

ETHANOL DRINKING AND DEPRIVATION ALTER DOPAMINERGIC AND
SEROTONERGIC FUNCTION IN THE NUCLEUS ACCUMBENS OF ALCOHOL-
PREFERRING (P) RATS.

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Running Title: Ethanol drinking alters monoamine function in P rats

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Abbreviations: extraction fraction (E_d), 1-(m-chlorophenyl)-biguanide (CPBG), artificial cerebral spinal fluid (ACSF), area under the curve (AUC)

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Abstract:

The alcohol deprivation effect is a temporary increase in the intake of, or preference for, ethanol following a period of deprivation which may result from persistent changes in key limbic regions thought to regulate alcohol drinking, such as the nucleus accumbens. The present study tested the hypothesis that chronic alcohol drinking under continuous 24-hr free-choice conditions alters dopamine and serotonin neurotransmission in the nucleus accumbens, and that these alterations persist in the absence of alcohol. Using the no-net-flux microdialysis method, the steady state extracellular concentration (point of no-net-flux) for dopamine was approximately 25 % higher in the adult female alcohol-preferring P rats given prior access to 10% ethanol, even after two weeks of ethanol abstinence, compared to the P rats given access only to water. However, the extracellular concentration of serotonin was approximately 35 % lower in animals given 8 weeks continuous access to ethanol compared to water controls and animals deprived of ethanol for two weeks. The effect of local perfusion with 100 μ M sulpiride (D_2 receptor antagonist) and 35 μ M 1-(m-chlorophenyl)-biguanide (CPBG) (5-HT₃ receptor agonist) on dopamine overflow were reduced approximately 33% in both groups of ethanol exposed P rats compared to water controls. Free-choice alcohol drinking by P rats alters dopamine and serotonin neurotransmission in the nucleus accumbens and many of these effects persist for at least 2 weeks in the absence of ethanol suggesting that these underlying persistent changes may be in part responsible for increased ethanol drinking observed in the alcohol-deprivation effect.

There is considerable evidence that the mesolimbic dopamine system is involved in mediating the reinforcing effects of ethanol and ethanol self-administration (see reviews by Koob et al., 1998; McBride and Li, 1998). A number of systems regulate the activity of this pathway, including auto-regulation by D₂ autoreceptors located on dopamine terminals and regulation by other neurotransmitter systems at the level of both the ventral tegmental area and nucleus accumbens.

The dorsal raphe serotonin system sends projections to both the ventral tegmental area and the nucleus accumbens (Parent et al., 1981). Microdialysis studies indicate that activation of serotonin projections from the dorsal raphe nucleus increases extracellular levels of dopamine in the nucleus accumbens (Yoshimoto and McBride, 1992), suggesting that activation of serotonin projections to the mesolimbic dopamine system can activate this system. Furthermore, local perfusion of the nucleus accumbens with a 5-HT₂ receptor (Bowers et al., 2000) or 5-HT₃ receptor (Campbell and McBride 1995) agonist significantly increased the extracellular levels of dopamine, suggesting that these two receptors are involved in regulating terminal dopamine release in the nucleus accumbens. There is pharmacological evidence that 5-HT₃ receptors may be involved in regulating alcohol drinking and that the effectiveness of the 5-HT₃ receptor antagonists depends upon the drinking conditions (McKinzie et al., 1998a, 2000; Rodd-Henricks et al., 2000a). For example, 5-HT₃ receptor antagonists are effective in reducing acquisition and maintenance of alcohol drinking under 24-hr free-choice alcohol drinking conditions, but they were relatively ineffective under alcohol relapse conditions, following a 2-week period of alcohol deprivation (Rodd-Henricks et al., 2000a). These latter results suggest that changes in 5-HT₃ receptors may have occurred during the deprivation period, which altered their response to the 5-HT₃ receptor antagonists. In addition, a preliminary study (Pommer et al., 2003) using

microinjections of a 5-HT₃ receptor antagonist into the shell of the nucleus accumbens, indicated that 5-HT₃ receptors in this limbic subregion are involved in regulating alcohol drinking of the alcohol-preferring P rat.

Limited data are available on the adaptation of these systems to repeated ethanol administration. Findings by Smith and Weiss (1999), using the no-net-flux quantitative microdialysis technique (Cosford et al., 1996; Justice, 1993; Parsons and Justice, 1992), indicated that 5 daily i.p. injections of 1 g/kg ethanol altered dopamine and serotonin neurotransmission in the nucleus accumbens of P rats. However, the neuroadaptive effects of parentally administered ethanol may be different from those where animals self-administer ethanol, as has been found with other drugs of abuse (Jacobs et al., 2003). Therefore it is important to examine the effects of chronic ethanol administration on these systems in animals that readily self-administer ethanol.

Alcohol-preferring P rats have been selectively bred from an outbred Wistar stock for high alcohol-seeking behavior (Lumeng et al., 1977). Under free-choice access to 10 % (v/v) ethanol and water, P rats will readily consume 5 g ethanol/kg body weight/day. Microdialysis studies indicate that alcohol drinking under scheduled access operant conditions can increase the extracellular levels of dopamine and serotonin in the nucleus accumbens of P and Wistar rats (Melendez et al., 2002; Weiss et al., 1993; 1996). A recent study suggested that chronic alcohol drinking under 1 hour limited access conditions by P rats reduced D₂ autoreceptor function in the nucleus accumbens (Engleman et al., 2003), but D₂ autoreceptor function in the nucleus accumbens has not been examined under 24 hour free-choice alcohol drinking nor has it been demonstrated if a reduction in D₂ autoreceptor function persists in the absence of ethanol.

The P line of rats also demonstrates a robust alcohol deprivation effect under chronic

free-choice drinking conditions (Rodd-Henricks et al., 2000b). The alcohol deprivation effect is a temporary increase in the intake of, or preference for, ethanol following a period of deprivation (Sinclair, 1972; Sinclair et al., 1973). Using the [14C]-2-deoxyglucose procedure to measure local cerebral glucose utilization rates as an index of functional neuronal activity, Smith et al. (2001, 2002) examined the effects of alcohol drinking, deprivation and relapse on local cerebral glucose utilization rates. These studies demonstrated that long-term alcohol drinking by P rats produced alterations in local cerebral glucose utilization rates in the limbic system, including the shell of the nucleus accumbens, and that some of these changes, including those in the shell of the nucleus accumbens, persisted in the absence of ethanol for as long as 2 weeks. These findings suggested that there are neuronal alterations within the limbic system that contribute to relapse drinking.

The present study was undertaken to determine the basal extracellular concentration and extraction fraction (E_d) for dopamine and serotonin in the nucleus accumbens to test the hypothesis that chronic alcohol drinking under continuous 24-hr free-choice conditions alters dopamine and serotonin neurotransmission in a key limbic region thought to regulate alcohol drinking, and that these alterations persist in the absence of alcohol. In addition, this study tested the hypothesis that alcohol drinking followed by deprivation produces alterations, different from that found with chronic drinking alone, in the function of nucleus accumbens D_2 autoreceptors and 5-HT₃ receptors, two important receptor systems regulating dopamine neurotransmission within the nucleus accumbens.

Methods

Animals:

Alcohol-naïve, adult female P rats from the 45th-49th generation weighing 177-326 g at the start of the experiment were individually housed in metal hanging cages in temperature and humidity controlled rooms. Female P rats were used in the present study because they were readily available at the time the studies were initiated and the size of female P rats allows for more consistent and accurate stereotaxic placements. Since the squads of rats were processed at different times, any effects of the estrous cycle should have been distributed over the different experimental conditions. Rats were maintained on a normal 12-hr light cycle (lights on 0700). Food and water were available ad libitum throughout the experiment. The animals used in this experiment were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996).

Alcohol-drinking Procedure:

Subjects were assigned to one of three groups. One group was the water control group, which received water as the sole source of fluid throughout the experiment. The second group was the alcohol non-deprived group, which received only water for the first 4 weeks, after which they were given continuous free-choice access in the home cage to 10 % ethanol and water for 8

weeks. The third group was the alcohol-deprived group, which received continuous free-choice access in the home cage to 10 % (v/v) ethanol and water for 6 weeks; at the end of the 6-week period, ethanol was removed for 2 weeks, then it was restored for 2 weeks before being deprived for a second 2-week period. Fluid intakes were recorded to the nearest 0.1 g by weighing the water and ethanol bottles every 24 - 48 hr. Body weights were recorded at the same time as the bottle weights. Ethanol intake was converted into average g ethanol/kg body wt/day.

Surgery:

Animals were anesthetized with 2 % isoflurane and, with the aid of a stereotaxic apparatus (David Kopf, Instruments, Tujunga, CA), were implanted unilaterally with 18-gauge stainless steel guide cannulas (Plastics One, Roanoke, VA) aimed 4 mm above the nucleus accumbens shell (A/P +1.7, L +2.2, D/V -3.8), according to the atlas of Paxinos & Watson (1998). The guide cannulas were secured to the skull with Cranioplastic cement (Plastics One, Roanoke, VA) and anchored with two stainless steel screws. Animals were allowed to recover for at least 5 days before probes were inserted. During the recovery period, animals were habituated to the clear Plexiglas microdialysis chambers (22.5 x 44.5 x 38.0 cm, W x L x H) for 2 days by bringing the animals into the experimental room for two-hour sessions (1100 - 1300) in the microdialysis chambers.

On the day before microdialysis, between 1000 and 1200, animals were briefly anesthetized, and a microdialysis probe was inserted and cemented into place. The loop style microdialysis probes were constructed as previously described (Kohl et al., 1998), having a 2-mm loop of active dialysis surface (Spectrum/Por 6 regenerated cellulose dialysis membrane, molecular weight cutoff 13,000 Da; Medical Industries, Los Angeles, CA) and extending 4 mm

below the guide cannula, into the nucleus accumbens. The alcohol-deprived group was run at the end of the second 2-week deprivation period. The non-deprived group was sampled at the end of the 8-week continuous alcohol-drinking period. The microdialysis experiments were conducted with animals from all 3 experimental groups run concurrently. During the last two weeks of the drinking period, rats in the non-deprived group were habituated to not having access to ethanol during the first 4 hr of the light cycle to ensure that ethanol was not present during microdialysis. This procedure has relatively little effect on total ethanol because P rats consume 75 - 80 % of their ethanol during the dark cycle (Murphy et al., 1986).

Dopamine No-net-flux:

The no-net-flux experiment and data analysis were conducted as previously described (Cosford et al., 1996; Justice, 1993; Parsons and Justice, 1992). The animals were placed in the microdialysis chambers and the microdialysis probes were perfused at a flow rate of 0.5 μ l/min with artificial cerebral spinal fluid (ACSF) (in mM, 140 NaCl, 3 KCl, 2.5 CaCl₂, 1 MgCl₂, 2 Na₂PO₄, 0.2 ascorbate; pH 7.4). The CaCl₂ concentration of 2.5 mM is higher than the 1.2 mM reported for CFS, however, the 2.5 mM CaCl₂ concentration has been used in microdialysis studies commonly and does not appear to produce anomalous results. After a 2-hr equilibration period, three baseline samples were collected every 20 min in microfuge tubes containing 2 μ l of 0.05 N perchloric acid. After collection of baseline samples, the nucleus accumbens was perfused, in random order, with ACSF containing 5, 10 or 20 nM dopamine for 100 min each. For each switch in dopamine concentration, the flow to probe was briefly interrupted and lines containing the new dopamine concentration in ACSF were attached to the inlet assembly. The new concentration of dopamine was perfused for an initial 20 min period and the sample

discarded to allow for equilibration of the dopamine concentration. An additional four 20 min samples were then collected and frozen for analysis of dopamine concentration by HPLC, after which the animals were switched to the next concentration of dopamine as described above. After perfusion with all three concentrations of dopamine, ACSF alone was returned for an additional 60 min (3 samples). Samples were immediately frozen on dry ice and stored at -70°C until assayed for dopamine.

Serotonin No-net-flux:

The procedures for the serotonin no-net-flux experiment are similar to those described above for dopamine, except samples were collected in microfuge tubes containing 2 μl of 0.2 mM EDTA, 0.33 mM L-cysteine and 0.05 mM L-ascorbic acid in 0.1 N acetic acid. After collection of baseline samples, the nucleus accumbens was perfused, in random order, with ACSF containing 0.2, 1.0, 2.0 nM serotonin for 100 min at each concentration. Again, the first 20 min sample was discarded to allow for equilibration of the serotonin concentration. An additional four 20 min samples were then collected and frozen for analysis of serotonin concentration by HPLC, after which the animals were switched to the next concentration of serotonin as described above. Following perfusion with serotonin, ACSF alone was returned for an additional 60 min (3 samples). Samples were immediately frozen on dry ice and stored at -70°C until assayed for serotonin.

Local perfusion of Nucleus Accumbens with Sulpiride:

Animals were placed in the microdialysis chambers and the microdialysis probes were perfused at a flow rate of 1.0 $\mu\text{l}/\text{min}$ with ACSF (in mM, 145 NaCl, 2.7 KCl, 1.2 CaCl_2 ,

1 MgCl₂; pH 7.4 with 0.1 N NaOH). After the 2-hr equilibration period, five baseline samples were collected every 30 min in microfuge tubes containing 5 µl of 0.1 N perchloric acid. After collection of the baseline samples, animals were perfused with ACSF containing 100 µM sulpiride, a selective D₂ receptor antagonist, and samples were collected every 30 min for the next 150 min. This perfusion rate (1.0 µl/min) and concentration of sulpiride were chosen because a previous study demonstrated that under these conditions 100 µM sulpiride reliably increased the extracellular levels of dopamine in the nucleus accumbens (Engleman et al., 2003). Samples were immediately frozen on dry ice and stored at -70 °C until assayed for dopamine content.

Local perfusion of Nucleus Accumbens with 1-(m-chlorophenyl)-biguanide (CPBG):

Animals were placed in the microdialysis chambers and the microdialysis probes were perfused at a flow rate of 0.5 µl/min with ACSF (in mM, 140 NaCl, 3 KCl, 2.5 CaCl₂, 1 MgCl₂, 2 Na₂PO₄, 0.2 ascorbate; pH 7.4). After the 2-hr equilibration period, three baseline samples were collected every 20 min in microfuge tubes containing 2 µl of 0.05 N perchloric acid. After collection of the baseline samples, animals were perfused with ACSF containing 35 µM CPBG, a selective 5-HT₃ receptor agonist, and samples were collected every 20 min for the next 60 min. This concentration of CPBG was chosen because a previous study demonstrated that 35 µM CPBG was a submaximal dose that reliably increased the extracellular levels of dopamine in the nucleus accumbens (Campbell and McBride, 1995). Following perfusion with CPBG, ACSF alone was returned and samples collected for an additional 120 min. Samples were immediately frozen on dry ice and stored at -70 °C until assayed for dopamine content.

Probe Placements

At the end of the experiment, 1 % methylene blue was perfused through the probe to mark the active membrane location. The animals were overdosed with CO₂, and the brains were removed and frozen. Brain sections (40 μm) taken around the probe location were stained with cresyl violet. Only animals with 80 % or more of the probe placements in the nucleus accumbens were included in the data analysis.

Determination of Serotonin and Dopamine levels in Dialysates

Microdialysate samples were analyzed for serotonin and dopamine levels by using microbore high-performance liquid chromatography with electrochemical detection. The samples from the dopamine no-net-flux, serotonin no-net-flux and the local perfusion of CPGB experiments were injected onto a reverse-phase microbore column (1.0 x 100 mm SepStik Spherisorb C18 column, 3 μm particle size, Bioanalytical Systems, West Lafayette, IN) with a Rheodyne injector (5 μl loop, Cotati, CA). For serotonin, the samples were separated by using a mobile phase composed of 100 mM sodium acetate, 0.5 mM EDTA, 1.25 mM sodium octylsulfonic acid, 10 mM NaCl, and 10.5% acetonitrile, pH 5.0 adjusted with glacial acetic acid, at a flow rate of 0.07 ml/min (Model 2350 pump, ISCO, Lincoln, NE). For dopamine, the samples were separated by using a mobile phase composed of 100 mM sodium acetate, 0.5 mM EDTA, 5.0 mM sodium octylsulfonic acid, 10 mM NaCl and 6% acetonitrile, pH 5.0 adjusted with glacial acetic acid, at a flow rate of 0.07 ml/min. Levels of serotonin and dopamine in the dialysates were determined by electrochemical detection (+550 mV potential, 0.5 nA sensitivity, EG&G Princeton Applied Research, Princeton, NJ) by using a 6-mm radial-flow, glassy-carbon electrode (Bioanalytical Systems, West Lafayette, IN). The injector, column, and electrode were

contained in a UniJet CC-6 cabinet (Bioanalytical Systems, West Lafayette, IN), which allows for very low dead volume and greater sensitivity when using smaller sample volumes. Output from the detector was sent to a microprocessor and integrated by using ChromPerfect Spirit (ver. 4.4.21, Justice Innovations, Palo Alto, CA). The detection limit was 20 pM for serotonin and 100 pM for dopamine.

The samples from the local perfusion of sulpiride were analyzed for dopamine content using a column switching system with a ten-port valve as previously described (Li et al. 1998; 2002). Samples were loaded into a 20 μ l sample loop and injected onto a clean-up column (BDS Hypersil C18, 3 μ m, 2 X 10 mm, Keystone Scientific, Bellefonte, PA), to remove late-eluting peaks, then diverted to an analytical column (BDS Hypersil C18, 3 μ m, 2 X 150 mm, Keystone Scientific, Bellefonte, PA). The samples were separated using a mobile phase consisting of 75 mM sodium phosphate (monobasic), 0.5 mM EDTA, 1.62 mM sodium octylsulfonic acid, 0.8% tetrahydrofuran and 10% acetonitrile, pH 3.0 adjusted with phosphoric acid, at a flow rate of 0.21 ml/min (Model 582, ESA Inc., Chelmsford, MA). The clean-up column was back flushed with mobile phase between samples (Model 2350, ISCO Inc., Lincoln, NE). Two 3 mm dual glassy carbon electrodes were used in series at potentials of +720 and +100 mV applied by an electrochemical detector (EG&G Princeton Applied Research, Princeton, NJ). Dopamine was detected at the second electrode at a sensitivity setting of 0.5 nA. Output from the detector was sent to a microprocessor and integrated by using ChromPerfect Spirit (ver. 4.4.21, Justice Innovations, Palo Alto, CA). The lower sensitivity limit for dopamine was approximately 100 pM.

Data Analysis:

Weekly averages for body weight and daily total fluid intake were obtained by averaging across days. Weekly averages of body weight and daily total fluid intake were compared by a mixed analysis of variance (ANOVA) with week as the repeated measure. To examine the alcohol deprivation effect, daily readings were taken for three days prior to removal of alcohol from the alcohol deprived group (baseline) and for five days after alcohol re-exposure. Ethanol intakes (g/kg/day), preference (% ethanol of total fluids) and total fluid intake (ml) were analyzed by a mixed ANOVA with day as the repeated measure. Significant interactions were further analyzed by orthogonal contrasts. Where appropriate, Student's t-tests were used to compare individual means.

Extracellular concentrations and E_d for dopamine and serotonin were obtained from multiple linear regression modeling using the SAS System for Windows ver. 8.02 (SAS Institute, Inc., Cary, NC). The net gain or loss of dopamine or serotonin from the probe ($\text{concentration}_{in}$ minus $\text{concentration}_{out}$) was plotted against the concentration of dopamine or serotonin perfused through the probe ($\text{concentration}_{in}$) to construct the regression lines. The concentration in was determined from a sample of perfusate just prior to its use. As animals from each group were run concurrently, each being perfused in random order with the transmitter of interest (dopamine or serotonin), each concentration was sampled three times throughout the day. $\text{Concentration}_{out}$ was the concentration of transmitter obtained in the microdialysis sample. Thus the y-axis represents the net gain or loss of dopamine or serotonin from the microdialysis probe (negative values representing diffusion into the probe and positive values representing diffusion away from the probe). The individual replicate data from all the animals in all groups were analyzed by multiple linear regression modeling to determine the E_d (slope) and x-intercept for each group.

ANOVA was used to compare the effect of drinking history on the slopes and x-intercepts. Where appropriate, Student's t-tests were used to compare individual means. All statistical comparisons for the no-net-flux experiments were done using the SAS System for Windows ver. 8.02 (SAS Institute, Inc., Cary, NC).

Sulpiride and CPBG baseline data are expressed as dopamine concentration in nM after correction for dilution by the perchloric acid preservative and were analyzed by one-way ANOVA. Time course data are expressed as percent of baseline. The data were analyzed by mixed ANOVAs with time as the repeated measure. Significant interactions were further analyzed by orthogonal contrasts. Where appropriate, Student's t-tests were used to compare individual means. In addition, area under the curve (AUC) was calculated using Prism ver. 3.0 (GraphPad Software, Inc., San Diego, CA). AUC was analyzed by one-way ANOVA, followed by Student's t-test to compare individual means, where appropriate. All statistical comparisons, except for the no-net-flux experiments, were done using SPSS for Windows, ver. 11.0.1 (SPSS, Inc., Chicago, IL)

Materials:

Ethanol was obtained from AAPER Alcohol (Shelbyville, KY). Isoflurane was obtained from Abbott Laboratories (North Chicago, IL). 1 % Methylene blue was obtained from LabChem, Inc (Pittsburgh, PA). Perchloric acid and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). Tetrahydrofuran, phosphoric acid, NaOH, acetonitrile (HPLC grade), NaH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1-octanesulfonic acid sodium salt, sodium acetate, L-ascorbic acid, L-cysteine·HCl·H₂O, ethylenediaminetetraacetic acid (EDTA, anhydrous), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, KCl, NaCl, dopamine·HCl, serotonin (serotonin creatine sulfate complex), sulpiride,

and 1-(m-chlorophenyl)-biguanide·HCl (CPBG·HCl) were obtained from Sigma-Aldrich (St. Louis, MO). Ethanol was prepared as a 10 % (v/v) solution in tap water.

Results

Figure 1 shows ethanol intake for the non-deprived and alcohol-deprived groups of P rats during the three days prior to ethanol removal from the alcohol-deprived group and for the first five days after ethanol re-exposure of the alcohol-deprived group. The alcohol-deprived group had an average daily intake of 5.9 ± 0.5 g/kg/day for the three days prior to ethanol removal whereas the non-deprived group had an intake of 5.6 ± 0.7 g/kg/day for this same three-day period. Analysis of variance of ethanol intake between the alcohol-deprived and non-deprived groups over the days before ethanol removal and after ethanol re-exposure for the alcohol-deprived group revealed a significant day x group interaction, $F(7, 161) = 2.44, p < 0.05$. Between groups analysis of intake on each day by independent Student's t-test revealed a significant elevation in ethanol intake on the first day of ethanol re-exposure of the alcohol-deprived group compared to the non-deprived group ($p < 0.01$). In addition, within subjects analysis also indicated that ethanol intake on the first day of ethanol re-exposure of the alcohol-deprived group was increased compared to its own baseline ($p < 0.01$).

Representative probe placements for the animals used in this study are shown in Figure 2. Only animals with verified probe placements in the nucleus accumbens were included in the data analysis. All probes were in the nucleus accumbens within approximately 1.0 mm of each other. Probes were placed between 0.7 and 1.7 mm rostral of Bregma, according to the atlas of Paxinos and Watson (1998). Most probe placements were in the shell plus core or in the shell alone. A few placements were primarily in the core with some overlap into the striatum. There were too few placements mainly in the core alone or shell alone to statistically compare with other placements. Therefore, data from all placements with 75 - 80 % of the probe within the nucleus accumbens were used for analysis.

The basal extracellular dopamine concentrations and *in vivo* dopamine E_d were, respectively, 6.4 ± 0.4 nM and 63 ± 4 % for the water control group, 7.8 ± 0.5 nM and 63 ± 5 % for the non-deprived group, and 8.4 ± 0.4 nM and 58 ± 4 % for the alcohol-deprived group (Fig. 3). Building a multiple linear regression model, there were no significant differences in the dopamine E_d (slope) between the groups, $F(2,291) = 0.44$, $p = 0.65$ (Fig. 3). Building a multiple linear regression model, but switching the variables to enable comparisons of dopamine concentrations, there was a significant effect of alcohol drinking history on the basal extracellular dopamine concentrations (intercept) $F(2,293) = 6.38$, $p < 0.005$ (Fig. 3). Post-hoc analysis revealed that the basal extracellular concentrations of dopamine were significantly elevated in the non-deprived and alcohol-deprived groups compared to the water control group, $p < 0.05$. The basal extracellular concentrations of dopamine were not significantly different between the non-deprived and alcohol-deprived groups.

The basal extracellular serotonin concentrations and *in vivo* serotonin E_d were, respectively, 0.92 ± 0.08 nM and 50 ± 8 % for the water control group, 0.60 ± 0.04 nM and 60 ± 4 % for the non-deprived group, and 0.90 ± 0.07 nM and 75 ± 9 % for the alcohol-deprived group. Building a multiple linear regression model, there was a significant difference in the serotonin E_d (slope) among the groups, $F(2,190) = 3.03$, $p < 0.05$ (Fig. 4). Post-hoc analysis indicated that the serotonin E_d was significantly higher in the alcohol-deprived group compared to the water control and non-deprived groups, $p < 0.05$. Building a multiple linear regression model, but switching the variables to enable comparisons of serotonin concentrations, there was a significant effect of alcohol drinking history on basal extracellular serotonin concentrations (intercept) among the groups, $F(2,192) = 4.63$, $p < 0.05$ (Fig. 4). Post-hoc analysis revealed that the basal extracellular concentrations of serotonin were significantly lower in the non-deprived

group compared to the water control and alcohol-deprived groups, $p < 0.05$. Basal extracellular concentrations of serotonin were not significantly different between the water control and alcohol-deprived groups.

The effects of local perfusion with the selective D₂ receptor antagonist sulpiride (100 μ M) on dopamine overflow in the nucleus accumbens are shown in Figure 5. Baseline DA levels, obtained by averaging the three baseline DA samples prior to sulpiride perfusion, were 1.00 ± 0.26 nM for the water control group, 0.93 ± 0.42 nM for the non-deprived group, and 0.78 ± 0.22 nM for the alcohol-deprived group. Comparison of baseline dopamine levels prior to sulpiride perfusion did not reveal any significant differences between the groups, $F(2,13) = 0.196$, $p > 0.05$. Local perfusion of 100 μ M sulpiride into the nucleus accumbens resulted in a significant increase in dopamine overflow in all three groups, $F(6,78) = 5.37$, $p < 0.001$. However, the effect of sulpiride on nucleus accumbens dopamine overflow was significantly reduced in the non-deprived and alcohol-deprived groups compared to the water control group as indicated by a significant effect of drinking history, $F(2,13) = 4.49$, $p < 0.05$. In addition, comparison of the dopamine AUC (Inset, Fig. 5) during the period of sulpiride perfusion showed that the increases in extracellular dopamine were significantly less in the non-deprived and alcohol-deprived groups as compared to the water control group, $F(2,13) = 4.29$, $p < 0.05$

The effects of local perfusion with the selective 5-HT₃ receptor agonist CPBG (35 μ M) on dopamine overflow in the nucleus accumbens are shown in Figure 6. Baseline dopamine levels, obtained by averaging the three baseline dopamine samples prior to CPBG perfusion, were 4.3 ± 0.5 nM for the water control group, 5.9 ± 1.2 nM for the non-deprived group, and 8.0 ± 1.3 nM for the alcohol-deprived group. Baseline dopamine levels were higher than those reported in the sulpiride experiment as a result of the higher perfusion rate used in the sulpiride

experiment compared to this study (see Methods). Comparison of baseline dopamine levels prior to CPBG perfusion revealed a trend for higher basal dopamine levels in the non-deprived and alcohol-deprived groups, but this did not quite reach statistical significance, $F(2,12) = 3.19$, $p = 0.08$. Local perfusion of 35 μM CPBG into the nucleus accumbens resulted in a significant increase in dopamine overflow in all three groups, $F(8,96) = 41.0$, $p < 0.001$. However, the effect of CPBG on nucleus accumbens dopamine overflow was significantly reduced in the non-deprived and alcohol-deprived groups compared to the water control group as indicated by a significant effect of drinking history, $F(2,12) = 4.92$, $p < 0.05$. In addition, using the AUC (Inset, Fig. 6) as a measure of the total overflow produced by CPBG indicated a significantly reduced dopamine overflow in the non-deprived and alcohol-deprived groups compared to the water control group, $F(2,12) = 4.60$, $p < 0.05$.

Discussion

The major findings of this study were that chronic alcohol drinking by female P rats produced significant alterations in the basal extracellular concentrations of dopamine and serotonin, and D₂ autoreceptor and 5-HT₃ receptor function in the nucleus accumbens (Figs. 3-6). Moreover, most of these changes persisted in the absence of alcohol (Figs. 3, 5 & 6). The extracellular concentrations of dopamine were higher in the nucleus accumbens of the non-deprived and alcohol-deprived groups compared to the water control group (Fig. 3), suggesting that alcohol drinking increased dopamine neurotransmission and that this increase persisted for as long as two weeks in the absence of alcohol. The results in the non-deprived group resemble data obtained by Smith and Weiss (1999), who administered 5 daily i.p. injections of 1 g/kg ethanol into male P rats. These results suggest that it may be a general pharmacological effect of repeated exposure to ethanol that is altering dopamine neurotransmission in the nucleus accumbens of P rats, rather than changes primarily associated with maintaining alcohol drinking. In addition, the dopamine no-net-flux data (Fig. 3) and the data on the local perfusion of the D₂ receptor antagonist sulpiride for the non-deprived group (Fig. 5) are in agreement with the results of Engleman et al. (2003) who reported reduced D₂ autoreceptor functioning in the nucleus accumbens of P rats after chronic alcohol drinking under 1-hr scheduled access. Reduced D₂ autoreceptor functioning in the nucleus accumbens should result in higher dopamine neurotransmission, which was observed in the present study (Fig. 3). Additionally, reduced D₂ autoreceptor function appears to be one factor contributing to higher dopamine neurotransmission in the nucleus accumbens of the alcohol-deprived group, as this reduced function persists in the absence of ethanol (Fig 5) and as evidenced by the group deprived of alcohol for 2 weeks still having elevated basal dopamine extracellular concentrations (Fig. 3).

Electrophysiological (Brodie and Appel, 1998; Brodie et al., 1990, 1995; Gessa et al., 1985) and microdialysis (Imperato and DiChiara, 1986; Melendez et al., 2002; Weiss et al., 1993) studies indicate that ethanol can activate the mesolimbic dopamine system. Chronic stimulation of dopamine release by ethanol may result in chronic activation of D₂ receptors (as well as other dopamine receptors) resulting in down-regulation of these receptors, including the D₂ autoreceptor, although perhaps one might have anticipated a compensatory increase in autoreceptor function to regulate synaptic levels of dopamine. Despite the higher dopamine neurotransmission in the nucleus accumbens of P rats in the non-deprived and alcohol-deprived groups, there were no apparent compensatory changes in dopamine transporter function, as indicated by the findings that the E_d values (as a measure of clearance) were similar among the 3 groups (Fig. 3).

There is ample evidence for the involvement of the mesolimbic dopamine system in mediating the reinforcing effects of ethanol and alcohol drinking (e.g., see review by Koob et al., 1998; McBride and Li, 1998). The higher basal dopamine concentrations in the nucleus accumbens may indicate that the mesolimbic dopamine system may be operating at a higher level of neuronal activity, which may help sustain high levels of alcohol intake in the P rat. Furthermore, if such a high level of mesolimbic dopamine neuronal activity persists in the absence of alcohol, this may be one factor promoting alcohol relapse. In the present study, the P rat readily demonstrated alcohol relapse drinking and a robust alcohol deprivation effect (Fig. 1). These findings are similar to previously published studies, which showed that P rats demonstrated relapse drinking and a robust alcohol deprivation effect under a variety of experimental paradigms (McKinzie et al., 1998b; Rodd-Henricks et al., 2000b). Although the constraints of the experimental design prevented measuring ethanol intakes after a second

ethanol re-exposure, previous studies show that repeated alcohol-deprivation results in higher ethanol intakes over a more sustained period when animals are re-exposed to ethanol (Rodd-Henricks et al., 2000b).

Although differences in extracellular dopamine concentrations between the groups were detected using the no-net-flux technique, differences were not detected between the treatment groups when looking at baseline dopamine levels in the sulpiride and CPBG studies. These differences are likely the result of the fact that dopamine concentration determined using the no-net-flux procedure are corrected for *in vivo* probe recoveries giving a more precise determination of basal transmitter levels. In the sulpiride and CPBG studies, the baseline values were not corrected for probe recovery, resulting in somewhat greater variability. This emphasizes the utility of quantitative microdialysis techniques for comparing small changes in transmitter levels between different treatment groups.

Chronic alcohol drinking by P rats reduced the extracellular concentration of serotonin in the nucleus accumbens (Fig. 4). This reduction occurred without a change in the E_d value (Fig. 4), suggesting that chronic alcohol drinking reduced serotonin neurotransmission in the nucleus accumbens. Acute ethanol injections increased extracellular serotonin levels in the nucleus accumbens (Yoshimoto et al. 1992), and oral alcohol drinking has been shown to increase the extracellular levels of serotonin in the nucleus accumbens of rats (Weiss et al., 1996). However, in the present study, ethanol was not present during the sampling. The study of Weiss et al. (1996) indicated that, during withdrawal, extracellular levels of serotonin and dopamine in the nucleus accumbens were reduced below normal and were restored with ethanol self-administration. Therefore, if withdrawal was a factor contributing to the reduction of the extracellular levels of serotonin in the non-deprived group, then a similar reduction in the levels

of dopamine would also be expected, but this was not the case (Fig. 3). The reduced serotonin neurotransmission in the nucleus accumbens of the non-deprived compared to the water control group is in agreement with the findings of Smith and Weiss (1999), who reported lower serotonin neurotransmission in P rats 24 hr after the last treatment of 5 daily i.p. injections of ethanol.

Following a 2-week deprivation period, the extracellular concentration of serotonin had returned to control levels (Fig. 4). However, because clearance of serotonin had increased during this period (Fig. 4), the net effect would be that higher extracellular serotonin turnover is occurring during deprivation. The results also suggest that increased clearance may be a compensatory response to the higher serotonin release. The alteration in serotonin neurotransmission, which occurs during the deprivation period, may be a factor contributing to alcohol relapse drinking and expression of the alcohol deprivation effect in P rats. It is not known whether the alterations in serotonin function are occurring at the level of the cell body in the dorsal raphe nucleus, within the terminal region, or a combination of both.

In the present study, the CaCl_2 concentration in the ACSF for the no-net-flux experiments was 2.5 mM, higher than the 1.2 mM CaCl_2 concentration reported for CSF. Therefore, the changes in both dopamine and serotonin extracellular levels with drinking history may represent changes in stimulated release instead of basal extracellular levels of the neurotransmitters. However, the values for dopamine and serotonin extracellular concentrations in the nucleus accumbens of P rats are in good agreement with those obtained in the same structure in P rats by Smith and Weiss (1999) who used a 1.2 mM CaCl_2 concentration in their ACSF.

There is an apparent reduced response of CPBG activation of 5-HT₃ receptors on dopamine release in the nucleus accumbens of the non-deprived versus the water control group

(Fig. 6), suggesting reduced functioning of the 5-HT₃ receptor regulating dopamine release. With the use of only a single concentration of the selective 5-HT₃ receptor agonist CPBG, it is not possible to determine whether the reduced function was a result of an alteration in receptor properties (i.e., affinity) or a reduction in the number of receptors. Electrophysiological studies indicate that ethanol enhances the depolarizing effects of serotonin at the 5-HT₃ receptor (Lovinger and White, 1991; Zhou and Lovinger, 1996). Microdialysis studies indicate that ethanol self-administration increases the extracellular levels of serotonin in the nucleus accumbens (Weiss et al., 1996). It is possible that under chronic ethanol drinking conditions the 5-HT₃ receptor may be chronically stimulated, resulting in its down-regulation. If this is the case, then the effect persists for at least 2 weeks because the response of CPBG in the alcohol-deprived group is similar to the response for the non-deprived group (Fig. 5). Pharmacological studies also suggested that alterations in the 5-HT₃ receptor persisted for several weeks in the absence of treatment (Rodd-Henricks et al 2000a).

If the mesolimbic dopamine system is involved in regulating alcohol drinking, then the results of the present study suggest that the influence of the 5-HT₃ system in regulating the activity of the mesolimbic dopamine system and thereby ethanol drinking may be reduced with chronic ethanol exposure. Consequently, this suggests that other transmitter systems or other neuroadaptations, such as the reduction in D₂ receptor function shown in the current study, may have been altered to produce the increased dopamine neurotransmission following chronic alcohol drinking (Fig. 3B). The reduced effectiveness of 5-HT₃ antagonists under relapse conditions (Rodd-Henricks et al. 2000a) may be a result of a combination of enhanced serotonin neurotransmission, reduced influence of the 5-HT₃ receptor on mesolimbic dopamine neuronal activity, and increased influence of other transmitter systems in elevating dopamine

neurotransmission.

Overall, the results of the present study suggest that chronic alcohol drinking by P rats increases dopamine neurotransmission, at least in part, by reducing D₂ autoreceptor function. Chronic drinking also decreases serotonin neurotransmission, and reduces 5-HT₃ receptor function in the nucleus accumbens. The effects of chronic alcohol drinking on dopamine neurotransmission, D₂ autoreceptor function and 5-HT₃ receptor function appear to be long-lasting and persist in the absence of alcohol. In addition, the dorsal raphe nucleus serotonin projections to the nucleus accumbens undergo additional alterations during the deprivation period that resulted in higher serotonin activity.

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Footnotes:

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Figure Legends:

Figure 1. Ethanol intake of non-deprived (open circles) and alcohol-deprived (closed squares) female P rats. Data are the means \pm SEM (n = 11-14). Alcohol-deprived P rats had six weeks of uninterrupted free-choice access to 10% ethanol and water prior to alcohol deprivation. Drinking levels were compared for the three days prior to removal of ethanol from the deprived group (baseline) and the first 5 days after ethanol re-exposure for the deprived group (re-exposure). ** p < 0.01 for ethanol intake in the alcohol-deprived group upon re-exposure versus intake in the non-deprived group on the same day.

Figure 2. Location of representative probe placements in the nucleus accumbens. Overlapping placements are not shown. Each line represents a probe placement. The active dialysis loop was 2 mm long. Probe placements for each of the three groups (water control, non-deprived and alcohol-deprived) are shown separately. Placements depicted on the left hemisphere represent probe placements for animals used in the no-net-flux experiments. Those shown on the right hemisphere represent probe placements for animals used in the sulphiride and CPBG studies. This was done for illustrative purposes only and does not represent the actual side in which probes were located. Adopted from the stereotaxic atlas of Paxinos and Watson (1998).

Figure 3. Determination of point of no-net-flux and E_d for dopamine for the water control, alcohol non-deprived and alcohol-deprived groups. The top panel shows the net gain or loss of dialysate dopamine concentrations in the nucleus accumbens as a function of dopamine concentration perfused through the microdialysis probe for the water control (open triangles; n =

7), non-deprived (open circles; $n = 5$) and alcohol-deprived (closed squares; $n = 6$) groups. Data are the means \pm SEM. The zero point intercepts indicate the basal extracellular concentrations of dopamine for the 3 groups. The slope yields the E_d . The middle panel represents dopamine concentrations at the point of no-net-flux for the water control, non-deprived and alcohol-deprived groups. *Indicates values for non-deprived and alcohol-deprived groups are significantly ($p < 0.05$) different than the water control group. The bottom panel shows the dopamine E_d for the 3 groups shown above. There were no significant differences between any of the groups.

Figure 4. Determination of point of no-net-flux and E_d for serotonin for the water control, alcohol non-deprived and alcohol-deprived groups. The top panel shows the net gain or loss of dialysate serotonin concentrations in the nucleus accumbens as a function of serotonin concentration perfused through the microdialysis probe for the water control (open triangles; $n = 6$), non-deprived (open circles; $n = 4$) and alcohol-deprived (closed squares; $n = 4$) groups. Data are the means \pm SEM. The zero point intercepts indicate the basal extracellular concentrations of serotonin for the 3 groups. The slope yields the E_d . The middle panel shows the serotonin concentrations at the point of no-net-flux for the water control, non-deprived and alcohol-deprived groups. *Indicates the non-deprived group is significantly ($p < 0.05$) different than the water control and alcohol-deprived groups. The bottom, panel shows the serotonin E_d for the 3 groups shown above. *Indicates the alcohol-deprived group is significantly ($p < 0.05$) different than the water control and non-deprived groups.

Figure 5. Time course of the effects of local perfusion of the nucleus accumbens with 100 μ M sulpiride on dopamine overflow in water control (open triangles), non-deprived (open circles), and alcohol-deprived (closed squares) P rats. Data are expressed as percent of baseline and represent the means \pm SEM (n = 5 each group). Baseline levels were determined by averaging the extracellular levels of dopamine in the three samples immediately preceding perfusion with 100 μ M sulpiride. Baseline extracellular dopamine levels did not significantly differ from each other. Inset: Area under the curve (AUC) for the 3 groups. *Indicates values for non-deprived and alcohol-deprived groups significantly lower ($p < 0.05$) than the water control group.

Figure 6. Time course of the effects of local perfusion of the nucleus accumbens with 35 μ M CPBG on dopamine overflow in water control (open triangles), non-deprived (open circles), and alcohol-deprived (closed squares) P rats. Data are expressed as percent of baseline and represent the means \pm SEM (n = 5 each group). Baseline levels were determined by averaging the extracellular levels of dopamine in the three samples immediately preceding perfusion with 35 μ M CPBG. Baseline extracellular dopamine levels did not significantly differ from each other. Inset: Area under the curve (AUC) for the 3 groups. *Indicates values for non-deprived and alcohol-deprived groups significantly lower ($p < 0.05$) than the water control group.

Figure 1

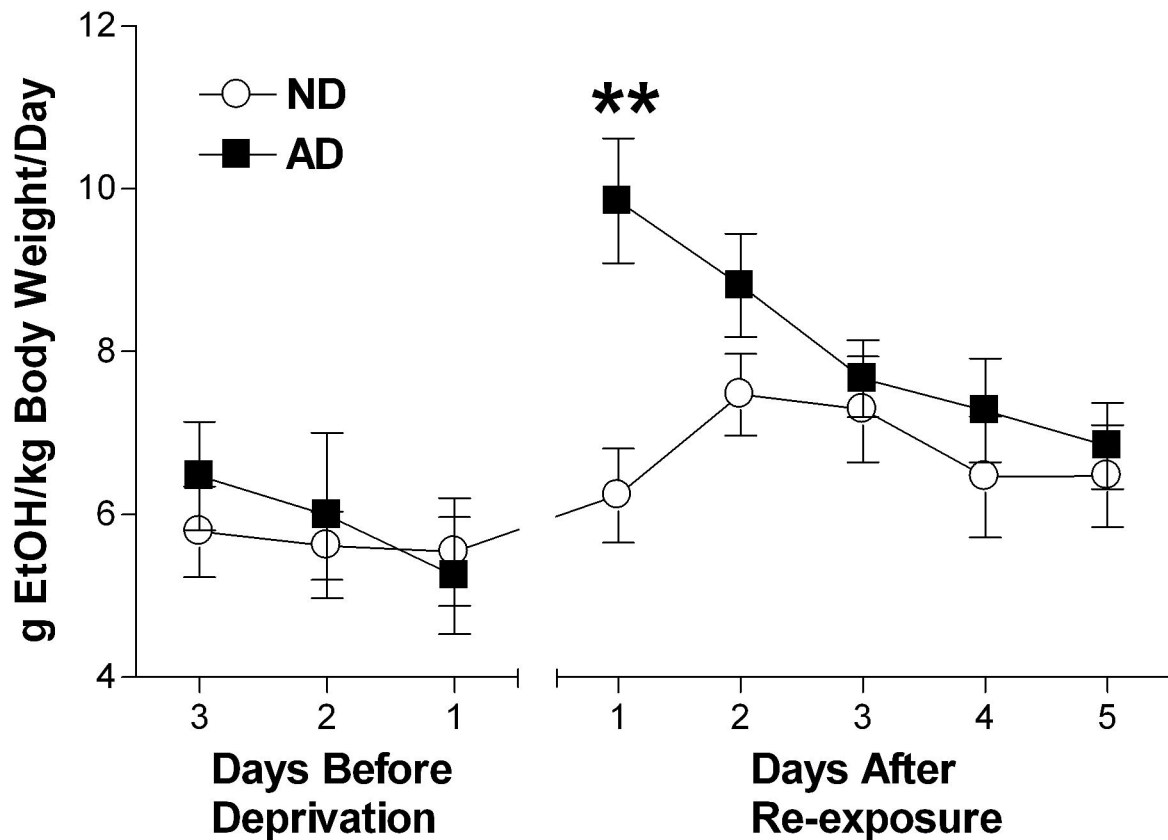


Figure 2

Water

Non-Deprived

Alcohol-Deprived

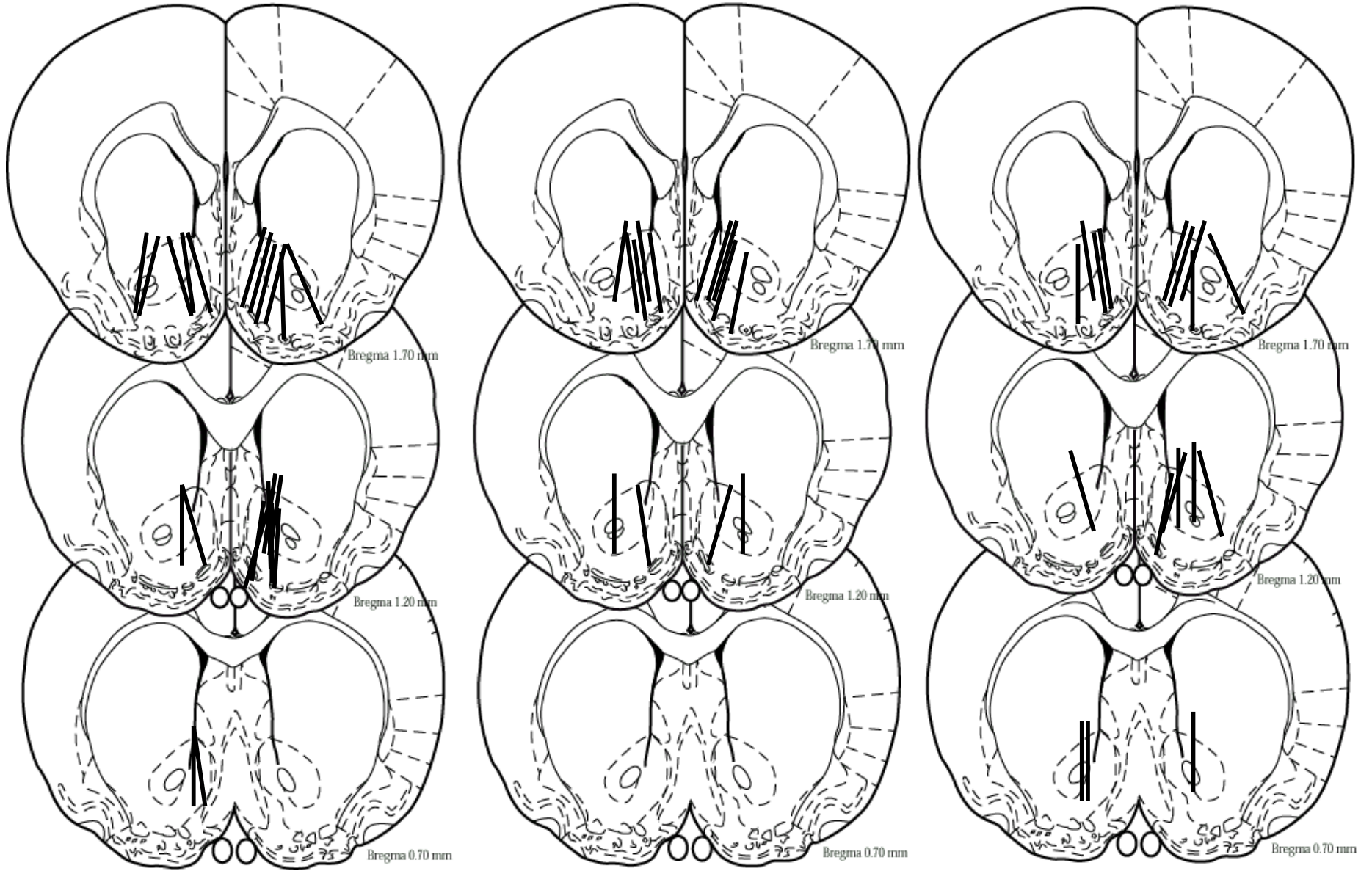


Figure 3

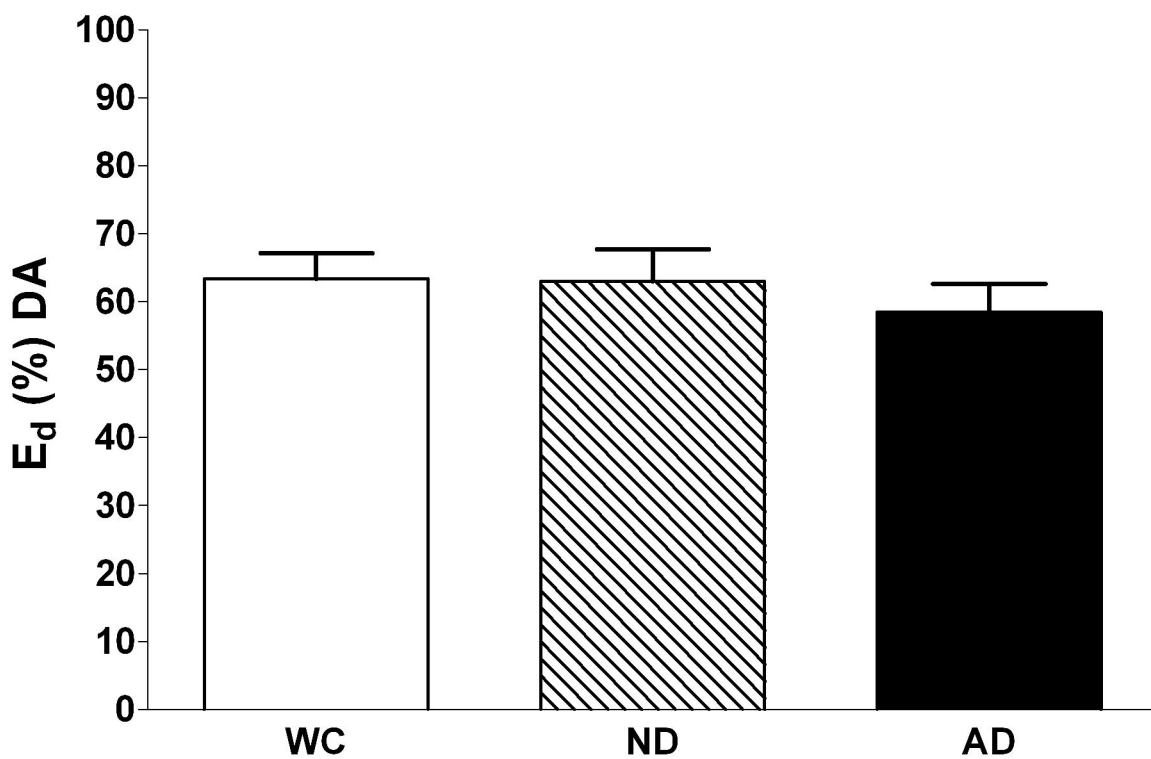
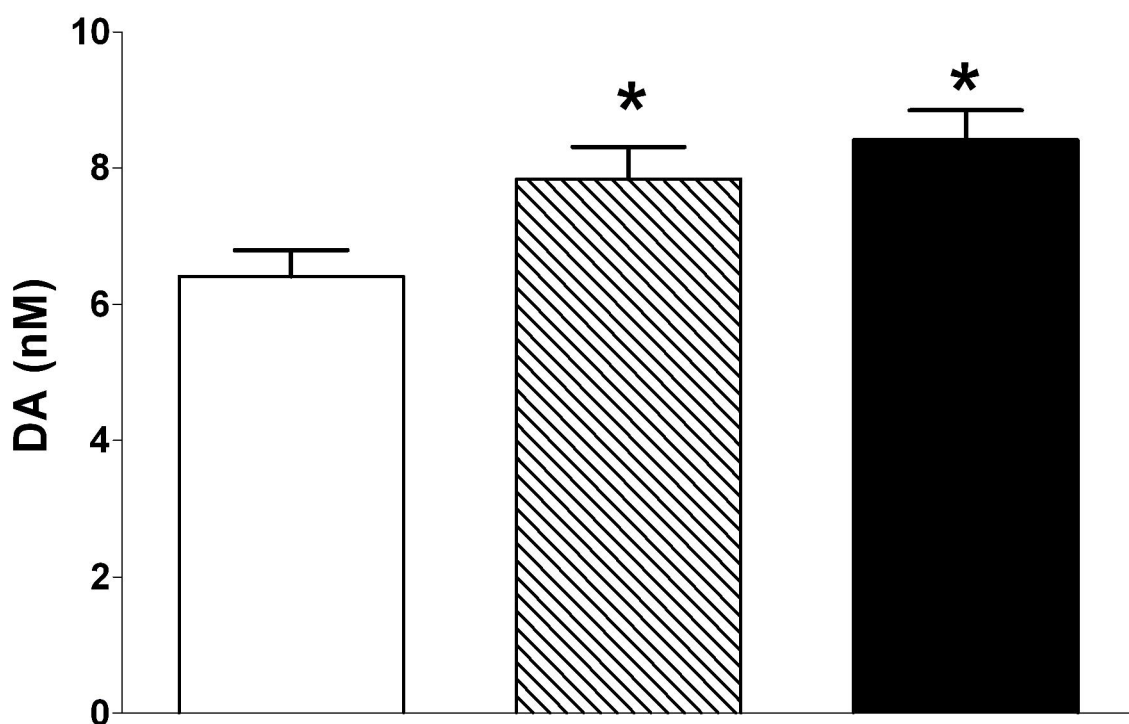
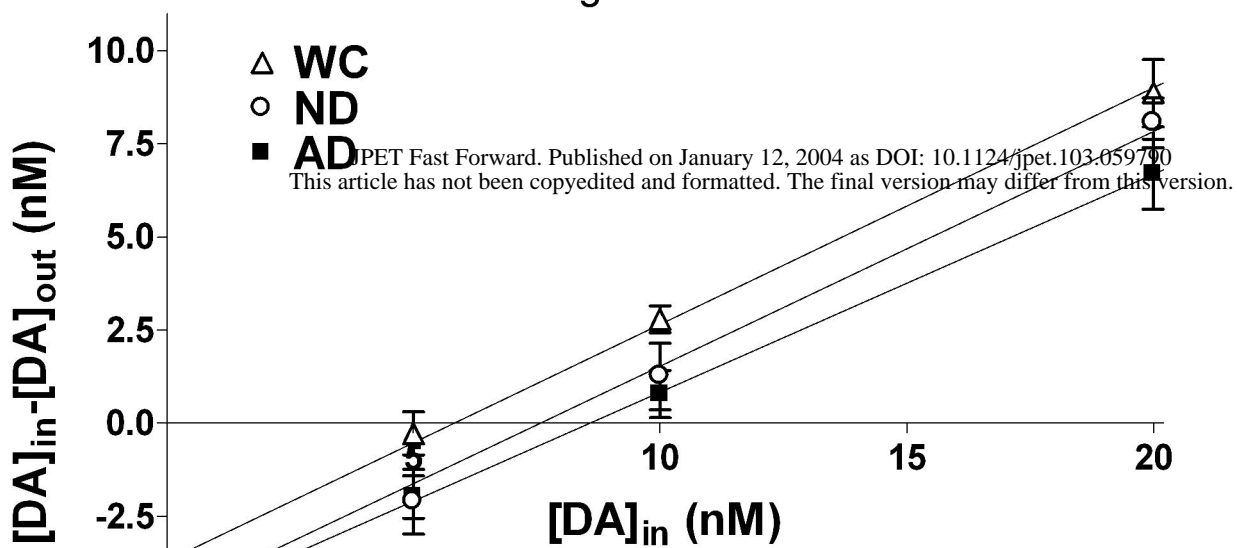


Figure 4

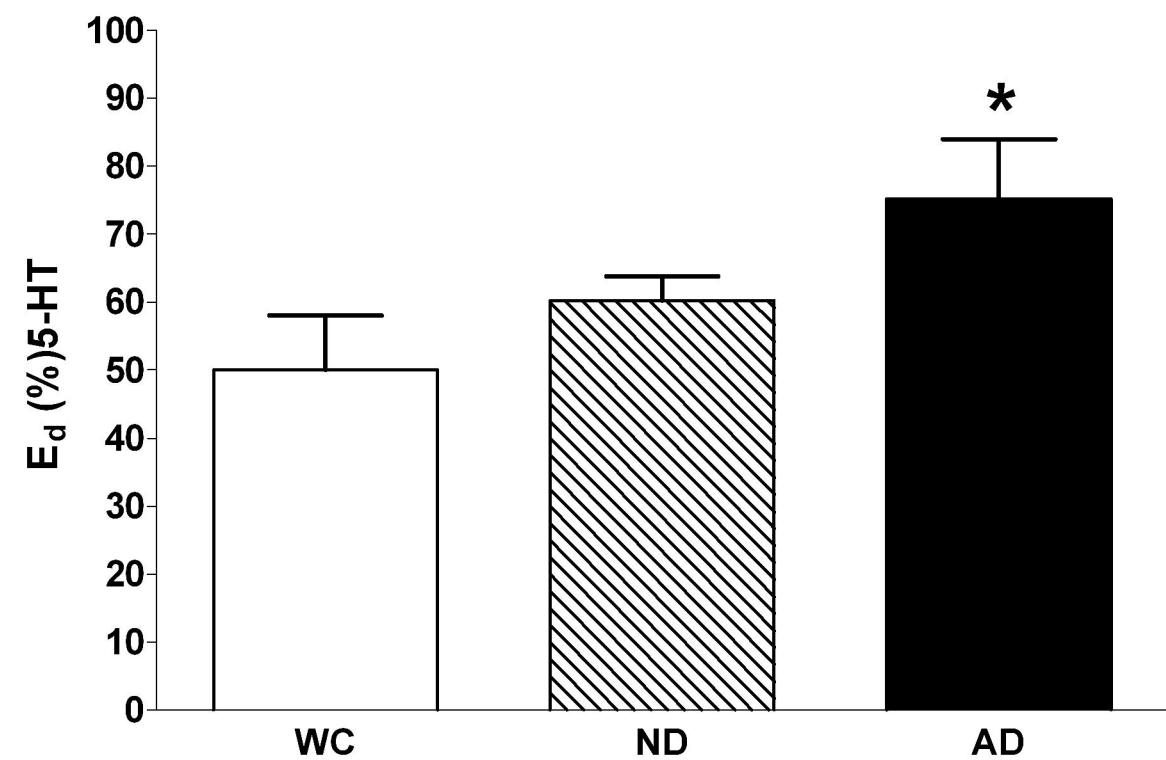
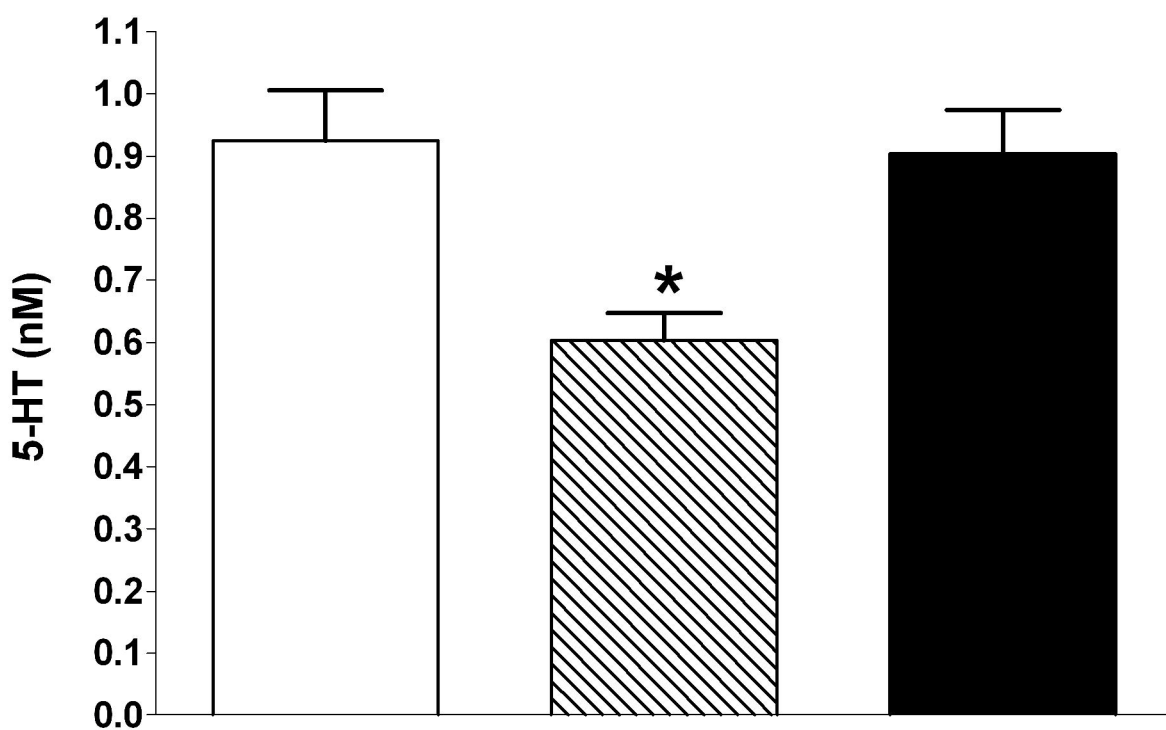
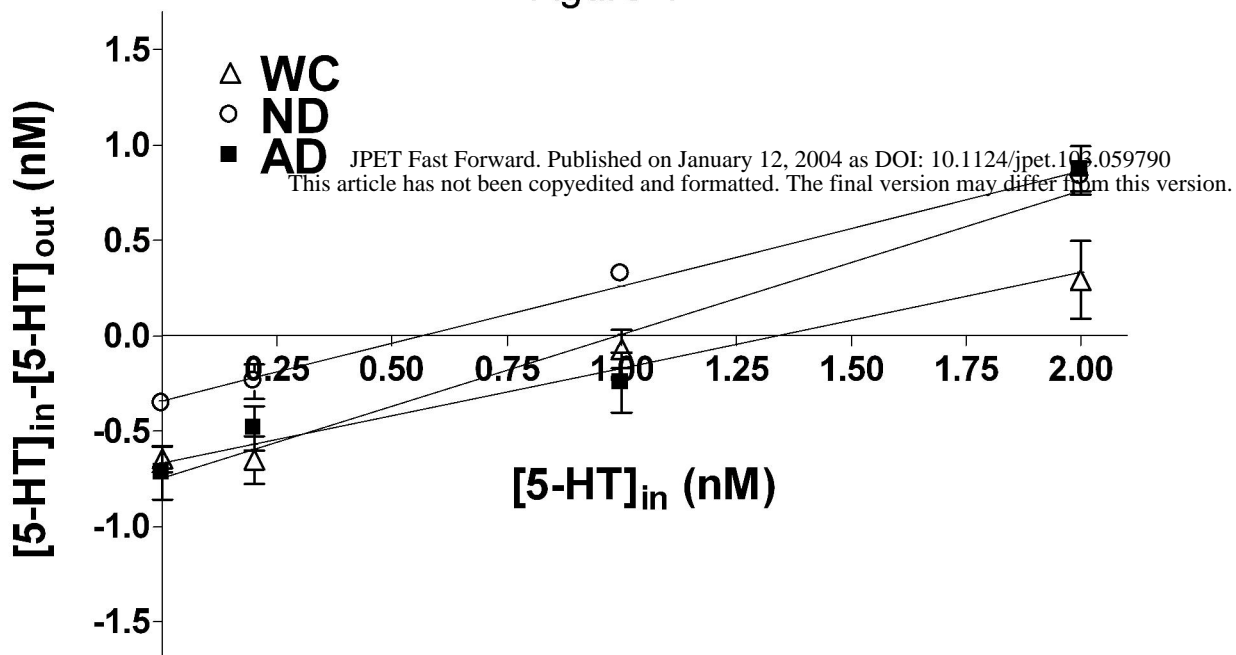


Figure 5

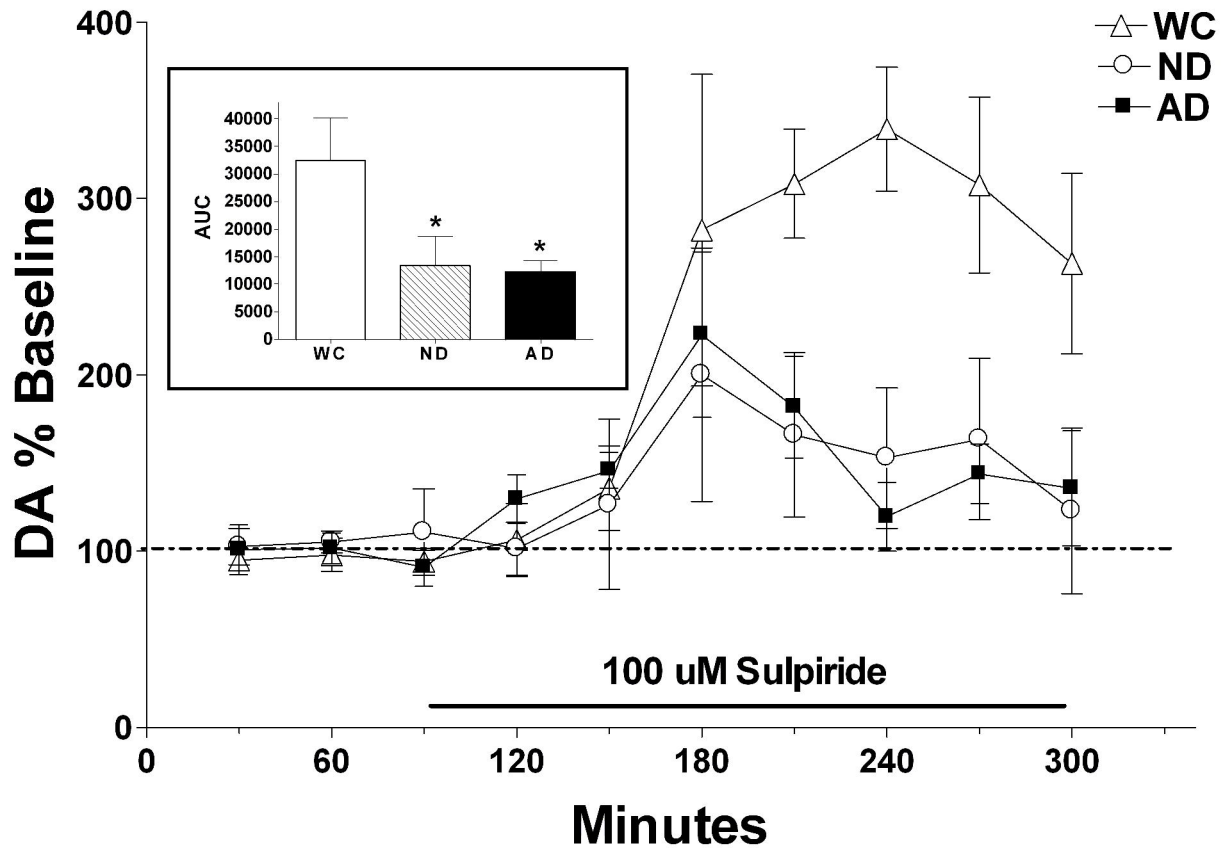


Figure 6

