Chronic Amphetamine and Effects on Behavior and DDR2 Protein Levels

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Abstract

There exists a need for improved medication adherence among schizophrenic patients. One method of ameliorating this risk factor may come from biodegradable antipsychotic implants, but a critical constraint in the study of this pharmacological solution is the development of a disease paradigm against which these implants might be tested. In this study, continuous subcutaneous amphetamine administration using Alzet® Mini osmotic pumps was tested in mice to evaluate the efficacy of this treatment method as a disease state. We hypothesize that chronic amphetamine will cause behavioral effects comparable to those found in schizophrenia through disruptions of the dopaminergic signaling pathway at the D_2 receptor. To test this hypothesis, behavioral analysis of Pre-pulse Inhibition (PPI) as well as Locomotor Activity were conducted. In addition, the levels of DDR2 protein were quantified after 2 weeks of chronic amphetamine to determine effects at the receptor level. Although behaviors did not change significantly over the period of this study, the levels of DDR2 protein, and thus D_2 dopamine receptors, did reduce among the amphetamine treatment groups signifying postsynaptic adaptation.

Introduction

Medication nonadherence in schizophrenia is an unfortunate complication of treatment with multiple causes spanning the realm of both social and pharmacological therapies. There are an enormous number of patients who struggle with this problem, with up to 55% of schizophrenia patients displaying significant difficulty with adherence to the rigors of treatment (Corriss et al., 1999). Poor family support as well as adverse drug side effects can lead to medication nonadherence, increasing the risk of relapse and rehospitalization (Robinson et al., 2002). The statistics are strongly against nonadherent patients, who are twice as likely to undergo rehospitalization from relapse, resulting in dramatic reductions in their quality of life (Svarstad et al., 2001). This unfortunate outcome of nonadherence also creates significant and unnecessary increases in health care costs, with an estimated $2.3 billion allocated to these patients annually (Menzin, Boulanger et al. 2003). While psychological and psychiatric therapies are both important factors in improving the outlook for schizophrenic patients, the inability of patients to completely comply with their oral prescriptions can also diminish the efficacy of these non-medical approaches.

A potential method of regulating the amount of drug available at the receptor could be to create an implant which would provide constant levels of drug. This could require one treatment over a six month period (Metzger et al., 2007) as part of an outpatient procedure rather than the 6-12 visits necessary for depot injections over this interval using either monthly haloperidol decanoate (Ortho-McNeil) or biweekly risperidone Consta® (Janssen). In addition to improving the ease of use for the patient, having constant levels of drug at the D_2 receptor, which is the site of action for many

schizophrenia medications, may in fact allow the necessary changes in gene expression to occur at the intra- and intercellular level, promoting stable activity and connectivity as a result. The current paradigm of an oral drug delivery system makes it difficult to reduce the constant flux in drug levels, and thus creates an environment which is not conducive to modifications in gene expression and may impede the ability of the postsynaptic cell to arrive at a stable state of intracellular signal transduction.

The dopamine D_2 receptor is an inhibitory G-protein coupled receptor (Gi) in which binding of dopamine reduces Adenylyl Cyclase activity, resulting in downregulation of cAMP production and a reduction in Protein Kinase A (PKA) activity. PKA is responsible for phosphorylation of multiple intracellular substrates with resulting early functional changes such as receptor phosphorylation as well as long term adaptations in gene expression through the cAMP responsive element (CRE) (Figure 1). The role of both amphetamine and antipsychotics on the modulation of the D_2 receptor makes it a viable candidate for study. The ability of this pathway to cause critical changes in connectivity and activity at the intra- and inter-cellular levels further suggest its importance as a therapeutic manipulation.

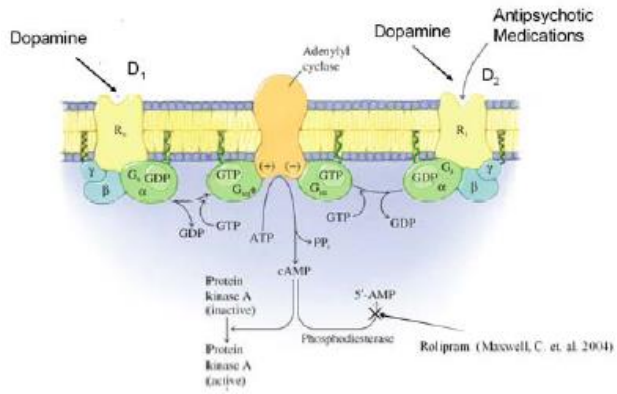


Figure 1: Schematic representation of the DA receptor-coupled adenylyl cyclase-cAMP pathway: All current antipsychotic drugs are believed to act through antagonism at the G-protein coupled D dopamine receptor. D_2 dopamine receptors are known to decrease Adenylyl Cyclase (AC) activity. AC is an enzyme that transforms adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which is a ubiquitous intracellular second messenger. Thus, antagonism of the D_2 receptor with antipsychotic medication leads to increased cAMP levels and subsequent regulation of PKA dependent intra- and inter-cellular events (Halene and Siegel, 2008). Consequently, agonism with amphetamine should lead to decreased cAMP and downregulation of PKA dependent activities.

In order to evaluate the efficacy of an implantable form of risperidone, an appropriate disease model needs to be created in order to simulate psychosis for the purposes of rescuing normal behaviors with implantable drug. A potential treatment candidate for this disease model is the continuous administration of subcutaneous amphetamine, a delivery method which has been well studied in rats but remains relatively uncharacterized in terms of behavioral and molecular effects in mice. We hypothesize that chronic amphetamine will cause behavioral effects comparable to schizophrenic conditions through disruptions of the dopaminergic signaling pathway at the D_2 receptor.

The purpose of this experiment is to assess changes after chronic amphetamine in mice, both in terms of performance in behavioral tests and in the level of receptor proteins. Specifically, the goal is to examine PPI performance as a measure of the pathway disruption and consequent neuroadaptations taking place at the D_2 receptor as evidenced by DDR2 protein levels. We performed an *in vivo* experiment using Alzet osmotic pumps to evaluate these changes.

Materials:

Species:

The study was performed in C57Bl/6J mice (Jackson Labs), a species which our group has extensive experience with. The animals were housed in ULAR compliant facilities and euthanized in CO_2 upon completion of the study. Serum concentrations were collected by extraction of the total blood volume from the animals at death (so as not to impact behaviors) for future study. Brain tissue was removed and dissected, then stored at -80° Celsius.

Behavioral alterations following chronic amphetamine administration in mouse have predictive validity for both efficacy and side effects in human. We

measured the ability to attenuate amphetamine-induced disruptions of PPI as a measure with predictive validity for antipsychotic efficacy (Swerdlow et al., 1994). Additionally, we will monitor baseline locomotor activity, catalepsy and rotorod activity as measures with predictive validity for motor side effects.

Pumps:

Animals were anesthetized with isoflurane and implanted with Alzet Minipumps (Durect), a small osmotic pump that delivers continuous infusion of solution, as well as a control implant identical in size and material to future risperidone implants. The pumps were primed for one week prior to surgery in either saline solution or amphetamine solution at various doses. Amphetamine conditions received subcutaneous delivery of chronic amphetamine for 2 weeks.

Control Implants:

Implants that contained no drug (polymer only) were subcutaneously inserted to control for the presence of a drug implant made from the same materials in subsequent experiments. These implants were fabricated with biodegradable poly lactide-co-glycolide (PLGA) in the Siegel lab prior to the study.

Methods

Experimental Groups:

Prior to behavioral testing, mice were divided into treatment and control groups and implanted with osmotic pumps primed with either Saline solution (4 groups), 1.2 mg/kg/day amphetamine, 2.4 mg/kg/day amphetamine, 4.0 mg/kg/day amphetamine, 8.0 mg/kg/day amphetamine, or 12.0 mg/kg/day amphetamine. Refer to Table 1 for study design. Total n=72.

Study Design	
Control Groups (n=8 for each)	**Treatment Groups (n=8 for each)**
Saline 1	1.2 mg/kg/day amphetamine
Saline 2	2.4 mg/kg/day amphetamine
Saline 3	4.0 mg/kg/day amphetamine
Saline 4	8.0 mg/kg/day amphetamine
	12.0 mg/kg/day amphetamine

Table 1. Study design includes 4 experimental controls which were not exposed to chronic amphetamine. Each control and treatment group were implanted with both Alzet Minipumps and PLGA implants in order to control for the effects of surgery on behavior.

Pre-Pulse Inhibition of Startle:

Prepulse inhibition of startle was tested in each group of mice once they had healed from implantation surgery. The animals were given three days to habituate to the test cages, and testing occurred on Day 11 of the study (11 days of chronic amphetamine in the treatment groups). Testing occurred according to the methods described in preliminary data and previously published studies by the PI (Connolly et al., 2003, Gould et al., 2004, Kanes et al., 2007, Halene and Siegel, 2008). The effects of chronic amphetamine (in the context of saline or amphetamine pump implants) on startle were interpreted as functional measures of disruptive amphetamine activity from implanted administration.

Locomotor Activity:

Locomotor activity was measured on Day 11 of the study in a "home cage" activity monitoring system (MedAssociates, St. Albans, VT) as previously published (Halene and Siegel, 2008). This system allows for a standard, clean home cage to be placed in a photobeam frame with two levels of sensors arranged in an 8-beam array strip with 1.25 inch spacing. A computer detection system monitors interruptions of the photobeams for the ambulations parameter. Total ambulations are determined by the number of photobeam interruptions an animal makes while moving about the cage. Data are recorded on Med Associates personal computer-designed software and monitored at 5-minute intervals for a total of 30 minutes per activity monitoring session.

Mice received 3 days of habituation to the apparatus prior to acquisition of data.

Tissue isolation and protein determination:

Each group of mice was sacrificed following 2 weeks of chronic drug administration. Mice were euthanized with CO_2 and brains were rapidly removed and dissected into four regions (cortex, hippocampus, striatum and cerebellum) and frozen in liquid nitrogen as previously described (Siegel et al., 2002). Brain regions were stored until needed at -80ºC.

Proteins were extracted from striatal tissue on ice for 45 minutes with occasional agitation. Striata were homogenized in homogenization buffer (25 mM Tris-HCl (pH 7.5) 2.0 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Samples were centrifuged at 800 × g for 10 minutes at 4˚C. Pellets were resuspended and solubilized in homogenization buffer. Supernatants were solubilized by sonicating (FISHER sonic desmembrator) on ice for 10 seconds. Triple detergent (.5% digitonin, .2% sodium cholate, and .5% NP-40) was added to the supernatant, which was then end-over-end shaken for 60 minutes at 4˚C. Total protein level was quantified by standard Bradford assay.

Western Blots:

Western blots were performed for quantitative analysis of D_2 receptor protein levels. Protein samples were prepared with 25% 4x NuPAGE sample buffer plus 10% reducing agent (Invitrogen) and heat shocked at 70oC for 10 minutes. Protein samples were subjected to electrophoresis in 10% acrylamide NuPAGE Tris-Glycine denaturing gels (Invitrogen). Gel electrophoresis was run at 200V for 1 hr. Samples were transferred to 0.4 μm pore PVDF membrane (Milipore) over a two hour period at 20V. The membrane was washed and then treated with polyclonal antibody

WR-3526, raised against amino acids 272-282 of the D_2 receptor protein (Research and Diagnostic Antibodies, Berkeley, Ca). Immunodetection of D_2 protein receptor was achieved by Pierce Fast Western Blot Kit (Invitrogen) chemiluminescent substrate phosphatase. Membranes were then wrapped in plastic and exposed to autoradiographic Kodak X-OMAT film (Sigma-Aldrich).

Quantification:

The intensity of each band will be quantified using a GS-700 densitometer and quantitative analysis software (BioRad, Hercules, CA) and expressed as a ratio to the corresponding band in control mice to yield a ratio of intensity.

Results:

All data was analyzed by grouping all 4 Saline Control Groups into one Saline Group (n=32) for comparison against each of the other treatment groups. Predicted changes in behavior for amphetamine treatment groups based upon known actions of amphetamine (and thus increased monoamine transmission) on the D_2 receptor pathway are a decrease in % PPI relative to control as well as an increase in Startle relative to control.

Pre-Pulse Inhibition of Startle:

Pre pulse inhibition of startle was increased with higher decibel stimuli, but between groups there was not a significant difference in the level of startle inhibition as measured by % PPI in Figure 2. The p-value for this experiment was .934, suggesting that there was almost no overt effect of amphetamine dose on the startle response in this behavioral test.

Startle, as measured by startle units, was also not significant between groups with a p-value of .076 (Figure 3). This is a critical component of the study, showing that there are no inherent differences between groups in their responses to

individual stimuli that would account for changes in their response to stimuli primed by a pre-pulse.

Locomotor Activity:

Locomotor activity was predicted to increase with greater doses of chronic amphetamine. While there was a general increasing trend, the data does not show significant increases in locomotor activity that correspond to increases in the level of chronic amphetamine delivery after 11 days of administration (p-value =.367). The results of this test are shown in Figure 4.

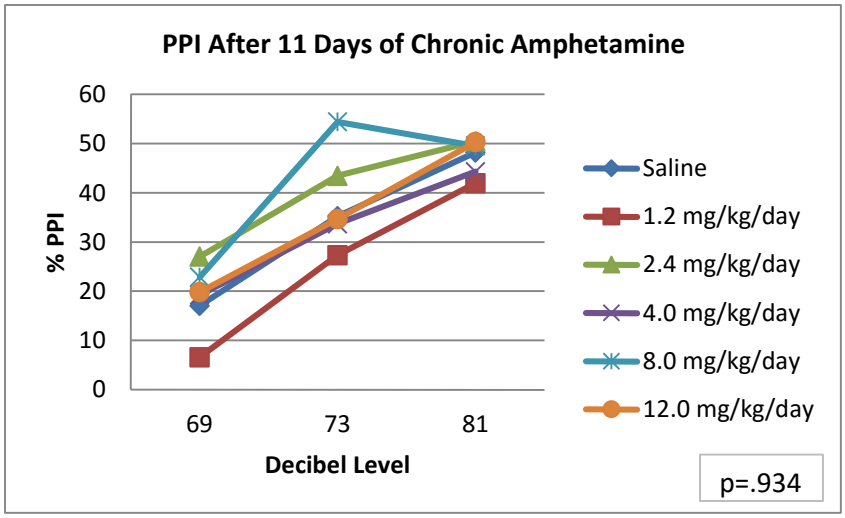


Figure 2. PPI over several decibel levels for each of the treatment groups and the saline control is shown at left. Points are based on the average %PPI for each of the groups, with Saline containing the average of all 4 Saline Control Groups. As calculated from the PPI data, across treatment groups there is no statistical difference in the impairment/amplification of inhibition of startle following a pre-pulse sound.

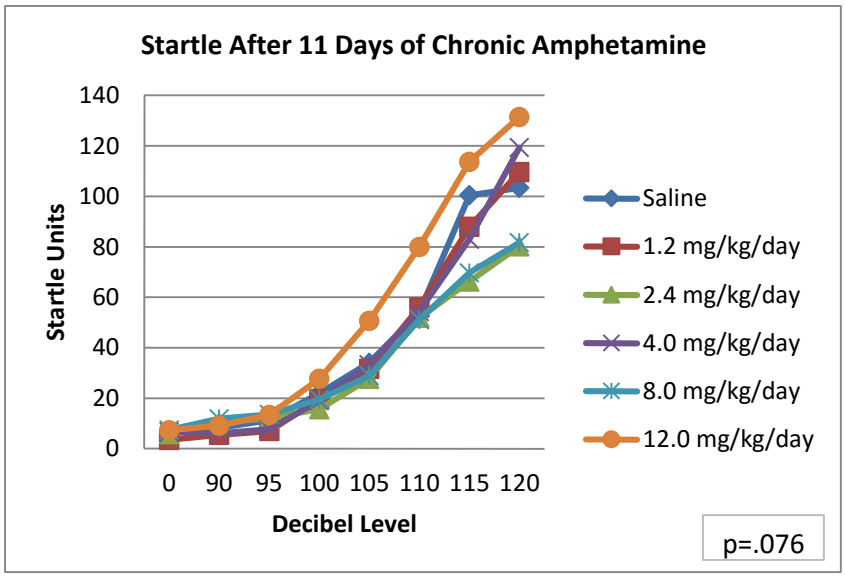


Figure 3. Average startle across groups shows no significant increase in startle for amphetamine treatment groups.

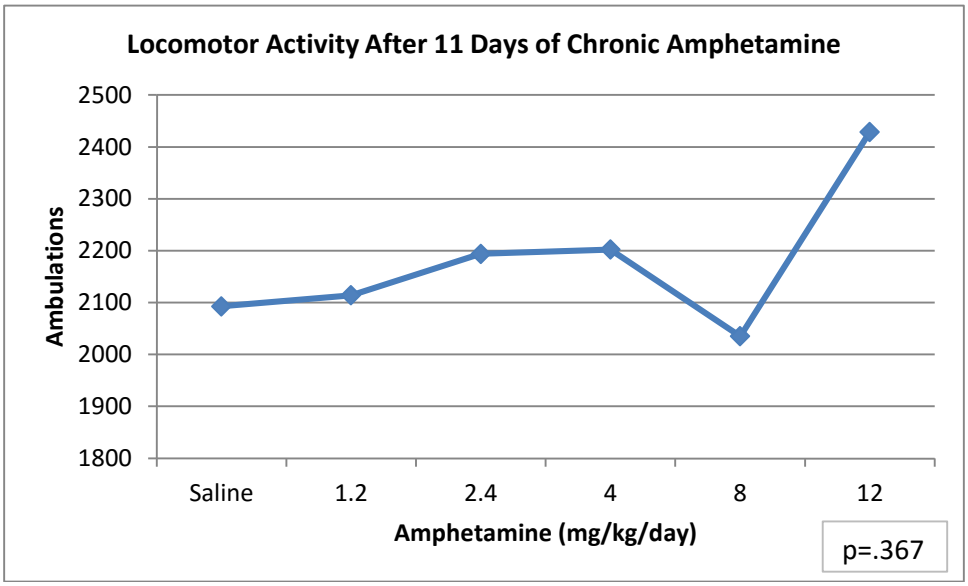


Figure 4. Locomotor Activity across treatment groups all showed increases in ambulations (interruptions of the home cage beams) except for the 8.0 mg/kg/day treatment group. These increases were not significant across groups, however.

DDR2 Levels:

It is apparent from the results of the three behavioral tests that the levels of monoamine disruption we tested did not affect PPI or Locomotor Activity significantly. This suggests an inherent adaptability of this pathway to small changes in monoamine levels. This may also result in long-term adaptations at the receptor level. The levels of DDR2 protein over the course of the experiment were quantified in an effort to characterize these adaptations as taking place at the D_2 receptor. All DDR2 measurements were normalized to the levels of β-actin protein, which was measured concurrently on the Western Blot. The results of this study can be seen in Figures 5 and 6 below.

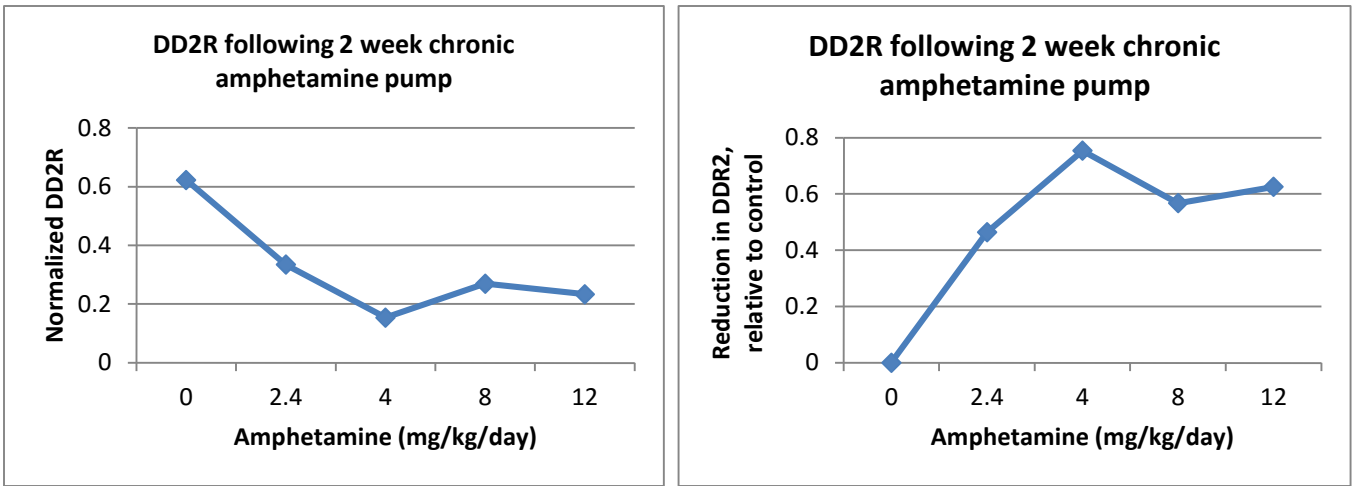


Figure 5,6. DD2R protein indicative of the levels of D_2 receptor decreased over the course of 2 weeks, both in normalized terms and with regard to percentage change relative to Saline. Note that in the graph of reduction in DDR2 levels, a reduction in 0.1 corresponds to a 10% decrease in protein levels.

Discussion

The extremely small changes in behavior exhibited by all chronic amphetamine groups in this study suggest that the experimental conditions we used to evaluate behavior require adjustment before being considered as a disease model of schizophrenia. Either the disruption in the signaling pathway which we created by perturbing the system with amphetamine were too small to have significant effects on behavior or the system's ability to adapt to these low level manipulations was comprehensive and rapid enough to counteract its effects. The former of these flaws points to the importance of a stable presynaptic mechanism of action, but based upon the results of our Western Blots, which show decreases in the levels of DDR2 protein with chronic amphetamine delivery, the focus of our study shifts toward this inherent ability of the system to adapt and compensate for these changes in monoamine levels. In addition, the rapidity of this adaptation is particularly intriguing, as changes as large as a 75% reduction in D_2 receptor levels in the striatum was possible for one of the groups over a 2 week period.

In relation to schizophrenia, this study suggests that when presynaptic perturbations exist the D_2 receptor pathway can adapt and compensate to a limited point. In real disease conditions, where presynaptic perturbations are extremely large, this pathway may not be able to adapt quickly or fully enough to compensate for these changes. This indicates the importance of promoting postsynaptic inter-and intra-cellular changes in connectivity and activity in the treatment of this disease by providing stability at the level of the receptor. If an effective implantable antipsychotic can provide this stability by facilitating postsynaptic adaptation through increases in PKA and thus changes in protein expression, the human brain may one day be able to adapt to the large scale changes in monoamine release caused by schizophrenia.

In order to further ascertain that these postsynaptic changes are critical to the ability to adjust to the symptoms of schizophrenia, one could run a time course experiment with risperidone or other antipsychotics and measure the serum levels of drug as well as the changes in D_2 receptor levels. In essence, this would remove the influence of monoamine production from the experiment and focus on changes at the receptor and downstream adaptations. The nature of this experiment would also improve our understanding of the speed with which adaptation takes place, as the study we conducted placed no real time constraints on this dynamic process.

Acknowledgements

This research opportunity was made possible by the Translational Research Laboratory at the University of Pennsylvania. Special thanks to Steve Siegel, M.D. Ph.D., for extending this opportunity to a new undergraduate researcher, and to Robert Lin, whose

guidance was invaluable throughout the scientific research process.

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