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Rapid Adaptation of Dopamine D2 Receptor Responses
in the Brain and Blood Following Acute
Ethanol

Ryan John Folsom

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science in Neuroscience

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ABSTRACT

Rapid Adaptation of Dopamine D2 Receptor Responses in the Brain and Blood Following Acute Ethanol

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Master of Science in Neuroscience

Dopamine (DA) D2 receptor expression parallels DA levels in the brain and these autoreceptors have been shown to be modulated by long-term ethanol exposure. We have previously demonstrated that ventral tegmental area (VTA) GABA neurons also express D2 autoreceptors (D2R), and that DA and D2R agonists markedly enhance the excitability of VTA GABA neurons, opposite to their well-known auto-receptor inhibition of DA neurons. Most importantly, D2R antagonists block ethanol inhibition of VTA GABA neurons and D2R expression in VTA GABA neurons down-regulates with chronic ethanol, as others have shown for whole VTA D2R expression.

The aim of this study was to evaluate short-term D2R adaptation in specific brain reward regions i.e., ventral tegmental area (VTA), nucleus accumbens (NAc), temporal lobe cortex, and also in peripheral white blood cells (WBCs) as a potential biomarker for brain DA. To accomplish these studies, we used quantitative RT-PCR to analyze rapid (within 2 hrs) changes in D2R expression from both brain and blood samples of rats from one of four *in vivo* treatment groups: saline, ethanol (2.5 g/kg, IP), eticlopride (1 mg/kg, IV), or quinpirole (0.1 mg/kg, IV). To verify the qRT-PCR effect we observed from tissue punches of the selected brain regions, we used immunofluorescence to quantify changes in D2R expression between the four treatment groups. To determine whether D2R adaptation in the blood was dependent on communication with the brain, we extracted blood samples and performed the same type of *in vivo* experiments *in vitro*.

We found that D2R expression was increased in the VTA with ethanol, eticlopride and quinpirole, increased in the NAc with ethanol but decreased with eticlopride and quinpirole, and decreased with ethanol and quinpirole in the temporal lobe cortex. In the *in vivo* blood experiments, D2R expression decreased in WBCs in all three drug treatment groups. *In vitro* blood experiments showed increased expression with ethanol treatment and decreased with eticlopride. When compared to saline treated animals, the immunofluorescence in the VTA suggests that D2R expression increased in ethanol and eticlopride, but decreased in quinpirole treated animals.

At this point, it is clear that D2R expression shows rapid adaptation when exposed to acute doses of ethanol and D2 targeting drugs in both the brain and blood. More evidence is needed through *in vitro* studies to determine whether a specific neuro-immune interaction is directing the changes seen in the blood and whether or not chronically exposed animals show significantly decreased D2R expression in the blood.

Keywords: ethanol, eticlopride, D2 dopamine receptor, VTA, NAc

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INTRODUCTION

The Mesolimbic Dopamine System

The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAc) is considered to be a key area in reward from natural behaviors such as feeding (Phillips et al., 2003), drinking (Agmo et al., 1995), and drug reward, including alcohol reward (Koob, 1996). Figure 1 depicts the theoretical connectivity of this pathway. Support for a role for this neural circuit in alcohol reward hinges on the evidence demonstrating that local injections of DA antagonists into the NAc and/or afferent circuit systems prevent ethanol self-administration in rats (Hyytia and Koob, 1995; Pettit et al., 1984; Roberts et al., 1996; Vaccarino et al., 1985). Moreover, rats will self-administer ethanol directly into the VTA (Gatto et al., 1994),

and an ethanol-induced increase of DA release in the NAc, detected by microdialysis, has been reported extensively (Di Chiara and Imperato, 1988; Weiss et al., 1993; Wozniak et al., 1991; Yoshimoto et al., 1992). Acute ethanol increases the firing rate of midbrain DA neurons both *in vivo* and *in vitro* (Brodie et al., 1990a; Gessa et al., 1985), and chronic ethanol reduces both

DA activity and release of DA in the NAc during withdrawal (Diana et al., 1993).

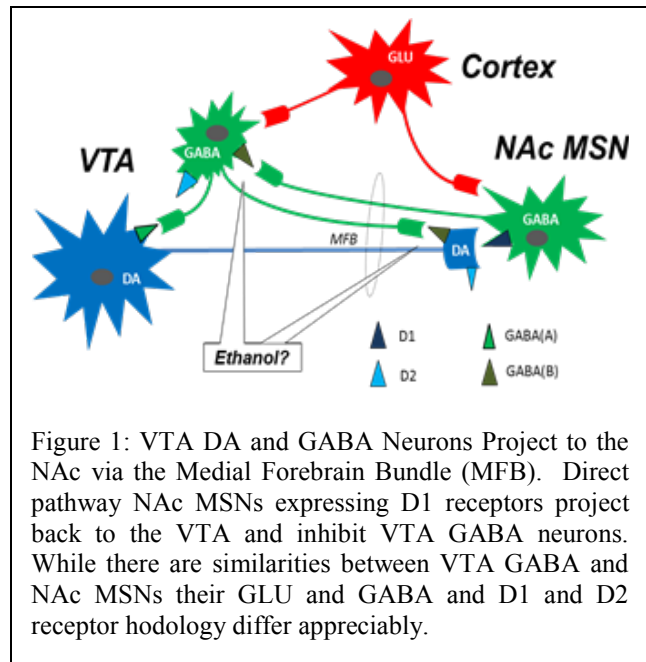


Figure 1: VTA DA and GABA Neurons Project to the NAc via the Medial Forebrain Bundle (MFB). Direct pathway NAc MSNs expressing D1 receptors project back to the VTA and inhibit VTA GABA neurons. While there are similarities between VTA GABA and NAc MSNs their GLU and GABA and D1 and D2 receptor homology differ appreciably.

D2 Dopamine Receptors as Biomarkers for Brain Dopamine Levels

Dopamine neurons in the SN and VTA are inhibited by DA via D2 autoreceptor

activation (for review see (Adell & Artigas, 2004)). Dopamine D2R expression in DA neurons in the midbrain that are involved in movement (i.e., substantia nigra; SN) and motivation (i.e., ventral tegmental area; VTA) is a well-known biomarker for brain DA levels, drug abuse and dependence. Currently, the levels of D2Rs in the

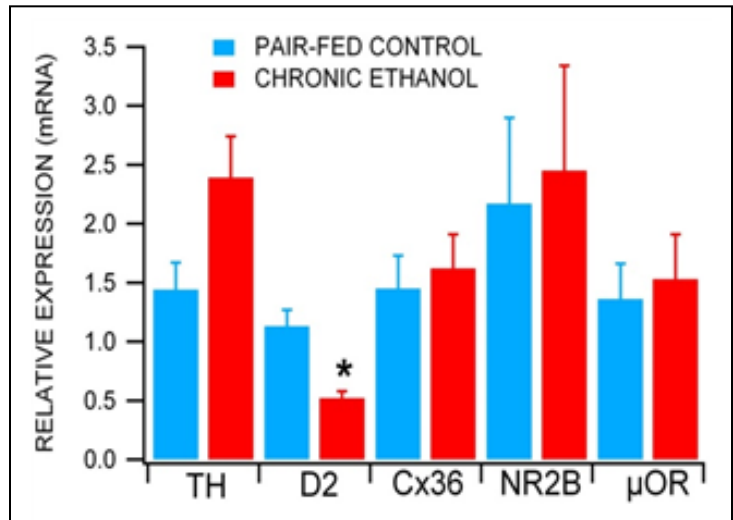
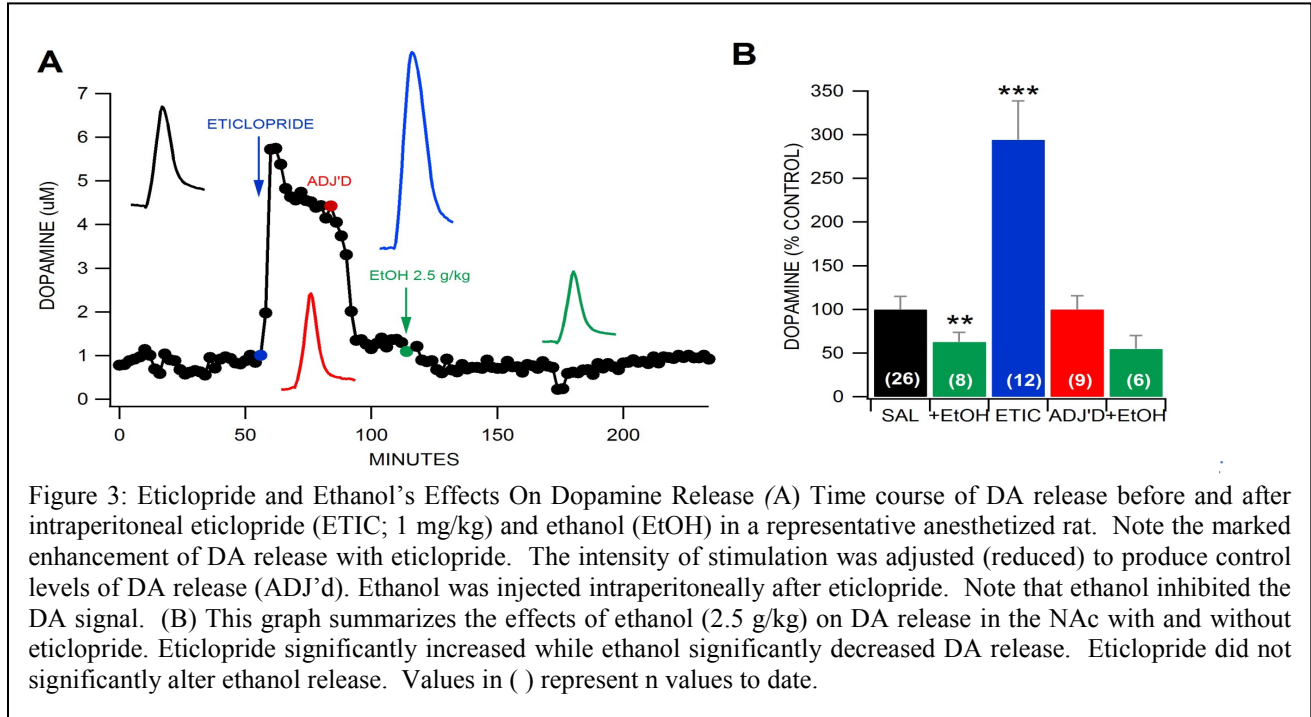


Figure 2: Chronic Ethanol Exposure Significantly Decreases D2R Expression. Chronic ethanol mice also demonstrate significant differences in TH expression, but for the intents and purposes of this study, we focused on the relative mRNA expression of the biomarker, D2.

brain can be studied with positron emission tomography (PET) and they correlate directly with brain DA levels. Many animal and human studies have demonstrated convincingly that D2R expression is directly proportional to DA levels in the brain with a lag of minutes to hours. The chronic abuse of stimulant drugs reduces DA D2R levels throughout the mesolimbic system (Figure 2) (Nader *et al.*, 2002). Thus, D2Rs are highly responsive to brain DA levels. However, PET scans are cost-prohibitive to be used in clinical practice. In addition to cost, invasiveness and radiation risk preclude PET scans as a routine clinical tool for assessment of D2R levels.

Electrophysiology of Dopamine Release and Tie to D2R Expression

Using fast scan cyclic voltammetry (FSCV) in mice and rats, we have found that DA release in the ventral striatum, the terminal region of VTA DA neurons, is reduced by moderately intoxicating doses of ethanol and markedly enhanced by systemic administration of



D2R antagonists (e.g., eticlopride; Figure 3). The enhancement of DA release by eticlopride is probably the most robust and reproducible effect we have observed in our studies of DA release. As eticlopride significantly enhances DA release, it will be used as a positive control for the studies outlined to evaluate D2R expression in the blood and brain associated with high levels of DA release. In preliminary studies, we found that D2R expression in the VTA is enhanced by eticlopride and reduced by ethanol, which is consistent with its well-known lag in DA release (Figure 4).

We also evaluated D2R expression in WBCs of rodents. Using flow cytometry and fluorescence immunocytochemistry, we have found that ~13% of WBCs in both rats and mice express D2Rs. Figure 5 shows D2R labeling in some WBCs. Using multiple fluorophores for different WBC types in mouse blood, we found that most of the D2R+ WBCs are activated monocytes. This is somewhat surprising considering the existing literature suggesting that

lymphocytes express D2 receptors.

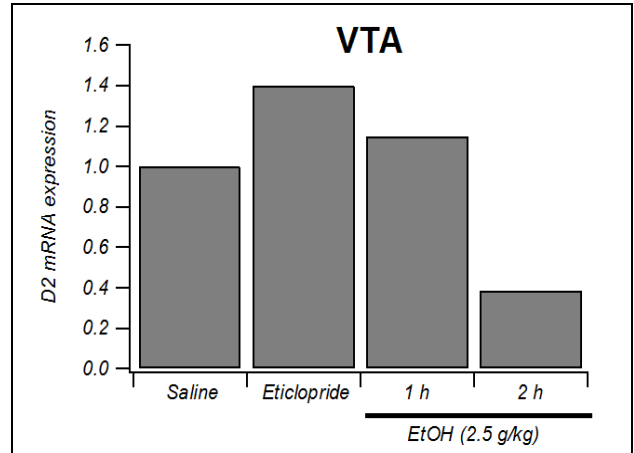


Figure 4. Ethanol and Eticlopride-Induced Change in D2R Expression: Preliminary. An intraperitoneal injection of eticlopride enhances D2 mRNA expression in the VTA while an IP dose of ethanol reduces D2 mRNA expression with a time course of 1-2 hrs.

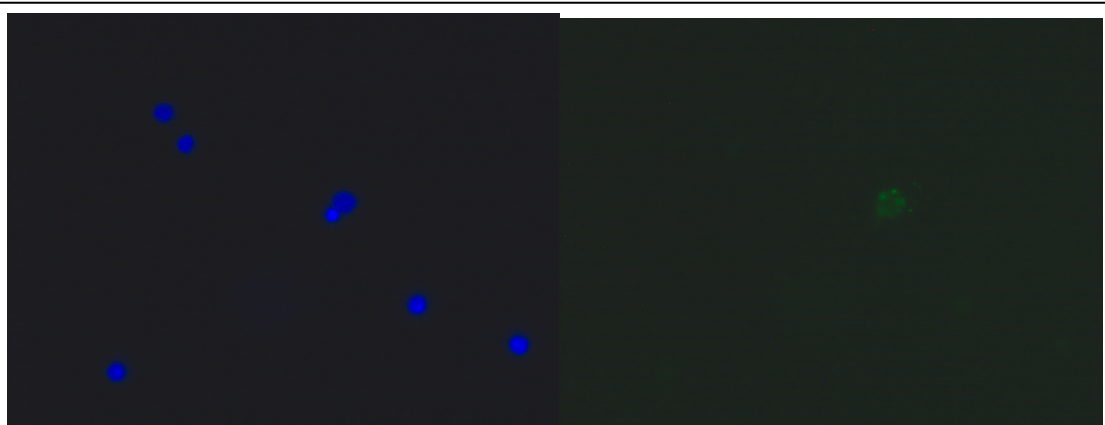


Figure 5: A Subpopulation of WBCs Express D2Rs. (A) Stain for all WBCs. (B) One of the WBCs in (A) expresses D2Rs.

RATIONALE AND HYPOTHESES

Markers of D2R expression are not only detectable in the brain but are also expressed in peripheral tissues, including blood, where DA appears to play a pivotal role in mediating communication between the nervous and immune systems (Basu & Dasgupta, 2000), and in particular via D2Rs on lymphocytes (Basu *et al.*, 2010). Of particular relevance, D2/D3 receptor expression is known to be enhanced in lymphocytes of schizophrenics (Ilani *et al.*, 2001), reduced in Alzheimer's disease (Barbanti *et al.*, 2000a), increased in migraineurs (Barbanti *et al.*, 2000b) and reduced in Parkinson's patients (Nagai *et al.*, 1996), albeit other DA receptors are increased in Parkinson's patients (Barbanti *et al.*, 1999). Thus, it has been suggested that DA receptor mRNA expression in circulating blood might reflect the DA receptor level in the brain (Ilani *et al.*, 2001) and might serve as a useful surrogate marker for more direct measurements of central receptor status, and thus by extrapolation, DA levels in the brain (Gladkevich *et al.*, 2004). A recent study has found the D2Rs are expressed on WBCs in humans and that their expression correlates robustly with psychostimulant use and cognitive deficits (Ersche *et al.*, 2011).

Critical DA-related gene products in the VTA, such as tyrosine hydroxylase (TH) are up-regulated (Ortiz *et al.*, 1995), and D2Rs are down-regulated (Rommelspacher *et al.*, 1992), 24 hr after withdrawal from chronic alcohol. We have shown that GABA neurons in the VTA, which regulate the activity of DA neurons, are activated by DA (Stobbs *et al.*, 2004; Lassen *et al.*, 2007) via D2 receptors (Steffensen *et al.*, 2008) and that D2Rs are down-regulated in VTA GABA neurons during withdrawal from chronic ethanol (Ludlow *et al.*, 2009).

Thus, the specific aim of this study was to determine the time course of adaptations in D2R expression and DA release in the mesolimbic DA system and in WBCs following systemic

administration of drugs known to modify DA release and chronic D2R expression; mainly, the D2R antagonist eticlopride, the D2R agonist quinpirole, and ethanol. The four experiments we employed to test this hypothesis were: 1) Examine acute eticlopride (positive control), quinpirole (negative control) and ethanol (addiction component) effects on DA release in the NAc and D2R expression in WBCs *in vivo* sampled every hour for two hours; 2) Examine acute eticlopride, quinpirole and ethanol effects on D2R expression in WBCs *in vitro*. This is the control experiment for direct effects on WBC D2Rs; 3) Examine acute eticlopride, quinpirole and ethanol effects on D2R expression in the VTA, NAc, temporal lobe cortex two hours after injection (acute, non-dependent condition); 4) Evaluate baseline D2R expression in the VTA, NAc and temporal lobe cortex sixteen hours after withdrawal from chronic intermittent ethanol exposure (dependent condition) in the alcohol vapor chambers (chronic, dependent condition).

MATERIALS AND METHODS

Animal Subjects and Surgical Procedure

Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University, which meet or exceed NIH guidelines. Adult male Wistar rats (PND 30-120) were used in this study. Once weaned at PND 21, all rats were housed in groups of two-three per cage and placed on a reverse light/dark cycle with lights ON from 8 PM to 8 AM. For DA FSCV recordings *in vivo*, rats were anesthetized using Isoflurane (MWI Veterinary Supply, Boise, ID) and placed in a stereotaxic apparatus. Anesthesia level was maintained at 1.5 % throughout the experiments. Body temperature was maintained at 37.4 ± 0.4 ° C by a feedback regulated heating pad. With the skull exposed, we drilled trephine holes for placement of stimulating and recording electrodes.

Carbon Fiber Electrodes and Fast Scan Cyclic Voltammetry

For voltammetry recordings *in vivo*, a 7.0 μm diameter carbon fiber was inserted into borosilicate capillary tubing (1.2 mm o.d.; A-M Systems, Sequim, WA) under negative pressure and subsequently pulled on a vertical pipette puller (Narishige, East Meadow, NY). The carbon fiber electrode (CFE) was cut under microscopic control with 150 μm of bare fiber protruding from the end of the glass micropipette. We back-filled the CFE with 3 M KCl. The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V vs Ag/AgCl using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 msec (i.e., 10 Hz) by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, Minneapolis, MN). Voltammetric recordings were performed and analyzed using LabVIEW (National Instruments, Austin, TX)-based customized software (Demon Voltammetry, (Yorgason et al., 2011). Stimulations were performed periodically every 2 min *in*

vivo. Dopamine levels were monitored for a stabilization period typically lasting 20 min. Once the stimulated DA response was stable for five successive collections, and did not vary by more than 10%, baseline measurements were taken.

Stimulation and Recording

For *in vivo* DA recordings, mice were anesthetized with Isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tejunga, CA). Bipolar, coated stainless steel electrodes were stereotaxically implanted into the medial forebrain bundle (MFB; -2.5 P, +1.9 L, -8.0-9.0 V), and a capillary glass-based CFE in the NAc core (+1.6 A, +1.9 L, -6.5-8.0 V). The MFB was stimulated with 60 biphasic pulses at 60 Hz (4 msec pulse width) at 2 min intervals. Stimulation and recording electrodes were positioned by fine stereotaxic control to optimize the release of DA at 1.5 mA stimulation intensity.

Drug Preparation and Administration

For *in vivo* experiments, eticlopride hydrochloride (Sigma Aldrich, St. Louis MO) was dissolved in physiological saline solution and administered at a dose of 1.0 mg/kg intravenously (IV). Ethanol (Decon Labs Inc., King of Prussia, PA) was dissolved in like manner, but into physiological saline solution at 16 % w/v and administered at a dose of 2.5 g/kg intraperitoneally (IP). Finally, quinpirole hydrochloride (Sigma Aldrich, St. Louis MO) was dissolved into physiological saline solution and administered at a dose of 0.1 mg/kg IV. These doses previously demonstrated robust, physiologically relevant enhancement and inhibition of DA release respectively.

qRT-PCR Methods

mRNA Isolation: Tissue punches were taken at 1 mm² from the VTA, NAc, and temporal lobe cortex. These samples were taken from rats treated with eticlopride, quinpirole or ethanol two hours post injection and also from saline-injected rats as a control. Samples were placed in 800ul of Trizol reagent (ThermoFischer Scientific, Waltham, MA) to halt mRNA degradation. The mRNA was extracted by chloroform, ethanol washes and centrifugation following the Trizol manufacturer's protocol. The mRNA concentrations were measured using mass spectroscopy and a reverse transcriptase reaction was run on the samples to convert the mRNA to cDNA.

qPCR: Two targets were examined by PCR, D2R as the varying gene of interest and 18S as the reference gene. The primers are designed for each target along with FAM-TAMRA primers for increased specificity. The samples were run for 60 cycles in triplicate.

Quantification: Quantification of the qPCR data was obtained using the $\Delta\Delta CT$ method. Averages of the triplicates were taken and the cycle numbers for 18S were subtracted from the D2R cycle numbers for each sample so they were comparable across samples. Average expression was obtained for each sample and all groups were normalized to the saline group.

Brain Slice Preparation for Immunofluorescence

Under sodium pentobarbital anesthesia (80 mg/kg, IP), rats from one of the four treatment groups were perfused with 100 ml of saline followed by 150-200 ml of 4 % paraformaldehyde (PFA) (Sigma Aldrich, St. Louis MO). The brains were then harvested and stored in 10 % sucrose and 4 % PFA solution at 4°C overnight, and then transferred to 30 % sucrose in 0.01M PBS for at least 48 hours. The fixed brains were then frozen in OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and coronal sections were taken at 30 μ m using a cryostat (ThermoFischer Scientific, Waltham, MA). Sections containing NAc, VTA, and

temporal lobe cortex were stored in 30 % ethylene glycol and 30 % glycerol in 0.01M PBS at -20 °C.

Staining Preparation and Imaging for D2R Immunofluorescence

Tissue samples were taken from the ethylene glycol, glycerol, and 0.01 M PBS solution and washed three times in 0.01M PBS. Between each washing, the tissue samples were placed on an Orbitron Rotator for five minutes (Boekel Scientific, Feasterville, PA). After washing, blocking solution comprised of 4% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA), 0.1% Triton X-100 (ThermoFischer Scientific, Waltham, MA), and 0.1 % bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in 0.01 M PBS was added and samples were placed on the orbitron for one hour at room temperature. Samples were then washed three more times using the same technique mentioned previously. Following the wash, we added 500 μ L of primary antibody rabbit polyclonal IgG anti-D2 (AB5084P, Millipore Corporation, Billerica, MA) at a dilution of 1:150 of the dilution buffer which consisted of 5ml 0.1M PBS, 5mg BSA, and 15 μ L Triton X-100. Samples were placed on orbitron rotator overnight (at least 20 hours) at 4 °C. After ample time for primary antibody binding, samples were washed three times for ten minutes each time in 0.01 M PBS. The secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (A11034, Invitrogen, ThermoFischer Scientific, Waltham, MA) was then added at a dilution of 1:500 and samples were placed on orbitron for two hours at room temperature. After secondary antibody binding, samples were washed three times for ten minutes each time with 0.01 M PBS. The stained tissue slices were then mounted on microscope slides and coverslip placed using Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, CA). All secondary antibody staining procedures were performed in dimmed lighting to prevent

photo-bleaching of the fluorescent antibody. Tissue was then viewed using an Olympus Fluoview laser scanning confocal microscope (Olympus America, Center Valley, PA).

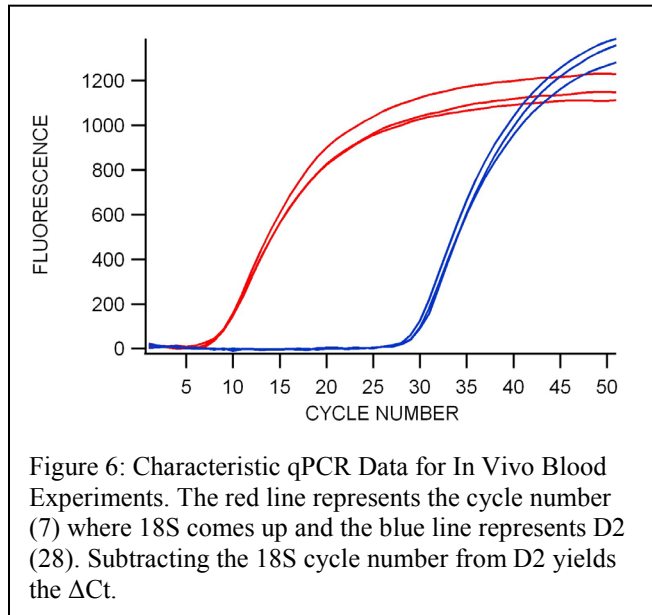
In Vitro Blood Experimentation Methods

Blood from naïve male rats was extracted using heparinized tubing and a heparinized syringe from a catheter placed in the jugular vein. The extracted amount (800 μ L) was then placed in a heparinized 1.7 mL RNase/DNase-free Eppendorf tube (BioExpress, Kaysville, UT). The extracted blood was then equally separated into 16 eppendorf tubes, each containing 50 μ L of heparinized-blood and 950 μ L buffer composed of 1 % HEPES (Sigma Aldrich, St. Louis MO), 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis MO) and RPMI-1640 (Sigma Aldrich, St. Louis MO) medium. The tubes were then centrifuged (5 kHz) at room temperature for 15 min. After centrifuging, the plasma layer was decanted and the sample was resuspended in 950 μ L of buffer solution. Then, the 16 tubes were separated into four treatment groups (saline, ethanol, eticlopride, or quinpirole), each comprising of four tubes. Saline, ethanol, eticlopride and quinpirole were all administered to their respective tubes at dosages that mirrored those of the *in vivo* experiments, which were 250 mg%, 5 μ M and 0.5 μ M respectively. The tubes were then incubated at 37 °C for two hours. After incubation, all tubes were centrifuged for 15 min at 5 kHz, the most superficial layer was removed, and the remaining blood sample was resuspended in 800 μ L Trizol Reagent and taken directly to the -80 °C freezer. mRNA isolation was performed as per *qRT-PCR Methods* above.

RESULTS

qRT-PCR Data for Brain Tissue and Blood Analysis

We took 0.79 mm² tissue punches from the NAc, VTA, and temporal lobe cortex of rats treated with saline, ethanol, eticlopride or quinpirole. This was accomplished by punching a 1 mm diameter blunt needle through a 1 mm thick tissue section. Using RT-PCR we were able to determine the changes in



D2R expression between the four treatments two hours after injection. Figure 6 shows representative qPCR data that illustrates typical cycle numbers where 18S and D2 are observed. Figure 7 represents tissue punch D2R expression at the three regions of interest: NAc, VTA, and temporal lobe cortex.

The VTA showed drastic changes in D2R expression between the treatment groups. The ethanol treated animals demonstrated a 494 % increase in D2R expression relative to saline. Eticlopride and quinpirole also showed marked changes in expression with eticlopride yielding a 36 % and quinpirole a 28 % increase in D2R expression. The NAc also demonstrated substantial, rapid changes in receptor expression. Again, the ethanol-treated animals experienced an average of 113 % increase in D2R expression, but eticlopride and quinpirole were merely 29 and 0.14 %, respectively, relative to saline. Finally, in the cortex, drastic decreases in D2R expression were seen in the ethanol and quinpirole treated animals with ethanol being 4.2 % and quinpirole being 1.3 % of saline expression levels.

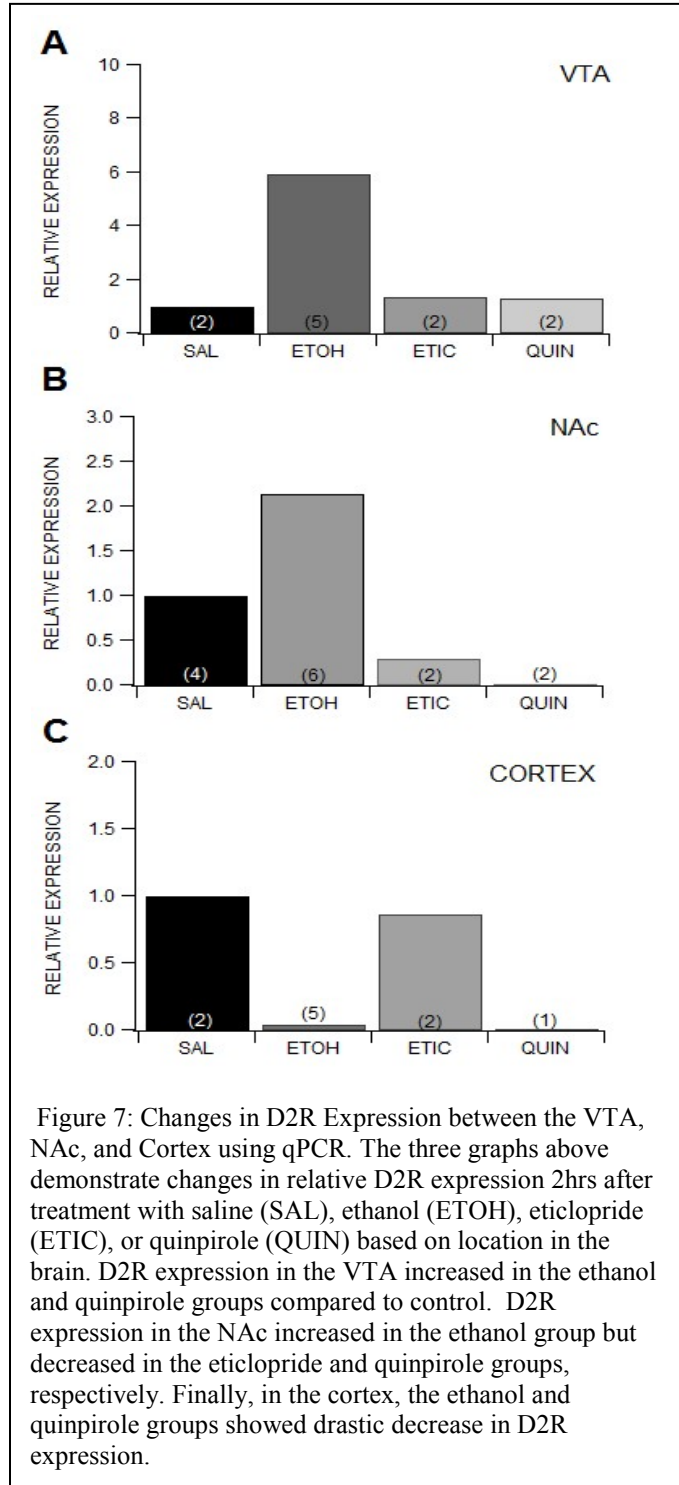
When looking to the periphery for drug effects on D2R expression, we see suggestive, but not statistically significant changes in D2R expression from WBCs of the *in vivo* experiments (Figure 8). Reasoning behind why such significant variation is seen in this experiment is explained more in the discussion.

Lastly, concerning the *in vitro* blood experiments, limited data suggests D2R expression on WBCs rapidly adapts even when communication between the nervous and immune systems cannot take place (Figure 9). Ethanol increased expression by 92 %, eticlopride showed no change, and quinpirole decreased by 53 % relative to saline controls.

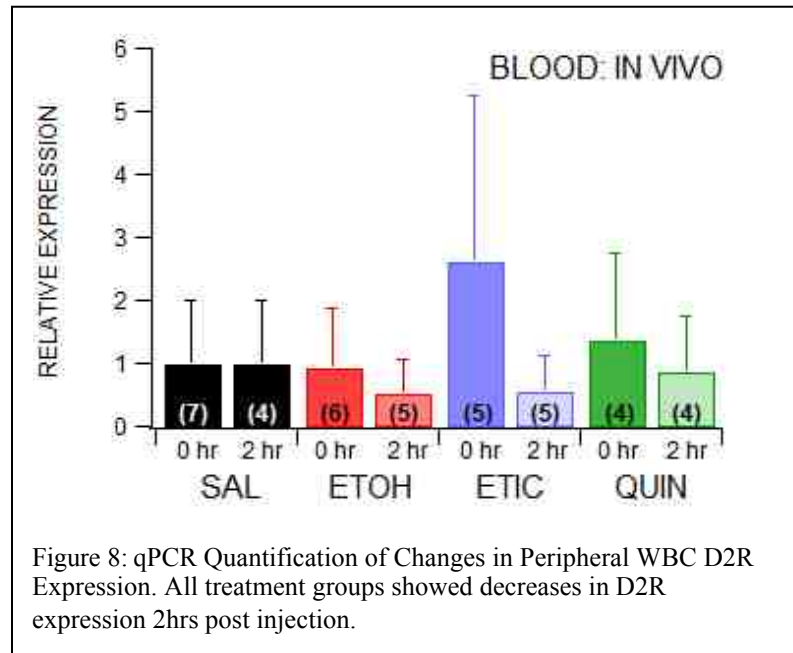
Differences in Immunofluorescence between Treatment Groups

We observed suggestive treatment effects in both the VTA and NAc (Figures

10 and 11). In the VTA, animals treated with ethanol and eticlopride demonstrated an increased amount of expression when compared to saline. Conversely, animals treated with quinpirole showed a slightly decreased amount of expression. These findings correspond with the data

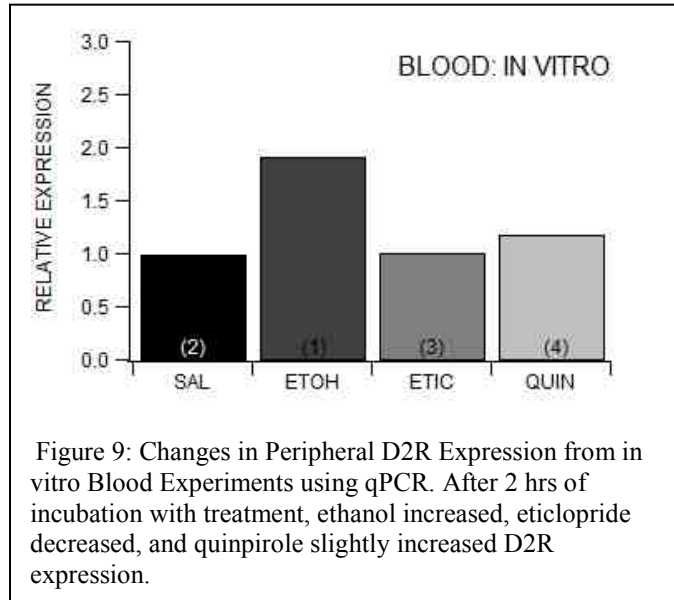


gathered above in the RT-PCR portion of this project and suggest that rapid adaptation of D2R is occurring in the brain.



DISCUSSION

As shown in our results, *in vivo* and *in vitro* D2R expression undergoes rapid adaptation in the brain and blood when exposed to acute doses of ethanol and other D2R targeting drugs. The fact that these changes were monitored using RT-PCR, specifically in blood from *in vivo* animals, suggests that peripheral D2R expression could be a biomarker of brain



DA levels. These findings are consistent with our preliminary electrophysiology data which demonstrated ethanol, eticlopride, and quinpirole's modulatory effects on brain DA levels.

The variation we observed in the blood experiments that was alluded to in the results was partly due to difficulty gathering and analyzing the RT-PCR data. The 18S values obtained in brain or blood tissue samples should be the same between experiments. In our case, the 18S values showed considerable variation between experiments, even though the values themselves were tight and accurate within experiments. Different methods of normalizing and analyzing this data might lead to more conclusive evidence of how D2Rs are changing in the blood of *in vivo* animals relative to the brain. Or, more effective, clean, and consistent methods of mRNA isolation might also decrease the variability of 18S values between experiments.

Tissue punch data revealed that in the VTA, ethanol, eticlopride, and quinpirole had a stimulatory effect on D2R expression. In the NAc, ethanol again increased D2R expression relative to saline controls, but eticlopride and quinpirole both decreased expression. Finally, in the temporal lobe cortex, ethanol and quinpirole had a significantly inhibitory effect on D2R expression. The purpose of using temporal lobe cortex tissue punches was to have a control reference for the NAc and VTA.

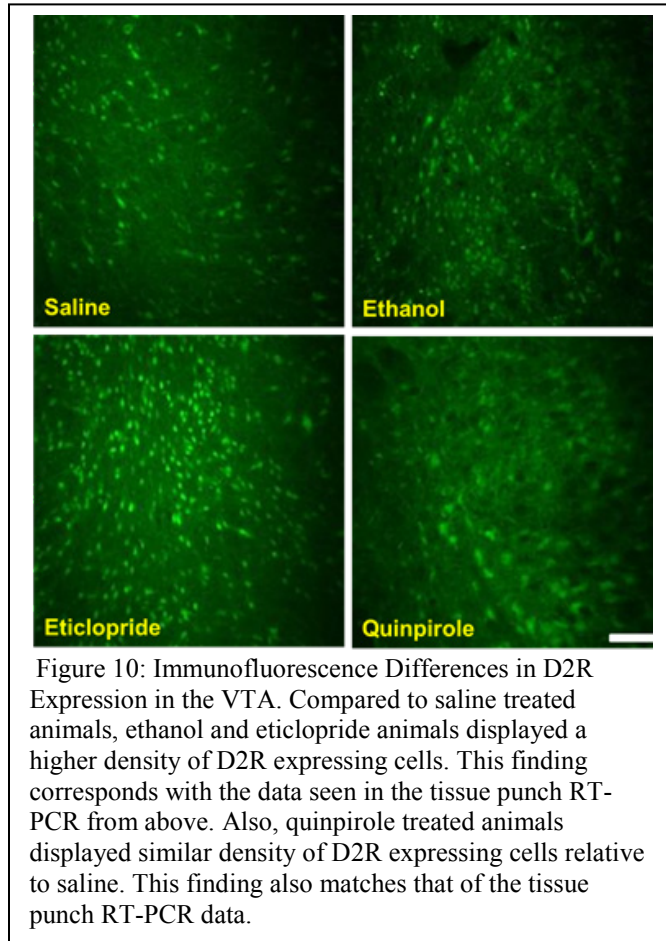


Figure 10: Immunofluorescence Differences in D2R Expression in the VTA. Compared to saline treated animals, ethanol and eticlopride animals displayed a higher density of D2R expressing cells. This finding corresponds with the data seen in the tissue punch RT-PCR from above. Also, quinpirole treated animals displayed similar density of D2R expressing cells relative to saline. This finding also matches that of the tissue punch RT-PCR data.

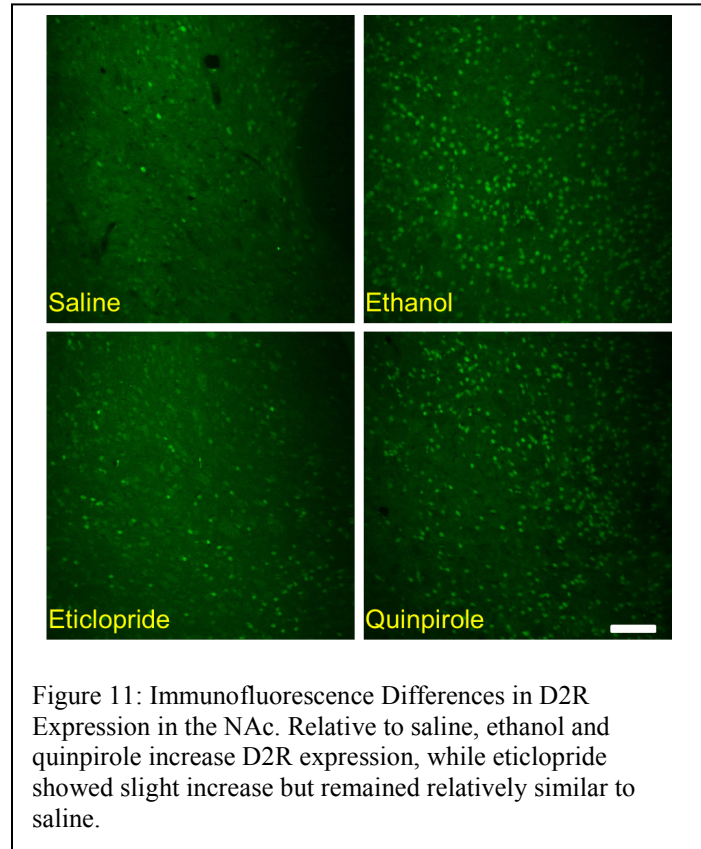
Unfortunately, this region of interest did not serve as that control. It is possible that D2Rs could have different functional responsibilities depending on location in the brain and that is why expression significantly changed. It is also possible that the punches were taken from what we thought to be temporal lobe cortex, but in fact were from another brain location which might utilize D2Rs in a way we are not familiar with.

Immunofluorescence (IF) also revealed changes in D2R expression in the brain. Our findings regarding saline and ethanol correlated with that of RT-PCR and verified that the changes we observed in these two treatment groups were consistent. There does appear to be some discrepancies between the RT-PCR and IF data. For example, RT-PCR data showed little change in D2R expression in the VTA of eticlopride treated rats while IF showed significant

increase. Also, RT-PCR showed a marked decrease in D2R expression in the NAc of eticlopride treated animals while IF showed similar, if not slightly increased, expression. As of now, we attribute this discrepancy to the small sample size of the eticlopride (n=2) and quinpirole (n=2) treated animals from RT-PCR. Until more data is acquired for these experiments, solid conclusions cannot be made.

We are still in the process of

gathering and analyzing *in vitro* blood data. The current data (Figure 9) suggests that in the absence of the nervous system, D2R expression on WBCs still adapts when exposed to drug treatment. The purpose of this adaptation is still largely unknown. However, considering that D2Rs are G-protein coupled receptors, binding of D2Rs on WBCs with agonists likely induce second messenger signaling mechanisms that lead to activation or inactivation of the cell. Indeed, activation of D2-like receptors enhances the production of interleukin-10, a cytokine that negatively regulates the function of effector T-cells (Butarelli *et. al*, 2011). This could occur with quinpirole activation of D2Rs. It may be that quinpirole binding leads to activated monocytes which can invade the brain. We are puzzled why WBCs undergo such marked down-regulation with eticlopride. This is contrary to what happens in the brain, at least with immunofluorescence, which shows that eticlopride is up-regulating D2Rs. Unfortunately,



mRNA expression does not seem to accurately reflect protein expression. For the intents and purposes of this project, what really matters is protein expression. In future studies, we will include double staining of D2Rs with neurons, astrocytes, microglia and other cell types to identify the purpose for D2R adaptation and whether or not there is a neuro-immune interaction dictating the changes that occur in the blood.

We are also in the treatment stage of acquiring animals chronically dependent on ethanol. The apparatus for these experiments was in use by another colleague for another project, which made acquiring this data before now impossible. When ready, animals will be sacrificed, the brains and blood will be analyzed, and more concrete data will help us determine whether or not peripheral D2Rs can be used as a biomarker of brain DA, as chronic ethanol treatment typically results in lowered DA release during withdrawal. We will study the rats 16-24 hrs after withdrawal from chronic ethanol exposure, which is the peak withdrawal period.

The main direction for this research is to find a strong, predictable correlation between peripheral D2R expression and central DA levels. As of now, our data suggests that rapid adaptation of D2Rs is occurring in WBCs *in vivo* and *in vitro*, but the discrepancy between brain and blood D2R expression shown by RT-PCR and IF leaves peripheral D2R tracking as a limited and potentially misleading method of monitoring brain DA levels. That being said, protein expression shown in figures 10 and 11 of *in vivo* animals seems to be more reliable, and less variant, than D2 mRNA levels in RT-PCR. Thus, IF data that shows an increase in D2R expression in the VTA after ethanol and eticlopride, and increase of D2R expression in the NAc after ethanol and quinpirole provides the most compelling evidence for rapid adaptation of D2Rs.

On a broad level, this project contributes to the existing literature which states that peripheral D2Rs are a potential biomarker for brain DA. This is the first demonstration of how

acute ethanol exposure is rapidly modulating D2R expression in the VTA, NAc, and WBCs as opposed to disease states and chronic drug abuse changing D2R expression. This project gives suggestive evidence for rapid adaptation of D2Rs in the brain and blood in response to drugs of abuse. Granted, addiction is not mediated by one receptor subtype alone, and the mechanisms behind addiction are substantially more complex, but D2R monitoring could provide clues for trends in addiction and treatment possibilities. The details of how cost-effective, accurate, and clinically useful it would be to track changing D2R levels via qPCR are still unknown, but it seems to be a more promising alternative to the current method of PET scanning.

Concerns, Pitfalls, Limitations and Alternative Approaches

For all of my experiments (tissue punches, blood mRNA analysis, immunofluorescence (IF), *in vitro* blood analysis), I had to learn and use new techniques. The research that sparked my interest in D2Rs came from findings using electrophysiology and DA neurochemistry in the brain. Almost all of my experiments utilized non-electrophysiological and neurochemical techniques and half of the experiments focused on D2Rs in the blood, or periphery. I am grateful this project required me to learn new techniques, but one consequence was that gathering meaningful data took more time, practice and patience than anticipated.

For example, for the *in vivo* tissue punch and blood mRNA experiments, my initial samples were frequently contaminated, which prevented the RT-PCR from yielding useful data. After troubleshooting my isolation technique and limiting variables that could introduce contamination, I was able to gather adequate, but not convincing, data.

Concerning IF, staining for membrane versus cytosolic proteins proved more difficult than anticipated. After changing our staining protocol several times and trying a new primary

antibody, we finally acquired the results we wanted. Now we need to perform additional experiments.

The technical difficulties we experienced also revealed conceptual limitations. It is clear that rapid adaptation occurred at two hours in both brain and blood samples, but could the adaptation be stronger at four or six hours? The literature on D2 expression makes it clear that, with chronic drug use, the strongest effect will occur days to weeks after treatment begins. One alternative approach would be to test D2 expression at several time intervals between two and ten hours to see when the maximum effect occurs.

One significant conceptual limitation that was not addressed by this project was the purpose of rapid adaptation of D2Rs in the periphery. Moving forward, double staining of brain tissue to show precisely where D2Rs are expressed under treatment conditions could lend insight to the reason for rapid adaptation in the brain and blood. Staining for D2Rs on astrocytes, activated microglia, and other types of cells before and after acute drug exposure could provide clues for why D2R adaptation occurs.

My final concern is whether we chose the best approach for testing D2R adaptation in the brain and blood. Concurrent western blots on central and peripheral D2R expression in addition to RT-PCR might have provided verification of the effect we saw and explained some of the variance. Fluorescence activated cell sorting can also be used to quantify changes in D2R expression, but changes in fluorescence are not as descriptive as numerical changes acquired using RT-PCR. Based off of methods in the literature and how others are testing D2R expression, I think we chose the best possible method.

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2013 Eun Young Jang^{1,3,#}, Yeon-Hee Ryu^{2,#}, Bong Hyo Lee¹, Su-Chan Chang¹, Mi Jin Yeo¹, Ryan J. Folsom³, Kwang Joong Kim¹, Chae Ha Yang¹, Scott C. Steffensen^{3,*}, Hee Young Kim.¹ Involvement of reactive oxygen species in cocaine taking-behaviors in rats. *Addiction Biology*.

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PRESENTED POSTERS

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