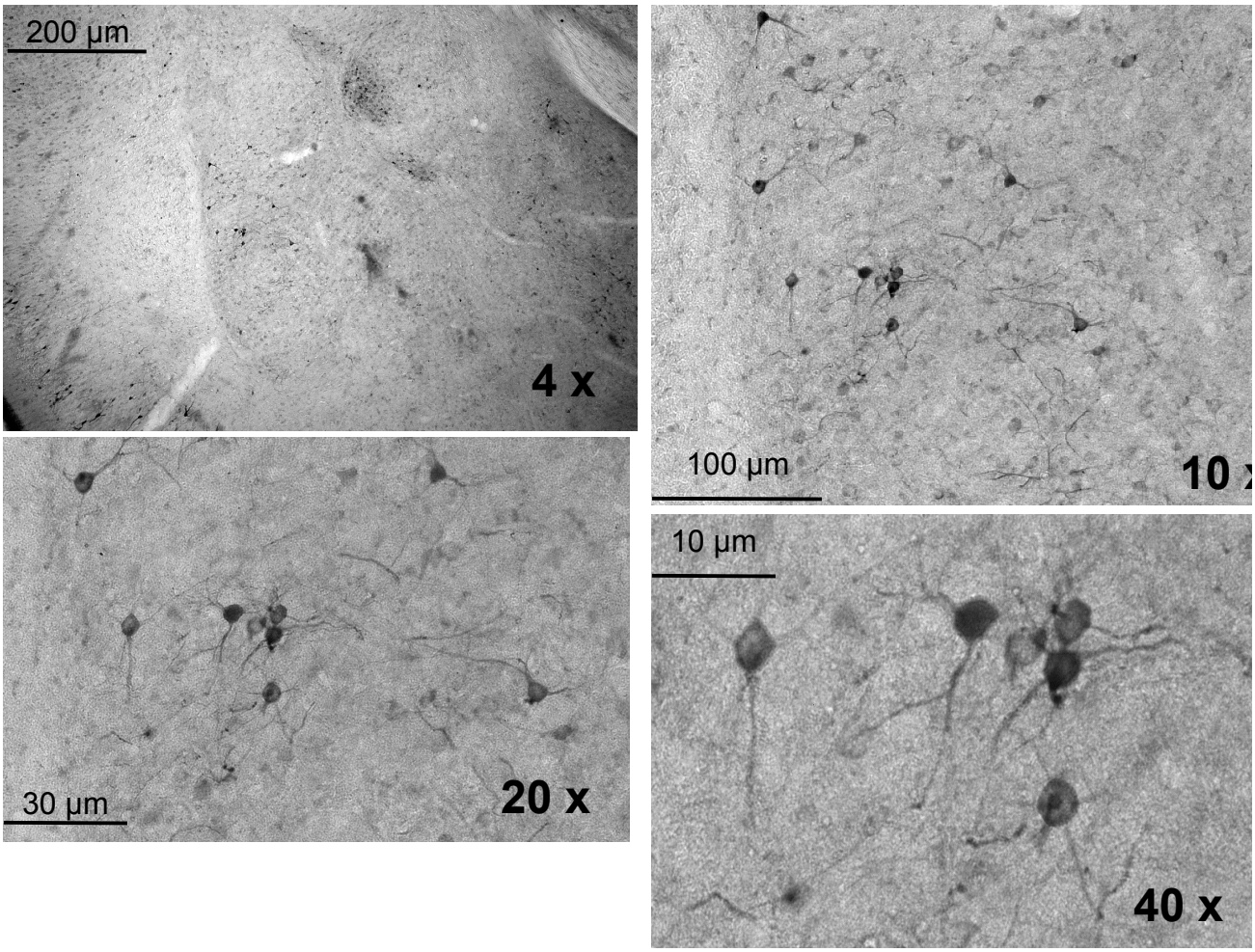


Figure 6: Representative Pictographs from the BLA
Adolescent rat injected with saline in the CPP chamber. Slice corresponds to Bregma 1.44 mm

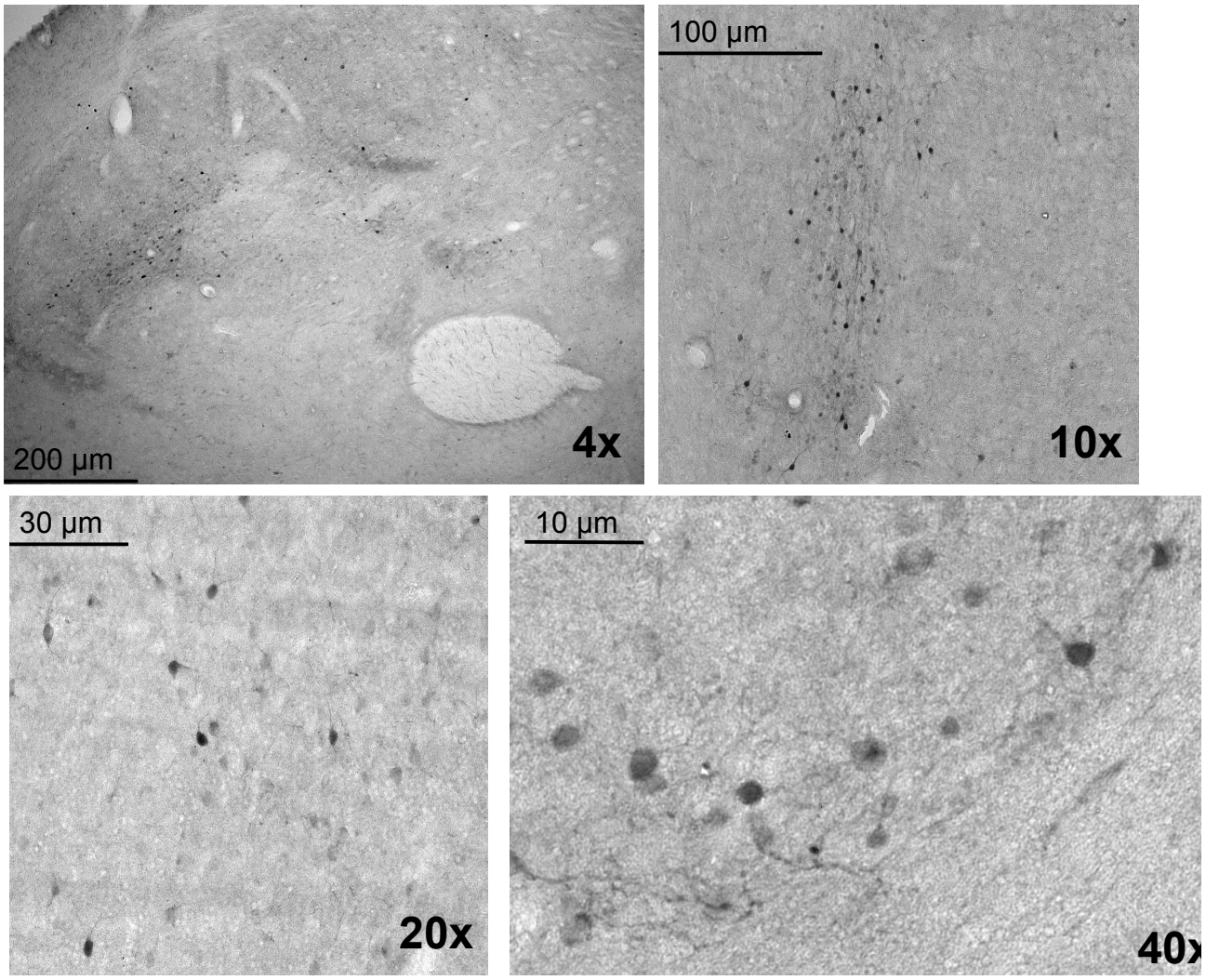


Figure 7: Representative Pictographs from the NAc
Adult rat injected with saline in the homecage. Slice corresponds to Bregma -2.16 mm

CPP				Nucleus Accumbens pMAPK Analysis			
Age	Drug	Dark Chamber Preference	*n*	Age	Drug	Context	*n*
Adult	Saline	High	19	Adult	Saline	Homecage	7
		Low	20			CPP	8
	Nicotine	High	22		Nicotine	Homecage	8
		Low	18			CPP	7
Adolescent	Saline	High	13	Adolescent	Saline	Homecage	6
		Low	21			CPP	6
	Nicotine	High	24		Nicotine	Homecage	8
		Low	17			CPP	6

Table 1: Total number of animals used for single trial nicotine CPP and histology

Data Analysis & Hypothesis

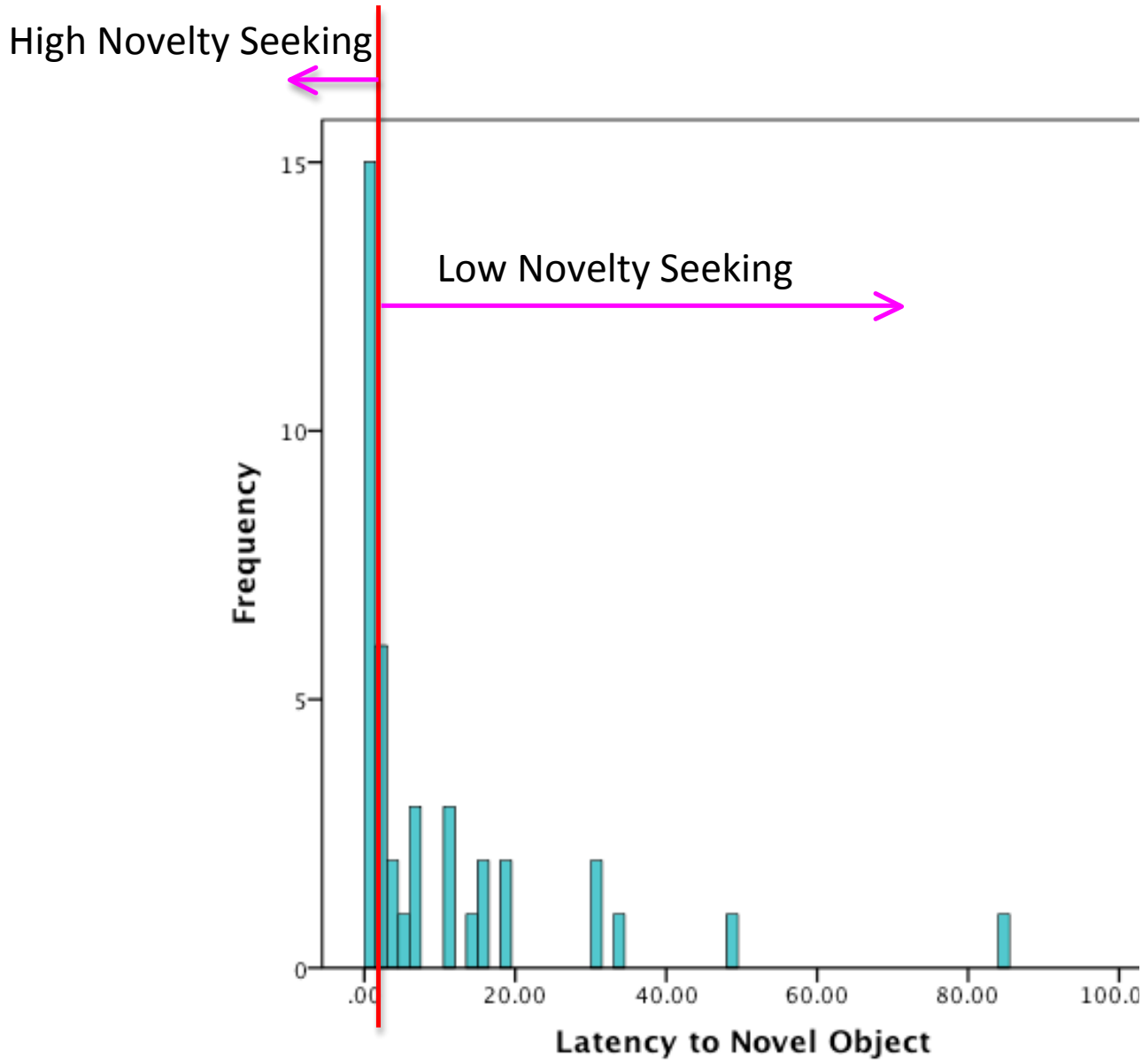
Previous observations from our lab speculated that time spent in the white CPP chamber during the CPP pretest was indicative of the strength of CPP induction following conditioning (Brielmaier et al., 2007). To test these observations, we used a Pearson's correlation to compare time spent in the white chamber during the CPP pretest and CPP difference scores. We found a significant correlation (r^2 = .46), and noted a difference in the correlation coefficient between adult and adolescent animals. A linear regression was used to verify whether age accounted for significant variance in the CPP pretest versus difference score correlation.

To characterize the relationship between CPP chamber preference and CPP induction, we correlated the time spent in the white CPP chamber on day 1 with EPM and NOR data. Specifically, we used Spearman's rho to compare the ratio of EPM open to closed arm entries with relative CPP pretest chamber preference. Based on the

similarities between the CPP chamber and a light- dark box, a standard measure of anxiety, we predicted that:

1. Decreased time spent in the white CPP chamber on day 1 would correlate with increased measures of anxiety indexed by the EPM: more unprotected versus protected head dips, more time spent in the EPM closed arms, and less entries into the EPM open arms.

A Pearson's correlation compared NOR latency to a novel object with relative CPP chamber preference. A one- way ANOVA analyzed the relationship between novelty seeking behavior (high versus low novelty seekers, based on a median split of the animals latency to a novel object during a NOR test session, Graph 1), CPP pretest scores and CPP induction. Outliers more than three standard deviations above the mean were removed from NOR analysis (n=2). Latency measures start from the beginning of video recording, not animal placement into the novelty chamber.



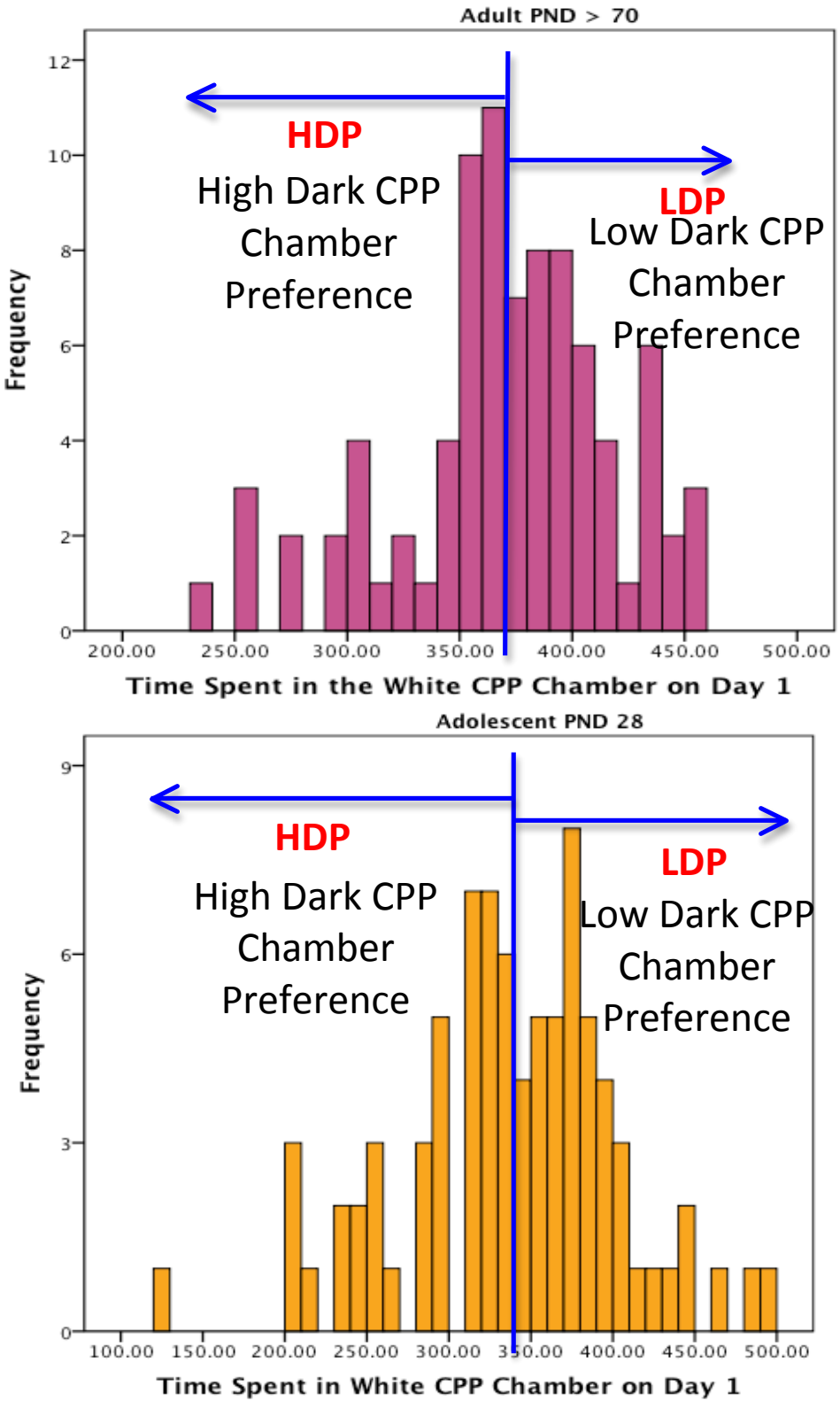
Graph 1: **Novelty Seeking Classification for NOR Test Session**

CPP induction was measured via difference scores. An individual difference score was computed for each animal, subtracting seconds spent in the white chamber on the pretest from the time spent in the white chamber on the posttest. Difference scores were analyzed using a 3 way ANOVA to determine the effect of conditioning drug (saline and

nicotine), relative dark CPP chamber preference (high versus low) and age (adolescent and adult).

Relative dark CPP chamber preference was classified based on time spent in the white CPP chamber. The number of animals assigned to either the relative low or high dark CPP chamber preference group are outlined in table 1. Animals in the relative high dark CPP chamber preference (HDP) group spent most of their time in the black CPP chamber on day 1; using a median split on time spent in the white chamber on day 1, HDP animals are those with the lower CPP white chamber time (graph 2).

Most of the animals in the relative low dark CPP chamber preference (LDP) group spent significantly more time in the black compartment, along with the HDP group. LDP animals are those that spent more time in the white chamber (based on a median split of white CPP chamber time on day 1) compared to the HDP group (graph 2). Animals were not excluded due to a significant preference for the white chamber on CPP day 1; these animals were included in the LDP group (n= 15: 12 adults [5 saline and 7 nicotine conditioned] and 3 adolescents [2 nicotine and 1 saline conditioned]).



Graph 2: Relative Dark CPP Chamber Preference Classification

Significant interactions were followed up with individual t- tests to determine the influence of chamber preference, age or drug conditioning on CPP difference scores. Based on previous findings in our lab (Falco et al., 2014; Brielmaier et al., 2007; 2012), we predicted that:

1. Adolescent animals conditioned with nicotine would form CPP, compared to saline conditioned adolescents
2. HDP adolescents conditioned with nicotine would form CPP compared to LDP adolescents conditioned with nicotine
3. Adult animals would not form CPP, regardless of drug conditioning

The role of MAPK signaling on single trial nicotine CPP was analyzed with a 2 x 2 ANOVA to investigate the effect of CPP conditioning drug (saline versus nicotine) and MEK inhibition (SL327 or vehicle) on CPP difference scores in either HDP or LDP adolescents. Since HDP adolescents formed significant single trial nicotine CPP, we hypothesized that

1. Only the HDP adolescent group would induce significant single trial nicotine CPP
2. MEK inhibition would only have an effect on the HDP adolescent group

Based on findings that MAPK inhibition attenuated drug induced CPP (Miller & Marshall, 2005; Valjent et al. 2004, 2006), for the HDP adolescent group, we hypothesized:

1. SL327 would abolish single trial CPP in the nicotine conditioned group
2. Vehicle pretreated, nicotine conditioned adolescents would form CPP

Primary neurons (non-spherical soma diameters between 12-20 μm) labeled immunopositive for MAPK antibodies were counted using Neurolucida software. Density measurements for pMAPK cells in the NAc core, NAc shell and BLA were compiled with the use of countour maps, based on the outline of these structures using Paxinos and Watson rat atlas (2007; figure 8 and 9), and traced at 4x magnification using a contour-tracing tool (Neurolucida, MBF Biosciences). The same set of countour maps were used in immunopositive cell counts for all animals.

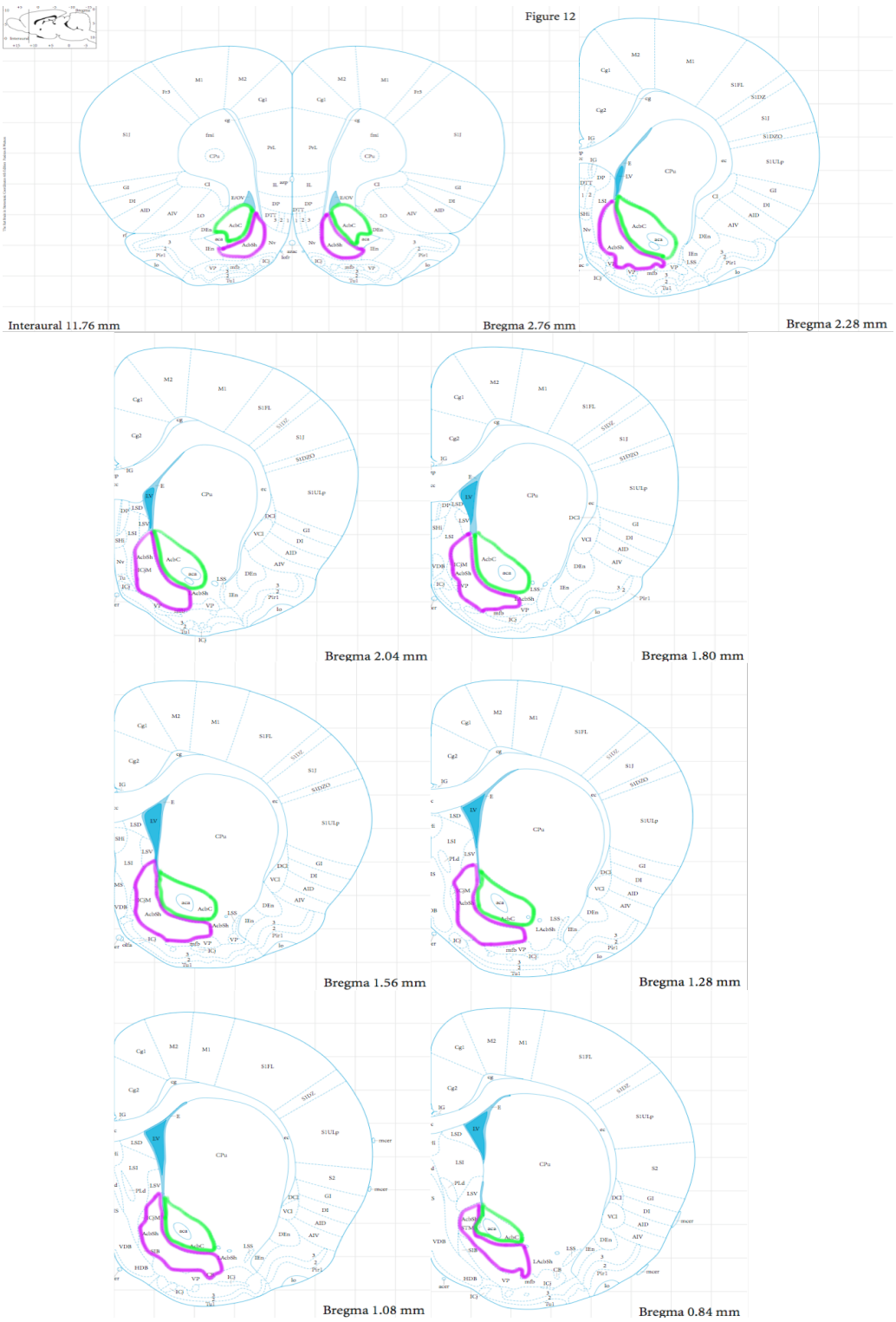


Figure 8: Representative Outlines from the NAc shell and core

NAc core (green) and shell (purple) contours that were included in cell counts spanned from Bregma 2.76 mm to 0.84 mm. The lateral NAc shell was not included in the analysis. Brain slice images were copied from Paxinos and Watson (2007).

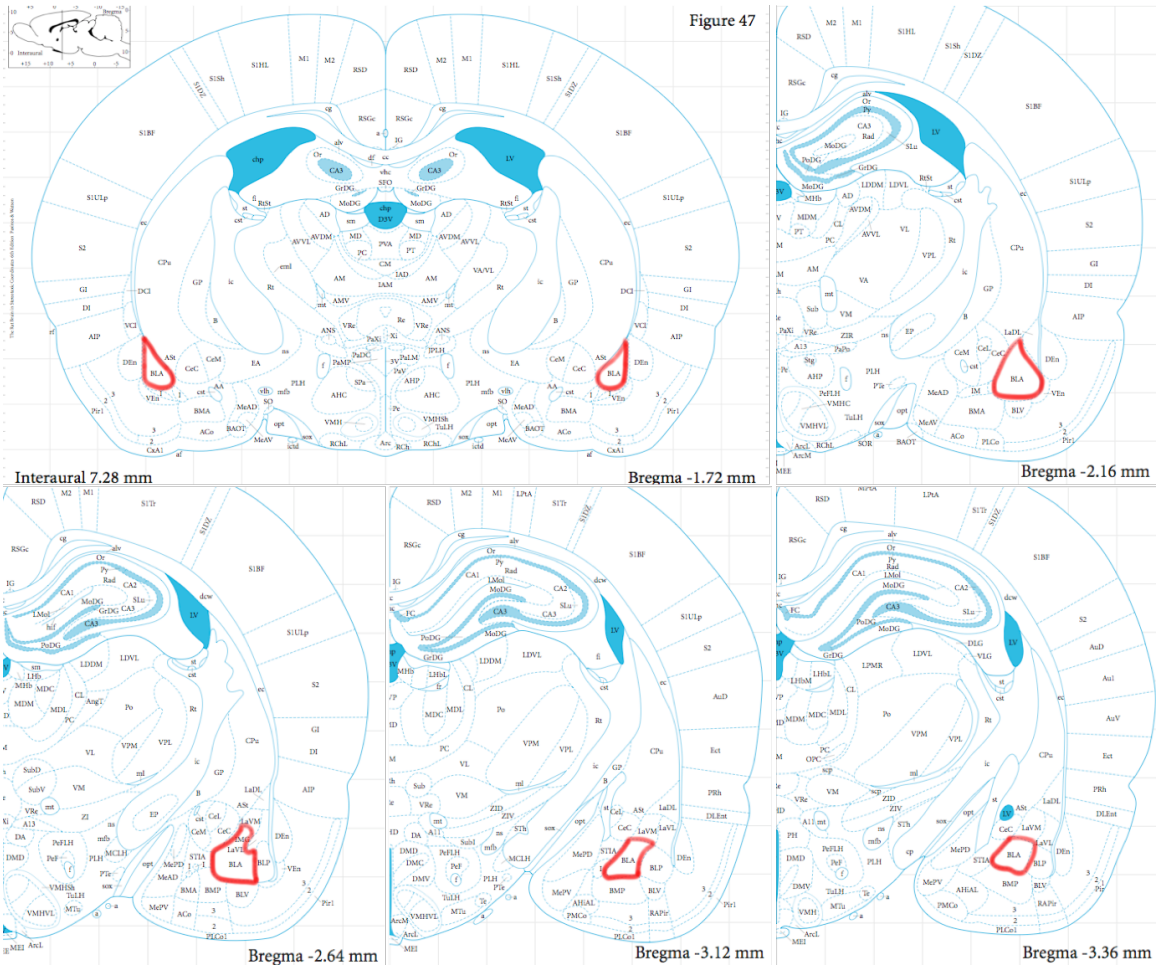


Figure 9: Representative Outlines from the BLA
BLA contours (red) that were included in cell counts spanned from Bregma -1.72 mm to -3.36 mm. Brain slice images were copied from Paxinos and Watson (2007).

A 3 way ANOVA examined the effect of age (adolescent versus adult), context (CPP conditioning chamber or homecage), and drug injection (saline and nicotine) on the number of pMAPK labeled cell in the NAc shell, NAc core and BLA. Trending interactions ($p < .10$) were followed up with individual t- tests to determine the influence

of context, age or drug injection on pMAPK labeled cells. In the NAc shell, NAc core, and BLA we predicted that:

1. Adolescents would have higher pMAPK labeled cells compared to adults, regardless of context
2. Adolescents injected with nicotine would have higher pMAPK labeled cells compared to adolescents injected with saline
3. Adolescents injected with nicotine in the CPP chamber would have higher pMAPK labeled cells compared to adolescents injected with nicotine in the homecage
4. Adolescents injected with nicotine in the CPP chamber would have higher pMAPK labeled cells compared to adults injected with nicotine in the CPP chamber

These a priori hypotheses were based on previous findings indicating that nicotine induces pMAPK activation in the ventral striatum and amygdala (Valjent et al., 2000), there is increased pMAPK activation in adolescents compared to adults (Spanos et al., 2012), nicotine CPP requires neural signaling proteins (Brunzell et al., 2009), and adolescents are more sensitive to the conditioning effects of a single nicotine session compared to adults (Brielmaier et al., 2007).

RESULTS: BEHAVIOR

Innate Behavioral Tendencies (EPM and NOR) and CPP

There was a significant, negative correlation between the time animals (both adult and adolescent rats) spend in the white CPP chamber on day 1 and CPP difference scores after nicotine conditioning, $r\,(67) = -.65$, $r^2 = .46$, $p < 0.001$. These results indicate that as animals spend less time in the white, typically non-preferred chamber on day 1, they form stronger associations between that context and nicotine exposure.

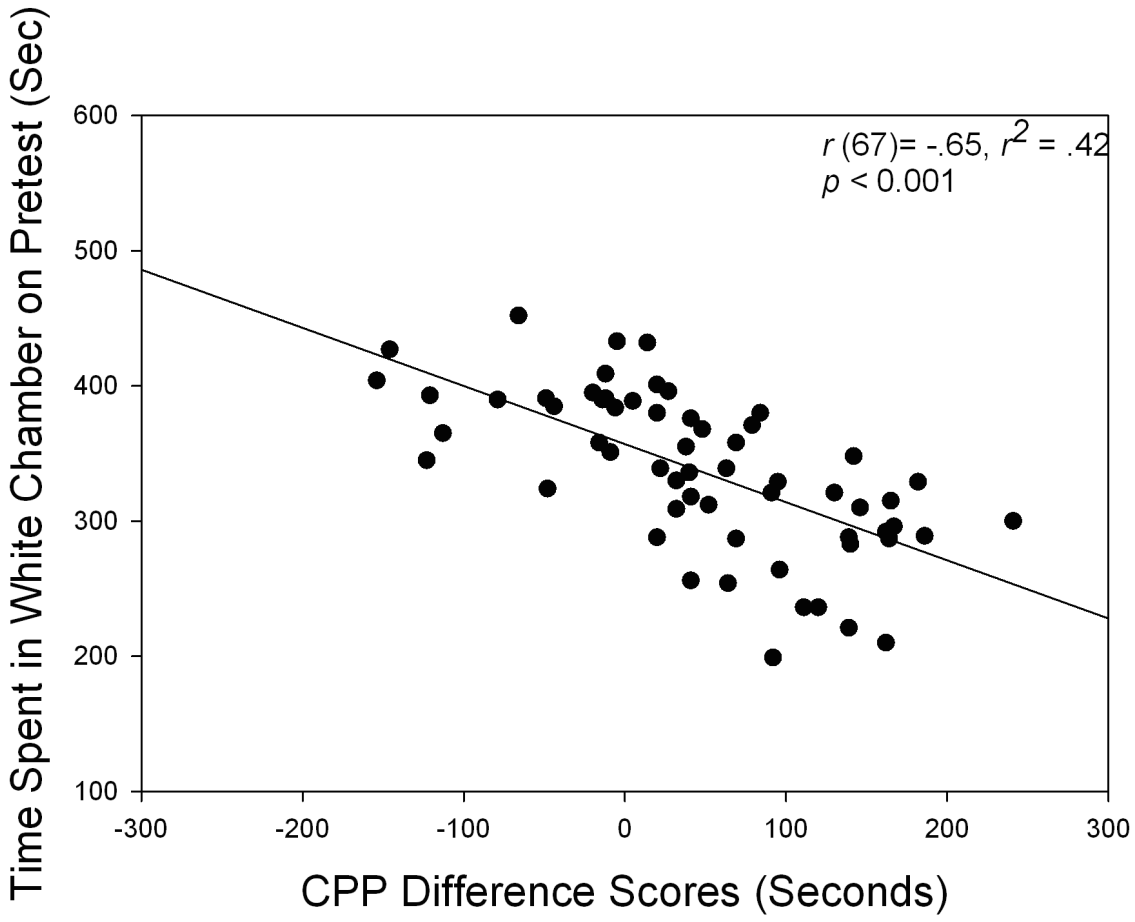


Figure 10: Rats spending less time in the white CPP chamber on day 1 form the strongest nicotine CPP
Pearson's *r* indicated a negative correlation between CPP difference scores (posttest- pretest time in white, drug paired chamber) and the time spent in the white CPP chamber on the pretest. We found that the shorter amount of time an animal (either adult or adolescent) spends in the white chamber during the CPP pretest day, the greater their difference scores are after nicotine conditioning.

We also examined the variance accounted for between CPP difference scores and CPP pretest time in the white chamber according to age after nicotine conditioning (Figure 10). Nicotine conditioned, adolescent animals (r^2 = .46) had higher variance accounted for by the relationship between CPP difference scores and CPP pretest time in the white chamber compared to adults (r^2 = .32) (figure 11). This difference, however, is not statistically significant (see table 2).

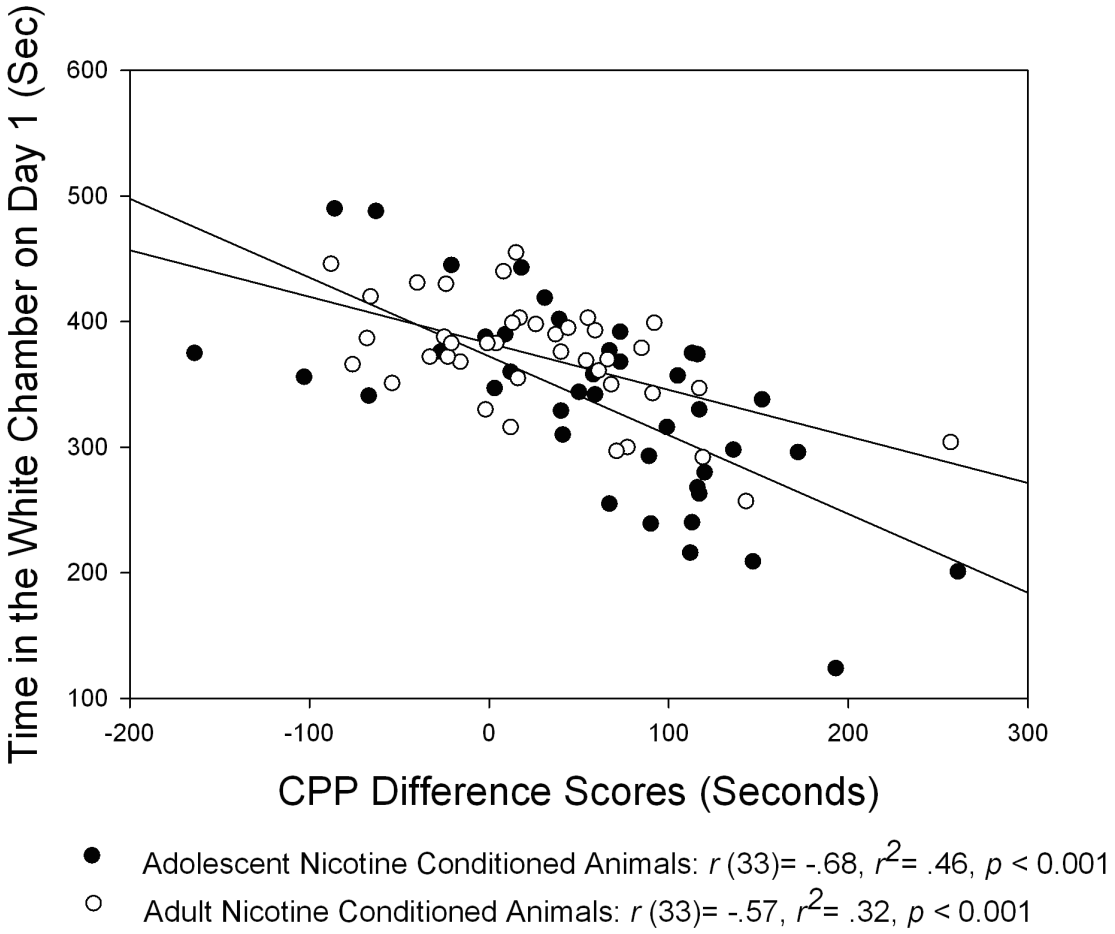


Figure 11: Time spent in the white chamber correlates significantly with CPP difference scores for nicotine conditioned adult and adolescent rats

Pearson's *r* indicated a negative correlation between CPP difference scores (posttest- pretest time in white, drug paired chamber) and the time spent in the white CPP chamber on the pretest.

	B	*SE B*	*β*
Model 1			
Constant	328.21	37.65	
Pretest White Time (Sec)	-0.80	0.10	-0.68*
Model 2			
Constant	324.15	43.12	
Pretest White Time (Sec)	-0.791	0.11	-0.68*
Age	3.029	15.29	0.19

Note $R^2 = .47$ for Step 1; $\Delta R^2 = .00$ for Step 2 ($p > 0.05$)

* $p < 0.001$

Table 2: Regression table comparing the coefficients of time spent in the white CPP chamber on day 1 and age on CPP Difference Scores

Time spent in the white CPP chamber on day 1 significantly predicts CPP difference scores for nicotine-conditioned animals, $p < 0.001$. For each unit increase in time spent in the white CPP chamber during the pretest, the CPP difference score decreases by a value of 0.8 seconds. When the predictor model adds age as a variable, there is no significant increase in variance explained in CPP difference scores, p>0.05.

Our two chambered CPP apparatus is similar to a light- dark box, an established anxiety model (see Hascoet et al., 2001 for review). As mentioned previously, the CPP apparatus has one white and one black compartment, similar to the white and black compartments found in a light- dark box. Due to these similarities, we examined whether time spent in the white CPP chamber on day 1 could also be used as a measure of anxiety. We predicted that increased time in the white CPP chamber on day 1 would correlate with decreased measures of anxiety indexed by the EPM. We found a positive trend towards a correlation between percent open arm entries (open arm entries divided by total arm entries; a measure of decreased anxiety) and time spent in the white CPP chamber on day 1, $r(18)_s = .375$, $p = 0.051$ (one- tailed, figure 12). Other anxiety variables tested in the EPM (percent open arm time, unprotected versus protected head dips) did not correlate with time spent in the white CPP chamber on day 1. Our results suggest that CPP chamber preference on day 1 is associated with a measure of anxiety- like responses.

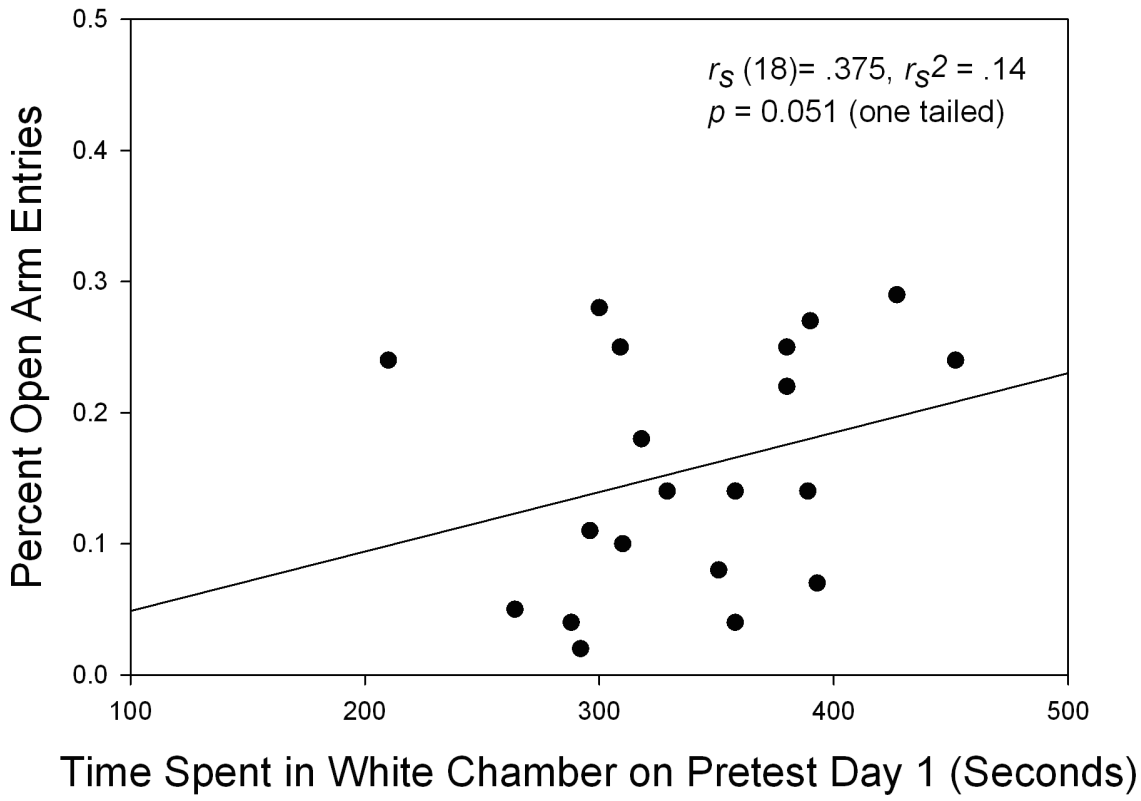


Figure 12: Animals spending less time in the white chamber on CPP pretest also have lower percent open arm entries on an Elevated Plus Maze
Spearman's rho (one- tailed) showed a trend towards a correlation between the time initially spent in the CPP white chamber and the percentage of open arm entries in the EPM (open arm entries/[open arm entries + closed arm entries]). We found that animals spending less time in the white CPP chamber tend to make less open arm entries.

To further define white CPP chamber preference on day 1, we also compared time spent in the white CPP chamber to NOR. Novelty seeking (or novelty learning), indexed by NOR, was correlated with time spent in the white CPP chamber on day 1. There was no significant relationship between the time adolescents spend in the white CPP chamber on day 1 and their latency to approach a novel object during a NOR test session, r (18)=

.27, $p = 0.101$ (figure 13). These results indicate that adolescent's latency to approach a novel object is not indicative of CPP dark chamber preference on day 1.

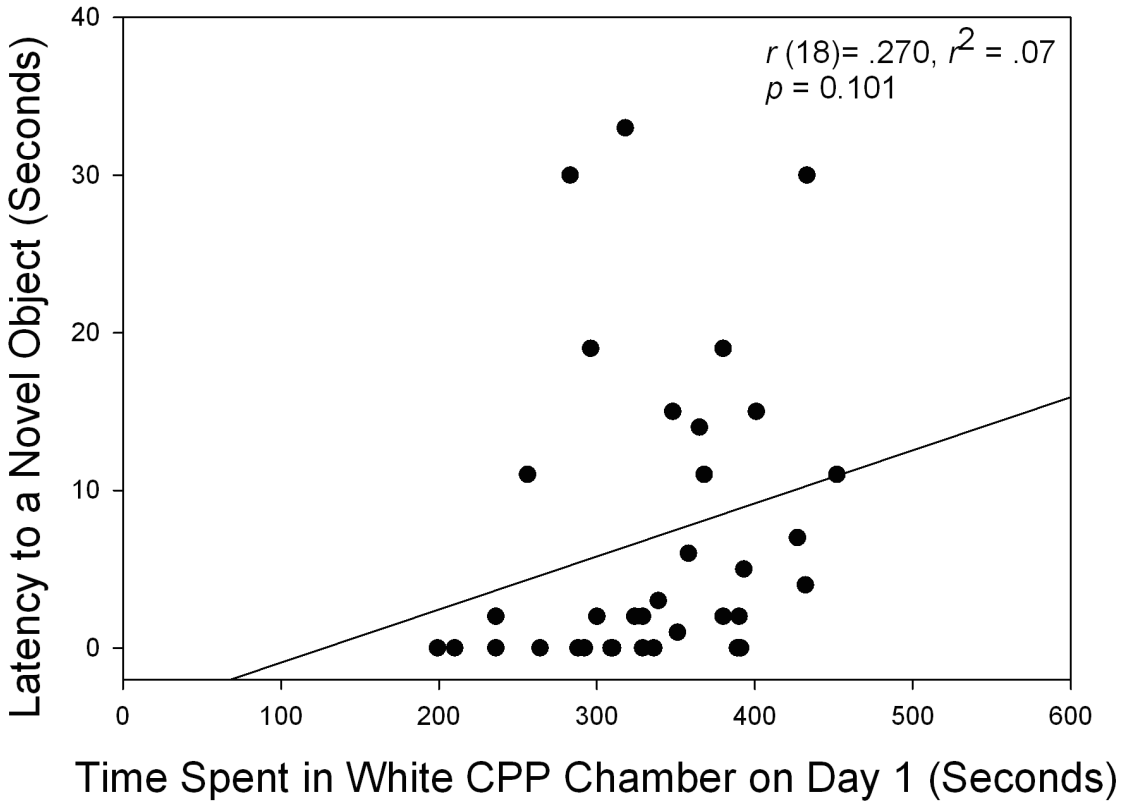


Figure 13: Adolescents that quickly approach a novel object tend to spend more time in a white CPP chamber
Pearson's *r* did not indicate a relationship between time spent in the white CPP chamber on day 1 and the latency to approach a novel object during the NOR test session, $p > 0.05$.

Latency to a novel object during an NOR test session is reflective of learning, but we were interested in using the same variable to compare CPP white chamber preference and novelty seeking. We used a median split on the latency to approach a novel object during an NOR test session to classify adolescents as either high or low novelty seekers. Adolescents with lower latencies towards a novel object were identified as high novelty

seekers and those with higher latencies towards a novel object were classified as low novelty seekers. There was a significant effect of novelty seeking on time spent in the white CPP chamber on day 1, F (1,37) = 7.78, p = 0.008 (figure 14). Our results indicated that low novelty seeking adolescents (M= 397.82, SE= 13.03) spent more time in the white chamber on pretest day compared to high novelty seeking adolescents (M= 342.90, SE= 14.20).

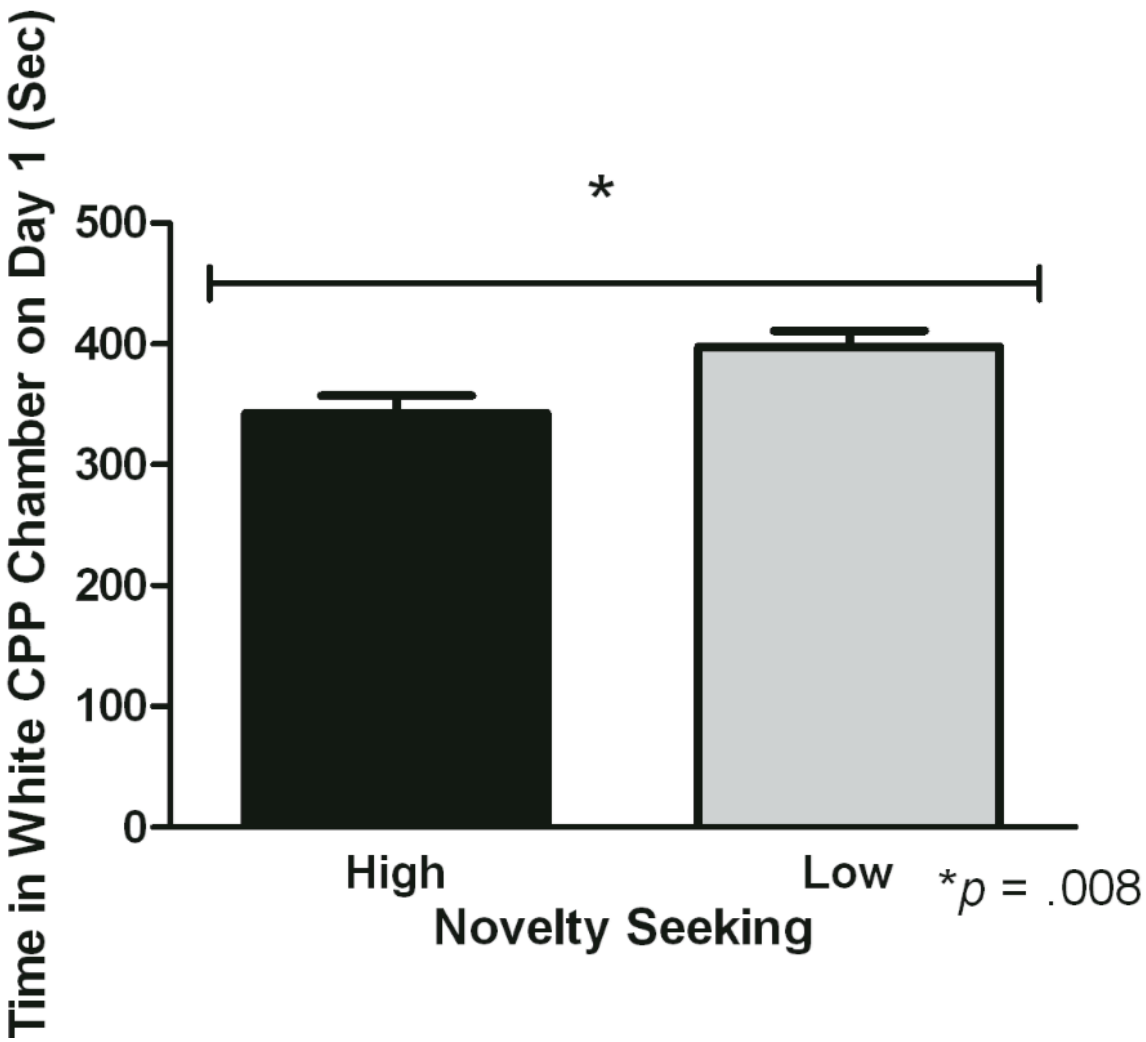


Figure 14: Adolescents that exhibit low novelty seeking behavior during an NOR test session spend more time in the CPP white chamber on day 1

Independent samples t test indicated a significant effect of novelty seeking on the time an adolescent rat spent in the white CPP chamber on day 1, $p < 0.05$. Low novelty seeking adolescents spent more time in the white CPP chamber on day 1 compared to high novelty seeking adolescents. Novelty seeking was classified using a median split on the latency to approach a novel object during a NOR test session. Error bars represent standard error.

In contrast to the relationship between novelty seeking and CPP pretest chamber preference, there was no significant effect of novelty seeking on CPP difference scores, $F(1,37) = 1.36$, $p = 0.251$ (figure 15). NOR latency does not appear to be related to either CPP pretest chamber preference or difference scores.

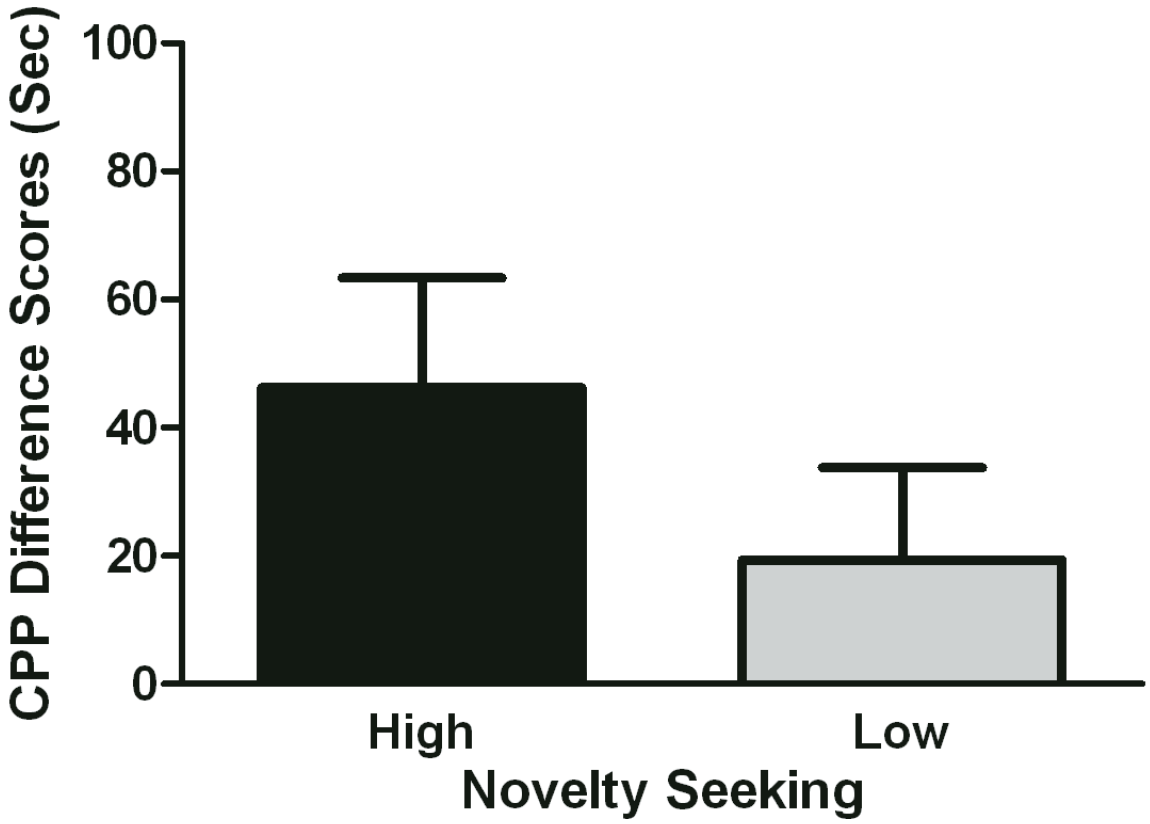


Figure 15: Novelty seeking behavior during an NOR test session is not related to CPP difference scores
Independent samples t test indicated there was no effect of novelty seeking on CPP difference scores, $p > 0.05$. Error bars represent standard error.

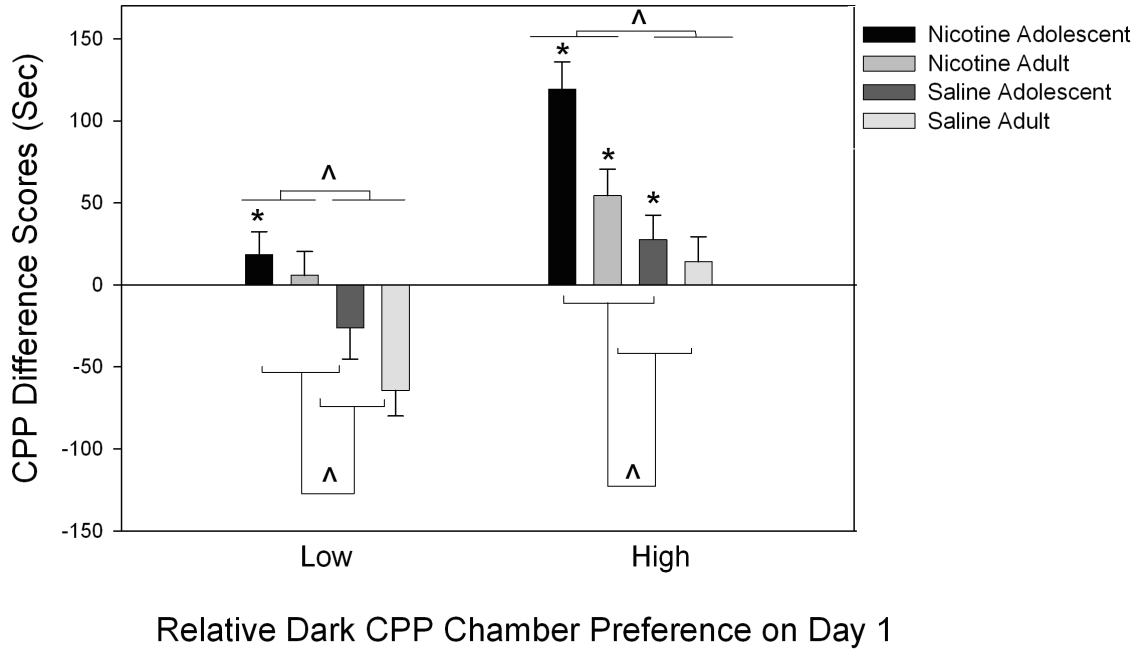
Single Trial Nicotine CPP

A median split on the time animals spend in the white CPP chamber on day 1 was used to classify animals as exhibiting either high or low relative dark CPP chamber preference. Animals spending more time in the white chamber were classified as relative low dark CPP chamber preference animals (LDP), and animals spending less time in the white chamber were classified as high dark CPP chamber preference animals (HDP). There was a significant main effect of age on CPP difference scores, $F(1,122) = 7.3$, $p <$

0.001 (figure 16). This indicates that adolescents (*M*= 33.25, *SE*= 9) spent more time in the white, non- preferred chamber on the posttest versus the pretest compared to adults (*M*= -0.07, *SE*= 8.7). There was also a significant main effect of conditioning drug on CPP difference scores, F (1,122) = 28.42, $p < 0.001$. This indicates that animals conditioned with nicotine (*M*= 49.8, *SE*= 8.6) spent more time in the drug-paired chamber compared to saline (*M*= -17.17, *SE*= 9.2) on the posttest day versus the pretest day. Additionally, there was a significant main effect of relative dark CPP chamber preference on CPP difference scores, F (1,122) = 29.9, $p < 0.001$. This indicates that animals exhibiting higher preference for the dark chamber, HDP, on day 1 (*M*= 50.61, *SE*= 8.8) spent more time in the drug paired chamber after conditioning compared to animals exhibiting lower relative preference for the dark CPP chamber (*M*= -18.04, *SE*= 8.9).

There was a significant interaction between relative dark CPP chamber preference, age and conditioning drug on CPP difference scores, F (1,122) = 4.45, p = 0.037 (figure 12). HDP, nicotine conditioned adolescents (*M*= 119.63, *SE*= 17.6) spent more time in the drug-paired chamber than LDP, nicotine conditioned adolescents (*M*= 18.52, *SE*= 16.17), as well as saline conditioned, HDP (*M*= 17.118, *SE*= 17.09) and LDP (*M*= -22.27, *SE*= 21.25) adolescents. These comparisons indicate significant nicotine CPP induction in adolescent animals. HDP, nicotine conditioned adolescents (*M*= 119.63, *SE*= 17.6) also spent significantly more time in the drug paired chamber compared to HDP, nicotine conditioned adults (*M*= 52.92, *SE*= 18.8), an effect not seen in saline conditioned, HDP animals. These comparisons indicate that relative dark CPP chamber

preference influences nicotine CPP depending on age. Interestingly, LDP, nicotine conditioned adults (*M*= 7.9, *SE*= 15.76) spent significantly more time in the white CPP chamber compared to LDP, saline conditioned adults (*M*= -76.31, *SE*= 17.62). Single trial nicotine CPP was not induced in LDP adolescents. The effect in LDP adults seems to be conditioned aversion, as evidenced by the significant shift in saline animals to spend less time in the white chamber on the posttest compared to the pretest day.



^ $p < 0.005$ (Main Effects)
* $p < 0.005$ (Interaction Terms)

Figure 16: Relative High Dark CPP Chamber Preferring Adolescents form Single Trial Nicotine CPP
3- way ANOVA indicated that single trial nicotine CPP was acquired in adolescents with high dark CPP chamber preference (HDP) that were nicotine conditioned compared to: HDP, nicotine conditioned adults (*); HDP, saline conditioned adolescents (*); and nicotine conditioned adolescents with relative low dark CPP chamber preference (LDP), $p < 0.05$. LDP nicotine conditioned adults spent more time in the white chamber compared to LDP, saline conditioned adults, $p < 0.05$. ^ denotes significant main effects of age (adolescent animals had higher CPP difference scores compared to adults, $p < 0.05$) and drug (nicotine conditioned animals had higher CPP difference scores compared to saline, $p < 0.05$). Relative CPP dark chamber preference was classified using a median split on the time spent in the white CPP chamber on the pretest day. Error bars represent standard error.

MAPK Involvement in Single Trial Nicotine CPP

Evidence for a relationship between MAPK signaling and single trial nicotine CPP in adolescence was examined using a MEK inhibitor (SL327) during CPP induction. There was no significant 3 way interaction between CPP conditioning drug, relative dark CPP chamber preference and MEK inhibitor on CPP difference scores, F (1,27) = 0.104, p = 0.75. There was a significant 2 way ANOVA for relative dark CPP chamber preference and MEK inhibition on CPP difference scores, F (1,27) = 4.683, p = 0.04. We predicted that MEK inhibition would abolish single trial nicotine CPP in HDP adolescents. By analyzing high versus low dark CPP chamber preferring adolescents separately, we found an effect of MEK inhibition on single trial nicotine CPP induction in HDP adolescent males.

In relative high dark CPP chamber biased adolescents, there was a significant main effect of conditioning drug on CPP difference scores, F (1,14) = 9.796, p = 0.04 (figure 17). Nicotine conditioned adolescents (M= 139.23, SE=24.83) spent more time in the drug-paired chamber on the posttest day versus the pretest day, compared to saline conditioned adolescents (M= 36.4, SE= 21.51). There was also an interaction between conditioning drug and MEK inhibitor on CPP difference scores in HDP adolescents, F (1,14) = 2.97, p = 0.05 (one- tailed). SL327 pretreated, nicotine conditioned adolescents did not form CPP compared to the SL327 pretreated, saline conditioned group. Vehicle pretreated, nicotine conditioned animals (M= 190.67, SE= 39.27) did spend more time in the drug-paired chamber compared to vehicle pretreated, saline conditioned adolescents (M= 31.2, SE= 26.11), indicating significant CPP induction. A directional test demonstrated that SL327 pretreated, nicotine conditioned adolescents (M= 87.8, SE=

30.41) spent significantly less time in the drug paired chamber compared to vehicle pretreated, nicotine conditioned adolescents (*M*= 190.67, *SE*= 39.27).

No effect of drug or MEK inhibitor pretreatment was seen in LDP adolescents, $p > 0.05$ (figure 18). These results show that disruption of MAPK activation via an MEK inhibitor abolishes single trial nicotine CPP in adolescents with relatively high dark CPP chamber preference, suggesting a modulatory role of MAPK in CPP induction.

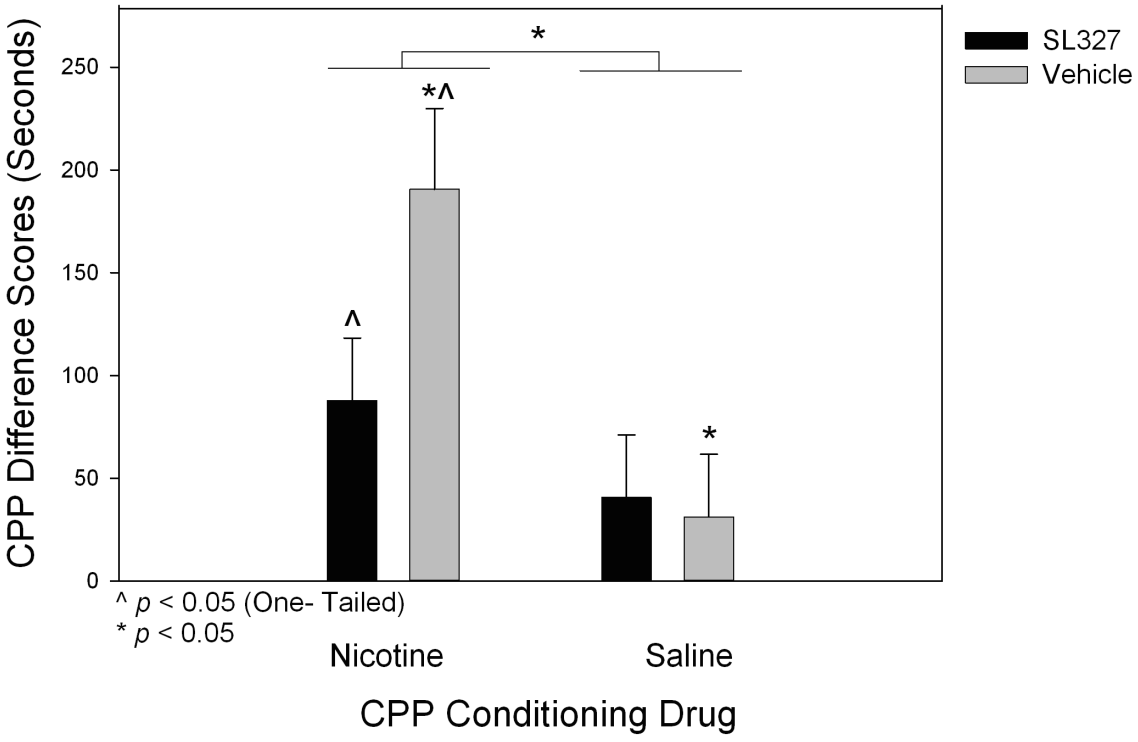


Figure 17: pMAPK inhibition, via peripheral injection of SL327, attenuates single trial nicotine CPP in adolescents with relative high dark CPP chamber preference
In animals that exhibited relative high dark CPP chamber preference (assessed via a median split on the time spent in white chamber on CPP day 1), there was a significant main effect of conditioning drug: animals that were conditioned with nicotine (n=48) spent significantly more time in the white chamber on the posttest day compared to the pretest day compared to saline conditioned animals (n= 28), $p < 0.05$. There was also an interaction between MEK inhibition and conditioning drug (one-tailed): vehicle pretreated animals (n= 38) spent significantly more time in the drug paired chamber on the posttest day after nicotine conditioning, compared to SL327 pretreated (one- tailed comparison; n= 30) and vehicle pretreated, saline conditioned animals, $p < 0.05$. Error bars represent standard error.

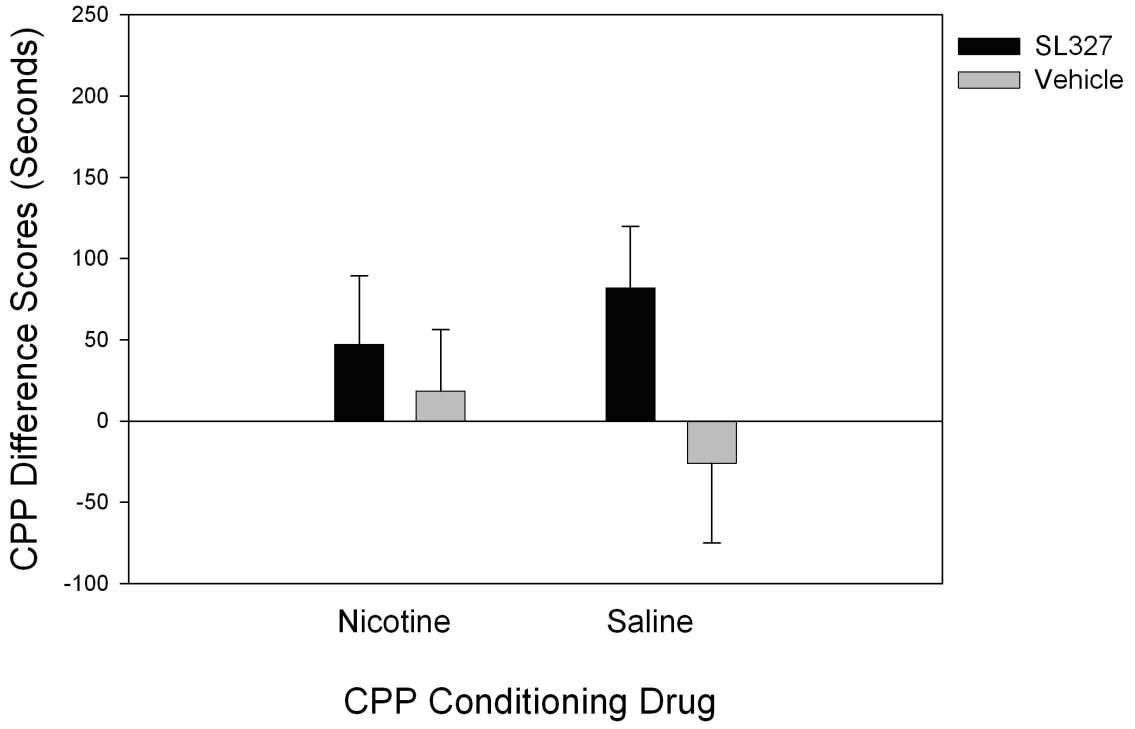


Figure 18: pMAPK inhibition, via peripheral injection of SL327, does not affect single trial nicotine CPP in adolescents with relative low dark CPP chamber preference
Animals that exhibited relative low dark CPP chamber preference on day 1 did not form CPP regardless of pretreatment with an ERK antagonist or nicotine conditioning, $p > 0.05$. Error bars represent standard error.

Summary

An animal's relative dark CPP chamber preference, exhibited during its initial exposure to the CPP apparatus, can predict the strength of CPP induction. Our findings demonstrate that the characterization of chamber preference as a measure of emotionality or novelty seeking is not easily discernable. It appears that these behaviors are related as concepts along a continuum that works to modulate nicotine- cue memory formation. Following single trial nicotine CPP, we found that HDP adolescents formed robust single trial nicotine CPP compared to LDP adolescents, and HDP adults. Interestingly, LDP

adults formed single trial aversion to the white CPP chamber. In HDP adolescents, we also found that an MEK inhibitor attenuates single trial CPP induction.

RESULTS: IMMUNOHISTOCHEMISTRY

pMAPK in the Nucleus Accumbens Shell versus Core

There was no significant difference in pMAPK density between the NAc shell and core, t (50) =1.158, $p > 0.05$ (figure 19). Our results indicate that pMAPK labeling in response to a single dose of either nicotine or saline does not differ in the shell (M= 45, SE= 20) compared to the core (M= 47, SE= 20).

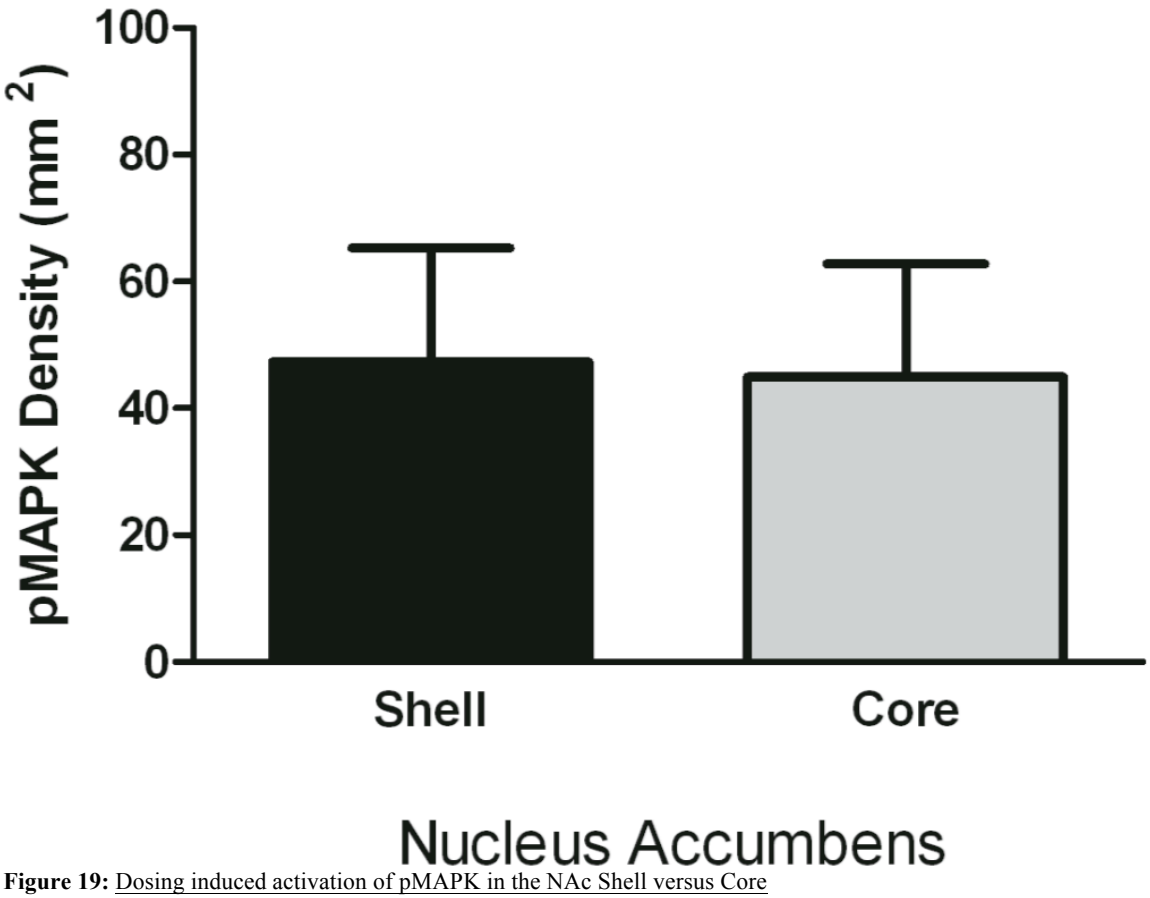


Figure 19: Dosing induced activation of pMAPK in the NAc Shell versus Core
Paired samples t test indicated that in response to a single injection of either saline or nicotine, there are no differences in pMAPK labeled cell counts between the nucleus accumbens shell and core in adults or adolescents, $p > 0.05$. Error bars represent standard error.

pMAPK, Age, and Drug Context in the Accumbens Shell, Core, and Basolateral Amygdala

In the NAc shell, there was no significant main effect of age, conditioning drug or exposure context on the number of pMAPK labeled cell, $p > 0.05$. There was a trend towards a significant interaction between all three variables on pMAPK labeled cell counts in the shell, $F(1,49) = 3.241$, $p = 0.08$ (figure 20). We hypothesized a higher amount of pMAPK labeled cells in the adolescent group administered nicotine in the CPP chamber compared to the homecage. This prediction was based on previous findings that

nicotine increases MAPK signaling (Valjent 2000; 2004). Based on this hypotheses, we followed up the 3-way interaction trend with a t test for the adolescent nicotine comparison. We found a trend indicating that adolescents exposed to nicotine in the CPP conditioning chamber (*M*= 363.14, *SE*= 58.4) had higher amounts of pMAPK labeled cells compared to adolescents exposed to nicotine in the homecage (*M*= 233.5, *SE*= 54.6). Unexpectedly, we also found that adults administered saline in the CPP conditioning chamber (*M*= 354, *SE*= 54.6) had significantly higher pMAPK labeled cells compared to adults administered saline in the homecage (*M*= 209.14, *SE*= 58.36). Also, contradicting one of our hypothesis, there was a trend for homecage adolescent rats to have a lower amount of pMAPK labeled cells (*M*= 233.5, *SE*= 121.3) after a single nicotine injection compared to saline (*M*= 441.5, *SE*= 38.69), $p = 0.09$. Our results suggest that pMAPK labeling differs between homecage and CPP chamber environments according to age and injection: levels differ in adolescence following a nicotine injection and in adult rats following a saline injection.

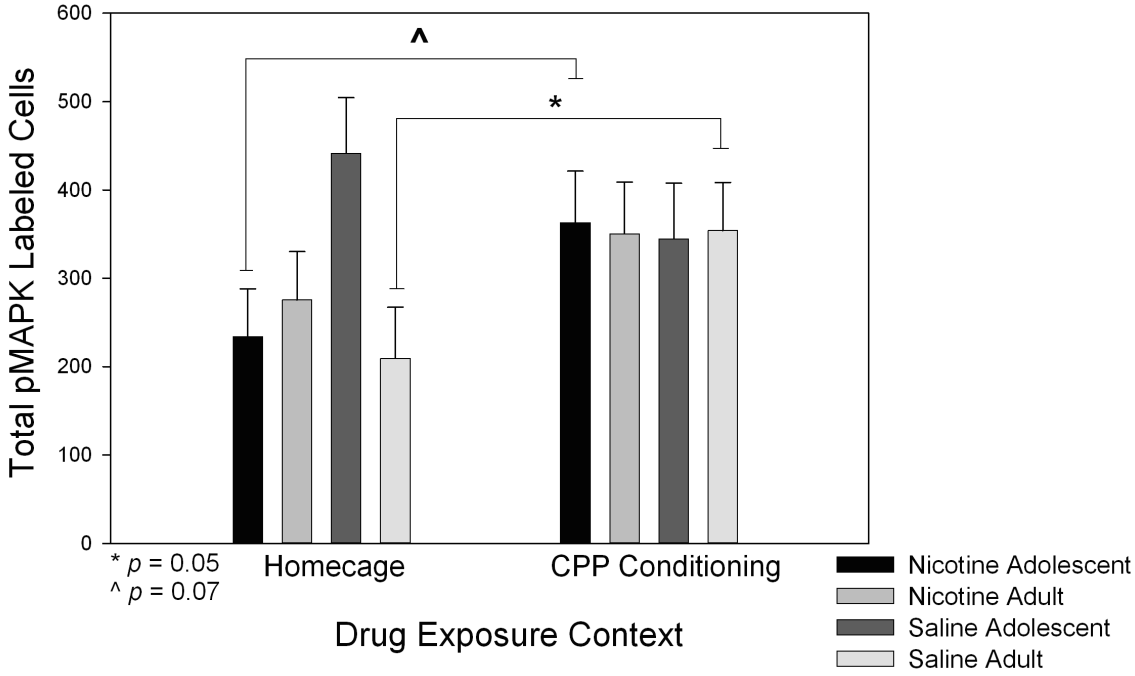


Figure 20: MAPK labeling in the Nucleus Accumbens Shell differs across Drug Administration, Age & Context
3 way ANOVA indicated a trend towards an interaction between age, drug, and context on pMAPK labeled cell counts in the nucleus accumbens shell, $p = 0.08$. Adults injected with saline in the CPP conditioning chamber had a higher amount of pMAPK labeled cells compared to adults receiving saline in the homecage. There was a trend for adolescents injected with nicotine in the CPP chamber to have a higher level of pMAPK labeled cells compared adolescents injected with nicotine in the homecage. Error bars represent standard error.

In the NAc core there was a significant main effect of injection context on the total number of pMAPK labeled cells, F (1,49) = 4.06, $p = 0.05$ (figure 21). Adolescent and adult rats had a significantly higher amount of pMAPK labeled cells when dosed in the CPP conditioning chamber (M= 178, SE=20) compared to the homecage (M= 122.5, SE= 19.68). There was no significant interaction between age, conditioning drug and exposure context on pMAPK labeling in the NAc core, F (1,49) = 1.76, $p > 0.05$. However, there was a trend in which adolescent saline pMAPK labeled cells (M= 206, SE= 68.6) in the homecage were higher than adult saline pMAPK labeled cells in the homecage (M= 78.3, SE= 23.57), p= 0.087. Our findings suggest that MAPK labeling

within the core is mediated by stimulus presentation in a particular context, regardless of drug or age.

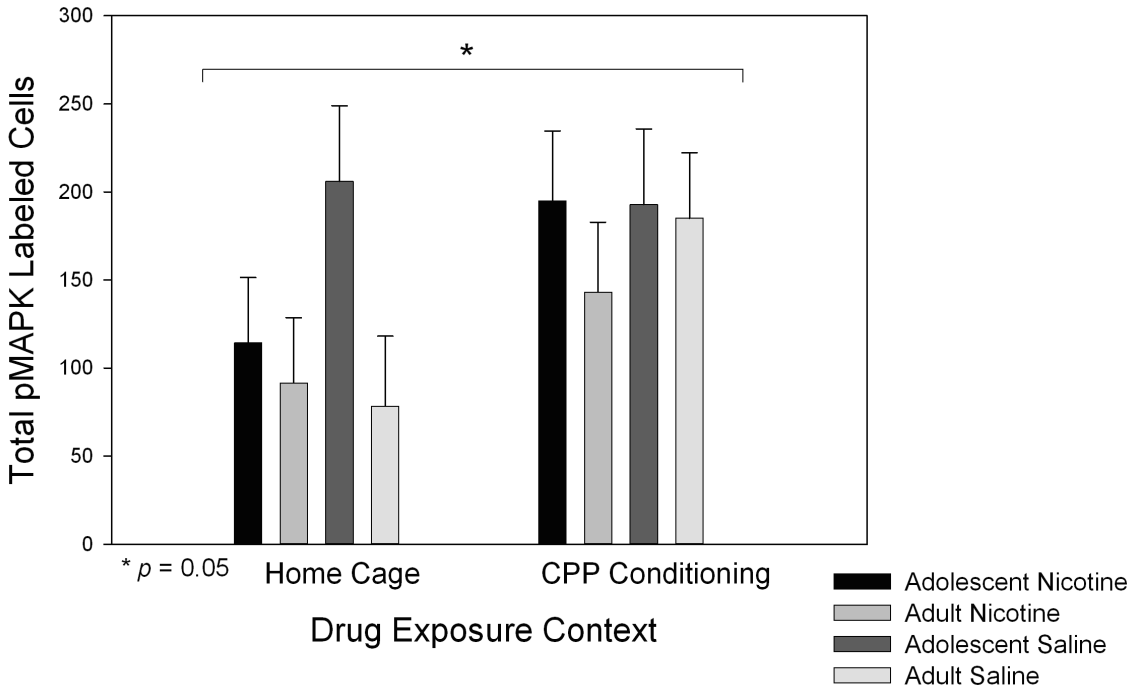


Figure 21: pMAPK labeling in the Nucleus Accumbens Core is dependent on the context of stimulus presentation
There was a main effect of injection context on total pMAPK labeled cell counts in the nucleus accumbens core, where labeling was greater in response to an injection in the CPP conditioning chamber compared to the homecage, $p < 0.05$. There was no interaction between drug, context, or age on pMAPK labeled cell levels in the accumbens core. Error bars represent standard error.

Within the BLA, in contrast to the effects seen in the NAc shell and core, there was no significant main effect of age, drug or context on the amount of pMAPK labeled cells, $p > 0.05$ (figure 22). Additionally, no significant interactions between these variables were found on the amount of pMAPK labeled cells in the BLA, $F(1,43) = .027$, $p > 0.05$. These results indicate that MAPK labeling within the BLA is not affected by differences in age, a single injection or context within our experimental parameters.

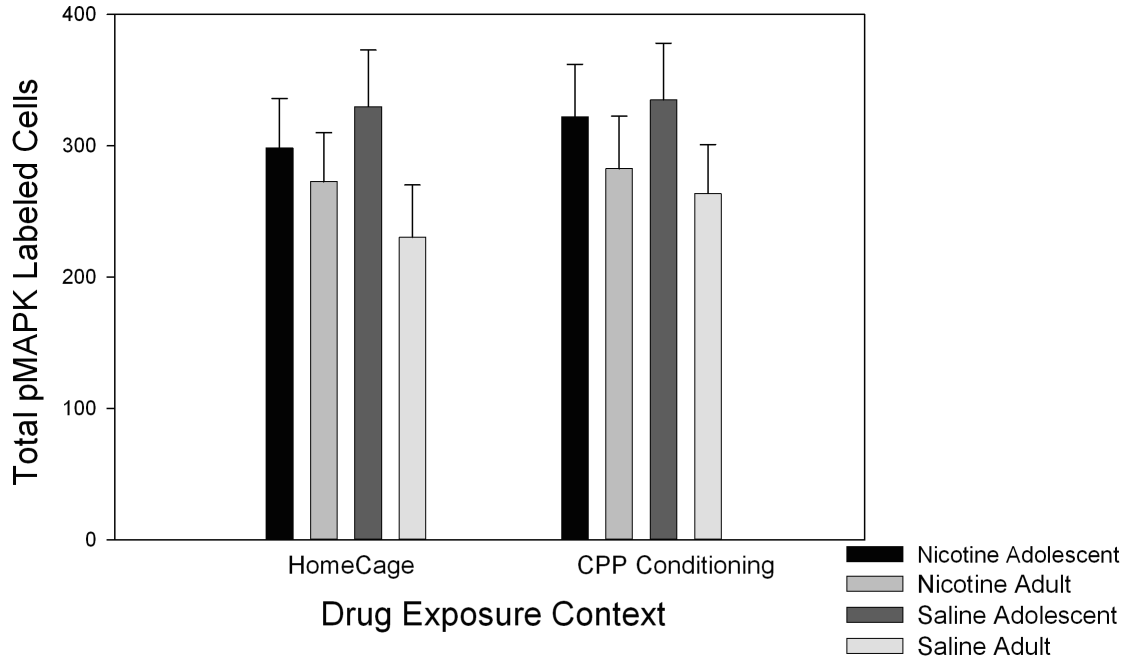


Figure 22: pMAPK Labeling in the BLA is Independent of Age, Drug & Context
There was no main effect or interaction between age, drug, or injection context on the total amount of pMAPK labeled cells in the basolateral amygdala, $p > 0.05$. Error bars represent standard error.

pMAPK across brain regions

As indicated previously, differences in pMAPK labeled cells that were influenced either by context or age were found in the NAc core and shell, but not the BLA. We did find a significant difference in pMAPK density between the NAc compared to the BLA that was not dependent on either age group (adolescent and adult) or drug (saline and nicotine), t (56) =5.03, $p < 0.001$ (figure 23). These results indicate that pMAPK cell labeling in response to a single injection is greater in the NAc (M= 106, SE= 17) compared to the BLA (M= 23, SE= 20). With our immunohistological protocol, we find that pMAPK labeling in the NAc is more sensitive to an injection compared to the BLA.

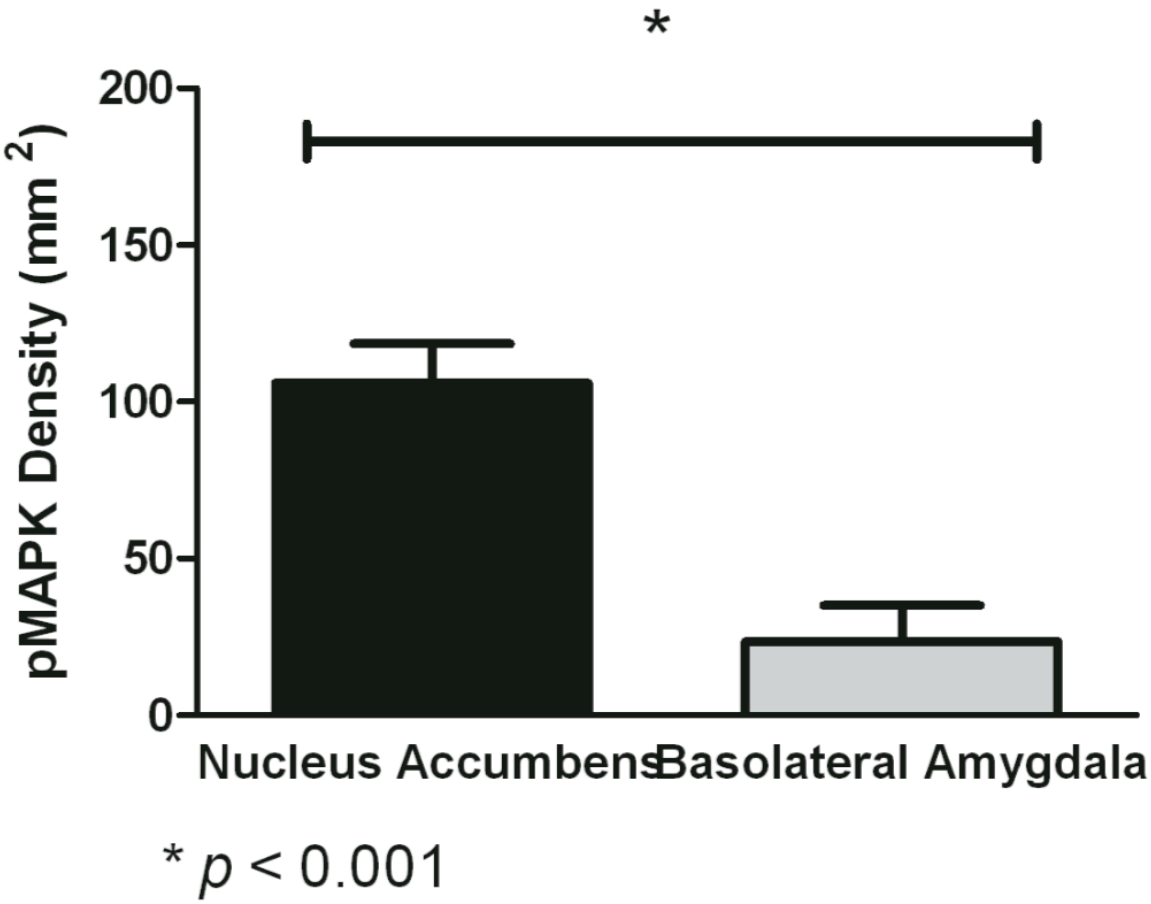


Figure 23: pMAPK labeling in the Nucleus Accumbens versus Basolateral Amygdala
Paired samples t test indicated that in response to a single injection of either saline or nicotine, there was a higher amount of pMAPK labeled cells in the nucleus accumbens compared to the basolateral amygdala, $p < 0.05$. Age and drug groups were collapsed in this analysis. Error bars represent standard error.

Summary

Within the NAc shell, we found a trend for a higher amount of pMAPK labeled cells after a single nicotine injection in adolescents within the CPP chamber compared to dosing in the homecage. A similar effect was significant in saline exposed adults; pMAPK labeled cell levels in the NAc shell increased in response to an injection in the CPP chamber compared to the homecage. The trend towards an age by drug by context

effect was not found in either the NAc core or BLA. In the NAc core, we found a general effect of injection context on pMAPK labeled cells: pMAPK labeled cell counts were higher in the CPP conditioning context compared to the homecage environment. Overall, these results suggest a unique pattern of MAPK activation in the NAc in response to a stimulus presentation within a CPP chamber.

CONCLUSION: BEHAVIORAL RESULTS

Using a biased, single trial nicotine CPP protocol, we replicated previous findings from our lab that show adolescent Sprague Dawley rats form nicotine CPP (Brielmaier 2007; 2008, Falco et al., 2014). As mentioned in Brielmaier et al. (2008), the time that an animal spends in the white CPP chamber on day 1 is correlated with the strength of the nicotine- context association established after conditioning. We found a similar relationship in our data, as nicotine CPP was induced in adolescents with a strong bias towards the dark CPP chamber.

CPP Dark Chamber Preference and Single Trial Nicotine CPP

We ran a series of experiments to define the behavior exhibited by both adolescent and adult animals in the CPP white chamber on day 1. We found a strong correlation between the time animals spent in the white CPP chamber on day 1 and CPP induction, measured using difference scores. As animals spend less time in the CPP white chamber during the pretest, they also have higher CPP difference scores after nicotine conditioning (figure 10). This relationship is observed in both adult and adolescent animals (figure 11). Interestingly, the relationship is equally strong across both age groups (table 2).

For our first set of experiments, we compared the time animals spent in the CPP white chamber during the pretest with their behavior in EPM. There is evidence to

suggest that emotionality, specifically anxiety, drives individuals to seek cigarettes and other forms of drug use, especially during adolescence (Hockenberry et al., 2011; Buckner et al., 2014). In animal models, anxiety like behavior augments nicotine seeking (Falco et al, 2014; Zarrindast et al, 2012; Smith & Aston- Jones, 2008). Our two-chambered, black and white CPP apparatus resembles a light- dark box, an established anxiety murine model. Due of this similarity, along with the relationship between emotionality and nicotine seeking, we postulated that the dark CPP chamber preference exhibited on day 1 of testing could be a measure of anxiety. We hypothesized that animals spending less time in the white chamber were exhibiting more anxiety- like behavior, as indexed in the EPM, and that anxiety- prone animals would form CPP to a greater extent than less anxious animals.

Our results did not fully support our hypothesis. The only correlation detected that approached statistical significance was the ratio of open arm entries to closed arm entries and CPP white chamber time (figure 12). The standard measure of anxiety using EPM is the ratio of time spent in the open arm time versus the closed arm (File, 2004; Carobrez & Bertoglio, 2005). While we found no significant correlation between percent open arm time and CPP pretest white time, a one- tailed trend did exist for percent open arm entries. This measure relates to decreased anxiety in animals (Acevedo et al., 2014). We found that increased open arm entries were related to increased time spent in the white CPP chamber on day 1, indicative of decreased anxiety. Since animals that spend the least amount of time in the white CPP chamber form more robust CPP, our EPM data tenuously suggest that more anxious animals form more robust CPP. Previous literature

has found that anxious animals form more robust cocaine CPP compared to non- anxious animals (Pelloux et al., 2009). However, since the relationship between CPP pretest time and EPM was not statistically significant, it is unlikely that we are detecting a pure measure of anxiety. A more definitive answer may have been reached by analyzing corticosterone levels before, during and after the initial CPP chamber exposure (Koob & Kreek, 2007). Since this data is not available, we can only state that dark CPP chamber preference exhibited on CPP day 1 is likely *related* to anxiety along a continuum with novelty approach behaviors.

Our second set of experiments looked at the relationship between adolescent CPP and NOR. NOR is a HC based, behavioral learning paradigm that is uniquely suited to our studies since it captures both memory mechanisms and the intrinsic reward of novelty in the environment (Antunes, 2012). Previous studies have demonstrated that novelty seeking, above and beyond anxiety, can predict nicotine consumption in animals (Abreu-Villaca et al., 2006). Since NOR gauges reward attributed to novelty (Salvetti et al., 2014), we used the latency to the novel object during a NOR test session as a measure of novelty seeking. This measure was correlated with both CPP pretest white time and CPP difference scores. We hypothesized that adolescents who quickly approached a novel object would spend more time in the CPP white chamber on day 1 and form robust CPP due to the interaction between rapid memory formation and novelty salience in NOR.

We found that the latency to a novel object was not correlated with CPP pretest white time (figure 13). When we used the latency towards the novel object as a measure of novelty seeking (by taking a median split on latency time), we found that high novelty-

seeking adolescents, those who quickly approached the novel object, spent significantly less time in the CPP white chamber on day 1 (figure 14). Novelty seeking was not, however, related to CPP difference scores (figure 15). Our findings contradict our anxiety results, as well as our hypothesis. Although previous research has found that increased novelty seeking enhances cocaine CPP (Vidal- Infer et al., 2012), amphetamine CPP (Klebaur et al., 1999), cocaine self- administration (Belin et al., 2001) and amphetamine self-administration (Klebaur et al., 2001), we did not find evidence of increased novelty seeking modulate of CPP behavior. High novelty seeking behavior is not required for reward seeking behavior, as increased responses to novelty have no effect on cocaine CPP (Gong et al., 1996).

In our findings, we saw that low novelty seekers were spending more time in the white CPP chamber, when the assumption was that high novelty seekers would be exploring the novel, white chamber at a higher comparative rate. This dissociation illustrates the difficulty in ascribing a singular concept to CPP dark chamber preference. It is possible that differences in emotional responses to novel environments drives novelty seeking initially (Clinton et al., 2012; Hayton et al., 2012; Aydin et al., 2012). The influence of physiological arousal to a novel environment is then diminished as the animal acclimates to the testing environment across days.

As with the EPM comparisons, we have to be cautious with classifying dark CPP chamber preference as novelty seeking because NOR is a memory test. Although not significant, high novelty seeking animals had higher CPP difference scores compared to low novelty seeking animals. It makes theoretical sense that animals quickly approaching

a novel object could form rapid, single trial nicotine CPP. Our high novelty-seeking animals were actively recalling the training object, and therefore approached the novel object at a quicker rate than low novelty seekers (Philpot & Wecker, 2008). We cannot separate the modulation of memory formation from novelty seeking with this task, so we cannot claim dark CPP chamber preference as either concept. In addition, we did not rule out the interactions of other behavioral confounds, such as impulsivity or activity.

Adult mice with low locomotor responses to an activity chamber do not form nicotine CPP (Bernardi & Spanagel, 2014). On the other hand, using locomotion in a CPP chamber as a measure of activity, Shimosato & Watanabe (2003) found that low activity, and not high activity, mice formed cocaine CPP. If locomotion was measured in our protocol, we could explore the effects of activity (high versus low novelty responders) on the formation of nicotine- cue memories.

Impulsive behavior in both animals and humans correlates with a vulnerability for cocaine and nicotine seeking behaviors (Belin et al., 2008; Diergaarde et al., 2012; 2008; Jentsch et al., 2014). The distinction between novelty seeking and impulsivity is also difficult to gauge. Novelty seeking does not correlate with the acquisition or self-administration performance for MDMA, but impulsivity did (Bird & Schenk, 2013). Impulsivity itself can be further subdivided into impulsive action and impulsive choice (Evenden, 1999), although these behaviors do not always correlate despite underlying the same construct (Broos et al., 2012). Rats that associate a conditioned stimulus with incentive salience are prone to impulsive action, but not impulsive choices (Lovic et al., 2011). Drug exposure also has differential effects on impulsive versus anxiety- like

behavior: ethanol exposure selectively increases impulsive behavior in adolescent rats, whereas it increases anxiety- like behavior in adult rats (Mejia- Toiber et al., 2014). These results are in accordance with previous research that finds adolescent mice are more impulsive than adults (Doremus- Fitzwater et al., 2012). If we measured impulsivity separately, using a five choice serial reaction time task, we could investigate age dependent differences in impulsive behavior, and how that relates to the formation of rapid, nicotine- cue memories (Robbins, 2002).

Since CPP pretest time encompasses elements of anxiety, memory and novelty seeking, and we cannot rule out the influence of other performance states, it would be misleading to title this approach/avoidance of the white chamber as a singular behavior. As such, we will classify CPP pretest time simply as relative dark chamber preference.

Single Trial Nicotine CPP

CPP chamber preference exhibited by animals during a CPP pretest is predictive of single trial nicotine CPP induction. We therefore used relative dark CPP chamber preference as a variable in our analysis of age dependent nicotine CPP. We found that relative high dark CPP chamber preferring (HDP) adolescents formed robust single trial nicotine CPP compared to HDP adults conditioned with nicotine (figure 16). Relative low dark CPP chamber preferring (LDP) adults significantly shifted their preference to the dark chamber after the CPP protocol.

Our findings that adolescent rats form single trial nicotine CPP are in accordance with previous results in our lab (Brielmaier et al., 2007; 2008; 2012). These results illustrate that adolescents are vulnerable to the reinforcing effects of nicotine. Nicotine

administration during adolescence has long-term neural morphological effects (McDonald et al., 2005; 2007, Bergstrom et al., 2010; 2008) and produces sensitization to other drugs of abuse, such as ethanol and cocaine (Roguski et al, 2014; Anker et al., 2011; Bracken et al., 2011; Philpot et al., 2014). Nicotine's hedonic reward is due to its effect on the mesocorticolimbic dopamine pathway, which is activated in response to pleasurable and rewarding stimuli (Balfour, 2009). Activation of the dopamine reward pathway increases the salience of changes that are occurring in the environment, increasing the motivation to repeat the behavior (Koob & Volkow, 2010). As nicotine binds to nicotinic acetylcholine receptors in the VTA, dopamine is released and activates the NAc (Leslie et al., 2013; Picciotto & Kenny, 2013; Changeux, 2010). The NAc, specifically the NAc shell, is responsible for the nicotine- cue association that is made in the environment (D' Souza et al., 2011; Ikemoto, 2007; Laviolette et al., 2008). Adolescents have a sensitized mesocorticolimbic dopamine pathway (Spear, 2010; Nixon & McClain, 2010), and so even a single nicotine administration is sufficient to alter drug-seeking behavior.

Our findings indicate that the underlying mechanism of dark CPP chamber preference affects responses to a single nicotine administration. Dark CPP chamber preference selectively influences single trial nicotine CPP in adolescents that spent the least amount of time in the white CPP chamber on day 1, based on a median split of total white CPP chamber time. As mentioned previously, CPP chamber preference may be reflective of an anxiety related response to the novel chamber. It is possible that in adolescent rats with high physiological responses to a novel, white CPP chamber, these

animals could also respond strongly to a single nicotine injection (Yu et al., 2014; Zou et al., 2014; Leão et al., 2012). For these animals, nicotine is likely activating both the dopamine reward pathway and dampening a stress-like response to an open chamber via regulation of the extended amygdala and prefrontal cortex (Andreasen et al., 2011; Bruijnzeel, 2012). Thus, our HDP adolescents conditioned with nicotine have an overwhelming shift in their chamber preference.

Since chamber preference also encompasses aspects of novelty memory, it is likely that arousal (or anxiety/stress) in HDP adolescents is enhancing memory induction (Counotte et al., 2011). Acute stress strengthens CPP (Bahi, 2013), fear conditioning (Reich et al., 2013; Smith et al., 2006) and Morris water maze (Hadad- Ophir et al., 2014) performance. Our results are therefore likely due to an interaction between nicotine and context specific activation.

On the other hand, LDP adolescents did not form CPP. The relative low dark CPP chamber-preferring animals are responding to the physiological arousal (or stress/anxiety) resulting from the novel, white CPP chamber differently than HDP adolescents. Instead of avoiding the white chamber, these adolescents relatively spend more time in the white chamber. As they are exposed to the CPP apparatus over testing days, their approach tendencies towards the white chamber decrease, as noted by their negative saline difference scores. In this case, a single nicotine administration is not sufficient to overcome the acclimation to the white chamber. It is possible that whatever psychological state is being expressed in LDP animals is protective during adolescence against the formation of rapid nicotine- cue associations that can lead to increased drug

seeking behaviors (Hammersley et al., 2013; Berridge & Arnsten, 2013). A multi- trial nicotine CPP protocol could test this hypothesis, in which repeated nicotine- context pairings could induce CPP in both adolescent dark CPP chamber preference groups.

While our results support the hypothesis that adolescents are more vulnerable to the rewarding effects of nicotine, we also found that LDP adults form an aversion to the white chamber after single trial CPP. Previous research has not focused on the direct influence of chamber preference in biased CPP designs, and so by taking a look at individual differences in chamber preference, we were able to detect a significant shift in dark chamber preference in LDP adults.

Adults are classified in an age group that encompasses a large developmental time span (Rice & Barone, 2000; Andersen, 2003), and differences in brain development affect drug related behavior (Spear, 2000). While previous protocols using older adults (P80-90) did not find a shift in chamber preference after CPP (Brielmaier et al., 2007; Belluzi et al., 2004), it is possible that our use of younger adults led to significant CPP induction. Unpublished observations from our lab consistently find that single trial adolescent nicotine CPP is day specific. If the animal is tested at P29 instead of P28, we no longer find significant CPP. Our adult cohorts did not arrive exactly at P61, and so we cannot be certain of the exact age at which they were tested. Therefore, differential maturation in our adult cohorts could have affected chamber exploration and conditioned aversion.

As seen in figure 16, saline conditioned adults drive the shift in chamber preference for LDP adults. Negative difference scores indicate that an animal spends

more time in the white chamber on the pretest compared to the posttest. LDP adults explored the novel, white CPP chamber on day 1. After multiple exposures to the testing apparatus, when given the choice to explore either chamber on the posttest day, saline conditioned adults did not approach the white chamber to the same extent they did during their first exposure. Nicotine exposure may have reversed this pattern of white chamber aversion in LDP adults. The lack of aversion in the experimental group may relate to nicotine's anxiolytic properties (Picciotto et al., 2002). It is likely that the white CPP chamber was not as aversive in the nicotine conditioned, LDP adult group because of it's association with an anxiolytic compound.

The intersection of CPP dark chamber preference, age and nicotine conditioning encompasses activity across multiple brain regions, including the striatum, amygdala, and VTA (Kalivas & Volkow, 2005; Koob, 2006). Differences in the maturation of synaptic connections between these critical brain regions, as well as age dependent activation of plasticity mechanisms may modulate the observed differences in single trial nicotine CPP (Spear & Varlinskaya, 2010).

MAPK Signaling and Single Trial Nicotine CPP

There was an effect of relative dark CPP chamber preference and MEK inhibition on CPP difference scores in adolescence. We found no effect of SL327 pre-exposure on nicotine CPP in LDP adolescents (figure 18). This finding was expected since we found that LDP adolescents do not form single trial nicotine CPP. A potential disruption of a learning related signaling cascade in the LDP adolescent group would not have an effect due to the lack of significant nicotine- cue memory formation.

We did find an effect of MEK inhibition and conditioning drug on CPP difference scores in HDP adolescents (figure 17). In adolescents with relative high dark CPP chamber preference, SL327 abolished single trial nicotine CPP compared to vehicle-pretreated adolescents. This result indicates that the MAPK pathway is involved in the formation of nicotine- context memory when adolescents tend to avoid an open, white chamber.

Our results are in line with previous findings that inhibition of MAPK signaling, via systemic injections of SL327 or intracranial infusions of U0126, decreases the strength of drug- cue associations (Valjent 2000; 2006; Miller & Marshal, 2005; Wells 2013; Li, 2012). Other studies have found that administration of SL327 prior to the presentation of a conditioned stimulus, such as the drug paired chamber (Groblewski et al., 2011) or a tone following fear conditioning (Matsuda et al., 2010), does not affect learning. These opposing findings have been attributed to differences in dose dependent effects across drug classes, peripheral side effects that are unavoidable with a systemic injection versus direct infusion, and differences in conditioning protocols (Groblewski, 2011). These experimental confounds may also explain why our omnibus F was not statistically significant. Nevertheless, Valjent et al. (2006) demonstrated that a single SL327 injection prior to cocaine priming dose in a previously cocaine paired CPP chamber eliminates cocaine CPP measured the next day. These results indicate that a single SL327 dose would be sufficient to abolish drug related plasticity, as was replicated in our study. It is also unlikely that our results were due to a lack of drug availability; peripheral injections of SL327 have been consistently proven to cross the blood- brain

barrier via histological analysis of MAPK protein levels in various brain regions (Valjent, 2000; Groblewski, 2011; Salzmann 2003).

SL327 affects MAPK activation levels in the striatum, NAc, VTA, amygdala, and HC (Valjent, 2000; Rajadhyaksha, 2004), and these brain regions are necessary for CPP induction (Gremel & Cunningham, 2008). SL327 inhibits the MAPK pathway, and also decreases levels of downstream MAPK targets such as the immediate early genes zif268 (Valjent, 2001), and transcription factors such as cFos, and fosB (Zhang et al, 2004), typically via activation of D1 receptors. Although specific pathways were not analyzed in our study, it is likely that SL327 inhibited the formation of a nicotine- cue association in HDP adolescents via a downregulation of ventral striatum activity.

Concluding Remarks

Relative dark CPP chamber preference influences single trial nicotine CPP in both adult and adolescent rats: adolescents with relatively high dark CPP chamber preference formed CPP whereas adults with relatively low dark CPP chamber preference shifted their preference towards the dark chamber after conditioning. In adolescent rats, relative high dark CPP chamber preference and single trial nicotine CPP are related to MAPK signaling; inhibition of the MAPK pathway abolished single CPP in HDP but not LDP adolescents. Our findings suggest that adolescents exhibiting decreased preferences for a novel, white, open CPP compartment are susceptible to the rewarding effects of nicotine. This susceptibility can be eliminated by a down regulation of MAPK. Adult rats with a relative initial preference for a novel, white, open CPP compartment are susceptible to developing aversion to that chamber after multiple exposures. Our results suggest the

importance of preexisting context preference across age groups in nicotine reward paradigms.

CONCLUSION: IMMUNOHISTOCHEMISTRY RESULTS

pMAPK Activation in the NAc Shell following a single injection

In the NAc shell, adult rats have an increased amount of pMAPK labeled cells in the CPP conditioning chamber following a saline injection compared to the homecage (figure 20). There is also a trend for nicotine-administered adolescents to have higher levels of pMAPK cell labeling following an injection in the CPP conditioning chamber compared to the homecage. Figure 20 also highlights a trend for homecage adolescents to have decreased levels of pMAPK labeled cells after a single nicotine injection compared to saline.

It is likely that our saline, adult cohort had higher levels of pMAPK labeled cells in the CPP chamber because the NAc is involved in the modulation of synaptic activity following a context specific stimulus presentation. Activity within the NAc accounts for differences in the hedonic value of novel stimuli presented across different environments (Bossert et al. 2007; 2009). Context specific locomotor sensitization to cocaine increases pCREB and pMAPK within the rat NAc, an effect not found following a cocaine injection in a novel, unpaired environment (Marin et al., 2009). As evidenced by the different pMAPK cell labeling profiles in the CPP chamber versus the homecage in both the NAc shell and core (discussed in the following section), the NAc is likely involved in encoding the significance of a stimulus presented in a novel context.

Our findings partially support our hypothesis that nicotine conditioned adolescents would have higher amounts of pMAPK labeled cells following an injection in the CPP chamber compared to the homecage. MAPK signaling is critical in the formation of drug- cue relationships (Cahill, 2014). Our results suggest a similar increase in synaptic activity, indexed via pMAPK counts, within a learning environment (CPP chamber) that may correlate with the induction of a context-cue association in adolescence.

In adolescents administered nicotine within the homecage context, we found a trend for decreased levels of pMAPK labeled cells compared to the adolescent saline group. Increases in MAPK activity are not consistently reported in response to drug administration or conditioned behavior. A single nicotine challenge following chronic variable stress in adult mice decreases pMAPK labeling in the NAc (Leao et al., 2012), although stress increases pMAPK levels in adolescent rats (Iñiguez et al., 2014). CPP induced via lateral hypothalamus stimulation does not cause differential levels of cFOS or pMAPK in the prefrontal cortex, VTA, or HC (Haghparast et al., 2011). It does, however, elicit pCREB increases in the prefrontal cortex and VTA, with a subsequent decrease in the HC (Haghparast et al., 2011). These findings suggest that although we found no overall effect in single trial CPP conditioning with pMAPK labeling, other activity related, neural proteins could modulate this effect, and thus went undetected with our testing protocol.

It is also possible that injection stress caused differences in adolescent pMAPK cell labeling in the homecage. Social stress causes pMAPK levels in the ventral tegmental

area to increase in adolescents (Iñiguez et al., 2014), and acute stress also causes pMAPK increases in the adult hippocampus (Ferland et al., 2014), hypothalamus and brainstem (Keshavarzt et al., 2014). It is possible that the increased pMAPK labeling in the adolescent saline homecage group were a result of increased stress from the animal's first exposure to a needle prick (Ryabinin et al., 1999). This effect was probably not detected in adults since adolescents are more susceptible to acute stress than adults (Toledo-Rodriguez & Sandi, 2007; Stone & Quartermain, 1997). In the adolescent group, nicotine, a known anxiolytic compound, may have counteracted the injection stress and resulted in decreased pMAPK labeling (Picciotto et al., 2002). Although acute and chronic stress activates neural activity within the BLA, our finding was limited to the NAc (Shors, 1999; Zhang & Rosenkranz, 2012). Some protocols analyze pMAPK cell labeling in the BLA 1-hour post stressor (Sarabdjiltsingh & Joëls, 2013). Since we analyzed tissue 30 minutes following injection, we may have missed a critical time point to detect stress effects in the BLA.

There are also age related differences in drug mediated pMAPK activation. During adolescence, moderate (1 g/kg) ethanol administration decreases pMAPK levels in the dentate gyrus, but increases levels in the BLA, an effect not found in adult mice (Spanos et al., 2012). A similar pattern of activation is found in both adolescents and adults with a high (3 g/kg) ethanol dose (Spanos et al., 2012). Since we only analyzed pMAPK labeled cells following a single dose of nicotine, it is possible that we may have missed a dose dependent effect on pMAPK labeling across age.

As mentioned previously, similar pMAPK labeling profiles were found in the NAc core. The interactions found in the NAc shell did not approach statistical significance in the NAc core due to greater variance in those pMAPK labeled cell counts. However, these analogous results are not surprising given that pMAPK density estimations in the NAc core and shell were not significantly different.

pMAPK Activation in the NAc Core after a Single Injection

In the NAc core, we found an effect of dosing context on pMAPK labeling after a single injection of either saline or nicotine (figure 21). There are higher amounts of pMAPK labeled cells in animals (adolescents and adults) injected and placed in the CPP conditioning chamber compared to the homecage. Although there was no significant effect of age on the amount of pMAPK labeled cells, there was a trend for adolescents to have higher baseline levels of pMAPK labeled cells compared to adults in the homecage setting.

Increased MAPK activation within the CPP conditioning chamber is likely related to the synaptic activity required to consolidate a stimulus- context association (Garcia-Carmona et al., 2013). MAPK signaling is necessary for long-term potentiation, and it has been implicated in nicotine induced synaptic activity within the HC (Philips et al., 2013; Welsby et al., 2009). Hypothetically, animals are not forming a drug- cue memory in the homecage setting, and so pMAPK cell labeling increases within our learning context match previous findings of CPP related, enhanced MAPK activity (Pan et al., 2011; Xu et al., 2012; Li et al., 2008). The caveat in this hypothesis is that there was no differential activation of pMAPK labeling across drug. If pMAPK levels were related to

the formation of a drug- cue relationship, then we would expect to see increased activation in nicotine-conditioned animals, which was not the case. Instead, the pMAPK labeling profile may relate to physiological arousal in response to a stressful stimulus occurring in a novel environment, such as an injection in the CPP chamber. The NAc is activated following novel and emotionally arousing stimuli (Green et al., 2006; Barrot et al., 2006; Fan et al., 2013). It is possible that our effects are not due to learning or novelty per se, but instead the interaction of an emotionally salient stimulus (the first injection experienced by the animal) in a novel environment (Ladurelle et al., 1995; Walsh et al., 2014).

Nicotine CPP is difficult to induce in animals, especially using a single trial protocol (Vastola et al., 2002; Shram et al., 2006). Perhaps the tentative nicotine- context association is an overall weaker memory compared to other forms of drug induced CPP. The similar levels of pMAPK labeling we found across age groups and drug may be a unique profile that is elicited during the initial stages of an associative memory formation. It is possible that we may have found differences in drug mediated pMAPK labeled cell levels if we analyzed tissue during the memory recall (CPP posttest). Also, since learning is taking place during a single training interval, rather than several, we may have missed the time-point for increased pMAPK activation that follows multiple conditioning sessions, as in other protocols (Cahill et al., 2014).

We also found that adolescent animals tend to have a higher amount of baseline pMAPK labeled cells compared to adults in the homecage, similar to previous findings in mice (Spanos, 2012). MAPK signaling is involved in many neural processes, including

cellular differentiation and growth (Cheng et al., 2013). Due to developmental changes occurring in the adolescent brain, we were not surprised to find higher levels of active MAPK signaling within the younger cohort (Spear, 2000). Increased levels of pMAPK signaling during this crucial developmental time period could relate to the increased vulnerability to drug reward seen during adolescence (Spanos et al., 2012). Unfortunately, our protocol did not allow us to reach such conclusions. Perhaps an analysis of pMAPK cell labeling after the CPP posttest, when the nicotine- context memory is being expressed, would have elicited an age dependent change in pMAPK labeling. Regardless, our findings in the NAc core, similar to those in the NAc shell, indicate that the NAc is involved in the regulation of context dependent stimulus exposure.

pMAPK Activation in the BLA after a Single Injection Across Contexts

A single nicotine or saline injection in either the homecage or CPP apparatus did not affect pMAPK cell labeling levels in the BLA across age groups (figure 22). As evidenced in the density comparisons for pMAPK labeled cell counts in the NAc versus the BLA, the BLA stands as our control region, since pMAPK labeled cell levels are significantly higher in the NAc after an injection of either saline of nicotine (figure 19). Using our testing protocol, pMAPK labeling is not ubiquitous, instead restricted to brain areas associated with the primary reinforcing effects of novel stimulus presentation. Novel environment exposure reverses stress induced pMAPK activation in the BLA (Yang et al., 2008). At least in the CPP chamber (novel environment) group, these

previous findings suggest that we did not see differential pMAPK labeling following injection stress due to remediation from novelty exposure.

Although the extended amygdala is involved in the mesolimbic dopamine reward pathway, during the initial drug exposure phases, structures such as the VTA and NAc take a primary role in the "drug liking" phase (Di Chiara, 2004; O'Dell, 2009). Following multiple exposures and after strong drug- cue associations have been established, the amygdala becomes essential in the maintenance of reinforcing and reinstatement behaviors (Chauvet et al, 2012; Brunzell et al., 2003). Therefore, it is possible that our testing parameters precluded a significant detection of pMAPK labeling within the BLA since we only looked for activation to a single drug dose. Since the BLA is involved in the expression of CPP behavior, it is possible to have seen an effect of pMAPK labeling if brains had been analyzed after the posttest, when the drug- cue memory was expressed (Hashemizadeh et al., 2014; Hetzel et al., 2012; Rademacher et al., 2010; Zarrindast et al., 2005). The specifics of our testing protocol, in terms of the timing for brain extraction following nicotine exposure, had been previously validated (Valjent et al., 2004; Brunzell et al., 2009; Zhai et al., 2008; Spanos et al., 2012). However, it is possible that we missed a critical time point in pMAPK cell labeling since alternate timing parameters have detected effects of pMAPK labeling in the BLA in response to behavioral learning paradigms such as fear conditioning, fear extinction, and CPP reconsolidation (Ding et al., 2013; Otis et al., 2013; Bergstrom et al., 2013; 2013; Fuchs et al., 2002).

The lack of differential pMAPK labeling in the BLA following a single drug administration, while surprising, is not implausible given that MAPK activation within

the BLA is not necessary for all types of drug induced CPP (Groblewski et al., 2011; Li et al., 2008). Our results likely indicate the specificity of our testing protocol to target brain regions critical during the initial exposure to a stimulus in novel environments.

Concluding Remarks

After a single injection in a CPP or homecage context, we found differences in the amount of pMAPK labeled cell in the NAc shell and core. In the adult NAc shell, we found increased levels of pMAPK labeled cells in the CPP chamber versus the homecage after a saline injection. There was a trend in the adolescent NAc shell for increased pMAPK labeled cell counts in the CPP chamber compared to the homecage after a single nicotine injection. Our findings suggest that pMAPK cell labeling levels within the NAc shell respond differentially to a stimulus in a novel versus familiar context. In the NAc core, we found higher amounts of pMAPK labeled cells in the CPP chamber compared to homecage, regardless of drug or age. This suggests that during an initial stimulus exposure, the environment modulates activity within the NAc core. We did not find an effect of age, context or drug on pMAPK labeling in the BLA, suggesting that within our protocol parameters, pMAPK labeling in the BLA is not regulating the response to single injections in a novel or familiar context. Across our regions of interest, we did not find that pMAPK labeling differed according to nicotine administration during CPP conditioning. These results suggest that after a single injection, pMAPK labeling does not differentially modulate the formation of a nicotine- cue association across age. Overall, our results suggest that in both adolescents and adults, the significance of an exposure to a novel stimulus is marked by activation of MAPK signaling within the NAc.

EXTENDED INTRODUCTION

Nicotine is one of the most heavily abused substances in the United States. Adolescents are known to initiate drug use with tobacco (Breslau et al., 1996), and most adult cigarette users began smoking during adolescence (Nelson et al., 1991). These trends suggest that cigarette use at an early age may be especially risky in terms of addiction liability (Breslau et al., 1996). Despite the implementation of drug prevention programs and details about the health risks related to smoking (U.S. Department of Health and Human Services, 2010), the rate of quitting success remains low (Center for Disease Control, 2000), demonstrating the addictive power of nicotine. While the study of the biological bases of drug addiction has made advances in recent years, understanding the neurodevelopmental trajectory of addiction during the adolescent developmental period is still lacking. The purpose of the proposed research is to identify molecular markers of brain change that underlie the propensity to seek nicotine during the adolescent development period.

One of the defining characteristics of drug addiction is a compulsion to seek and take drugs, along with the loss of control in limiting its intake (American Psychological Association, 2000). Adolescents undergo a series of developmental neural changes that prime their susceptibility for compulsive drug seeking. They exhibit a sensitized ventral striatal reward pathway, which increases their perception of the positive affect associated

with nicotine (Bava & Tapert, 2010). Additionally, adolescents have attenuated inhibitory control circuits, stemming from the immaturity of prefrontal cortices (Casey & Jones, 2010). Our lab has shown that nicotine influences long-term changes to neurons in the medial prefrontal cortex that are specific to adolescence, suggesting that nicotine alters the development of reward (Bergstrom et al., 2010).

The learned associations between drug-related, environmental stimuli and the rewarding 'high' further support addictive behaviors in cigarette users. Our lab found that adolescent rats establish a learned preference for context after a single nicotine- context pairing, an effect that did not generalize to adults (Brielmaier et al., 2007). This suggests that adolescents form stronger associations between drug related cues and their environment compared to adults.

The formation of long term associative memories, such as the ones involved in CPP, are initiated by the activation of molecular signaling cascades that ultimately result in new gene expression and long terms synaptic changes. One of these pathways, the mitogen activated protein kinase cascade (MAPK), is involved in the establishment of synaptic plasticity associated with long-term memory formation (Adams et al., 2000). This proposal will first illustrate the role of MAPK in memory functions and then introduce CPP as a valid measure of drug reward. Briefly, adolescence will be explored as a vulnerable time period in addiction and methods will be discussed as to how we can empirically ascertain the relationship between nicotine induced CPP and MAPK across age.

MAPK and Associative Learning

During learning, activation of NMDA glutamate receptors results in calcium influx to the neuron (Atkins et al., 1998). Calcium acts as a second messenger, activating a series of protein kinases families, notably protein kinase C (PKC). Activation of PKC triggers the MAPK pathway, which coordinates gene expression changes within the nucleus (Davis et al. 2000). MAPK phosphorylates specific transcription factors, such as CREB, which is involved in HC- dependent long-term memory formation (Sweatt, 2001).

The MAPK pathway is often conceptualized as a MAP kinase module due to the "consistent appearance of 3- kinase cascades" (Cobb & Goldsmith, 1995). The modules are seen to carry information to effector proteins and "coordinate incoming information from parallel signaling pathways" (Cobb & Goldsmith, 1995). The standard MAP kinase module is made up of three protein kinases, as mentioned previously, that act sequentially within one phosphorylation cascade: a MEKK (MEK activator), a MEK (MAP kinase activator), and a MAP kinase (Cobb & Goldsmith, 1995). MEKs are dual specificity kinases that trigger the activation of MAPKs by phosphorylating a threonine (Thr) and a tyrosine (Tyr) in their activation loop (Girault, 2007). MAPKs are subdivided into three large subgroups based on their activation sequence. This project will focus on the relationship between one of the MAPK modules, p44/p42 MAPK, which is part of the extracellular signal related kinase (ERK 1/2) subfamily (Schramek, 2002). The MAPK/ERK family is characterized by a Thr-Glu-Tyr motif in the activation loop, and this module has been identified as "important" in cell growth regulation, as well as neuronal plasticity (Girault, 2007).

The “best characterized” method of MAPK activation is via growth factors (Seger & Krebs, 1995) and illustrated in Figure 20. Binding of a growth factor to its receptor leads to the activation of the protein tyrosine kinase within the cytoplasm. It autophosphorylates, and recruits adapter proteins such as GRB2. GRB2 then activates mSOS, a guanine nucleotide exchange protein. mSOS catalyzes GDP to GTP via the Ras G-protein. Ras then recruits the protein kinase Raf to the membrane, which phosphorylates and activates the dual-specific protein kinase MEK (MAPKK) (Adams et al., 2000). In addition, certain G protein receptors can trigger MAPK activation, including alpha 1-adrenergic, alpha2-adrenergic, muscarinic, dopamine 2 receptors, and AMPAr, all via Raf stimulation (Gutkind, 1998). Alpha 7 nicotinic acetylcholine receptors (nACHr) also activate MAPK pathways via activation of CaMKII (Gubbins et al., 2010). An activated ERK1/2 can then phosphorylate numerous substrates at serine/threonine protein kinase sites within the cell, including the nucleus where they play a role in the activation of protein synthesis (Girault, 2007).

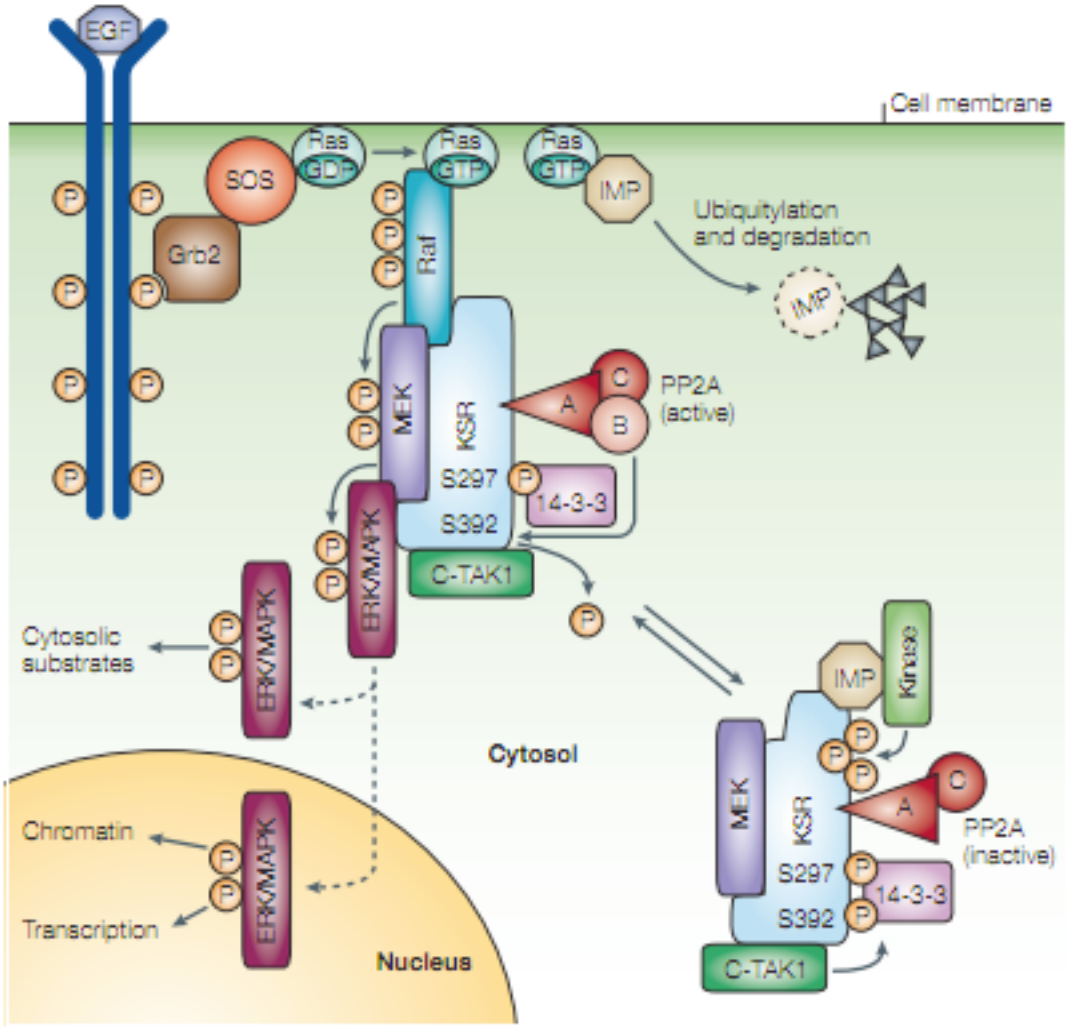


Figure 24: MAPK cascade triggered by an epidermal growth factor
Taken from Kolch, 2005

The MAPK cascade, in terms of learning and memory functions, "serves to funnel" extracellular signals into "common downstream targets" to produce a "coordinated" output at the cellular level (Sweatt, 2001). The "diversity" found in MAPK activation via PKA or PKC suggests that these kinases exert a role in whether MAPK modulates short versus long-term memory (Sweatt, 2001).

English and Sweatt (1996) provided the first evidence that the MAPK cascade is involved in memory. They found that ERK2 is activated after NMDAr stimulation in the CA1 area of the HC. Tetanic stimulation of the Schaffer-collateral inputs to the CA1 also led to the activation of ERK2 in area CA1. Later, the authors also found that ERK2 is required for the induction of stable long-term LTP through the use of intraventricular injections of PD098059, a specific ERK inhibitor (English & Sweatt, 1997). ERK2 is also involved in both NMDAr dependent and independent LTP in the dentate gyrus of the rat (Coogan et al., 1999). ERK has also been shown to be involved in LTP in the insular cortex synapses, and this pathway is hypothesized to be involved in taste memory (Berman et al., 1998).

MAPK has been found to be involved in several associative learning paradigms, such as fear conditioning (FC) (Atkins et al., 1998). During FC, an animal associates a tone (cued FC) or an environment (contextual FC) with an electric shock, and after training the tone or environment induces freezing behavior. Contextual FC is hippocampal dependent, while cued FC is amygdala and hippocampal dependent (Flinn, 2009). ERK2 phosphorylation increased in the rat HC within two minutes of training and lasted for up one hour in both the cued and contextual paradigms. Peripheral injections of another ERK inhibitor, SL327, inhibited the freezing behavior, demonstrating the necessary presence of MAPK proteins in order for learning to occur (Atkins et al., 1998).

MAPK also plays a role in spatial memory, another form of hippocampal dependent learning that is classically tested using the Morris Water Maze. In this paradigm, animals undertake a “search strategy to locate a platform hidden under an

opaque water pool" (Selcher et al., 1999). SL327 injected mice took longer to find the hidden platform compared to controls and performed worse on a probe trial test compared to controls. A probe trial examines the animal's use of their "search strategy" in the quadrant where the platform used to be located (Selcher et al., 1999). The same effect was found after intrahippocampal infusions of PD098059 in rats (Blum et al., 1999).

MAPK activation is not consistent across brain regions or age groups. During cued FC extinction training, the CS (tone) is presented without the US (shock). Under normal circumstances, the animal forms a new memory that the tone no longer predicts a shock and does not freeze to the CS. Extinction training is both prefrontal, specifically infralimbic cortex, and amygdala dependent (Flinn, 2009). Preadolescent (P24), adolescent (P35) and adult (P70) rats all exhibit normal FC and extinction following training as mentioned previously. Adolescent rodents, however, do not maintain extinction learning on the day following training (Kim et al., 2010). This deficit was correlated with decreased phosphorylated MAPK levels in the infralimbic cortex of adolescent animals. With increased extinction training the adolescent animals maintain extinction learning past day 2, which also correlated with increased MAPK activation in the infralimbic cortex (Kim et al., 2010).

Beyond classical learning paradigms, MAPK signaling has also been implicated in addiction. Drug seeking behaviors consist of a set of learned patterns; associations that are made between drug cues, the environment (both physical and social), reward, and withdrawal. Several brain structures are known to play a role in drug addiction, namely

the HC, ventral striatum (including the nucleus accumbens and ventral tegmental area), and the extended amygdala. MAPK activation increases in the ventral tegmental area in response to cocaine exposure (Valjent et al, 2000). Dopamine- 1 receptors are known to modulate the rewarding effects of drugs, and D1 antagonism blocks MAPK phosphorylation in response to cocaine exposure. Additionally, MAPK inhibition via SL327 blocked cocaine induced locomotion, indicating a role for MAPK in drug related behaviors (Valjent et al, 2000). However, MAPK activity is not ubiquitous in its modulation of drug related learning. ERK activation is not involved in ethanol (Groblweski, 2011) or morphine dependence models (Mouledous, 2007). In addition, its role in nicotine related plasticity has yet to be investigated.

Conditioned Place Preference

As mentioned previously, CPP is a paradigm used to measure drug reward in laboratory animals (Bardo & Bevins, 2000). In theory, CPP reflects the animal's preference for a particular situation due to the contiguous association between that context and a drug stimulus (Tzschentke, 2007). CPP follows a simple protocol that beings with a pretest, in which the animal is allowed to move freely between the contexts of a two or three- chambered testing apparatus while their baseline activity is being monitored. Afterward, conditioning sessions involve exposing the animals to the drug stimulus and confining them to one chamber of the apparatus. CPP is established through the use of a post-test, in which the animal is again allowed free access to the entire chamber while their activity is monitored. The amount of time spent in the chamber

previously paired with the drug stimulus is quantified and the results are compared with those from the pretest.

The drug- context pairing can follow either of two paradigms. In an unbiased CPP design, the researcher presupposes that the "contribution of a subject's initial preference for a given chamber" is negligible and therefore context cues are counterbalanced to initially provide "equivalent" side preference (Calcagnetti, 1993). Subjects are then randomly assigned to drug conditioning on either side of a two or three-chambered apparatus. On the other hand, a biased CPP procedure takes into account that subjects can display an initial side preference. The bias is incorporated into the basis of establishing a baseline preference measure, thus drug pairings only occur in the non- preferred chamber. Biased CPP designs have been argued to be a more "conservative" (Calcagnetti, 1993) and "effective" (Le Foll & Goldberg, 2009) measure of reward, compared to unbiased designs, because the drug association must be sufficiently rewarding in order to overcome avoidance and produce a shift in preference.

An advantage of CPP is the ability to test for reward in a drug- free state. Researchers can avoid confounds of drug mediated alterations in locomotion and effectively test the association between drug cues and motivation (Bardo & Bevins, 2000). Some researchers believe biased and unbiased CPP test different context induced motivational states. It is thought that the shift in preference to the initially non- preferred chamber in a biased paradigm is accounted for by the reduction of anxiety rather than increased reward (Brielmaier et al., 2008). Nicotine is known to have anxiolytic effects (Kobiella et al., 2011; Cohen et al., 2009), and thus the animal's shift in preference may

be due to nicotine's interaction with their initial aversion, rather than endogenous reward states (Le Foll and Goldberg, 2005). However, counterbalanced nicotine pairings in a two-chambered apparatus did not yield significant nicotine CPP, whereas nicotine pairings in the non- preferred chamber did (Brielmaier et al., 2008). If the results from a biased CPP paradigm were due to reduction of avoidance, both the paired and counterbalanced groups should have "demonstrated similar mean difference scores, " in other words, more time spent in the non-preferred, white chamber (Brielmaier et al., 2008).

Another advantage of CPP is its ability to demonstrate drug context conditioning across drug classes. Additionally, under the correct conditions, this paradigm is sensitive enough to detect place preference differences across age groups. In an unbiased paradigm, adolescent (P28) rodents form single trial nicotine CPP, in contrast to their older adolescent and adult (P38, P90) counterparts (Belluzi et al., 2004). As mentioned previously, work in our lab found evidence of single trial nicotine CPP induction in early adolescent (P28) but not adult animals (P77) using a biased CPP paradigm (Brielmaier et al., 2007). Using a higher conditioning dose of nicotine (0.6 mg/ kg versus 0.5 mg/kg used in the Belluzi (2004) and Brielmaier (2007) experiments) over 8 training days also yielded an age difference in a biased CPP protocol: adolescents (P28) formed a bias for the nicotine paired chamber whereas adults (P58) did not (Vastola et al., 2002).

MAPK & CPP

During CPP, a learned association is made between distinct environmental cues and a drug after a series of pairings between the context and drug injection. There is an

increase in MAPK phosphorylation both a day and a week after CPP training with morphine (Li et al, 2001). Over half of the neurons that stain positively for MAPK express NMDAr, suggesting a calcium-regulated activation of MAPK. Additionally, infusions of an ERK inhibitor "abolishes" place preference and NMDAr antagonists suppress MAPK activation (Li et al, 2001).

Similarly, cocaine CPP also activated both MAPK and CREB in the nucleus accumbens shell, a subregion of the accumbens related to reward (Miller & Marshall, 2005). Infusions of an ERK inhibitor into the accumbens immediately after memory recall (CPP posttest) "blocked" MAPK activation and place preference when the animals were tested the next day, and two weeks later (Miller & Marshall, 2005). Thus, it seems that MAPK is necessary for the initial formation of the place preference memory, as well as reconsolidation of that memory. Taken further, these experiments suggest the MAPK is required for the overall consolidation and expression of a drug- cue memory via LTP like mechanisms. Direct infusions of nicotine into the HC can elicit LTP (Tang et al., 2009). Induction of nicotine- dependent LTP requires MAPK activation, specifically the p42/p 44 isoforms (Wang et al., 2001).

LTP induced in response to nicotine exposure and CPP has also been linked to calcium induced protein synthesis within the postsynaptic neuron. In mice, nicotine CPP has been associated with increases in phosphorylated CREB in the ventral tegmental area, nucleus accumbens and pedunculopontine nucleus (Walters et al., 2005). Disruption of normal CREB activity via a viral- mediated gene transfer of a negative CREB isoform disrupts nicotine place preference (Brunzell et al., 2009). Nicotine CPP induction and

long term maintenance, tested as drug primed reinstatement following an extinction period, also induces CREB phosphorylation in rats within the accumbens, VTA, prefrontal cortex, amygdala and HC (Pascual et al., 2009). Therefore it seems that activation of CREB is required for the formation of place preference, and protein synthesis within the nucleus accumbens regulates the motivational value of the drug-paired chamber. Increases in synaptic function within the amygdala and HC may facilitate the formation of long-term memories associated with the value of the conditioned stimulus (drug paired chamber). Although there is an increasing amount of work being conducted that explores the relationship between signaling cascades and drug related associative conditioning in adulthood, relatively little has been done in the way of exploring these links in the developing brain. The goal of this project is to determine whether there is a potentiation of MAPK activity within younger groups that could account for differences in drug seeking during adolescence. In a very general sense, MAPK would be used in this project as a marker for altered synaptic activity in relation to the formation of single trial nicotine CPP.

Adolescent Drug Use

There has historically been a rising trend in adolescent substance abuse, especially in terms of cigarette and alcohol use (Kenkel et al., 2001). Often times, adolescent drug use escalates, with roughly a quarter of the self reported "non-heavy drinkers" in 10^{th} grade later reporting being heavy drinkers by 12^{th} grade (Public Health Service, 1990). The average age at which illicit substance use began reported by adult addicts is sixteen, with over half beginning between the ages of fifteen to eighteen (Chambers et al., 2003).

Although adolescent experimentation is seen to occur with nicotine, alcohol, marijuana, or cocaine (Chambers et al., 2003), my project will focus on adolescent nicotine exposure since smoking onset is uncommon after age twenty- five (Breslau et al., 2001). Thus, smoking provides a prime example of drug seeking that initiates early in life and is maintained over the lifespan. Adolescents are initially drawn to smoking due to positive media presentation regarding smoking in movies and television shows, along with its ubiquitous availability (Kenkel et al., 2001).

Early onset nicotine has long-term consequences on drug-taking cessation. There is an increased likelihood for successful cigarette cessation if smokers initiated smoking after age thirteen (Breslau & Peterson, 1996). Beyond age thirteen, the "percentage of quitting increased with increasing age at first cigarette" (Breslau & Peterson, 1996). While there have been several programs implemented to deter teens from drug use, these have had little success (Kenkel et al., 2001). Adolescent behaviors, such as increased novelty seeking and risk taking seem to foster experimentation with drugs. This early exposure to drugs could affect the development of normal synaptic circuitry, and prime the adolescent for further drug use, an effect that could last into adulthood.

P*roposed Project*

Adolescence has been distinguished as a period of particular vulnerability to addiction, including drug cued- learning, and MAPK activation is related to formation of these associative memories. Thus, the aim of my dissertation is to determine whether differential MAPK activation during adolescence (compared to adulthood) is related to

the formation of a drug- cue relationship by looking at place preference following a single exposure to nicotine.

The purpose of examining the potential molecular underpinnings of addiction is ultimately to suggest targets for treatments that may prevent the development of addiction or reverse the changes imposed on the brain by early drug exposure. Studying the mechanisms involved in the formation of drug- cue memories is of utmost importance, given that the significance of memories formed during the first cigarette exposure in adolescence has strong effects on the development of nicotine dependence (DiFranza et al., 2004). Understanding these links would elucidate the ability of nicotine to modulate goal directed behavior during adolescence.

Immunohistochemistry Procedure

Slides were cover-slipping after tissue dehydration in ethanol concentrations of 50%, 50%, 95%, 100%, and 100%.

Negative Tissue Control

In order to assess the presence and pattern of non- specific staining of the antibody to proteins other than phospho- pMAPK, a non- immune serum control will be run on select tissue slices. As stated previously, every other tissue slice will be run for pMAPK immunohistochemistry. Those slices not processed for phospho-pMAPK will be run in a mock staining procedure using the exact parameters as stated above in "Immunohistochemistry Procedure," except that the primary antibody will be replaced with non- immune rabbit serum (Normal Rabbit IgG, 1:250 dilution, Cell Signaling).

Hypothesis

Based on previous work in our lab (Brielmaier 2007; 2011), I expect to replicate the behavioral phenomenon in which the adolescent group, and not the adult group, shifts their preference to the nicotine-paired chamber. The adolescent group, and not the adult group, will quickly form a long lasting association between the distinct training environment and the rewarding effects of nicotine. Since MAPK signaling has been implicated in both cellular development and long-term memory formation, the induced place preference shift in the adolescent-nicotine group should correlate with increases in phosphorylated MAPK. This increase in immunopositive MAPK within the adolescent-nicotine group is expected to be significantly different from baseline levels recorded from open field controls, adolescent-saline controls, or the adult cohort (nicotine and saline conditioned). These control groups are not hypothesized to learn a specific drug- cue relationship.

MAPK activation will be assayed in three specific brain structures. The nucleus accumbens core and shell relate to the reward conditioning effects of nicotine, therefore I expect to find an increase in pMAPK in these two areas. The basolateral amygdala is being used a negative control, and thus I expect to find no increase in pMAPK counts. I do not expect to find any significant staining values in the negative control tissue since the immunohistochemistry procedure has been validated previously in a separate experiment (Bergstrom et al., 2011)

Finally, it is also possible, although unlikely due to recent research findings, that I find no MAPK activation within groups. If there were an absence of pMAPK activation, it would have served to run a double labeling experiment. In this case, I could detect not

only the activation of pMAPK, but also determine the influence of another protein within the MAPK signaling cascade. In this instance, a possible candidate could have been a more downstream effector target such as c-Fos or CREB. A comparison of the percent of activated to non activated pMAPK using double labeling and fluorescence could also unmask a differences between groups that were obscured by absolute counts of phospho-pMAPK. Regardless, an absence of pMAPK activation could still suggest the interesting possibility that one- trial nicotine CPP is a unique behavioral paradigm that involves different neural substrates compared to other CPP models.

EXTENDED RESULTS

Normal IgG Serum Controls

There was a significant difference between p44/42 MAPK cell labeling and normal rabbit IgG cell labeling in the nucleus accumbens, $t(56) = 14.34$, $p = 0.00$ (figure 25). Our results illustrate that p44/42 MAPK cell labeled counts ($M = 447.32$, $SE = 31.07$) are significantly greater than control serum ($M = 4.5$, $SE = 0.25$) responses in the nucleus accumbens, demonstrating antibody specific staining with our immunohistochemistry protocol.

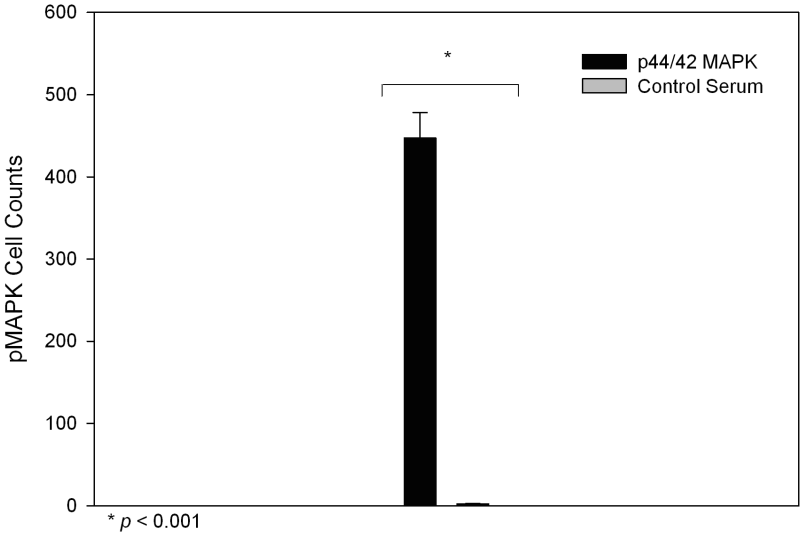


Figure 25: P44/42 MAPK labeling in the Nucleus Accumbens versus Normal Rabbit IgG labeling:
Paired samples t test indicate that in response to injection exposure, there are a higher amount of p44/42 MAPK labeled cells in the nucleus accumbens compared to normal rabbit IgG serum labeled cells, $p < 0.05$. Error bars represent standard error.

There was also a significant difference between p44/42 MAPK and normal rabbit IgG cell labeling in the basolateral amygdala, $t(56) = 20.79$, $p = 0.00$ (figure 26). Our results illustrate that there is a significantly higher amount of p44/42 MAPK labeled cells (M= 447.32, SE= 31.07) compared to control serum (M= 2.37, SE= 0.2) labeled cells in the basolateral amygdala, demonstrating antibody specific staining with our immunohistochemistry protocol.

Figure 26: P44/42 MAPK Labeling in the Amygdala versus Normal Rabbit IgG Labeling
Paired samples t test indicate that in response to injection exposure, there are higher amounts of p44/42 MAPK labeled cells in the basolateral amygdala compared to normal rabbit IgG serum labeled cells, $p < 0.05$**.** Error bars represent standard error.

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