Identification of Gene Expression Changes in Relation to a Sensitized Motor Response or Signs of Tolerance Due to Chronic D-Amphetamine Exposure

Identification of Gene Expression Changes in Relation to a Sensitized Motor Response or Signs of Tolerance Due to Chronic D-Amphetamine Exposure

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Abstract

Drug addiction is a disorder in which people continue to escalate drug use despite the presence of negative consequences. The repeated use of drugs of abuse is a major risk factor in drug addiction. There is a compulsive component of drug addiction that may stem from the effects of drugs of abuse on the dorsal striatum. The dorsal striatum is known to be a brain region that is critical for habit formation. Varying patterns of gene induction in the dorsal striatum are correlated with the intensity of drug-induced repetitive behaviors known as stereotypies. It is challenging to disrupt these repetitive behaviors once initiated and they resemble extreme habits. We compared stereotypy and mRNA sequence changes in the striatum of mice injected repeatedly with D-amphetamine, a habit-forming drug that induces long-lasting changes in behavior.

With the administration of D-amphetamine, the transcriptome response presented a parallel to the behavioral response. On the first day of D-amphetamine administration, there was high variability in the locomotor and gene induction responses when comparing drug-injected mice against the vehicle treated control mice. Following one week of daily D-amphetamine treatment, all of the mice presented highly similar behavioral responses consisting of immediate hyperlocomotion followed by strong stereotypy. In a parallel treatment group that was being used for RNA sequencing, it was shown that the genes that were significantly changed were consistently and strongly upregulated among all of the mice. However, by treatment day 21 most of the significantly changed genes at earlier time points showed downregulation compared to vehicle treated controls. Furthermore, mice treated for 21 days with D-amphetamine presented significantly shorter periods of stereotypy compared

to mice that were treated for seven days. This behavioral observation is in line with the development of tolerance to the stereotypy inducing effects of D-amphetamine. Mice treated with D-amphetamine for 7 days to induce the most severe stereotypy showed the most upregulation of immediate-early genes, relative to mice treated for 1 or 21 days.

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Chapter I

Introduction

Amphetamines are classified as psychomotor stimulants with both therapeutic and abuse potential. While this compound can be used as therapy for specific conditions, it is better known for its high potential for addiction among users. Researchers are still trying to understand why some individuals are more vulnerable to drug addiction compared with others. Individuals addicted to amphetamine may experience compulsions for the drug or relapse without necessarily presenting overt signs of dependence or withdrawal symptoms (Hyman et al., 2006). Amphetamines have the ability to initiate behavioral changes, gene expression changes, or even neurotoxic events in the central nervous system by increasing levels of biogenic amines, especially dopamine (McGinty et al., 2008). Hyperactivity, stereotyped movements, and cognitive impairments are just a handful of the effects observed in human patients that are also present in animal models of chronic psychomotor stimulant administration (Berman et al., 2009).

Past studies have reported that rodent models treated repeatedly with D-amphetamine exhibit three distinct, sequential behavioral phases. The first and last phases (wearing-on and wearing-off periods) are characterized by hyper locomotor activity such as running. The middle phase involves stereotyped movements for varying amounts of time. These motor stereotypies consist of restricted and repetitive movements such as chewing or head-bobbing. Such stereotypies reflect extremely repetitive and inflexible behaviors that might reflect hi-jacking of the habit system (Graybiel, 2008).

Protocols utilized by research groups interested in the effects of D-amphetamine on rodent models include different doses of D-amphetamine, various administration methods, timelines, and frequencies. I proposed to test a protocol to maximize stereotypy during acute and long term administration periods. During acute administration of D-amphetamine, it may be possible to observe variability in the onset and duration of each portion of the tri-phasic motor response as the subject becomes behaviorally sensitized. As chronic drug administration continues, it may be possible to observe the behavioral transition from a sensitized response to the transient effects of tolerance via a reduction in stereotypy time and severity.

After successful induction of D-amphetamine triggered stereotypies, I planned to examine the changes on immediate early gene expression in the striatum for each subject across multiple time points. Past studies have searched for gene expression changes in relation to routes of drug administration and sensitization to the locomotor response, however, immediate early gene changes have not been profiled specifically in relation to stereotypic behaviors. The goal is to identify potential behavioral variations across subjects involving sensitization/severity of stereotypies/tolerance and how transcriptome profiles may be correlated.

Definition of Terms

"ADHD": Attention deficit hyperactivity disorder.

"Basal Ganglia": This brain region consists of a group of subcortical nuclei. This structure can be broken down into multiple regions including the ventral striatum, dorsal striatum, globus pallidus, substantia nigra, and subthalamic nucleus. The basal ganglia plays a role in various functions including control of voluntary movements and habits.

"D-amphetamine": A psychomotor stimulant with addictive properties.

"Dopamine": An organic chemical that serves as a neurotransmitter in the brain.

"ERK": This term stands for extracellular signal-regulated kinases.

"FXYD2": This term stands for FXYD domain containing ion transport regulator 2. It is part of the FXYD family of transmembrane proteins. It is involved in the encoding of sodium/potassium-transporting ATPase subunit gamma.

"6-OHDA": The abbreviation for 6-OHDA dopamine. This compound is known to selectively destroy catecholaminergic nerve endings and cell bodies in the brain.

"MAPK": This term stands for mitogen activated protein kinase.

"NDUFA3": This term stands for NADH ubiquinone oxidoreductase subunit A3. This gene is believed to be related to metabolism and respiratory electron transport pathways.

"NR4A1-3": This term stands for nuclear receptor subfamily 4 group a member 1-3

"RASD1": This term stands for ras related dexamethasone induced 1. This gene encodes a member of the Ras superfamily of small GTPases and is induced by dexamethasone.

"RPKM": This term stands for reads per kilobase of transcript per million mapped reads.

"Sensitization": The amplification of behavioral and motivational responses to a constant drug dose over time.

"Striatum": This is a brain region that is part of the basal ganglia. The ventral striatum consists of the nucleus accumbens and the dorsal striatum consists of the caudate nucleus and putamen.

"Tolerance": A person's diminished response to a drug usually involving chronic use.

Background of the Problem

Chronic exposure to psychomotor stimulants, such as D-amphetamine, may cause the onset of drug induced sensitization and the deterioration of goal-directed behaviors due to

alterations in the cortico-basal ganglia circuit (Leyton and Vezina, 2014; Volkow et al., 2009). Repeated administrations may even produce tolerance to some drug effects, presumably, as a result of homeostatic adaptations (Hyman et al., 2006). Once a sensitized state is reached, behavioral responses may be amplified even when the dose of the compound is not increased (Weidenauer et al., 2016; Robinson and Camp, 1987). After being abstinent from D-amphetamine for long periods of time, subjects may remain in a sensitized state that places them at a long lasting risk for relapse (Leyton and Vezina, 2013; Robinson and Camp, 1987). Phenotypes such as the amphetamine induced sensitized state have been shown to closely resemble manic phenotypes in neuropsychiatric disorders such as schizophrenia (Featherstone et al., 2007).

In the context of learning, stimulation in the form of attention, motivation, and thought can lead to localized or brain wide changes along with behavioral alterations. D-amphetamine affects the mesocorticolimbic pathway by elevating extracellular dopamine through the inhibition of dopamine transporter reuptake and by additional release through reverse transport of dopamine (Koob et al., 1998). During a state of addiction, mesolimbic and nigrostriatal dopamine neurotransmissions are heavily affected as psychomotor stimulants enhance brain activity in the dopamine pathways (Canales, 2005). Even in the face of negative consequences, addicted individuals continue to present compulsions for drug seeking and drug administration behavior (Chao and Nestler, 2004). It is also becoming evident that risk factors, such as the presentation of increased dopamine responses to emotionally intense stimuli during development, may cause one to be susceptible to impulsive, reward seeking behaviors. As impulsive and reward seeking behaviors become

paired with drug related cues, an individual may veer towards drug conditioning and sensitization (Leyton and Vezina, 2014).

Several studies provide evidence for the interactions between the dopaminergic cell bodies of the ventral tegmental area in the midbrain and the neurons of the nucleus accumbens in the limbic forebrain in relation to drug induced sensitization and compulsive drug seeking behavior (Canales, 2005; Chao and Nestler, 2004; Sutton et al., 2003). These brain regions along with the basal ganglia, which is composed of the caudate nucleus, putamen, nucleus accumbens, and the globus pallidus, are also involved in the reward pathways of the brain. When the reward pathways are faced with repeated doses of D-amphetamine, the effects of natural rewards stimulated by external elements such as food are rapidly diminished and unable to arouse the reward pathways to normal levels (Nestler, 2002). In addition, the use of addictive drugs may also initiate the formation of long term memories involving cues or associations related to substance abuse and these memories may cause one to remain vulnerable to addiction or relapse over long periods of time (Berke and Hyman, 2000; Nestler, 2002).

The striatum is critical for elements in decision making such as action initiation and action selection. One study has provided evidence that imbalances in specific striatal circuits have the potential to initiate and release fixed behaviors and stereotypies during drug induced states in a rodent model (Canales and Graybiel, 2000). Stereotypies are a common symptom in rodent models treated repeatedly with D-amphetamine. Stereotypies can take form as motor stereotypies or cognitive stereotypies. Motor stereotypies are characterized by the repetition of a single or specific set of motions, whereas cognitive stereotypies involve inflexible patterns of attention or emotion (Canales and Graybiel, 2000). In both cases, the

basal ganglia seems to be one of the key brain regions implicated. Researchers have also shown the involvement of the striatum by presenting attenuation of psychomotor stimulant induced hyper locomotor activity and motor stereotypies through the ablation of dopaminergic nigrostriatal neurons using 6-OHDA in rats (Creese and Iverson, 1972; Fibiger and Zis, 1973). Dopaminergic system dysfunction has also been confirmed in human users of D-amphetamine. More specifically, researchers have identified decreased levels of striatal dopamine D2/3 receptor binding along with abnormal dopamine release and function compared to healthy patients (Schrantee et al., 2015).

Psychomotor stimulants have also been shown to induce morphological and immediate early gene expression changes in the striatum for several targets (Berke and Hyman, 2000; Canales and Graybiel, 2000; Kalivas and O'Brien, 2008; McGinty et al., 2008). Immediate early gene expression patterns may vary during different time points of psychomotor stimulant induction paradigms in rodents, including sensitization (Chao and Nestler, 2004; McGinty et al., 2008; Unal et al., 2009). Contextual information has also been implicated in the formation of differential patterns of immediate early gene expression after psychomotor stimulant administration (Badiani et al., 1998; Engelke et al. 2017; Uslaner et al., 2003).

Chapter II

Materials and Methods

Experimental procedures involving animal subjects were conducted in compliance with the MIT Committee on Animal Care, which is an AAALAC accredited institution. Experimental procedures have taken into account guidelines from the *Guide for the Care and Use of Laboratory Animals*, which is available to ensure that animals subjects are used in a humane manner for approved scientific applications.

Mice for behavioral experiments were male mice that were group housed. The strain of mice was 129 Sv/Jae S4 mice. Their ages ranged from 8 to 10 months old. Within the animal holding facility, mice were maintained on a normal laboratory lighting cycle with lights turning on at 7 am and turning off at 7 pm. All mice had ad libitum access to food pellets and water. All behavioral tasks were conducted between the hours of 10 am and 5 pm. Mice were only excluded from studies if they presented signs of illness or poor body condition scores.

During the first experiment, mice were treated with D-amphetamine in order to induce behavioral sensitization and stereotypic behaviors using defined procedures (Crittenden et al, 2014). Observed behaviors included locomotor activity, rearing, and stereotypy while under the influence of D-amphetamine or vehicle treatment. We expected variations in the response to D-amphetamine across mice in terms of sensitization profiles, severity of stereotypies, and the onset of potential tolerance effects. We tracked and observed how these profiles shifted throughout the drug administration period. The vehicle for all animal experiments was 0.9% sterile saline. D-amphetamine (Sigma Aldrich) was formulated

each treatment day in combination with 0.9% sterile saline to a stock concentration of 0.7 mg/mL. Mice were treated with D-amphetamine at a dose of 7 mg/kg or vehicle via intraperitoneal injections (10 ml/kg) for 1, 7 or 21 consecutive days. A total of twelve mice were a part of this cohort.

Open field behavior measurements were taken in enclosed square chambers (TruScan monitor, Coulbourn Instruments) measuring 25.4 cm for each transparent plastic wall. The open field arena was outfitted with two sets of sensor rings stacked on top of one another along the perimeter of the arena. The dual sensor ring was comprised of multiple photobeams that allow for detection in the X and Y planes. Movement detection was recorded by the TruScan software including distance travelled and time spent rearing. Prior to drug administration procedures, all mice were habituated to 0.9% sterile saline injections in the activity chambers. On drug administration days, mice were be placed into the activity chambers for 20 minutes initially. Afterwards they were treated with D-amphetamine or vehicle via intraperitoneal injection and then they were monitored for an additional 140 minutes. 80 minutes after D-amphetamine or 0.9% sterile saline treatment, each mouse was filmed for two minutes to capture stereotypic behaviors. Behaviors such as air sniffing, floor sniffing, wall sniffing, locomotor movements, and undefined confined stereotypy were quantified by using J-Watcher. J Watcher is a Java based software that is used for quantitative analysis of behavior.

The second experiment involved tissue collection for RNA-sequencing in order to identify specific transcript level changes in striatal samples after D-amphetamine exposure. Based on previous studies, it was likely that transcript level changes would be detected in these samples. However, if it is possible to associate specific genes or correlate gene

expression patterns to identify certain phases of behavior as a result of D-amphetamine exposure is currently unknown. We expected potential variations in the mouse tissue samples depending on the number of consecutive days of D-amphetamine or vehicle administration. There were three different drug administration time lengths: 1 day, 7 consecutive days, or 21 consecutive days. During each day of treatment, mice either received D-amphetamine at a dose of 7mg/kg or vehicle via intraperitoneal injections inside of the activity monitors described above. Each group consisted of three mice for a total cohort size of 18 mice. On the final day of drug or vehicle treatment for each group, mice were given a lethal dose of Euthasol (pentobarbital sodium and phenytoin sodium by intraperitoneal injection) 15 minutes after D-amphetamine or vehicle injection. In the following five-minute period, the caudate-putamen were dissected and frozen on dry ice. All of the tissue samples were homogenized in Tri Reagent from Sigma Aldrich and followed by a RNA precipitation procedure using chloroform and isopropanol. The RNA samples were purified using the Qiagen RNEasy kit and DNAse-treated as well. RNA integrity values based on Bioanalyzer data (Agilent Technologies Inc.) were between 7.8 and 9.0 for all samples. Total RNA samples were processed using the TruSeq RNA sample preparation kit (Illumina, Inc.) by the MIT BioMicro Center and sequenced by the HiSeq2000 (Illumina).

RNA-seq analysis was completed according to the procedure described in Vashishtha et al., 2013, PMID 23872847. Reads were mapped to the mm9 assembly of UCSC known genes for *Mus musculus* (http://genome.ucsc.edu/, Fujita et al., 2011) and a database of splice junctions using the Bowtie alignment program with setting --best -m1 -v2. To measure transcript abundance, the number of sequence reads in constitutive exons in the coding sequence of a gene were summed and then normalized, to account for gene length and depth

of sequencing, according to the total reads per kilobase of transcript per million mapped reads. Thus, changes in constitutive exons, but not splice-form specific changes, were identified. Raw counts were evaluated for differential expression using the R package DESeq with a 10% false discovery rate cutoff and log2 difference of 0.5 between amphetamine and saline injected mice. Outliers were further excluded by restricting the residual variance quotients to less than 10. Gene expression is represented in tables and heat maps as reads per kilobase of exon per million uniquely mapped reads (RPKMs). Comparisons for genes with an average RPKM < 1 in both the saline group and in the amphetamine comparator group were considered unreliable and are not reported.

Chapter III

Results

Amphetamine Induced Sensitization to Locomotor Response in Mice

Mice treated with D-amphetamine in our study displayed three phases of behavior during open field testing. Initially, mice presented a period of rapid ambulatory movements and then they gradually transitioned into a period of confined stereotypic (Figure 1). Eventually the mice returned to a state of locomotion within the open field chambers. Interestingly, with repeated administration of D-amphetamine across consecutive days, changes were observed in the average duration and intensity for portions of the triphasic motor response (Figure 1). These changes in behavioral activity in response to drug administration were visible across all animals; however, variability in the response is present in these mice. This is true even as the mice are derived from an isogenic background in addition to being age and sex (male) matched siblings. (Figure 1) The variability that is observed across treated animals may parallel how drug response, drug addiction, and vulnerability to drugs of abuse may vary in humans and other animal models of drug addiction.

When amphetamine is administered in humans at therapeutic doses, the drug half-life ranges between six to eight hours. In mice, the clearance rate is much faster translating to a half-life of about twenty to fifty minutes (Fan and Hess, 2007). The amphetamine treated mice also displayed sensitization to the secondary locomotor phase and this finding may be coinciding with the time that the drug effects are beginning to diminish. By day seven of D-amphetamine treatment, the distance traveled by the mice was significantly increased during the secondary locomotor phase compared to the first days of drug administration (Figure 1).

In addition, D-amphetamine treated mice were transitioning from the confined stereotypic phase to a secondary locomotor phase earlier with progressive treatment days (Figure 1).

Using a random effects state-space model, we investigated this progressive change in the onset of the secondary locomotor phase to test for significant changes in distance traveled by the treated mice across multiple days. Results from the model showed that in comparison to day one of D-amphetamine treatment, mice presented a significant increase in total distance traveled on day six at about the 105-110 minute post-injection time-point (Figure 1). As we continued to treatment day twelve, the D-amphetamine treated mice were beginning to transition out of the confined stereotypy phase even earlier than before as there was a significant increase in the distance traveled at the 70-75 minute time point (Figure 1). Based on these observations, the secondary locomotor phase was appearing progressively sooner under chronic D-amphetamine administration. Overall, sensitization to the initial locomotor response after D-amphetamine treatment appeared during initial treatment days and continued throughout the treatment period. The onset and degree of this behavioral response stabilized across the treatment period while the secondary locomotor response continued to appear progressively earlier as treatment with D-amphetamine continued.

Following 21 days of treatment with D-amphetamine, the mice were subjected to a withdrawal period in their home cages for 40 days without drug treatment. After the withdrawal period, the mice were provided with a challenge dose of 7 mg/kg D-amphetamine. Post injection, the sensitized response to D-amphetamine emerged again during the initial locomotor phase but in this case the mice traveled significantly more during the 5 minutes after drug administration compared to the distances traveled on day 1 of drug treatment (Figure 1). In addition to changes during the initial locomotor response on the challenge day,

we also observed that the transition time point from confined stereotypies to the secondary locomotor phase were almost in line with the results from treatment day I with D-amphetamine. This finding may indicate that the tolerance to the prolonged stereotypy is not stable across withdrawal.

Tolerance to D-Amphetamine Induced Stereotypies During Repeated Treatment

In order to better understand stereotypy duration and type of confined behavior, we filmed each mouse at the 80-minute time point after D-amphetamine administration – this time point spans the transition from stereotypic behaviors to the secondary locomotor phase. After each filming session we rated their stereotypic behaviors and computed the total time engaged in stereotypic behaviors and the length of each stereotypy bout. Typically, the stereotypic behaviors identified consisted of sniffing or licking at a single position on the wall or floor of the activity chamber. Based on this analysis, we identified a significant increase in the total time engaged in stereotypic behaviors on treatment days two and seven when compared to the first day of D-amphetamine treatment (Figure 2), at the 80 minute post-treatment time-point. Furthermore, the average duration of each individual stereotypic episode was significantly longer on treatment days two and seven when compared to day one of D-amphetamine treatment (Figure 2). By day twenty-one of treatment, the average stereotypy scores of the mice, at 80 min after drug injection, had fallen to the levels on day one of treatment. Based on the results from our stereotypy ratings and average distanced traveled data across multiple weeks of treatment with D-amphetamine, the duration of intense stereotypic behavior slowly decreases with progressive treatments. This provides evidence for the development of tolerance to prolonged amphetamine-induced stereotypy

during repeated administration of D-amphetamine.

Sensitization to Amphetamine-Induced Rearing Behavior

Mice also showed sensitization to rearing behavior induced by D-amphetamine treatment. The rearing counts were counted automatically by the activity chambers based on infrared beam breaks in a vertical plane. The rearing behavior that we measured differed from the confined stereotypy in that the time spent rearing rose across the first week of treatment and remained high for the duration of the daily treatments (Figure 4). On challenge day of D-amphetamine however, the time spent rearing had dropped and was similar to day one (Figure 4).

Activity Chamber Location-Preference for Confined Stereotypy

During the drug administration period, we analyzed the location of each animal within the activity chambers in order to check the potential for place preference behavior during engagement in confined stereotypies. Across the first twenty-one days of D-amphetamine treatment, we determined the average coordinate location for each individual mouse within the activity chambers. Then we plotted the location of each individual mouse against their twenty-one day average location to test for the level of correlation between to the two locations within the activity chambers (Figure 3). We found that each mouse spent increasing amounts of time, across the first week of treatment, at a specific location within their activity chamber. The same preferred location for each mouse was maintained throughout the duration of repeated drug administration. In order to check whether the place preference of each individual mouse was dependent on drug intoxication, we measured the

correlation between their preferred locations prior to and after drug-injection for each treatment day. We found no correlation between the preferred location of the mice before drug injection to their preferred location after drug injection (Figure 3). These findings parallel our observations that the mice showed idiosyncratic preferred locations for engagement in stereotypy, normally in one corner of the cage. However, when mice were provided with a 40 day hiatus from drug treatment until the challenge day, there was no correlation between the preferred location calculated during daily drug treatment and challenge day treatment.

Gene Expression Changes in the Caudate-Putamen Identified by RNAseq

Gene expression experiments were performed in order to identify the changes in gene expression caused by the administration of D-amphetamine. More specifically, we aimed to better understand the gene expression changes related to sensitization and tolerance to D-amphetamine induced motor behaviors. We studied gene expression changes in mice that were treated with acute or chronic D-amphetamine using the same paradigm as those mice involved in our behavioral studies. In order to include a baseline transcriptome comparison, we administered 0.9% sterile saline vehicle to three groups of mice serving as the control groups treated in parallel with their drug-treated counterparts. In order to habituate the mice to the injections, all mice within the study were given three daily injections of saline prior to the first day of D-amphetamine or control saline injections. In order to observe potential differences in gene expression profiles with varying administration timelines, the D-amphetamine or 0.9% saline treatments varied in duration from 1, 7, or 21 consecutive days of injections. Each saline and D-amphetamine group prepared for the RNAseq study included

3 mice. Thus, a total of 18 mice were treated in preparation for RNA sequencing. On the final day of treatment, mice were injected with a terminal dose of pentobarbital fifteen minutes after the last D-amphetamine or saline treatment. Then striatal tissue was dissected out and snap-frozen within five minutes. Therefore, our sequence data should provide the number of polyadenylated RNAs at approximately twenty minutes after D-amphetamine or saline administration.

Mice injected with only a single dose of D-amphetamine provide insight into gene expression changes that occur during the acute response to drug administration. Mice injected with repeated D-amphetamine should include acute response changes in addition to stable baseline changes from prior drug exposures.

Quality control analysis of the sequencing data showed that approximately 80% of the sequence reads mapped to a unique site in the genome, 1% of sequence reads were mapped to intronic regions, and fewer than 0.1% of sequence reads mapped to intergenic regions. Of the 18,670 genes that were represented, 11,200 had RPKM values > 1 in at least one sample, a cut-off that we have used previously to increase the validity of the calculated fold change. For genes meeting this criterion, we compared RPKM values between the mice treated with amphetamine for either 1, 7 or 21 days and the group of all saline-treated mice. In order to pinpoint the changes that are specifically caused by D-amphetamine treatment, we excluded genes that showed expression changes among the three saline-treated groups.

The gene expression analysis revealed a wide variety of gene expression changes as a result of D-amphetamine treatment across multiple timelines. During the first exposure to D-amphetamine, the acute response presented significant gene expression changes for 44 genes at twenty minutes after drug administration compared to saline-injected controls. Of these

genes, 86% showed increases and 14% showed decreases in expression. On day seven of D-amphetamine treatment, there were only 31 genes with significant changes in gene expression and again most (87%) of these were increases. Compared to the changes on day I, we noticed that the fold increase was higher and more consistent across all mice after seven days of D-amphetamine administration. Strikingly, in caudate-putamen samples from mice that were treated with 21 consecutive days of D-amphetamine, many more transcripts were downregulated (47% down), relative to days I and VII of treatment. In summary, these data show that there are transcriptomic changes in the striatum that seem to parallel the behavioral changes that occur in response to acute vs. short-term vs. longer-term D-amphetamine treatment. Relative to acute drug treatment, the increase in gene expression as well as the stereotypy became stronger and more consistent across individual mice sampled. With prolonged 21-day D-amphetamine treatments however the stereotypy period became shorter and the proportion of genes that were upregulated was diminished.

Chapter IV

Discussion

The goal of this study was to elucidate the genes that are responsible for behavioral differences in genetically identical mice with chronic D-amphetamine treatment or saline treatment. Through this study, we were able to better understand the sensitization profiles to D-amphetamine along with the gene expression level changes for specific genes that may correspond to certain phases of the behavioral responses to drug treatment. In relation to disease modeling, the amphetamine sensitization model is believed to model the positive symptoms experienced by schizophrenia and with repeated, long term exposure there is the potential to lead to psychosis (Featherstone et al., 2007)

Amphetamine is an indirect dopamine agonist that can stimulate a wide variety of signaling cascades in the striatum. Depending on the strength of the drug stimulus, these signaling cascades can react in various ways as they have different subcellular locations (McGinty et al., 2008). In our study, a group of wildtype mice were treated with D-amphetamine or saline for twenty-one consecutive days. Through our gene expression analyses of tissue collected from these mice, alterations to the Mitogen-activated protein kinase (MAPK) cascades were very clear throughout the treatment period. MAPKs are a highly conserved family of protein kinases that play critical roles in cell proliferation, differentiation, survival, and apoptosis. With repeated psychomotor stimulant administration, MAPK cascades are known to undergo changes in activation (McGinty et al., 2008 / Takaki et al, 2001).

In our studies we observed upregulation of four MAPK activated phosphatases also

known as dual specificity phosphatases (DUSP-1, 4, 5, and 6). These MAPK activated phosphatases have been known to provide negative feedback to multiple MAPK subfamilies including, ERK, JNK, and P38. Extracellular signal regulated kinases are known to induce cell growth, proliferation, and gene transcription. c-Jun N-terminal kinases are originally known as stress activated protein kinases due to their response to environmental stressors, cytokines, and growth factors. P38 is known to induce apoptosis and activation of transcription factors as a result of stressors (Takaki et al., 2001). SPP1 is an example of a gene that is dependent on ERK for activation. In our studies, SPP1 activation was increased with acute D-amphetamine administration. With repeated dosing up to 7 days, the level of activation was unchanged. However, by day 21 of drug administration, SPP1 levels decreased.

In our groups of D-amphetamine treated mice, two widely characterized MAPKs, ERK1 (44 KDa) and ERK2 (42 KDa), were activated by psychomotor stimulants in D1 dopamine receptor expressing neurons. This class of neurons is a part of the direct striatal projection pathway. Pharmacological and genetic perturbations to ERK signaling interferes with motor responses to psychomotor stimulants. ERK signaling may be involved in the process of behavioral sensitization after chronic administration of psychomotor stimulants. One study compared D-amphetamine pre-treated rats and saline pre-treated rats being administered D-amphetamine as a challenge dose and they revealed that 2 hours after drug administration, phospho-ERK levels remained high in the drug pre-treated rats while phosphor-ERK levels in the saline pre-treated rats returned to baseline levels (Shi and McGinty, 2007).

RASD1 was upregulated on all treatment days and is associated with repression of

MAPK cascades, including repression of ERK cascades in the striatum (Shi and McGinty, 2007). RASD1 encodes for Dexras1, which belongs of the RAS superfamily of small GTPase and can be strongly regulated by hormones (Thapliyal et al., 2014). It has also been shown that Dexras1 plays an important role in the regulation of circadian rhythms by adjusting the responsiveness of the mammalian master clock, located in the suprachiasmatic nucleus, by modulating photic and nonphotic stimuli (Thapliyal et al., 2014). Myd116 is another example of a gene that was upregulated on all treatment days and it is involved in endoplasmic reticulum stress induced cell death.

On day 7 of D-amphetamine treatment very few genes were exclusively dysregulated. However, TH (Tyosine hydroxylase) was significantly upregulated on day 7 of treatment. TH encodes for an enzyme that is involved in the conversion of tyrosine to dopamine. Researchers studying the midbrain and dopaminergic function have shown that chronic administration of D-amphetamine can lead to depletion of striatal dopamine and neurotoxic events in rodent brains (Bowyer et al., 1998). Furthermore, studies suggest that the neurotoxic effects of amphetamine are targeted mainly to the axons and terminals and not the soma of dopaminergic neurons (Bowyer et al., 1998). On day 7 of drug treatment during our studies, amphetamine induced stereotypies were more severe and longer lasting. In parallel, we discovered that gene expression changes were also stronger and less variable in mice treated for 7 days with D-amphetamine.

The NR4A family of genes also presented a wide variety of changes across chronic amphetamine treatment. NR4A1-3 is a group of immediate early genes that can be activated in several ways including, activation of G-protein coupled receptors, tyrosine receptor kinases, and direct activation of intracellular protein kinase pathways (Hawk and Abel, 2011).

This particular gene family is highly critical in the development of dopaminergic neurons in the ventral tegmental area and substantia nigra (Perlmann and Wallen-Mackenzie, 2004). It is also implicated in psychiatric disorders such as schizophrenia (Xing et al., 2006 / Chen et al., 2001). Data from RNA-seq analysis showed a significant increase NR4A1 on day 7 of D-amphetamine treatment.

There were a total of twenty-five genes that were downregulated in any D-amphetamine treated groups. The genes are as follows: Kcnh7, Wars2, 8430408G22Rik, T2bp, Grin2b, Igfbp6, Car10, Acpl2, Tmem10, Fxyd2, Eif2c4, Zfp109, Tmc4, Grin2a, Zfp772, Mlh1, Npas1, Paqr6, Cpne9, Spp1, Cyp2s1, Hapln4, Slc25a18, Vamp1, and Ndufa3. From this subset of genes, two genes showed significant downregulation with D-amphetamine administration. These genes are FXYD Domain Containing Ion Transport Regulator 2 (Fxyd2) and NADH Dehydrogenase Ubiquinone 1 Alpha Subcomplex 3 (Ndufa3). Fxyd2 was found to be downregulated on day 7 in mice treated chronically with D-amphetamine and may be associated with very severe stereotypic behaviors on day 7. This gene is located on chromosome region 11q23 in humans, which along with 11q22 and 11q24 are regions that are strongly linked to schizophrenia (Choudhury et al., 2007). While the function of Fxyd2 is not well established in the brain, the highest mRNA levels of Fxyd2 in the central nervous system are located in the dorsal root ganglion (Wang et al., 2015). A recent study involving Fxyd2 transgenic knockout mice suggests that Fxyd2 plays a role in chronic inflammatory pain through an interaction with alpha-1NKA (Wang et al., 2015). Ndufa3 was found to be significantly downregulated on day 21 in mice treated chronically with D-amphetamine. Ndufa3 is known for its role in mitochondrial respiratory chain complex 1 (Rak and Rustin, 2014).

Performing a particular behavior repeatedly may eventually lead to automatization which can potentially emerge due to experience dependent plasticity in the basal ganglia (Graybiel, 2008). It is common for animals to automatize behavioral routines such as grooming. In some cases, however, repetitive behaviors that serve no clear purpose can be potential markers for neuropsychiatric illness or addictive states. These abnormally repetitive behaviors are referred to as "stereotypies" and they occur in numerous neurological disorders. Our study presents that mice undergo sensitization to D-amphetamine induced stereotypies and also develop a preference for a specific location for stereotypy expression. With progressive treatments over a long time course, mice began to develop a tolerance to D-amphetamine induced stereotypy, which is also observed in rat models receiving multiple amphetamine injections over time (Segal et al., 1980). When provided a withdrawal period, the mice returned to presenting a highly sensitized stereotypy response to D-amphetamine administration. Next generation sequencing experiments were carried out in order to pinpoint potential differences in mRNA transcripts among mice that sensitized to D-amphetamine induced stereotypies versus mice that developed tolerance with repeated treatments and the relevant controls. A number of transcripts were identified that were altered only in the group that sensitized to D-amphetamine induced stereotypies and not the other treatment groups.

Rat studies involving *in vivo* microdialysis measurements show a correlation between the duration of amphetamine administration and the levels of extracellular dopamine in the dorsal striatum. More specifically, rats treated with amphetamine in an acute manner presented high levels of extracellular dopamine whereas rats with longer amphetamine treatment regimens presented lower levels of striatal dopamine. Dopamine levels in the ventral striatum and nucleus accumbens remained the same, which suggests that the

transition to building a tolerance against amphetamine induced stereotypies may be partially mediated by diminished dopamine release in the dorsal striatum. These findings align with past studies providing evidence that amphetamine induced stereotypy is dependent on dopamine terminals in the dorsal striatum whereas the increase in locomotor behavior caused by amphetamine administration are dependent on dopamine terminals in the nucleus accumbens through the use of location specific lesions (Kelly et al., 1975).

After analyzing the behavioral response after D-amphetamine administration on the challenge day, it was clear that locomotor sensitization was stable across the withdrawal period of our study. However, the tolerance to stereotypic behaviors was not stable across the withdrawal phase. These results indicate that the co-occurrence of amphetamine sensitization and tolerance is conserved in mice. Therefore, this evidence suggests that distinct molecular mechanisms underlie sensitization and tolerance. These interpretations are based on mouse behavior data that was averaged across mice per treatment group. Throughout the study, we did observe variations between mice within the same treatment group. Therefore, each individual mouse experienced and presented varying degrees of sensitization and tolerance to D-amphetamine induced behaviors.

Locomotor behaviors and stereotypic behaviors in response to D-amphetamine are mutually exclusive events. Due to this fact, one may interpret our claim regarding the development of tolerance to stereotypic behaviors as a behavior that is part of the late phase of the locomotor response to drug treatment. One argument against this idea is based on the response of the mice following D-amphetamine withdrawal. We see evidence for the stability in locomotor sensitization across the withdrawal, as the early peak for locomotor activity on challenge day was near maximum after drug administration. The peak representing late phase

locomotor activity is low on challenge day, which may be due to the reemergence of stereotypic behaviors in an unstable and variable manner.

With progressive treatment days, there appeared to be a divergence in the plasticity of these two patterns of behavior. Throughout all treatment days, the peak amplitude of locomotor response was maintained. The period of stereotypic behaviors, however, became gradually shorter to the point where there was a complete change over to the secondary locomotor phase. In conclusion, locomotor sensitization was stable across three weeks of D-amphetamine treatment whereas the stereotypic behaviors began to exhibit tolerance after only one week of D-amphetamine treatment.

Overall, the transcriptome responses to D-amphetamine paralleled the behavioral data in several ways and it was clear that drugs of abuse elicit repetitive locomotor behaviors and confined stereotypies as we moved forward with progressive treatment days. We identified several gene expression changes associated with D-amphetamine induced sensitization to stereotypies, which may help us to better understand the basis for these repetitive behaviors in relation to neurological disorders and addictive states.

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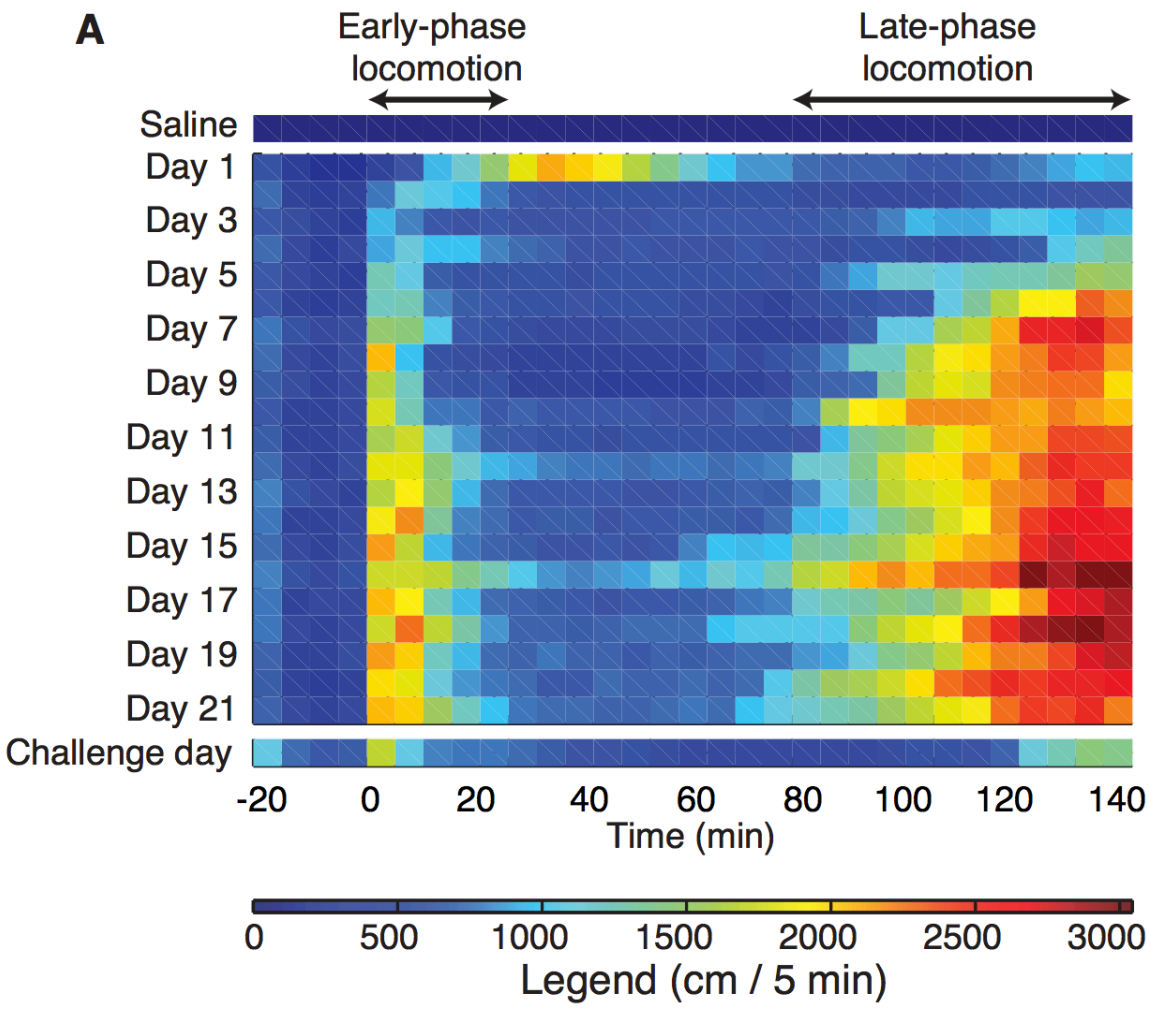
Appendix

Figure 1.



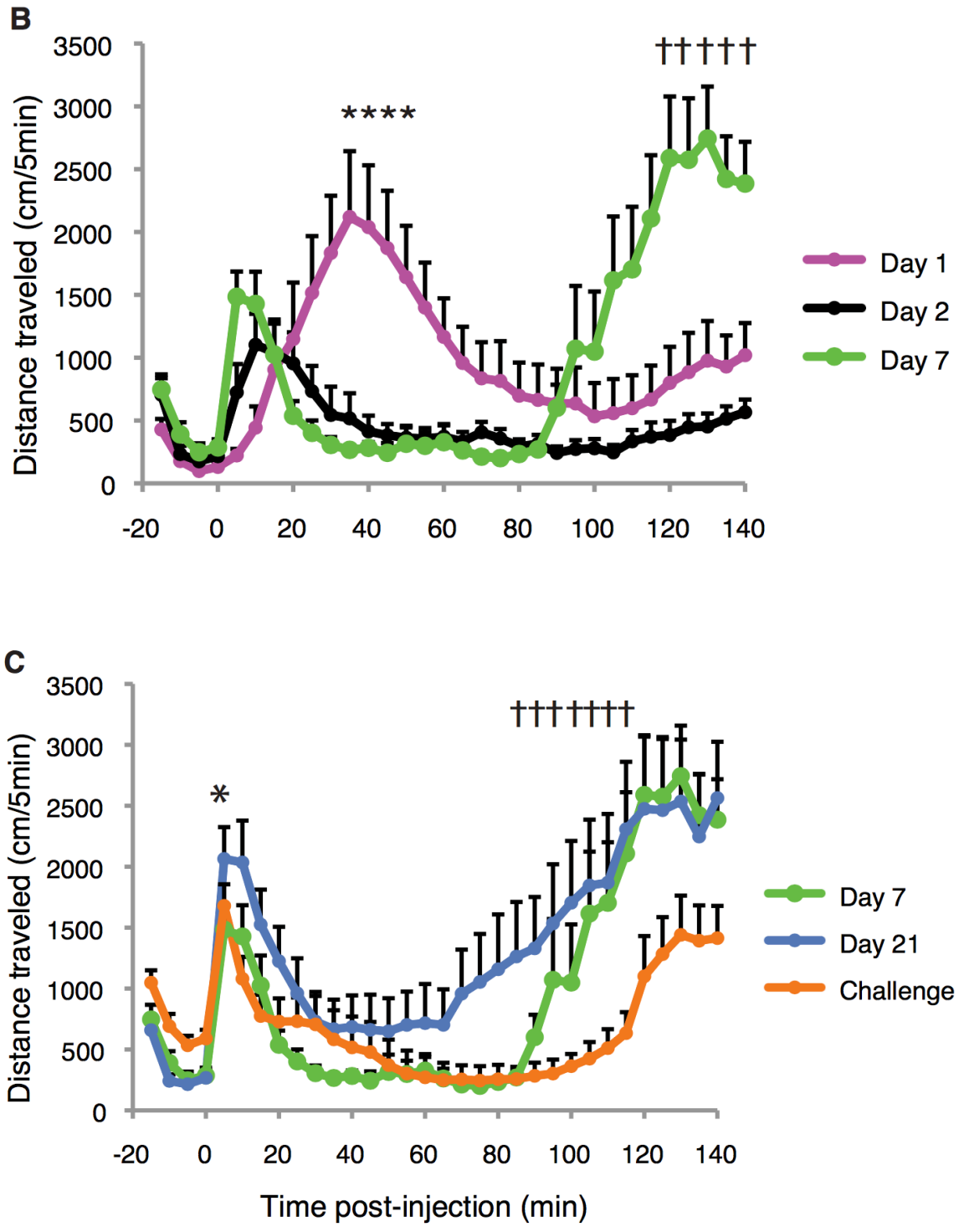
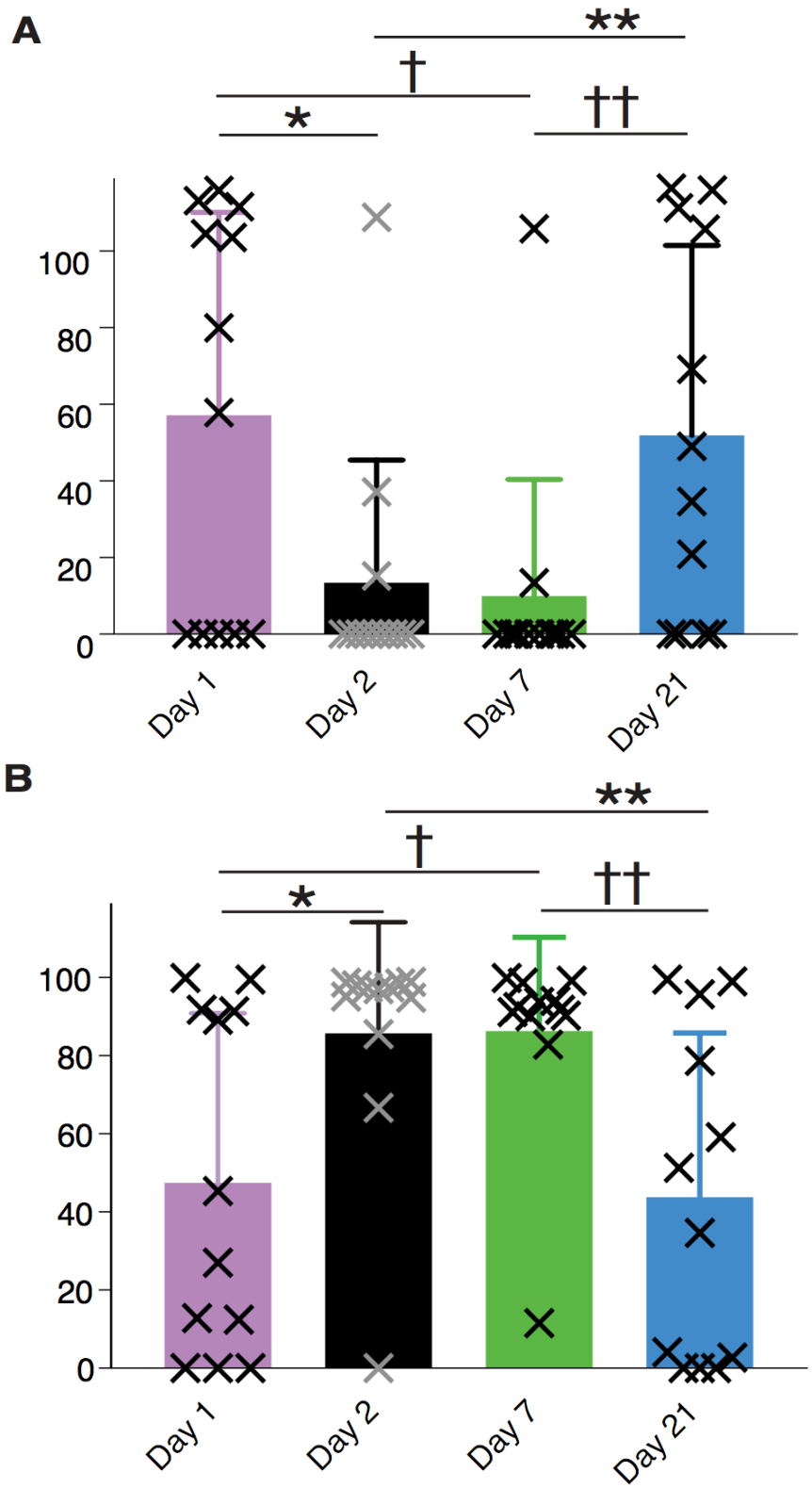


Figure 1. Measurements of ambulatory distance traveled across the last saline-treatment day and following amphetamine-treatment days. A) Each row presents a treatment day with automated distance traveled measured in 5 min bins for 20 min pre-injection and 140 min post-injection. Injections occurred at time = 0. Severe stereotypy begins after day 1 of D-

amphetamine treatment and occurs in the period between early- and late-phase locomotion. The interval of repressed locomotion diminishes with prolonged treatments but recurs on the challenge day. B) Line graphs showing the average distance traveled before and after D-amphetamine injection on days 1, 2, and 7. Paired, 2-tailed t-tests showed that there was significantly less mid-phase locomotion by the mice on treatment days 2 and 7 than on day 1 (* $P < 0.005$ at every point marked in the comparison between day 1 and day 7 or day 1 and day 2). By day 7, the mice showed sensitization of late-phase locomotion, relative to day 1 (†$P < 0.005$ at every point marked in the comparison between Day 1 and Day 7. C) By treatment Day 21, the interval of repressed locomotion was shorter owing to earlier resumption of locomotion. On the challenge day, mice traveled significantly farther than on day 1 during the early phase (*$P = 8 \text{ X } 10^{-6}$ by 2-tailed, paired t-test compared to Day1 data shown in panel (B)). During the late phase locomotion period, mice did not locomote as much as on Day 21 (†$P < 0.03$). Averages and +SEM are shown ($n = 12$ mice).

Figure 2.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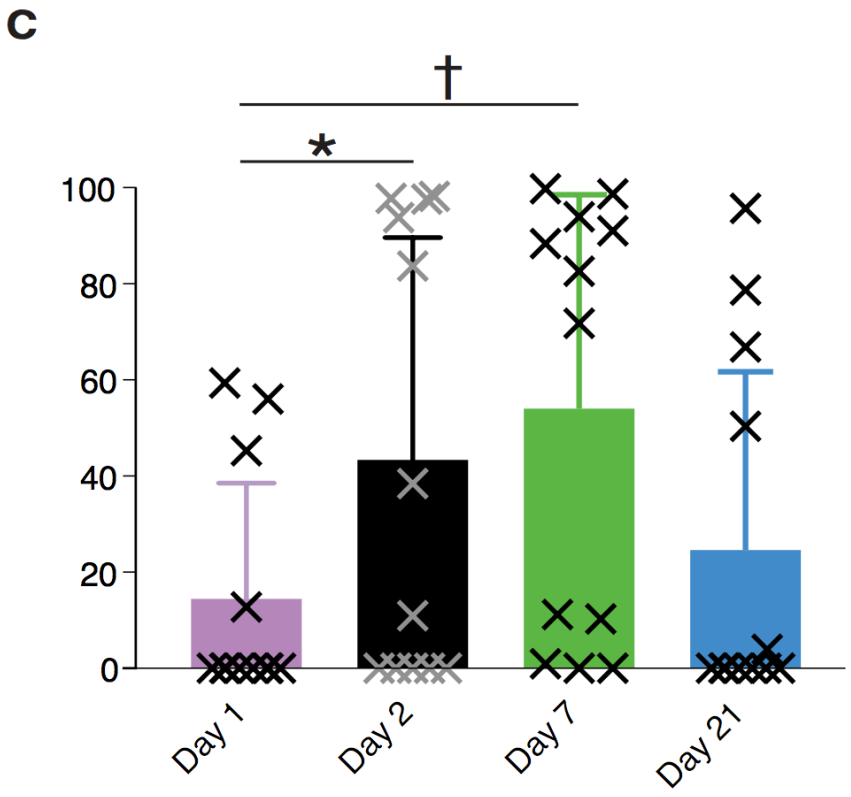

Figure 2. Mice show sensitization to confined stereotypy by the second day of D-amphetamine injection and tolerance by the 21st day of treatment. Plots show the percent of time engaged in A) locomotion, B) any confined stereotypy, and C) confined sniffing or licking at the wall, based on rating of behaviors at the 80 - 82 min. post-injection time period. In A) $*P = 0.02$, $\dagger P = 0.008$, $**P = 0.01$, $\dagger\dagger P = 0.01$, B) $*P = 0.01$, $\dagger P = 0.008$, $**P = 0.004$, $\dagger\dagger P = 0.005$, and in C) $*P = 0.02$, $\dagger P = 0.003$, by 2-tailed, paired t-tests.

Figure 3.

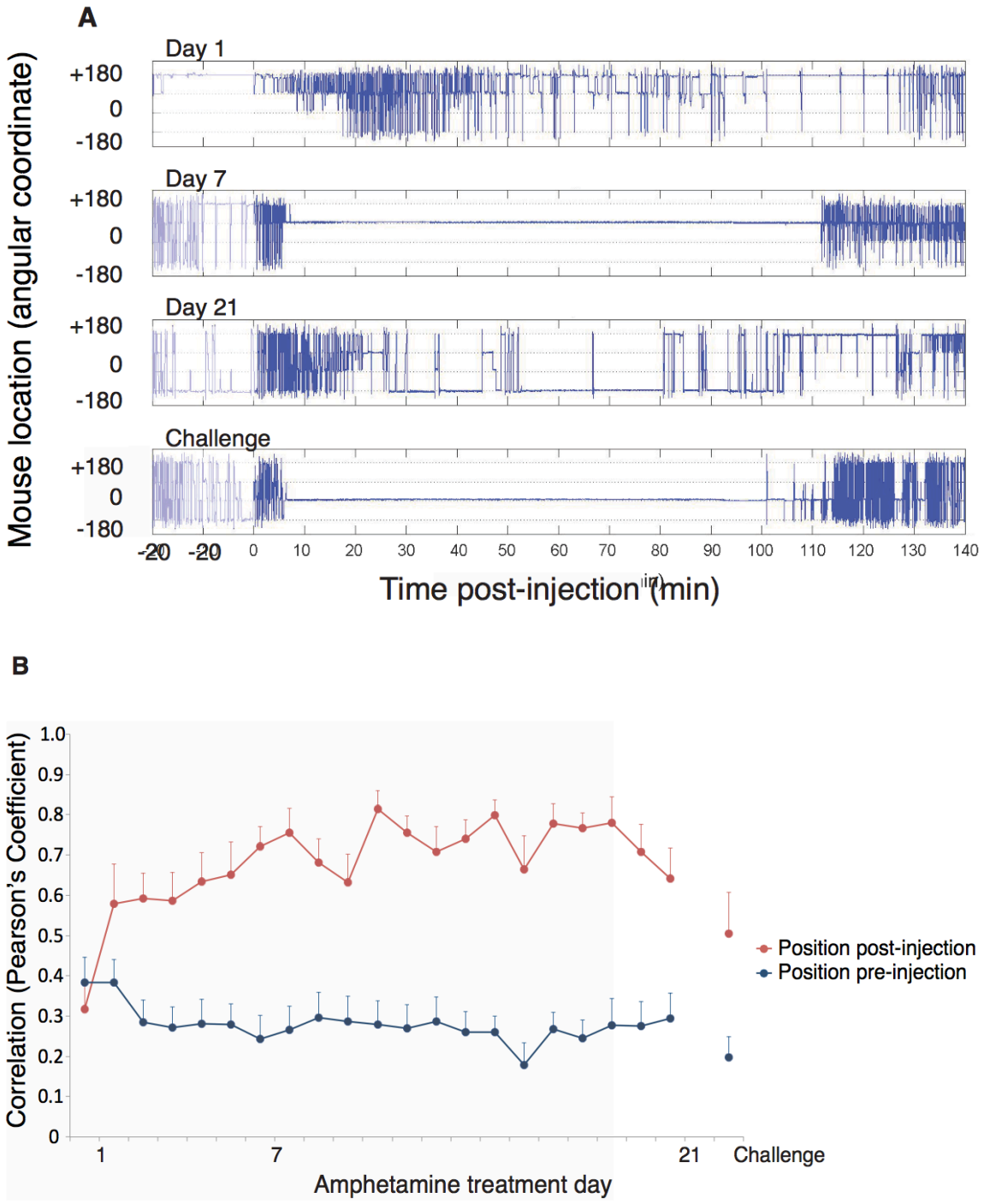

Figure 3. Mice show a preferred location for stereotypy across continuous treatment. A) Example of stationary vs. circling behavior of a single mouse on D-amphetamine treatment days 1, 7, 21 and challenge day. An angular coordinate deflection from -180^0 to $+180^0$

(vertical lines) represents a full revolution around the cage. B) The coordinate position data for each mouse on each D-amphetamine-treatment day was plotted against the average coordinate position across all days and the average correlations were plotted. Beginning on the second day of drug treatment, there was a high correlation, reflecting a relatively constant favored location across days. The correlation before drug injection was low, indicating that the mice did not have a preferred location before drug injection.

Figure 4.

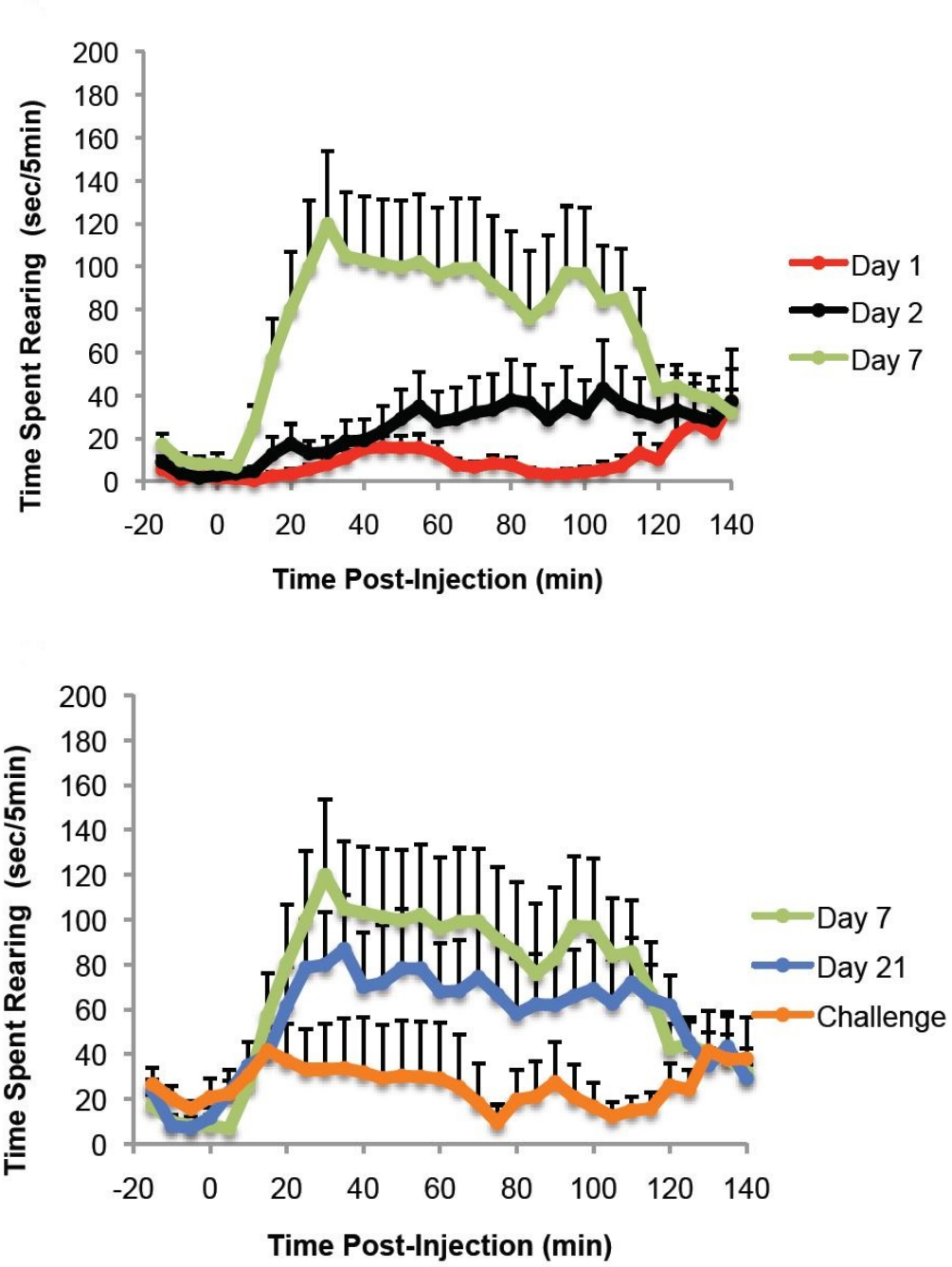

Figure 4. Measurements of average time spent rearing across all of the amphetamine-treatment days including challenge day. A) Each line shows a treatment day (1, 2, and 7) with automated rearing counts measured for 20 min pre-injection and 140 min post-injection. Injection occurred at time = 0. Rearing behavior begins after day 1 of D-amphetamine treatment and occurs during a time of confined stereotypy with little to no locomotion. B)

Each line shows a treatment day (7, 21, and Challenge) with automated rearing counts measured for 20 min pre-injection and 140 min post-injection. Injection occurred at time = 0. Rearing behavior begins after day 1 of D-amphetamine treatment and occurs during a time of confined stereotypy with little to no locomotion.

Figure 5.

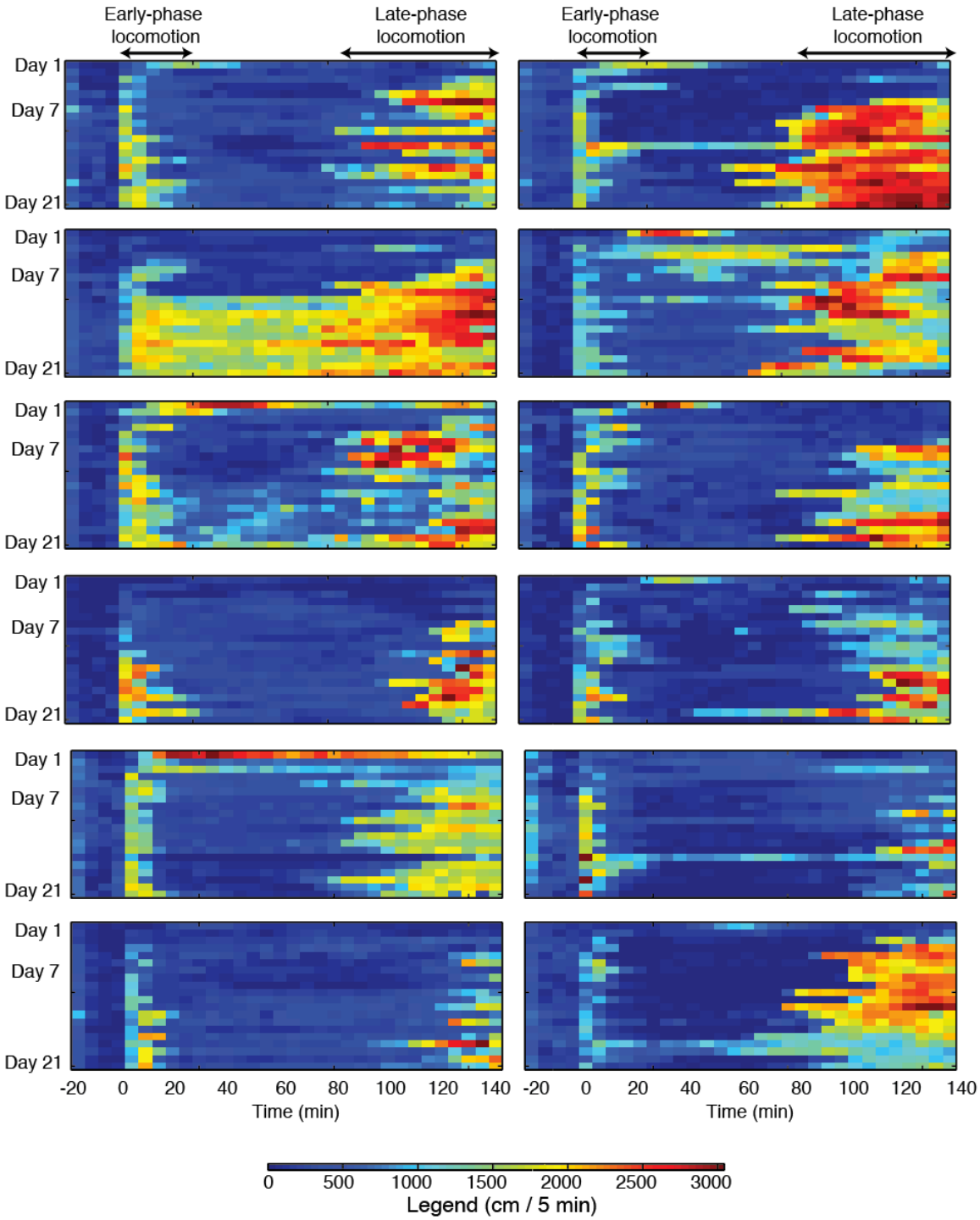

Figure 5. Heat maps of the ambulatory distance traveled measurements for each mouse reflect interanimal variability in sensitization rate. D-amphetamine treatment days 1 – 21 are shown, minus day 16 data for which some data were lost.

Figure 6.

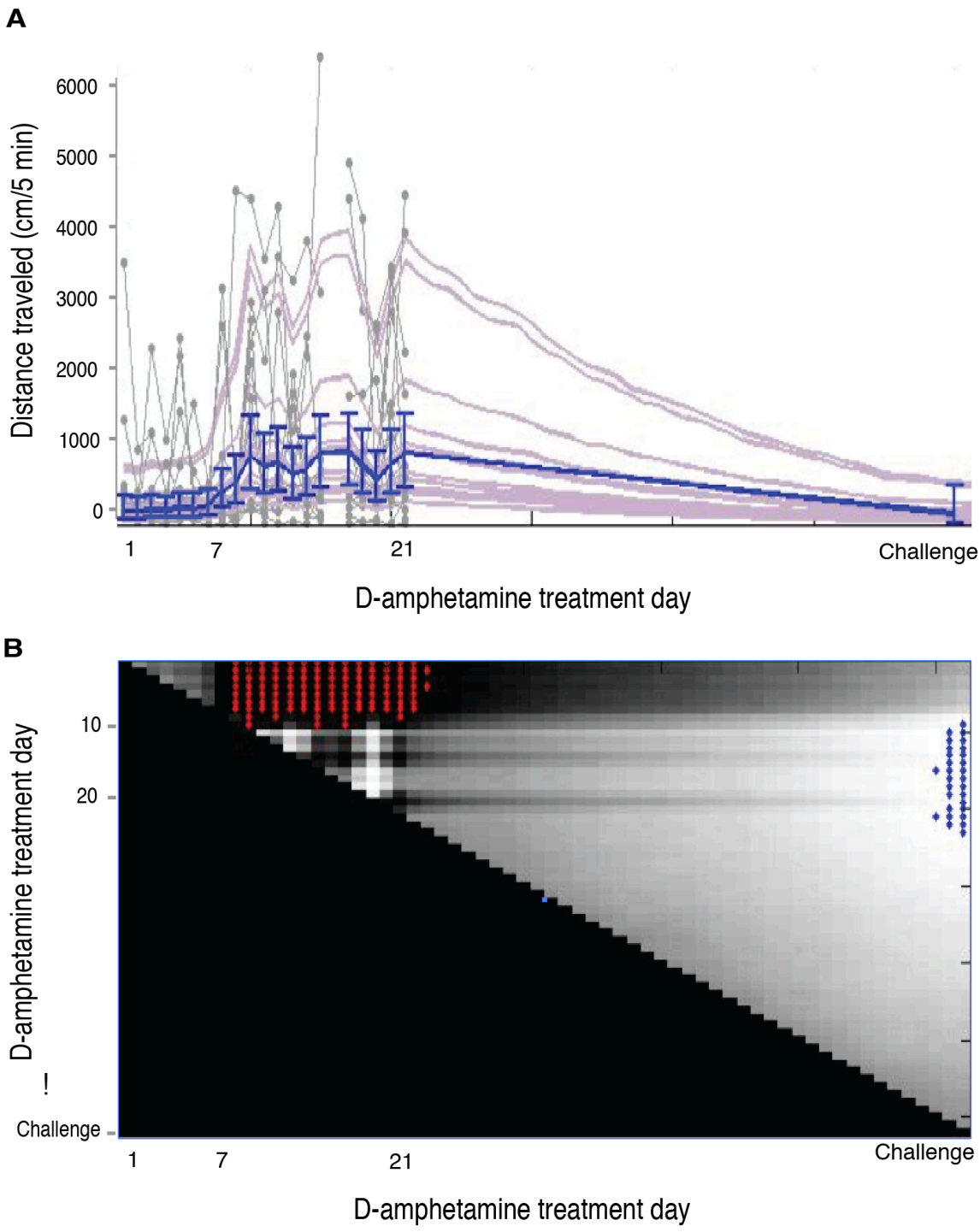

Figure 6. Model of distance traveled data shows significant increase in late-phase locomotion begins on D-amphetamine treatment day. A) Evaluation of the distance traveled in the 80 –

85 min time bin is plotted across days. The raw data from each of the 12 mice is plotted as gray dots joined by lines and estimates of individual fit to distance travelled are shown as light purple lines. The group median estimate with 90% credible intervals is shown by the blue line. B) Day-by-day comparison of the state-space fit to the group estimates of distance travelled shown in panel A. Each point on the surface represents the probability that the group estimate at the day shown on the x-axis is higher than the day shown on the y-axis. When the surface is very light colored then the probability is very low. When it is blue-highlighted this means the probability is less than 0.005. Conversely when the color is dark then the probability that the day on the x-axis is higher than the day on the y-axis is close to 1. When it is red- highlighted this means the probability is greater than 0.995.