

NEUROBIOLOGICAL BASIS OF SELF-INJURIOUS BEHAVIOR: FOLLOWING
CONVERGENT PATHWAYS TOWARD NOVEL PHARMACOTHERAPY

By

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To doing things the right way, maybe I'll try that next time

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LIST OF ABBREVIATIONS

3MT	3-methoxytyramine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
6-OHDA	6-hydroxydopamine
ACTH	adrenocorticotropic hormone
BS ³	bis(sulfosuccinimidyl) suberate
COMT	catechol-O-methyl transferase
CRH	corticotrophin releasing hormone
CSF	cerebrospinal fluid
DA	dopamine
DHPG	3-methoxy-4-hydroxyphenylglycol
DOPAC	3,4-dihydroxyphenylacetic acid
EDTA	ethylenedraminetetraacetic acid
Fmr1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
GABA	gamma-aminobutyric acid
GPe	external segment of the globus pallidus
GPI	internal segment of the globus pallidus
HPA axis	hypothalamic pituitary adrenal axis
HPLC	high pressure liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HR	high responder
HVA	homovanillic acid
I	intracellular
LE	Long Evans

LR	low responder
LSD	least significant differences
MAO	monoamine oxidase
MeCP2	methyl CpG-binding protein
MHPG	3-methoxy-4-hydroxyphenylglycol
NE	norepinephrine
NTS1	neurotensin receptor 1
OCD	obsessive compulsive disorder
OCT3	organic cation transporter 3
OPA	o-phthalaldehyde
PVN	paraventricular nucleus
Rai1	retinoic acid-induced 1
RM-ANOVA	repeated measures analysis of variance
S	surface
SEM	standard error of the mean
SNC	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SNRI	serotonin and norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
VTA	ventral tegmental area

Abstract of Dissertation Presented to the Graduate School
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Self-injury is a debilitating feature of neurodevelopmental disorders, including autism-spectrum disorders and genetic disorders like Lesch-Nyhan, Prader-Willi, Rett, and Fragile X syndromes. Self-injury is a devastating disorder that involves not only bodily injury (even as severe as broken bones and amputation of tongues, lips, and fingers) but also leads to exclusion of the self-injurer from social activities and cognitive therapies and causes severe distress for family and caretakers. As such, reducing self-injury in these populations would positively impact overall health, socialization, cognitive function, and relationships with family, friends, and caregivers. Furthermore, understanding the neurobiological basis of this behavior disorder will help to elucidate genetic variables that predispose vulnerability and gene-environment interactions that promote the development of self-injurious patterns of behavior.

To elucidate neurobiological changes associated with the induction and expression of self-injurious behavior, an analysis of several brain regions and circuits was conducted using a rat model of self-injury. In this model, pemoline, an indirect monoamine agonist, is administered repeatedly across days, which causes the development of repetitive behavior and self-biting in rats. Repeated stress exposure increased the severity of pemoline-induced self-injury, suggesting that the biochemical

stress response may cross-sensitize with the stimulant properties of pemoline to potentiate self-injury. Relatedly, endogenous stress responsiveness correlated with the vulnerability to develop pemoline-induced self-injury. Pain thresholds of pemoline-treated rats were also evaluated and were found to be significantly greater than the pain thresholds of vehicle-treated rats. The pemoline-induced elevation in pain thresholds did not correspond to the development of pemoline-induced self-injury, which indicates that altered sensory processing is not necessary or sufficient for pemoline-induced self-injurious behavior, though pain-related brain pathways may be dysregulated by pemoline treatment. An extensive analysis of the neurochemistry of several brain regions was undertaken and revealed significant depletion of intracellular dopamine in the striatum that corresponded temporally with the onset and maintenance of pemoline-induced self-injury. Parallel increases in extracellular monoamine metabolites were also found, suggesting that pemoline administration caused elevations in synaptic monoamine content. Taken together, these projects have implicated the important role of dopamine depletion in the striatum for the development of pemoline-induced self-injury, the impact of pemoline administration on pain responsiveness, and the consequence of stressful experiences on pemoline-induced self-injury. Neurotensin is a modulatory neuropeptide that causes release of stress hormones, as well as striatal dopamine, and mediates a form of morphine-independent analgesia. Considering the foregoing findings, neurotensin appeared to be a likely candidate to contribute to pemoline-induced brain changes and self-injurious behavior. Examinations of striatal neurotensin content and the efficacy of pharmacological challenges on pemoline-induced self-injury using neurotensin-related drugs confirmed this hypothesis. Intracellular neurotensin levels were significantly higher in the striata of pemoline-treated rats. Additionally, a neurotensin agonist was

shown to increase the severity of pemoline-induced self-injury, whereas an antagonist lessened all measures of pemoline-induced self-injury. The convergence of findings suggests that neurotensin is an important modulator of self-injurious behavior and may be an important target for pharmacotherapy.

CHAPTER 1 INTRODUCTION

Self-Injurious Behavior

Many abnormal behaviors are exhibited by people with psychiatric, neurological, and developmental disorders, but none are as maladaptive and devastating as self-injurious behavior. In the context of intellectual handicaps these behaviors are typically repetitive, cause mild to moderate injury, and are overtly expressed. These differ somewhat from the self-harm behaviors enacted by individuals with psychiatric or emotional problems that are more likely to be performed using rituals (Favazza, 1998), in times of heavy emotional burden (Klonsky, 2007), or where the self-injurious behaviors and injuries are usually hidden (Madge et al., 2008). Though the self-injury in developmentally disabled populations and in individuals with normal intelligence differs in some ways, the occurrence of self-injury during times of negative affect and arousal and its function to reduce these feelings (Klonsky, 2007; Kemp et al., 2008) are quite consistent across the two groups.

Our work describes the neurobiological basis of self-injury in an animal model that is presumptively a more closely related analogue to the self-injury seen in individuals with developmental disorders, rather than to those exhibited by individuals with psychiatric and emotional disturbances (e.g. borderline personality disorder, schizophrenia, post-traumatic stress disorder). As such, we will mainly focus on the phenomenology and neurobiology of the self-injurious behavior expressed by those with intellectual and developmental disabilities. It has recently been suggested that the relationship between genotype and phenotype may be stronger in intellectual and developmental disabilities compared to psychiatric disorders (Ernst et al., 2010). The

onset of psychiatric disorders and the consequent self-injurious behaviors are heavily influenced by severe emotional and environmental events (e.g. sexual, drug, and/or alcohol abuse) that may complicate the direct relationship between genotype and phenotype. Some researchers are beginning to postulate that understanding the genetic underpinnings of self-injury in the intellectually disabled may lead to advances in the genetics of vulnerability for self-injury and suicide in other populations (Ernst et al., 2010). So although our focus is predominately on the self-injurious behaviors of those with intellectual and development disabilities, we are encouraged by the possibility that our work may also elucidate the neurobiological basis of all self-injury.

Clinical Phenomenology of Self-Injurious Behavior

Self-injurious behavior is described as any behavior directed toward the self that causes tissue damage or has the potential to cause damage. This definition is quite general but has been widely used, though also scrutinized, over the last several decades (Schroeder et al., 1980). In general, estimates of the population prevalence of self-injurious behavior range from 1.7% to 65.9% of the intellectually disabled. These estimates vary considerably because definitions of self-injury are inconsistent across studies; some include mild self-injury whereas others only report the incidence of moderate to severe self-injury (for review see Rojahn and Esbensen, 2002). In a study of 596 self-injurers receiving services for being intellectually disabled (IQ less than 70), 40% of the self-injurious sample was profoundly intellectually disabled (IQ below 20), 49% were severely intellectually disabled (IQ between 20-50), and 11% were mildly disabled (IQ between 50-70). Furthermore, 51% lived in hospitals, 28% lived in small health service agencies or residential schools, and 21% lived at home with their families. Over half of the cases showed more than one type of self-injurious behavior

and skin picking/scratching, self biting, head punching/slapping/banging were seen in over 30% of all cases (Oliver et al., 1987). Other self-injurious behaviors include hair pulling, eye gouging, pinching, rubbing and stuffing orifices (Schroeder et al., 1980). The skin directed behaviors are typically focused on the head (mostly face), hands (usually the back of the hands), and the legs (Symons and Thompson, 1997; Symons et al., 1999). Self-injurious behaviors occur in both distinct, singular episodes or as continued bouts (Kroeker et al., 2004). It is the repetitiveness of the self-injurious behavior (i.e. continued bouts that can last hours with only very short pauses; Thompson and Caruso, 2002) and the intensity of these behaviors that contribute to the devastating nature of the behavioral phenomenon. For example, when the biomechanical dynamics of head hitting of several individuals were analyzed, the impact of the self-hitting blows was found to be similar to the force generated by a boxer's jab (Newell et al., 1999; Newell et al., 2002). Considering that these behaviors can occur in continued bouts every day for years, the devastating impact of these behaviors on the health and well-being of the self-injurer and their loved ones cannot be overstated.

Emergence of self-injurious behavior

The emergence, maintenance, and assessment of self-injurious behavior have been described most thoroughly from a behavioral perspective. Several researchers (Guess and Carr, 1991; Kennedy, 2002) suggest that the emergence of self-injury stems from normal rhythmic behavior that is displayed by normally developing infants (e.g. body rocking and head banging). These benign behaviors typically fade with the development of more functional motor skills (Ryan, 1983). Because these behaviors are exhibited by normally developing infants, the authors describe these rhythmic behaviors as biologically based and internally regulated. In a later stage of development these

stereotyped rhythmic behaviors in children with intellectual disabilities may continue to be expressed, and function as an adaptive correction for an environment that is either under- or over-stimulating. Repetitive stereotyped behaviors at this level come under the control of operant conditioning, by being paired with positive or negative reinforcers, and lead to the emergence of self-injurious behavior. A recent experimental analysis of repetitive behavior, potentially injurious behaviors, and self-injurious behaviors in children with intellectual disabilities found a close temporal relationship between these behaviors (Petty et al., 2009), which suggests a developmental relationship between them.

Maintenance of self-injurious behavior

Carr and Smith (1995) expanded upon these behavioral theories by further outlining variables that impact the expression of self-injurious behavior. Namely, these variables are 1) the consequences of the behavior (i.e. conditioned positive or negative reinforcers), 2) antecedent stimuli that predict whether or not the consequences of the self-injurious behavior will follow, and 3) setting events, including biological setting events. These authors focused solely on physical ailments as the potential biological setting events. Taken together, according to this behavioral theory, self-injury is likely to occur during a period of physical discomfort (the biological setting event; when the individual is experiencing an ear infection, fatigue, allergies, etc.), in the presence of a trigger stimulus (the antecedent; when presented with a task, for example), and when self-injury has previously been associated with reinforcement (the consequences; for example, escape from the demand to do the task). This behavioral model of self-injurious behavior is stimulating and lays the foundation for the neurobiological

concomitants of these three variables to be mapped onto the expression of self-injurious behavior.

Although the emergence and maintenance of self-injurious behavior have mostly been described in behavioral terms, several authors have proposed theories wherein neurobiology can interact with social consequences to affect the expression of self-injury. Oliver and colleagues (1993) argued that operant conditioning may be the process that maintains the self-injury, while neurobiological or neurochemical factors may predispose the individual to develop this behavioral pathology. Furthermore, Thompson et al. (1995) propose that self-injurious behavior is an endogenous means of neurochemical self-administration and have outlined several parallels between self-injury and drug self-administration. These parallels include the efforts exhausted to exhibit the behavior and the neurochemicals involved in both drug taking and self-injury (dopamine, opioids, and stress hormones).

The emergence and development of stereotypy and self-injury in children have received much more attention than has the continued expression of these behaviors or their potential natural remission throughout development. Very few studies have focused on prevalence rates of self-injury in adults and there have been no examinations of potential changes in self-injury frequency, intensity, location, or maintaining variables throughout the adult years. Cooper and colleagues (2009) found that almost a third of their adult self-injurious sample had stopped self-injuring after a two year follow-up of their initial assessment. On the contrary, using a cross-sectional design, Cohen and colleagues (2010) found the prevalence of self-injury was constant in their sample of adults with intellectual handicaps in their twenties, thirties, forties,

fifties, and sixties, suggesting no significant remission in their sample of 2,345 adults. Another cross-sectional study of nearly 700 people with a diagnosis of autism found a significant negative correlation between prevalence of self-injury and age, although this correlation may have been skewed by the high prevalence of self-injury in kids 2-4 years old and the low incidence of self-injury in adults 41+. Self-injury prevalence rates of their autistic sample between 5-40 years of age appear relatively stable (Esbensen et al., 2009). Although there are very few studies of self-injurious behavior across the lifespan, it appears that the prevalence is quite stable and that this behavioral pathology is not just a disorder of early childhood.

Assessment of self-injurious behavior

The preponderance of evidence suggests that social consequences have a significant impact on the expression of self-injurious behavior (Iwata et al., 1994, Kahng et al., 2002). As such, assessments of self-injury, its functions, and the benefit of behavioral treatments have been developed by applied behavior analysts who study the effects of social consequences on human behavior (Vollmer et al., 2009). For therapists trying to reduce self-injury it is tremendously important to assess the function of the behavior, not just the incidence of the behavior.

Several assessment methods have been developed, each of which assay the conditions in which the self-injury is expressed and the subsequent events that follow the self-injury. Indirect methods of assessment are sometimes used in which questionnaires or checklists are completed by parents, caregivers, or teachers and do not involve any direct concurrent observations of the self-injurer. Descriptive analyses of self-injury involve many sessions of direct observations in several different, but naturally occurring environments to correlate the presence or absence of particular antecedent or

subsequent events with the expression of self-injury. Functional analysis, as opposed to descriptive analyses and indirect assessments, is an experimental manipulation of the environment, which allows for systematic testing of different antecedent events and reinforcers and can elucidate the functional relationships between the behavior and the social environment. During these assessments, self-injurious behavior may be provoked by different testing situations, which helps to determine the antecedent and consequent events that maintain self-injury in the natural environment. The testing conditions in a functional analysis of self-injury include an “attention” condition, an “escape” condition, and an “alone” condition (Iwata et al., 2002). A large review of many functional analyses has provided a general characterization of the proportion of self-injury that is maintained by different consequences. In a sample of 152 self-injurers, Iwata and colleagues (1994) found that self-injury was maintained by social negative reinforcement in 38% of self-injurers (measured in the escape condition), social positive reinforcement controlled self-injury in 26% of their sample (assayed in the attention condition), while another 26% of the sample showed automatically reinforced self-injury (revealed in the alone condition). Multiple reinforcers controlled the self-injury of 5% of the sample, and the data from the remaining 5% of self-injurers could not be interpreted. These data suggest that functional analyses provide interpretable results on the functions of self-injury for a large proportion of individuals and that behavior can be clearly mapped on to particular social or automatic consequences when assessed in systematic, experimental conditions. Additionally, the information provided by the functional analyses aid in treatment selection and efficacy. The effectiveness of reinforcement based interventions is significantly improved when the treatment selection is based on the precise

information provided by a functional assessment (Kahng et al., 2002; Mace and Mauk, 1995).

In summary, theories on the emergence and maintenance of self-injurious behavior, as well as the assessment methodologies have been predominantly developed by behavioral researchers. They have set forth theories of self-injury that are behavioral in nature, but are also compatible with the hypothesis that neurobiological processes may contribute to the emergence, development, and maintenance of self-injurious behavior.

Comorbid behaviors and related behavioral pathologies

Self-injury is considered part of a class of behaviors termed “destructive” or “challenging” behaviors, which also includes aggression and property destruction. The expression of self-injury, aggression, and destruction correlate well with each other in individuals with intellectual handicaps (Matson and Rivet, 2008), have been found to be maintained by similar social functions (Petty et al., 2009), and show close temporal association when both challenging behavior and self-injury are part of the individual’s behavioral repertoire (Petty et al., 2009). In fact, pharmacological trials typically demonstrate overlapping effects on both self-injurious behavior and aggression, be it significant reductions of both behaviors or no effect on either behavior (Parikh et al., 2008). These data suggest that common genetic vulnerability, social functions, and neurobiology are involved in the expression of this class of challenging behaviors.

This group of challenging behaviors has been proposed to be part of a spectrum of abnormal repetitive behaviors, including stereotypies, compulsions, impulsions, and tics (Bodfish and Lewis, 2002). When compared to individuals who do not exhibit self-injury, those that do exhibit self-injury are more likely to also express stereotypy

(Bodfish et al., 1995), compulsions (Bodfish et al., 1995), and severe tics (Mathews et al., 2004). Moreover, a compulsive behavior hypothesis of self-injury has been proposed, based on the similarities between the self-injurious behaviors exhibited by individuals with intellectual handicaps and the compulsive behaviors seen in those with Obsessive Compulsive Disorder (OCD; King, 1993). This hypothesis is framed around the fact that a significant proportion of self-injurers engage in self-restraint (Powell et al., 1996) in an apparent attempt to keep from self-injuring. King's compulsive behavior hypothesis (1993) outlines parallels between the attempts of people with OCD to refrain from engaging in their compulsions to the self-restraint behaviors of self-injurers. Consistent with this hypothesis, Powell and colleagues (1996) found that self-injurers who exhibit self-restraint are more likely to display compulsions than are self-injurers who do not attempt self-restraint. Interestingly, self-restraint attempts did not prevent eventual self-injurious behavior, which is consistent with the inevitable occurrence of compulsive behavior in people with OCD.

Effects of self-injurious behavior on self and others

Self-injurious behavior can cause significant physical harm to the self-injurer. The most severe injuries include broken bones, amputation (e.g. tongue, lips, and fingers), and disfigurement (e.g. cauliflower ear). Less severe self-injury can also be quite harmful causing bruising, swelling, and secondary infection of the injury site. Self-injury can also be functionally impairing, leading to exclusion from educational and socialization activities and thereby further limiting the opportunities of self-injurers. Family members and caregivers are also significantly impacted by the self-injurious nature of these behaviors (Tausig, 1985). These people report high rates of stress (Sarimski, 1997) and feelings of sadness and despair (Bromley and Emerson, 1995).

Dealing with self-injury and the comorbid challenging behaviors is also a common factor for staff turnover in residential facilities (George and Baumeister, 1981). Furthermore, society pays a significant cost for the support and treatment of self-injurers; at last estimate the federal burden was approximately \$3 billion per year (National Institutes of Health Consensus Development Conference Statement, 1989).

Clinical Syndromes

Numerous syndromes associated with mental retardation and neurobiological dysfunction exhibit self-injury, but many others generally do not. For instance, self-injury is not a common phenotypic trait of Down syndrome or Williams syndrome. This suggests that self-injury is not due to just any aberration in brain development, but may be due to specific deficits in normal brain function. The developmental syndromes that are most commonly associated with self-injurious behavior are Lesch-Nyhan, Rett, Prader-Willi, Fragile X, Smith-Magenis, and Cornelia de Lange syndromes, as well as autism. These disorders differ in terms of genetic causes and comorbidities, but share the self-injurious phenotype and may provide clues to a common neural dysfunction.

Lesch-Nyhan syndrome

The prevalence of Lesch-Nyhan syndrome is approximately 1 in 380,000 live births (Crawhall et al., 1972) and is considered a rare disease by the Office of Rare Diseases at the National Institutes of Health. Lesch-Nyhan syndrome is caused by a mutation in the *Hprt* gene (located at Xq26), that causes little to no functioning of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme in any tissue type in the body, including the forebrain nuclei that comprise the basal ganglia, where HPRT enzyme activity is normally the highest (Kelley, 1968). HPRT is responsible for purine salvage, and without proper functioning of this enzyme purine recycling is disregulated

and purines are instead converted into uric acid. Diagnostic criteria for Lesch-Nyhan syndrome include virtually complete HPRT deficiency, hyperuricemia (high levels of uric acid in the blood) in association with hyperuricosuria (excessive amounts of uric acid in urine; Nyhan, 1968).

Phenotypically, Lesch-Nyhan syndrome is associated with severe motor problems (dystonia, choreoathetoid movements), intellectual disabilities, aggression, and self-injury. Nearly all individuals with Lesch-Nyhan syndrome bite some part of their body; the most commonly injured sites are the tongue, lips, fingers and/or arms (Anderson and Ernst, 1994). This self-injury may begin as early as the primary teeth grow in. In an attempt to prevent the self-biting, many people with Lesch-Nyhan are fitted with mouth guards (Cauwels and Martens, 2005) or the teeth are excised (Hall et al., 2001).

Anderson and Ernst (1994) completed one of the most thorough analyses of self-injury in Lesch-Nyhan syndrome. They found individuals with Lesch-Nyhan syndrome self-injured in multiple different ways, with the average person exhibiting seven different forms of self-injury. Self-biting was the most common, followed by hitting their arm, leg or head on a doorway, snapping their head backwards, head banging, placing their feet under the wheels of their moving wheelchair, placing their fingers in the moving spokes of their wheelchair, or eye gouging. These alarming findings were replicated by Robey et al. (2003). Most individuals are fitted for arm splints or other restraints to decrease these self-injurious behaviors (Ball et al., 1985; Letts and Hobson, 1975; Torres and Puig, 2007). Even though the individuals with Lesch-Nyhan syndrome have a seemingly unending compulsion to self-injure, it has been shown that they have normal pain

sensitivities and that they will actually beg and plead for restraints to be returned if they are removed (Nyhan, 2002).

Rett syndrome

Rett syndrome is an X linked dominant disorder that occurs in girls at a rate of between 1:10,000 (Hagberg, 1985) and 1:22,000 (Kozinetz et al., 1993). Rett syndrome is associated with a mutation in the *methyl CpG-binding protein (MeCP2)* gene. The MeCP2 protein binds to methylated CpG nucleotides in the DNA and is involved in epigenetic regulation of gene expression. Rett syndrome is the most common cause of intellectual disability in girls. Rett syndrome involves normal development for the first 9 to 18 months of life, followed by severe regression including deceleration of head growth, social withdrawal, loss of any acquired speech, and motor and gait problems. There is also loss of appropriate hand usage and an onset of hand stereotypies characterized by midline, chest level claspings, wringing, and washing-like hand motions. Self-injury occurs in about half of the girls with Rett syndrome and hand biting and mouthing are the most common types of self-injurious behaviors (Sansom et al., 1993), though the hand stereotypies can also be so extreme as to produce lesions.

Prader-Willi syndrome

Prader-Willi syndrome occurs in approximately 1:15,000 births and is caused by abnormal expression of genes around the 15q11-13 chromosome region. In humans, the maternal copy of this chromosomal region is hypermethylated and therefore inactive. Approximately 70% of Prader-Willi cases are caused by a large deletion in the 15q11-q13 region of the paternally derived chromosome. In another 20% of Prader-Willi syndrome patients, two maternal chromosomes are transmitted (uniparental disomy). The normal imprinting of the maternal alleles remain, leaving no transcription of genes

in this imprinted region. Other cases of Prader-Willi syndrome are caused by microdeletion of the imprinting center near the 15q11-13 region or the translocation of genetic material affecting the transcription of genes in the critical locus.

Prader-Willi syndrome is associated with muscle hypotonia during infancy and later in development hyperphagia, obesity, irritability, anxiety, anger, obsessive-compulsive symptoms, intellectual disability, and self-injury are exhibited. Self-injury is seen in approximately 80% of individuals with Prader-Willi syndrome and it most commonly consists of skin picking that targets the legs, head, and arms (Symons et al., 1999). The severity of self-injurious behavior is significantly different between individuals with the paternal deletion and the uniparental disomy. Those with the deletion subtype exhibit more severe self-injury compared to those with uniparental disomy (Dykens et al., 1999; Hartley et al., 2005; Symons et al., 1999). The genetic cause of these differences has yet to be elucidated.

Fragile X syndrome

The prevalence of Fragile X syndrome is approximately 1:4,000 males and 1:9,000 females. Fragile X syndrome is caused by a genetic mutation on the X chromosome at Xq27.3. This region is associated with the *Fragile X mental retardation 1* gene (*Fmr1*). This mutation involves CGG trinucleotide repeats, the number of which increases through maternal generational transmission. These CGG repeats are found in the 5' untranslated region of the first exon, which leads to hypermethylation of the promoter region and to little or no transcription of the FMR1 protein (FMRP; Feng et al., 1995). FMRP is a RNA binding protein and is important for gene translation repression. FMRP controls local protein synthesis that is initiated with cellular activity and without

normal functioning of FMRP protein the amount of gene translation is significantly increased, leading to alterations in synaptic homeostasis.

Fragile X syndrome is the most common cause of intellectual handicaps and the most common genetic cause of autism (described below). Fragile X boys typically show moderate intellectual disabilities, while girls vary between mild intellectual handicaps to normal intellectual functioning. The mutation in FMRP is associated with abnormal brain development and Fragile X individuals show eye gaze aversion, hyperactivity, social anxiety, aggression, and/or self-injurious behavior (Reiss and Dant, 2003; Hall et al., 2008). Nearly half of all individuals with Fragile X syndrome exhibit self-injury. These self-injurers typically express more than one form of self-injurious behavior, which include self-biting, head hitting, skin picking, hair pulling, head banging, and skin scratching. Interestingly, the area of self-injured surface is negatively correlated with FMRP protein levels (Symons et al., 2003a).

Smith Magenis syndrome

Smith Magenis syndrome is quite rare, affecting 1:25,000 people. This syndrome is caused by a sporadic deletion involving the *retinoic acid-induced 1 (Rai1)* gene on the short arm of chromosome 17. Two types of genetic mutations have been characterized in individuals with Smith Magenis syndrome. Ninety percent of cases involve large deletions at 17p11.2 that involves many genes. The remaining 10% of individuals with Smith Magenis syndrome have small deletions of 17p11.2 that only includes the *Rai1* gene. RAI1 protein is proposed to be part of the transcription machinery (Elsea and Girirajan, 2008).

The behavioral phenotypes associated with Smith Magenis syndrome include intellectual disability, hypotonia, attention deficit hyperactivity disorder, seizures,

attention seeking, stereotypy, and self-injury. The stereotyped behaviors exhibited by individuals with Smith Magenis syndrome are not associated with other common genetic disorders and consist of self-hugging and repetitive page turning (Dykens and Smith, 1998). Nearly all people with Smith Magenis syndrome exhibit self-injurious behaviors, which do resemble typical self-injury topographies. These include self-biting, head banging/slapping, skin picking, hair pulling, pulling finger and toenails, and inserting objects into orifices (Finucane et al., 2001).

Cornelia de Lange syndrome

Cornelia de Lange syndrome occurs in approximately 1:10,000 people and is associated with several types of mutations in any one of three genes that code for cohesion-associated proteins. Sixty percent of cases are caused by mutations in the *Nipbl* gene located on chromosome 5. Another 5% of cases are due to mutations in one of two genes, *Smc1a* (on the X chromosome) or *Smc3* (on chromosome 10). The molecular bases of the other 35% of Cornelia de Lange cases have not been identified. The process of cohesion is responsible for the proper segregation of chromosomes during mitosis and meiosis and researchers are beginning to understand the critical role of these and other cohesion-associated proteins in the expression of many different genes (Liu and Krantz, 2009).

Individuals with Cornelia de Lange syndrome exhibit intellectual disability (80% of individuals exhibit moderate to profound handicap), autistic-like behaviors, compulsions, hyperactivity, attention deficits, depression, sleep disturbances, aggression, and self-injurious behavior (Berney et al., 1999). Self-injury is expressed by 50-60% of individuals with Cornelia de Lange syndrome and the behaviors typically involve self-biting, skin picking, banging self against objects, and body poking (Oliver et al., 2009).

Autism

As opposed to many of the disorders listed above, autism is a syndrome that is defined solely by the expression of particular behaviors, not by molecular characterization of genetic mutations. The three behavioral domains that are diagnostic for autism are impairments in social interaction, communication deficits, and restricted repetitive behaviors. The most recent estimates indicate that 1 in every 110 children (and 1 in every 70 boys) has an autism diagnosis. Although autism is not diagnostically defined as being linked to one particular genetic cause, it is highly heritable with heritability estimates at nearly 90%. Five to fifteen percent of individuals with autism have a single gene disorder (e.g. Fragile X syndrome, Rett syndrome) and rare mutations and copy number variants have been found in small percentages of autistic cases. The genes most often found to be associated with autism cluster into categories of genes involved in cell growth, proliferation, and differentiation and pre- and post-synaptic proteins that maintain synaptic connectivity (Pinto et al., 2010). This genetic heterogeneity may be responsible for the clinical heterogeneity (described below), although environmental insults and gene-environment interactions probably also contribute to the highly variable autism phenotype (Herbert, 2010).

Variability is characteristic of all the features of autism. In regards to the three diagnostic behavioral domains, social interaction difficulties can range from impairments in nonverbal behaviors (e.g. eye gaze, gestures) to failure to develop appropriate peer relationships. Communicative abilities vary greatly between people with autism. Many people with autism are completely nonverbal, whereas others exhibit incessant stereotyped and repetitive language. Level of intelligence is also variable among people with autism. Some exhibit above average intelligence whereas others are intellectually

disabled. Restricted repetitive behaviors and interests also vary greatly and range from insistence on sameness, repetitive language, circumscribed interests, object attachments, repetitive facial movements, tics, motor stereotypies, repetitive manipulations of objects, and self-injurious behavior (Turner, 1999). Self-injurious behavior is exhibited by approximately half of autistic individuals and about 15% express severe self-injury (Baghdadli et al, 2003). The prevalence of autism is much higher in boys, however in regards to self-injury, autistic females are more likely to express self-injury and self-injure more severely than boys with autism (Cohen et al., 2010; Esbensen et al., 2009).

Neurobiology and Neurochemistry of the Clinical Syndromes Associated with Self-Injurious Behavior

As detailed above, there is no obvious similarity in the genetic basis between any of the syndromes that exhibit self-injurious behavior. Of the syndromes where the genetic and molecular pathologies have been identified, the deficits range from dysfunctions of DNA replication, DNA methylation, mRNA transcription, protein translation, and purine salvage. This range of molecular pathologies is quite striking considering the consistency of the phenomenology of the self-injurious behaviors across syndromes. With the understanding that the neurobiological basis of adaptive behavior is predicated on the normal functioning of entire brain circuits, it is probable that the differing molecular pathologies across these syndromes contribute to a similar dysregulation of the output of the brain's behavioral circuits and thusly share common behavioral dysfunction.

The neural circuit most likely involved in the expression of self-injurious behavior is the cortico-basal ganglia loop because of its role in cognition, emotion, and movement

(Bodfish and Lewis, 2002; Cromwell and King, 2004). The basal ganglia has several different circuits that mediate motor behavior, oculomotor movements, cognition, personality, and motivation/emotions (Visser et al., 2000). Each of these circuits differs slightly by the area of cortex that innervates the particular subcortical regions of the basal ganglia input nuclei, the pallidal output nuclei, and the thalamic projections. Though in general the neuroanatomy of the cortico-basal ganglia loop consists of cortical inputs to the striatum (caudate, putamen, and nucleus accumbens), which project to the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). These two nuclei, the GPi and SNr, are functionally equivalent and are generally grouped together when describing striatal output regions. The outputs from the striatum to the GPi/SNr take one of two paths. One path is direct from the striatum to the GPi/SNr and is termed the “direct pathway.” The other path is indirect and is termed the “indirect pathway.” The indirect pathway includes relay projections to the external segment of the globus pallidus (GPe) and on to the subthalamic nucleus. From the GPi/SNr, projections go to the thalamus and then to the cortex. The neurochemistry of the cortico-basal ganglia loop consists mainly of glutamate, gamma-aminobutyric acid (GABA), and dopamine. Descending cortical projections to the striatum are glutamatergic. The direct pathway neurons from the striatum to the GPi/SNr are GABAergic, as are the indirect pathway neurons from the striatum to the GPe. The indirect pathway neurons from the GPe release GABA onto the relay neurons of the subthalamic nucleus, and those neurons that project from the subthalamic nucleus to the GPi/SNr are glutamatergic. The output from the GPi/SNr to the different nuclei of the thalamus is GABAergic, whereas the neurons that project from the thalamus to the

cortex release glutamate. This neuroanatomical and neurochemical organization is responsible for differing effects of the direct and indirect pathways on cortical activation. Activation of the direct pathway causes disinhibition of the thalamic neurons and results in excitation of the cortex. Likewise, activation of the indirect pathway increases the inhibition of the thalamic neurons, which results in a reduction of glutamate signaling to the cortex, and therefore, decreased activation of the cortical neurons. The cortico-basal ganglia loops can be modulated by dopamine signaling from neurons originating in the substantia nigra pars compacta (SNc). Dopamine has different effects on direct and indirect pathway neurons, which is mediated by the different dopamine receptor subtypes. Direct pathway neurons in the striatum contain mostly D1-type dopamine receptors (D1 and D5 subtypes). Dopamine binding to these receptors activates stimulatory G-proteins, which then stimulate adenylate cyclase and its second messenger systems. Indirect pathway neurons in the striatum contain mostly D2-type dopamine receptors (D2, D3, D4 subtypes). Dopamine binding to these receptors activates inhibitory G-proteins, which then suppresses adenylate cyclase and its second messenger systems. Furthermore, basal ganglia function can also be modulated by other neurotransmitters (e.g. serotonin and acetylcholine) and neuropeptides (Angulo and McEwen, 1994).

The direct and indirect pathways are parallel pathways. Proper expression of basal ganglia-mediated behaviors depends on the appropriate balance of activity from these two antagonistic tracts. Neuroimaging techniques and postmortem brain analyses suggest that the development and/or functioning of the cortico-basal ganglia pathways may be disregulated in individuals with neurodevelopmental disorders associated with

self-injury and potentially mediate the vulnerability to develop self-injurious behavior and its continued expression (Boddaert et al., 2004; Casanova et al., 1991; Eliez et al., 2001; Ernst et al., 1996; Gothelf et al., 2008; Haas et al., 2009; Harris et al., 1998; Hoefft et al., 08, Hollander et al., 2005; Jellinger et al., 1998; Lloyd et al., 1981; Menon et al., 2004; Rassin et al., 1982; Saito et al., 1999; Sears et al., 1999; Subramaniam et al., 1997; Wenk et al., 1991; Wong et al., 1996; Wong et al., 1998). Unfortunately these studies were not designed to look at the neurobiological differences between self-injurers and non-injurers. As such, these neuroimaging and postmortem brain studies reveal abnormalities in cortico-basal ganglia circuits in neurodevelopmental disorders where self-injury is commonly expressed, but the mechanisms that underlie the actual vulnerability or the etiology of self-injurious behavior has not be systemically examined. Since the prevalence of self-injurious behavior is highest in Lesch-Nyhan syndrome, nearly 100%, analyses of this population provide the most potentially relevant information regarding the neurobiological basis of self-injury that is currently available.

Neuroimaging is difficult to perform in individuals with neurodevelopmental disorders, but several research groups have succeeded in performing this technique in self-injurious populations. Wong et al. (1996) found that the volume of the caudate nucleus was smaller in five subjects with Lesch-Nyhan syndrome. No volumetric differences were found in the putamen. Harris et al. (1998) replicated this finding in the caudate and were able to find reduced putamen and total brain volume using magnetic resonance imaging in individuals with Lesch-Nyhan syndrome.

Dopaminergic dysfunction has been the most prevalent finding in analyses of post-mortem brain tissue, neuroimages (i.e positron emission tomography binding

studies), cerebrospinal fluid (CSF), and blood plasma in individuals with Lesch-Nyhan syndrome. These deficits have been seen from the levels of the amino acid precursor to dopamine, tyrosine, all the way to the dopamine receptor expression level. Lower tyrosine levels have been found in almost all brain areas that have been assayed (e.g. putamen, several regions of cortex; Rassin et al. 1982). Decreased levels of synthesizing enzymes, tyrosine hydroxylase and dopa decarboxylase, that are responsible for the conversion of tyrosine to dopamine have been found in the caudate and putamen of individuals with Lesch-Nyhan syndrome (Lloyd et al., 1981). Ernst et al. (1996) also found lower enzymatic activity of dopa decarboxylase in the caudate, putamen, frontal cortex and ventral tegmental area (VTA). Not surprisingly, dopamine levels are lower in the caudate, putamen, external segment of the globus pallidus, and the nucleus accumbens in post-mortem Lesch-Nyhan syndrome brains (Lloyd et al., 1981). This finding in the caudate was later replicated by Saito et al. (1999). Levels of the dopamine metabolite homovanillic acid (HVA) have also been found to be lower in Lesch-Nyhan syndrome brain (Lloyd et al., 1981) and in CSF (Jankovic et al., 1988), compared to controls. These lower levels of dopamine metabolite are associated with increased activity of the dopamine catabolizing enzyme, monoamine oxidase (Lloyd et al., 1981). All of these differences were found in dopaminergic projection areas (i.e. regions with axon terminals) and not in dopaminergic cell body regions (Lloyd et al., 1981; Saito et al., 1999). Moreover a reduction in binding to the dopamine transporter was identified in the caudate and putamen (Wong et al., 1996). These findings suggest that there is a functional loss of dopaminergic axon terminals and not a loss of dopaminergic cell bodies in Lesch-Nyhan syndrome. Conversely, increases in

dopamine receptor expression (D₁ and D₂) have been identified in the caudate and putamen in individuals with Lesch-Nyhan syndrome, which suggests there is dopaminergic receptor supersensitivity (Saito et al., 1999).

The roles of the other monoamines in Lesch-Nyhan syndrome have not been fully characterized. Lloyd et al. (1981) found elevated levels of serotonin and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the putamen. Jankovic et al. (1988) also found increased 5-HIAA levels in the CSF. No differences in norepinephrine have been identified in Lesch-Nyhan syndrome versus controls (Ernst et al., 2000). However, decreased norepinephrine metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) has been found in plasma (Ernst et al., 2000). Additionally, massive increases in epinephrine have been found in the plasma of individuals with Lesch-Nyhan syndrome, which may indicate dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis or the adrenal gland's response to HPA axis activation (Ernst et al., 2000). There are no reports of other markers of HPA axis functioning (i.e. adrenocorticotrophic hormone or cortisol) in Lesch-Nyhan syndrome.

In Rett syndrome, reduced caudate volume has been shown consistently (Casanova et al., 1991; Subramaniam et al., 1997), and significant reductions in gray and white matter in the cortical dopaminergic projection area, the prefrontal cortex, have also been found (Subramaniam et al., 1997). Postmortem findings of dopaminergic dysfunction have been inconsistent (Roux and Villard, 2010). Tyrosine hydroxylase immunoreactivity is lower in the dopaminergic cell body regions in the substantia nigra (Jellinger et al., 1988), suggesting reduced monoamine synthesis. Wenk et al. (1991) found lower dopamine concentrations in the putamen and cortex of girls with Rett

syndrome (Wenk, 1995). Furthermore, several other postmortem studies have found lower concentrations of dopamine, serotonin, norepinephrine, HVA, and 5HIAA in several brain regions (Brucke et al., 1987; Riederer et al., 1986). However, in a follow up study with additional subjects, Wenk and colleagues were unable to find significant differences in any of their measures of dopaminergic functioning, including dopamine and HVA concentrations (Wenk, 1996), and Lekman et al. (1989) found no differences in dopamine, serotonin, norepinephrine or their metabolites in the caudate or putamen in four women with Rett syndrome. Obtaining consistent results regarding dopamine transporter levels and functioning has also been a challenge. Wenk found decreased density of dopamine transporter sites in the caudate and putamen in one set of patients (Wenk, 1995), but was unable to replicate this finding in another group of patients (Wenk, 1996). Wong et al. (1998), however, found a significant reduction in dopamine transporter binding in the caudate and putamen. Amino acid receptor levels are another inconsistent result in Rett syndrome. Blue et al. (1999) reported significant reduction in the ionotropic glutamatergic AMPA receptor levels in the putamen, and increased GABAA receptor in the caudate, while Wenk et al. (1993) found no differences in any ionotropic glutamate receptor subtype in the cortex or basal ganglia structures of Rett and control postmortem brains.

Neuroimaging studies of individuals with Prader-Willi syndrome are few. One study reported small orbitofrontal cortex volume (Ogura et al., 2010), while findings of no differences in caudate volume is consistent across a couple studies (Ogura et al., 2010; Yamada et al., 2006). Furthermore, the only monoamine result reported in Prader-Willi patients indicates higher HVA and 5HIAA concentrations in the CSF of

Prader-Willi patients compared to healthy controls (Akefeldt et al., 1998). Interestingly, the deleted region of the paternal 15q11-q13 chromosome associated with Prader-Willi syndrome contains genes for three subunits (GABRA5, GABRB3, GABRG3) of the GABAA receptor (Meguro et al., 1997). This suggests that GABAergic functioning may be reduced in the cortico-basal ganglia pathways of Prader-Willi syndrome patients. Significantly elevated GABA plasma levels have been found in individuals with Prader-Willi syndrome patients, which may be a homeostatic response to receptor hypofunctioning (Ebert et al., 1997).

Several neuroimaging studies have revealed larger caudate volumes in patients with Fragile X syndrome compared to controls (Eliez et al., 2001; Gothelf et al., 2008; Hoefft et al., 2008). Caudate volume is negatively correlated with FMRP levels and positively correlated with the presence of aberrant behaviors as assessed by the stereotypy subscale of the Aberrant Behavior Checklist (Gothelf et al., 2008) in those with Fragile X syndrome. Functional brain imaging analyses have also shown reduced activation of the basal ganglia during a response inhibition task in individuals with Fragile X syndrome (Hoefft et al., 2007; Menon et al., 2004). Activation of the basal ganglia during this task is positively associated with *FMR1* gene expression (Menon et al., 2004). Additionally, diffusion tensor imaging has shown increased fiber density in the neuronal tracts from the ventral frontal cortex to the striatum in males with Fragile X syndrome (Haas et al., 2009). These neuroimaging findings again suggest dysregulation of the cortico-basal ganglia loop in a population with high prevalence of self-injurious behavior.

There is very limited information regarding the neuroanatomical and neurochemical basis of Smith Magenis and Cornelia de Lange syndromes. The only relevant study to date found less gray matter in regions of the putamen and globus pallidus, as well as lower blood flow to these regions in individuals with Smith Magenis syndrome (Boddaert et al., 2004). The anatomical and functional deficits in the Smith Magenis brain may mediate self-injurious behavior in this population, though much more work will need to be done to confirm this role. Research and publication regarding Cornelia de Lange syndrome have not yet moved beyond description of phenotype and genotype, so the neurobiological basis of this disorder or the associated aberrant behaviors like self-injury are completely unknown.

The only physiological finding associated with self-injury in autism was made by Kolevzon and colleagues (2010). They found that whole blood serotonin levels were inversely correlated with the expression of self-injurious behavior. It is unclear what this peripheral effect has on brain pathways or if it is a more general marker of altered development of serotonergic systems, both central and peripheral (Whitaker-Azmitia, 2005). Additionally, increased caudate volume is a consistent finding in autism compared to normal controls, and caudate size correlates positively with repetitive behavior (Hollander et al., 2005; Sears et al., 1999). This is a result of continued caudate enlargement throughout development in the autistic brain whereas caudate size begins to decline in the normal controls beginning in adolescence (Langen et al., 2009). Autism is an incredibly heterogenous disorder and obtaining consistent results across subgroups (high functioning/low functioning) and development is a challenge. In

respect to elucidating the neurobiological basis of self-injurious behavior, autism may not be the ideal population in which to study.

In summary, although a single particular neurobiological dysfunction has not been elucidated in the different disorders that exhibit self-injury, there is some evidence to suggest that overactivity of the cortico-basal ganglia loops is a common pathology. Fewer dopamine terminals and striatal postsynaptic receptor supersensitivity has been suggested in Lesch-Nyhan and Rett syndromes. Decreased GABAA receptor functioning in Prader-Willi syndrome may cause dysregulation of direct versus indirect pathway activation and lead to over-excitation of the cortico-basal ganglia loops. Conversely, enhanced cortical glutamatergic input to the basal ganglia may be important for the expression of self-injury in Fragile X syndrome. Furthermore, inconsistent results in postmortem studies of each of these syndromes are common and may be due to age differences between subjects, and the methods used to collect, dissect, or process the brain tissue.

Therapeutic Strategies for Self-Injurious Behavior

The therapeutic strategies for the reduction of self-injurious behavior have historically involved either behavioral therapies or pharmacological interventions. Assessment of combined treatment plans has rarely been documented (Durand, 1982; Luiselli, 1986) and a large scale clinical trial of any combined treatment for self-injury has never been reported. The understanding that psychotropic medication can alter an individual's sensitivity to environmental events is beginning to receive notice (Kennedy et al., 2001). This suggests that pharmacotherapy for self-injury can not only change the dysregulated neurochemistry but may also be able to change the impact of reinforcers within the environment that contribute to the expression of self-injurious behavior. This

is an interesting idea that will hopefully receive significant attention by both basic and behavioral researchers.

Pharmacotherapies

The pharmacological targets of the drug therapies that have been evaluated in self-injurers are consistent with neurochemical pathways that are dysregulated in self-injurious populations. Targets of these drugs include dopaminergic, serotonergic, glutamatergic, and GABAergic systems, including their synthesis enzymes, neuronal transporters, and receptors. There are several limitations to the reports of pharmacotherapeutic efficacy of these drugs. Very few have been evaluated in clinical trials; most include very small sample sizes, many report no placebo controls, the patients are often taking several other psychotropic medications, and data are provided by researchers who are not blind to the drug status of the patients and often include only retrospective analyses of the patient's charts. Across studies the results are often inconsistent and the positive response rate is variable. Despite this, these studies still contribute to our understanding of the neurobiological basis of self-injury and these drugs have the potential to reduce self-injury in some sufferers.

The class of atypical antipsychotics has shown the greatest efficacy in reducing destructive behaviors, including self-injury. Olanzapine is a common atypical antipsychotic with a 5HT₂/D₂ receptor antagonism profile and has shown reasonable efficacy in reducing self-injurious behavior in individuals with intellectual disabilities (Janowsky et al., 2003a; McDonough et al., 2000) and pervasive developmental disorders (Potenza et al., 1999). Aripiprazole is a newer atypical antipsychotic and exerts 5HT_{2A} antagonism and D₂ partial agonism. Aripiprazole has been evaluated in a large double blind, randomized, placebo controlled study with people with autism and

provided significant improvement of several behaviors including self-injury, as measured by the irritability subscale of the Aberrant Behavior Checklist (Owen et al., 2009).

Furthermore, risperidone (a 5HT_{2A}/D₂ antagonist) reduces irritability (including aggression and self-injury) in children with autism (McCracken et al., 2002) and has shown efficacious effects on self-injurious behavior in individuals with Lesch-Nyhan syndrome (Allen and Rice, 1996) and intellectual disabilities (Cohen et al., 1998).

A number of selective serotonin reuptake inhibitors (SSRIs) have been evaluated for their efficacy at reducing self-injurious behavior. Fluoxetine (Ricketts et al., 1993), paroxetine (Snead et al., 1994), sertraline (Hellings et al., 1996; Luiselli et al., 2001), and the serotonin and norepinephrine reuptake inhibitor (SNRI), venlafaxine (Carminati et al., 2005), have shown some efficacy at reducing self-injury in small studies.

Interestingly, these same drugs (King et al., 1991; Denys et al., 2003) have all been reported to increase self-injurious behavior in OCD patients who did not self-injure before the SSRI/SNRI treatment. A sufficient explanation of these paradoxical effects has yet to be uncovered but they suggest a very important role of serotonin modulation on basal ganglia function (Kelland and Chiodo, 1996; Di Matteo et al., 2008) and its consequences on the expression of self-injurious behavior.

Antiepileptic drugs have also been used to reduce self-injurious behavior with some success. In several small studies, valproate reduced aggression and self-injury in patients with intellectual disabilities (Kastner et al., 1993; Ruedrich et al., 1999).

Valproate is generally regarded as a GABA agonist (increasing GABA levels by inhibiting metabolism and increasing synthesis), but also dampens neuronal excitability through actions on ion channels, alters several intracellular signaling pathways, and

regulates gene expression through epigenetic modifications (Terbach and Williams, 2009). Topiramate also reduces self-injury in individuals with intellectual handicaps (Janowsky et al., 2003b) and those with Prader-Willi syndrome (Shapira et al., 2002). Like valproate the pharmacology of topiramate is broad and includes inhibition of sodium and L-type calcium channels, as well as increases in GABAA receptor activity and antagonism of glutamatergic AMPA receptors (Shank and Maryanoff, 2008). The complicated pharmacology of these antiepileptic drugs does not elucidate the particular neurobiological basis of self-injurious behavior but does support the notion that increased basal ganglia output to the cortex plays a significant role in the production of self-injury.

Naltrexone, an opioid receptor antagonist, has been used to treat self-injury for several decades. Its utility has been disputed, but a recent review of studies evaluating the effectiveness of naltrexone on self-injurious behavior revealed a significant number of reported cases (nearly 80%) were responders to naltrexone treatment (Symons et al., 2004). The efficacy of naltrexone corresponds to the theory that individuals engage in these maladaptive behaviors for the release of endogenous opioids that follows the self-injurious episode and that recurrent self-injurious behavior is a way in which the self-injurers can get a "fix" for their own addicted endogenous opioid systems (Sandman, 1990). According to this opioid theory, administration of naltrexone blocks the effects of the self-injury-mediated opioid release and the frequency of self-injury then fades. Opioids and dopamine interact in many ways within basal ganglia structures, including modulation of presynaptic neurotransmitter release, neuronal activity, and by way of their postsynaptic receptors (Churchill and Kalivas, 1996). It is unclear which of these

mechanisms is important for the reduction of self-injury, but a role for endogenous opioidergic systems in self-injurious behavior is indicated.

Behavioral therapies

Studies of behavioral therapies are unfortunately plagued by some of the same limitations as their pharmacotherapeutic counterparts. These studies use very small samples, typically do not include information regarding treatment generalization, and rarely describe the continued efficacy of the therapy upon follow-up (DeLeon et al., 2002). Despite the limitations of these studies, behavior therapies offer significant reduction in behavior in a large majority of cases, at least while they remain under the care of the behavioral therapist. These behavior therapies range from communication training (Carr and Durand, 1985), extinction procedures (Zarcone et al., 1993), differential reinforcement strategies (Vollmer and Iwata, 1992), and noncontingent reinforcement treatments (Fischer et al., 1997).

Animal Models of Self-Injurious Behavior

Animal models of self-injurious behavior have provided important information regarding the neurobiological basis of self-injury. These models allow researchers to decipher which neurobiological dysfunctions found in the human populations are central to the expression of self-injury and which are not. These animal models utilize a wide array of techniques to induce self-injury including environmental deprivation, neonatal lesions, and pharmacological manipulations. A major strength of these animal models is that phenomenologically they are quite similar to the human behavioral pathology; they involve repetitive behaviors, typically self-biting, that can cause injury.

Although in most of the animal models of self-injury the behavior is induced by drugs, self-injury is commonly seen in animals living in labs, farms, zoos, and human

households. In this respect, these animal models represent species typical behaviors, which make them ideal to model human pathology (Insel, 2007). Furthermore, it is interesting that many of the mouse models that have been developed to model the genetic neurodevelopmental syndromes do not exhibit self-injury. For example, the mouse models of Lesch-Nyhan syndrome (*Hprt* knockout; Kasim and Jinnah, 2002), Fragile X syndrome (*Fmrp1* knockout; Price et al., 2007), and Prader-Willi syndrome (imprinting center deletion; Relkovic et al., 2010) do not exhibit spontaneous self-injurious behavior. Consequently, the mouse models that typically receive the most attention for construct validity in regards to the human disorders do not adequately model the behavioral pathology. In light of this, other animal models must be studied in order to further our understanding of the neurobiological basis of self-injurious behavior.

Nonhuman Primate Models

There are two nonhuman primate models of self-injury. One involves social isolation rearing for the first several months of life; the other allows for social rearing until weaning, followed by single housing in laboratory facilities for use in biomedical research. In both models only a subset of the rhesus monkeys demonstrates self-injurious behavior. Several aberrant behaviors are associated with the isolation rearing model, including stereotypy, pacing, circling behavior, aggression, apathy, indifference, withdrawal, fear, and self-directed behaviors, including huddling and self-injury (Goosen, 1981). Adult rhesus monkeys raised in isolation have significant loss of dopaminergic axon terminals and dopamine depletion (Martin et al., 1991). Dopamine receptor supersensitivity has also been suggested by their exaggerated behavioral response to the D1 and D2 receptor agonist, apomorphine (Lewis et al., 1990). No differences in CSF measures of serotonin metabolite (5HIAA), dopamine metabolite (HVA), or

norepinephrine metabolite (MHPG) have been found between self-injurious and non-injurious rhesus monkeys (Lewis et al., 1990).

A subset of the rhesus monkeys raised socially, but single housed as adults also exhibit self-injurious and aggressive behaviors, though these monkeys typically do not demonstrate a lot of the other aberrant behaviors seen in the isolation reared monkeys. Again, these self-injurious rhesus monkeys do not differ from non-injurious monkeys on measures of 5HIAA, HVA, or MHPG. There is some evidence that the self-injury in this model is mediated by a hyposerotonergic state. Administration of the serotonin precursor, L-tryptophan, reduces self-injury (Weld et al., 1998) as does treatment with fluoxetine (a SSRI) and a 5HT1a receptor agonist, buspirone (Fontenot et al., 2005). In addition, a specific polymorphism in the tyryptophan hydroxylase 2 gene, which codes for an enzymatic protein involved in serotonin synthesis, was recently found to correlate with a history of self-injurious behavior (Chen et al., 2010).

The roles of stress and anxiety have received a lot of attention in this rhesus monkey model. The prevalence of self-injury in these single-housed monkeys is related to the occurrence of several stressors including the age in which they were isolation housed and the number of minor veterinary procedures they've undergone (Lutz et al., 2003). Furthermore, self-injurious rhesus monkeys have increased stress-induced hormone release and less negative feedback of the body's stress response system, the HPA axis (Tiefenbacher et al., 2004). In addition, stressors, such as footshock (Gluck et al., 1985) and housing relocation (Davenport et al., 2008), significantly exacerbate the incidence of self-injurious behavior in monkeys. Rhesus monkeys with a history of self-injury also vocalize more and exhibit a greater number of threat behaviors (Novak et al.,

2002). There is also evidence that the self-injury seen in a subset of self-injurious monkeys is anxiety related. Self-injurious behavior is increased upon administration of an anxiogenic drug, FG7142 (Major et al., 2009), and reduced by an anxiolytic drug, diazepam (Tiefenbacher et al., 2005) in a subset of self-injurious monkeys. Both of these drugs bind to the benzodiazepine site on the GABAA receptor.

Taken together, the neurobiological findings from the nonhuman primate models of self-injurious behavior further implicate dopamine and serotonin depletion as well as a potential role for dysregulated GABAergic signaling. The sensitized stress pathways in self-injurious rhesus monkeys might also potentiate these dysregulated systems to increase the incidence of self-injurious behavior in stressful situations.

6-Hydroxydopamine Model

As described previously, significant dopamine depletion is found in post-mortem striatal tissue of individuals with Lesch-Nyhan syndrome (Lloyd et al., 1981). To model this early dopamine depletion, the dopaminergic systems of neonatal rats are lesioned with the neurotoxin, 6-hydroxydopamine (6-OHDA). As compared to rats that are lesioned as adults, the adult rats lesioned as neonates display self-injurious behavior when given a dopamine agonist like L-dopa (Breese et al., 1984). Consistent with most of the syndromes associated with self-injurious behavior, both dopamine depletion and activation of sensitized postsynaptic receptors are required for the induction of self-injury. Experiments using specific dopamine receptor agonists reveal that the D1 receptors mediate the expression of self-injury (Criswell et al., 1992; Sivam, 1989). Vulnerability to develop dopamine agonist-induced self-injury in the lesioned rats seems to be due to the magnitude of the dopaminergic lesion and the adaptive changes in D1 receptor binding in the midbrain. Self-injurious 6-OHDA treated rats have more

substantial depletion of striatal dopaminergic neurons and increased D1 receptor binding in the SNr compared to non-injurious 6-OHDA-treated rats (Yokoyama and Okamura, 1997). Interestingly, there are no differences in D1 receptor mRNA levels (Duncan et al., 1993), protein levels (Sivam et al., 2008), or binding rates (Yokoyama and Okamura, 1997) in the striatum of self-injurious versus non-injurious rats. However, significant differences in phosphorylation status of intracellular signaling molecules (p38MAPK) and transcription factors (CREB) have been found, suggesting that the self-injurious behavior is mediated by changes in gene transcription and cellular functioning within the striatum (Sivam et al., 2008), which are induced through activation of the D1 receptors.

Several characteristics of the 6-OHDA model closely resemble characteristics of Lesch-Nyhan syndrome. L-dopa induces self-injury in 6-OHDA rats and exacerbates self-injury in individuals with Lesch-Nyhan syndrome (Jankovic et al., 1988). Also, levels of serotonin are increased in the striatum of 6-OHDA rats (Sivam et al., 2008; Towle et al., 1989), similar to Lesch-Nyhan syndrome brains (Lloyd et al., 1981). Another similarity between Lesch-Nyhan syndrome and the 6-OHDA lesioned rat is that the self-injurious behavior seen in both is potentiated by stress (Anderson and Ernst, 1994; Stodgell et al., 1998). Furthermore, risperidone reduces self-injury in the 6-OHDA model (Allen et al., 1998) and in Lesch-Nyhan syndrome (Allen and Rice, 1996). Another interesting finding is that dopamine levels in the lesioned striatum are increased with a procedure (fixed ratio discrimination) that is similar to the behavioral interventions used for human self-injurious behavior (Stodgell et al., 1996; Loupe et al., 2002). This

suggests that behavioral interventions can rescue dopaminergic deficiencies, which may be the reason for their therapeutic efficacy in self-injury.

Bay K 8644 Model

Bay K 8644 is an L-type calcium channel activator. L-type calcium channels are predominately located in the striatum, cortex and hippocampus (Jinnah et al., 2003; Tanaka et al., 1995) and are voltage gated, indicating that only after the cell membrane is depolarized do the L-type channels open to allow a transient influx of calcium. Bay K 8644 increases the calcium influx during neuronal membrane depolarization. Mice given Bay K 8644 show impaired locomotion, dystonia, spasticity, self-injurious behavior, and aggression (Jinnah et al., 1999). These Bay K 8644-induced behaviors are relatively short-lived. They present within 10 minutes of Bay K 8644 subcutaneous administration, peak at 20-30 minutes and are absent by 50-120 minutes after injection (Jinnah et al., 1999). The behavioral effects (Jinnah et al., 1999) and c-fos expression (Jinnah et al., 2003) induced by Bay K 8644 are prevented by pre-administration of the L-type calcium channel antagonist nifedipine, which suggests that all effects are due specifically to activation of the L-type calcium channel. The roles of serotonin and dopamine on Bay K 8644-induced self-injury have been studied. Generally speaking, drugs that increase synaptic levels of serotonin and dopamine increase self-injury in the Bay K 8644-treated mice and drugs that deplete serotonergic or dopaminergic systems decrease self-injury (Kasim et al., 2002; Kasim and Jinnah, 2003). To determine whether the effect of Bay K 8644 was related to presynaptic or postsynaptic mechanisms, Kasim et al. (2006) used microdialysis to measure the effects of Bay K 8644 on dopamine release in the striatum. They found no increases in extracellular dopamine concentrations in the striatum of Bay K 8644-treated mice. Likewise, they found that antagonists for the dopamine D1/5 and

D3 receptors attenuated the self-injurious behavior in Bay K 8644-treated mice. This suggests that the self-injury-inducing effects of Bay K 8644 are due to postsynaptic calcium channels that are associated with D1/5 and D3 receptors, and not due to presynaptic effects of Bay K 8644. Calcium influx through L-type calcium channels, which is enhanced by Bay K 8644 administration, activates calcium/calmodulin kinase and MAPK pathways and stimulates CREB-mediated gene expression (Rajadhyaksha and Kosofsky, 2005). These pathways were found to mediate self-injurious behavior in the 6-OHDA model and may also cause the self-biting seen in Bay K 8644-treated mice. In addition, D1 receptor activation enhances L-type calcium channel functioning (Surmeier et al., 1995). This suggests that supersensitive D1 receptors in human self-injurers may lead to a potentiation of L-type calcium channel function and enhanced intracellular signaling and CREB-mediated gene expression. In summary, this model reconfirms that dopamine, and perhaps serotonin, are involved in the expression of self-injurious behavior and that there may be some therapeutic promise for the use of nifedipine in reducing self-injury in humans (Blake et al., 2007). Because of the short-lived behavioral effects of Bay K 8644, however, studies of chronic L-type calcium channel activation and its effects on neurotransmitter content and function have not been completed.

Methamphetamine Model

Methamphetamine, an indirect monoamine agonist, which reverses the monoamine transporters and causes significant release of monoamines into the synapse, while depleting vesicular stores (Sora et al., 2009), dose dependently induces self-injurious behavior in mice (Kita et al., 2000; Halladay et al., 2003). Self-injury in this model is acute, mostly occurring only after the first injection of methamphetamine, not

after any subsequent injection (Kita et al., 2000; Kuroda et al., 2010). The induction of self-injury in the methamphetamine model corresponds to significant reduction in dihydroxyphenylacetic acid (DOPAC) levels and increases in HVA levels in the striatum (Kita et al., 2000). Methamphetamine-induced self-injury in mice (particularly the BALB/c strain) is lessened by co-administration of a D1 antagonist, a glutamatergic NMDA receptor antagonist, and a serotonin precursor (Shishido et al., 2000). These authors also found that a D2 antagonist and naloxone did not reduce self-injurious behavior in this model. These results suggest that self-injury in this model is mediated by increased synaptic dopamine and glutamate, and decreased serotonin. More specifically, D1 receptor functioning, as opposed to D2 receptor functioning, appears to play a larger role in mediating methamphetamine-induced self-injury. Interestingly, the efficacy of the serotonin precursor is consistent with the reduction in self-injurious behavior in the nonhuman primate model (Weld et al., 1998), and is in accord with the findings that SSRIs are effective pharmacotherapies for some self-injurers (Hellings et al., 1996; Luiselli et al., 2001; Ricketts et al., 1993; Snead et al., 1994). Furthermore, consistent with the dopaminergic terminal loss exhibited by individuals with Lesch-Nyhan syndrome (Wong et al., 1996), methamphetamine causes significant reduction in dopaminergic terminals, which may be caused by a combination of dopamine-mediated (Kita et al., 2000) and glutamate-mediated (Mori et al., 2007) oxidative stress related neuronal damage. Information from the methamphetamine model of self-injury supports the significant role of dopamine terminal damage in the expression of self-injurious behavior, as well as critical mediation by D1 receptors and glutamatergic and serotonergic functioning.

Muscimol Model

Bilateral injections of the GABAA agonist, muscimol, into the SNr induce stereotypy and self-injurious behavior in intact rats (Baumeister and Frye, 1984) and in rats that have neonatal 6-OHDA lesions (Breese et al., 1987). These effects support our hypothesis that increased excitation of the cortex from the cortico-basal ganglia loops is involved in the expression of self-injury. Enhancing the inhibition of the SNr, through GABAA agonism, may potentially decrease the inhibition of the thalamic relays, and could lead to increased excitation of the cortex. This mechanism, though consistent with the neurobiology and neurochemistry of the cortico-basal ganglia loop, is only hypothetical because the effect of muscimol on self-injury expression is not reduced by a GABAA receptor blocker and is actually potentiated by a GABAA antagonist (Baumeister and Frye, 1984). This suggests that non-GABAA-related effects of muscimol, or the GABAA antagonists, may be responsible for the expression of self-injurious behavior in this model.

Pemoline Model

Pemoline is an indirect monoamine agonist (Cromwell et al., 1996; Fuller et al., 1978; Gilbert et al., 1978) that induces self-injurious biting in rats when administered in one high dose (Muller and Hsaio, 1980) or with repeated lower doses (Kies and Devine, 2004; Mueller et al., 1986). Although the pharmacological targets of pemoline are the monoamine transporters, pemoline-induced self-injury is not associated with changes in dopamine, serotonin, or norepinephrine transporter levels (Mueller et al., 1986). Consistent with data from human self-injurers, dopamine, serotonin, glutamate, and GABAergic signaling are involved in the expression of pemoline-induced self-injurious behavior, as evaluated by pharmacological challenges. Haloperidol (a D2 antagonist),

risperidone (a 5HT₂/D₂ antagonist), and valproate (an antiepileptic) each reduce pemoline-induced self-injury (Muehlmann et al., 2008; Mueller and Nyhan, 1982) and human self-injury. Pemoline-induced self-injury is also reduced by the glutamate antagonist MK-801 (King et al., 1995; Muehlmann and Devine, 2008). Interestingly, the SSRI paroxetine significantly potentiates self-injury in the pemoline model, suggesting an important role for synaptic serotonin in the expression of self-injury. Consistent with other animal models of self-injury there are differences in vulnerability to develop pemoline-induced self-injury wherein some rats exhibit self-biting, and others do not. Cromwell and colleagues (1997) showed that the rats that did develop pemoline-induced self-injury had differential changes in postsynaptic responsivity to dopamine-modulated glutamatergic signaling. Self-injurious rats showed increased striatal depolarizing postsynaptic potentials induced by dopamine application, whereas pemoline-treated non-injurious rats and control rats had decreased depolarizing postsynaptic potentials. These differential postsynaptic responses were normalized with the application of D₁ and D₂ antagonists, as well as by MK-801. Taken together, the pemoline model appears to demonstrate predictive validity and the individual differences in vulnerability that are seen in the human syndromes that exhibit self-injurious behavior. The individual differences in behavioral responses to pemoline also seem to be based on specific cellular neuroplastic changes that are mediated by neurotransmitter systems within the cortico-basal ganglia pathways that are altered in human self-injurers.

Summary

Each of these animal models has contributed significantly to our understanding of the neurobiological basis of self-injurious behavior. Information from each model has

supported the hypothesis that cortico-basal ganglia pathways are dysfunctional in self-injurious animals, and most have indicated a role of over activation of the direct basal ganglia pathway that is modulated by D1 dopamine receptors. The pursuit of elucidating the particular neurobiological dysregulation responsible for the expression of self-injury can be undertaken using any of these models. We have chosen to use the pemoline model in our investigations. Previously in the lab a dose response analysis of the self-injury-inducing effects of pemoline was completed (Kies and Devine, 2004), and we have demonstrated its predictive validity (Muehlmann et al., 2008) and generalizability (Blake et al., 2007).

The projects described herein continue our characterization of the model including the roles of pain processing, stress responsiveness, and stress exposure on the expression of pemoline-induced self-injurious behavior. We have also elucidated the neurochemical changes associated with the onset and maintenance of self-injury in several key brain structures. Lastly we will describe our investigation into the role of a neuropeptide, neurotensin, in pemoline-induced self-injurious behavior and the efficacy of neurotensinergic drugs on self-biting behavior. These projects have expanded our understanding of the pemoline model of self-injury and have elucidated a novel pharmacotherapy for the treatment of self-injury in humans.

CHAPTER 2 CHANGES IN PAIN RESPONSIVENESS DURING PEMOLINE ADMINISTRATION

Background

Behavioral theories of self-injurious behavior contend that self-injury is maintained by positive or negative reinforcement (Favell et al., 1982). This suggests that these reinforcers outweigh the aversive aspects of self-injuring, including any pain involved with the injurious actions and the resulting tissue damage. Pain is difficult to assess in people with self-injurious behavior as the prevalence of self-injury is inversely associated with level of intellectual ability in individuals with neurodevelopmental disorders (i.e. self-injury is most common in individuals with severe and profound intellectual disability; Saloviita, 2000). Research on a large cohort of individuals with intellectual disabilities found that those with more severe intellectual handicap were most likely to exhibit pain insensitivity or pain indifference, according to their caregiver (Biersdorff, 1994), though measuring pain thresholds in those who are cognitively impaired is problematic (Biersdorff, 1991).

Unfortunately, research from individuals of normal intelligence has not clarified the relationship between the perception of pain and self-injurious behavior. Paradoxically, self-injury is exhibited by individuals with average intelligence both in the absence of pain perception and in the presence of chronic pain. Individuals, who have suffered severe spinal cord injuries causing loss of sensory input and motor control of their limbs, sometimes self-injure to the point of amputation. The self-injurious behavior in these individuals with normal intelligence consists of self-biting and typically initiates long after the spinal cord injury (Frost et al., 2008). Conversely, self-injurious behavior

has been reported in people of average intelligence with chronic pain where the self-injury was directed toward the painful site (Mailis, 1996).

Studies of pain responsiveness in individuals with neurodevelopmental disorders associated with self-injurious behavior have also not clarified our understanding of these issues. In general, girls with Rett syndrome have reduced pain reactivity (Downs et al., 2010). Rett syndrome patients also have lower levels of substance P in CSF compared to normal controls (Matsuishi et al., 1997). Substance P is a neuropeptide involved in the transmission of pain information from the periphery and decreased CSF substance P is associated with centrally-mediated analgesia (Zubrzycka and Janecka, 2002). Furthermore, individuals with Prader-Willi syndrome consistently show lower pain sensitivity in response to thermal and painful stimuli (Priano et al., 2009), compared to normal controls. Severe self-injury is also seen in people with congenital insensitivity to pain. A description of their self-injurious actions has not been published and this genetic syndrome is associated with intellectual disabilities, which may also contribute to the expression of self-injury (Zafeiriou et al., 2004). Pain reactivity measures in kids with autism have been reported as heightened (Nader et al., 2004) and diminished (Tordjman et al., 2009) in response to the same stimulus (venipuncture). These inconsistent reports may be due to inadequate sample sizes, subjective measurement of facial reactivity, or to the heterogeneity of nearly all autism phenotypes.

In a study of children with severe intellectual handicaps, Breau and colleagues (2003) found that the prevalence of self-injurious behavior was equal in children with and without chronic pain. They also found no differences in the expression of pain in self-injurers and non-injurers. These findings suggest that the expression of self-

injurious behavior is not specifically associated with painful experience, though many of the clinical syndromes associated with self-injury have reduced pain reactivity, as measured by subjective testing.

Furthermore, in an effort to better characterize potential aberrant pain processing in individuals with intellectual disabilities, Symons and associates have moved from only studying subjective pain reactivity in these potentially nonverbal populations to adding analyses of peripheral markers of pain fibers and inflammatory responses. In comparison to normal controls, Symons et al. (2008) found further spacing between epidermal nerve fibers and higher substance P levels in the uninjured forearm of self-injurers. However, when later compared to cognitively impaired non-injurers, self-injurers had more epidermal nerve fibers and comparable substance P fiber density in a non-injured site (Symons et al., 2009). Furthermore, self-injurers were more responsive to sensory stimulation than the non-injurers were and sensory reactivity was positively associated with epidermal nerve fiber density in all subjects. Self-injurers, however, had higher levels of mast cell degranulation, compared to IQ matched non-injurers, though mast cell density was equal between the two groups. Mast cell degranulation is initiated by substance P release from sensory fibers and causes release of secretory granules that contain high levels of proteases, lysosomal enzymes, and cytokines, which mediate immune and inflammatory responses (Pejler et al., 2010). These results suggest that among intellectually disabled individuals, self-injurers are more reactive to sensory stimulation than their non-injurious counterparts, which may be a result of higher levels of epidermal nerve fibers and mast cell degranulation.

In summary, most studies of pain in intellectually disabled populations suggest decreased sensitivity relative to normal controls; however peripheral markers of sensory systems indicate self-injurers are more similar to controls than non-injurers are. This suggests that pain processing and reactivity may not play a significant role in the expression of self-injurious behavior and further supports our hypothesis that central mechanisms, such as dysregulated cortico-basal ganglia function, are responsible for the development and maintenance of self-injury. To further investigate the relationship between pain responsiveness and self-injurious behavior, we evaluated pain thresholds using the hot plate test in pemoline-treated rats during the non-injurious and self-injurious phases of repeated pemoline administration.

Methods

This project consisted of two experiments. The first experiment was designed to test Long Evans (LE) rats on the hot plate apparatus at escalating temperatures to evaluate which temperature was most appropriate for repeated testing. The rats used in this experiment did not receive pemoline or vehicle injections. After choosing an appropriate temperature and test duration, we initiated the second experiment wherein we analyzed pain thresholds in pemoline- and vehicle-treated rats throughout the five days of injections.

Experiment 1: Escalating Thermal Pain Testing in Drug Naïve Rats

Animals

Twenty four male LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in a climate controlled vivarium with a 12h/12h light/dark schedule (lights on at 7:00a.m.). Standard laboratory rat chow (Lab Diet 5001) and tap water were available *ad libitum*. The rats were pair-housed in standard

polycarbonate cages (43 cm x 21.5 cm x 25.5 cm) during six days of acclimation to the housing facility. All of the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were pre-approved by the Institutional Animal Care and Use Committee at the University of Florida.

Hot plate testing

The hot plate apparatus (10 x 10 x 12in) was enclosed by clear Plexiglas sides and a lid. After six days of acclimation to the housing facilities, rats were habituated to the hot plate apparatus (Hot Plate Analgesia Meter, Columbus Instruments) each day for four days. Beginning at approximately 4:00 p.m., each rat was individually placed on the hot plate at 36°C for 10 min. Three days after the habituation period ended, the hot plate testing began in which the rats were tested once every third day at one of the following ascending temperatures: 36°C for 10 min, 44°C for 10 min, 47°C for 3 min, 49°C for 3 min, and 51°C for 1 min. All hot plate sessions were videotaped at two camera angles and scored for licking and guarding behavior. Licking was defined as any oral contact on the hindpaws. Guarding was defined as either a withdrawal of the hindleg with a protracted return or quickly stamping the hindleg up and down.

Experiment 2: Thermal Pain Testing in Pemoline- and Vehicle-Treated Rats

Animals

Thirty six male LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in the same conditions as the rats in Experiment 1.

Hot plate testing habituation

After six days of acclimation to the housing facilities, rats were habituated to the hot plate apparatus each day for four days (as in Experiment 1). Beginning at

approximately 4:00 p.m., each rat was individually placed on the hot plate at 36°C for 10 min.

Drug

Pemoline (2-amino-5-phenyl-1,3-oxazol-4-one; Spectrum Chemicals, New Brunswick, New Jersey) was suspended at a concentration of 50 mg/ml in peanut oil. In order to get the pemoline into suspension the solution was kept stirring at room temperature overnight.

Drug treatment

Beginning the morning after the last hot plate habituation session, the rats were weighed and injected subcutaneously with either pemoline at 150 mg/kg (n=18) or peanut oil vehicle (n=18) each day for five days. These injections were administered at the nape of the neck and either flank on a rotating basis.

Assays of self-injury, stereotypies, and locomotion

The rats were visually inspected each time they were injected (approximately 9:00 a.m.) and then again every evening (approximately 4:00 p.m.), and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Any rat with an open lesion was immediately euthanized.

Night-vision cameras were focused on the cages of the rats (one camera per cage), and 5-minute time samples were recorded once every three hours over the entire day. The duration of self-injurious oral contact, rearing, locomotion, and stereotypy were quantified during each videotaped interval by a trained observer. Self-injurious oral contact was defined as all oral contact that stayed fixed on any one body part for longer

than 2 seconds. This was differentiated from grooming, which is oral contact with any part of the body that continues to move from site to site on the body (e.g. oral contact with the forepaws, that then moves up each forelimb and continues to the ventrum, and the contact is not sustained at any spot on the body for longer than 2 seconds). Rearing was counted each time a rat lifted both forepaws off the cage floor for at least 3 seconds. A subsequent rear was only counted after the rat resumed and maintained contact with the cage floor for at least 1 sec. Locomotion was counted by sectioning the video image of the cage into thirds (i.e. drawing lines on the television monitor dividing the cage into three equal parts along its length) and tallying the number of times the rat's forepaws crossed these lines. However, a subsequent crossing of that line was only counted after the rat crossed the other line. Thus, each locomotor count represents a minimum of 14.3 cm traversing of the cage. Since individual rats differed in the specific forms of stereotypy they expressed, and some stereotyped behaviors were expressed only at very low levels, the stereotypy measures were summed. Scored stereotypic behaviors included duration of stereotyped bob/lick, wherein the rat either bobbed its head in a stereotyped manner or licked the side or floor of the cage, and the duration of stereotyped digging, sniffing, or burrowing through the bedding. The durations (in seconds) of rearing, self-injurious oral contact, and stereotypy were summed over the entire day (i.e. from the eight video samples) and divided by the total number of seconds recorded, 2400 seconds. For example, the duration measures on day 1 cover the percent of time the rat was exhibiting that behavior during the recording periods between the first pemoline injection and just prior to the second injection, thereby covering the entire 24 hours following the first pemoline injection.

Hot plate testing

Each afternoon (approximately 4:00 p.m.) during pemoline or vehicle administration, each rat was tested on the hot plate apparatus at 47°C for 3 min. The test sessions were recorded and licking and guarding behaviors were scored in the same manner as in Experiment 1.

Statistical analyses

Differences between the pemoline group and the vehicle group in latency and frequency of licking/guarding, and duration of oral contact, rearing, locomotion, and stereotypies were each evaluated using repeated measures analyses of variance (RM-ANOVA). The frequency of licking and guarding was combined together as is standard in hot plate testing (Mogil et al., 1996). Between-groups differences in all these dependent measures were treated as statistically reliable when the p-values were less than 0.05. Significant effects for the lick/guard latency and frequency measures were further analyzed with pre-planned Fisher's least significant difference (LSD) post-tests.

Three pemoline-treated rats were euthanized before the end of the experiment because they had open lesions. In these cases, the missing data were replaced by repeating the final score that was attained for each dependent measure through the end of the experiment. This strategy was used to avoid the potential that the group means would over- or under-estimate the licking and guarding latency and frequency when the most severe self-injurers were terminated.

Results

Experiment 1

In drug naïve rats, lick/guard latency decreased with increasing temperatures (Fig. 2-1A). Frequency of lick/guards was also related to temperature (Fig. 2-1B),

though the duration of testing was different for most temperatures making direct comparisons difficult. After scoring these data we chose 47°C for our hot plate testing temperature for repeated evaluations. This intermediate temperature was selected because it allowed for potential pain threshold changes in either direction without the complication of either ceiling or floor effects. We were also confident that repeated hot plate testing at this temperature would not cause tissue injury to the rats' paws.

Experiment 2

Pemoline-induced self-injury was first exhibited on day 3, and by day 6 all rats had injuries (Fig. 2-2A). Self-injurious oral contact was initially seen on day 2 and remained high throughout days 3, 4, and 5 (time effect: $F_{(4,136)} = 28.54$, $p < 0.0001$; drug effect: $F_{(1,136)} = 86.08$, $p < 0.0001$; time x drug interaction effect: $F_{(4,136)} = 28.53$, $p < 0.0001$ Fig. 2-2B). Vehicle-treated rats showed very low levels of rearing, locomotion, and stereotypies and exhibited no self-injurious oral contact or self-injured tissue. In the pemoline-treated rats, rates of rearing and locomotion were highest on day 1 (Fig. 2-3A,B), followed by peak in the rate of stereotypies on day 2 (Fig. 2-3C). RM-ANOVA effects for drug, time, and drug x time interactions were significant for all measures of ancillary behaviors, in comparison to vehicle-treated rats (rearing: time effect: $F_{(4,136)} = 36.28$, $p < 0.0001$; drug effect: $F_{(1,136)} = 42.60$, $p < 0.0001$; time x drug interaction effect: $F_{(4,136)} = 27.79$, $p < 0.0001$; locomotion - time effect: $F_{(4,136)} = 28.17$, $p < 0.0001$; drug effect: $F_{(1,136)} = 60.51$, $p < 0.0001$; time x drug interaction effect: $F_{(4,136)} = 22.42$, $p < 0.0001$; stereotypies - time effect: $F_{(4,136)} = 8.061$, $p < 0.0001$; drug effect: $F_{(1,136)} = 162.9$, $p < 0.0001$; time x drug interaction effect: $F_{(4,136)} = 8.453$, $p < 0.0001$).

Repeated pemoline administration at 150mg/kg/day diminished the expression of pain-related behavior on a 47°C hot plate. Lick/guard latency at 47°C was significantly

increased with pemoline treatment (Fig. 2-4A). The RM-ANOVA revealed a significant effect of drug on lick/guard latency ($F_{(1,136)} = 6.424$, $p < 0.05$), but no significant effect of time or time x drug interaction. Consistent were the effects of pemoline found on the frequency measure of licking/guarding behavior (Fig. 2-4B). The RM-ANOVA showed a significant effect of drug ($F_{(1,136)} = 18.87$, $p < 0.001$), without significant effects of time or time x drug interaction effects. In fact, these non-significant effects that were suggested by the RM-ANOVAs that failed to reach statistical significance for time or time x drug interactions reveal that pain thresholds at 47°C changed relatively little during the five days of daily testing in either group. To examine the potential relationship between elevated pain thresholds and pemoline-induced self-injurious behavior, we analyzed the mean lick/guard latencies and frequencies on the third experimental day when exactly half of the pemoline-treated rats exhibited self-injury and the other half did not (Fig. 2-2A). Two-tailed t-tests found no significant differences in lick/guard latency ($t_{(16)} = 0.827$, $p = 0.4204$; Fig. 2-4C) or frequency ($t_{(16)} = 0.4024$, $p = 0.6927$; Fig. 2-4D) between self-injurers and non-injurers. These data suggest that there is no direct relationship between pain sensitivity and the induction of self-injury in the pemoline model.

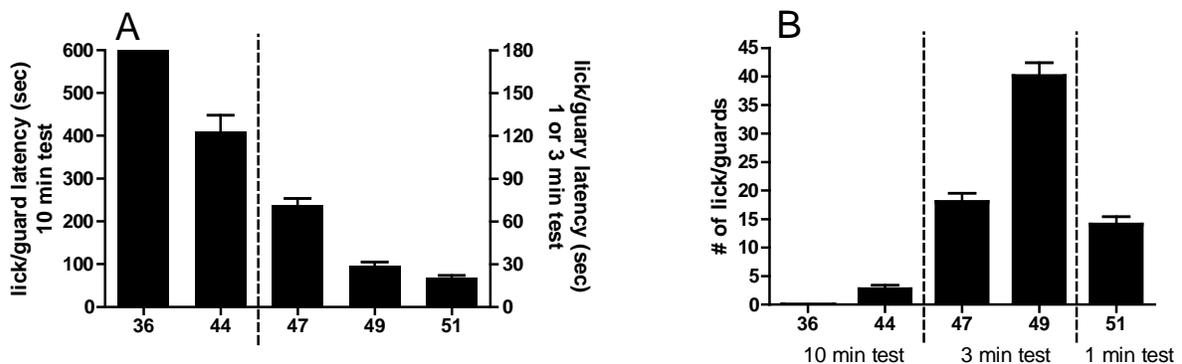


Figure 2-1. Hot plate test behaviors of drug naïve rats during stimulation at escalating temperatures. Latency to lick/guard in response to the temperature of the hot

plate decreased as temperatures were increased (A). Frequency of lick/guard behaviors increased as temperatures were increased (B). All values are expressed as group means \pm standard error of the mean (SEM).

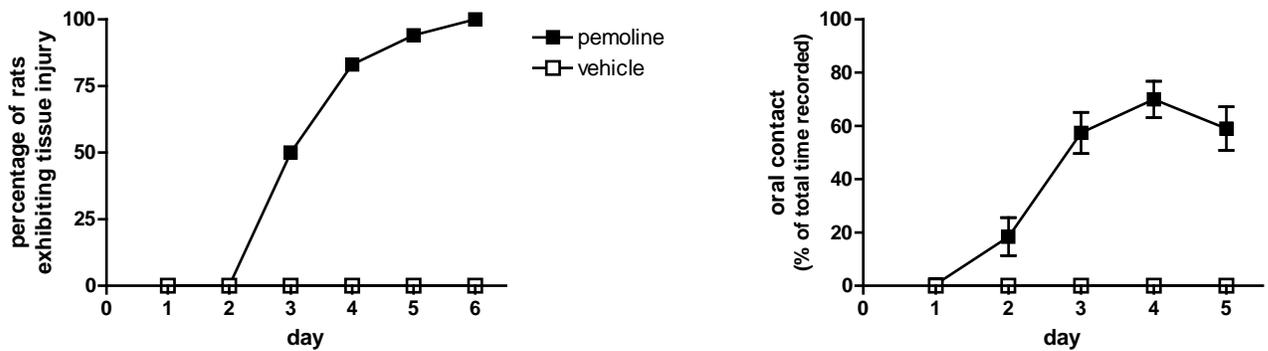


Figure 2-2. Pemoline-induced self-injurious behavior. Incidence (A) and duration (B) of pemoline-induced self-injurious behavior increased across days.

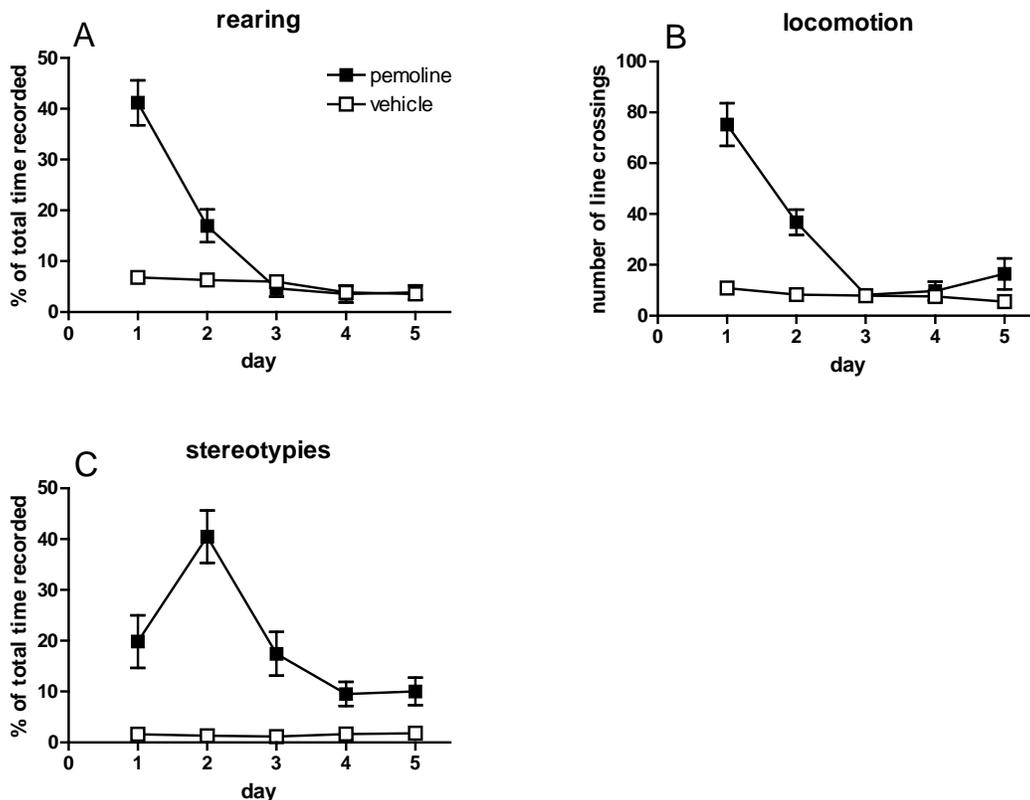


Figure 2-3. Rates of home-cage behaviors of pemoline- and vehicle-treated rats. Pemoline-treated rats exhibited more rearing (A), locomotion (B), and

stereotypies (C), compared to vehicle-treated rats. All values are expressed as group means \pm SEM.

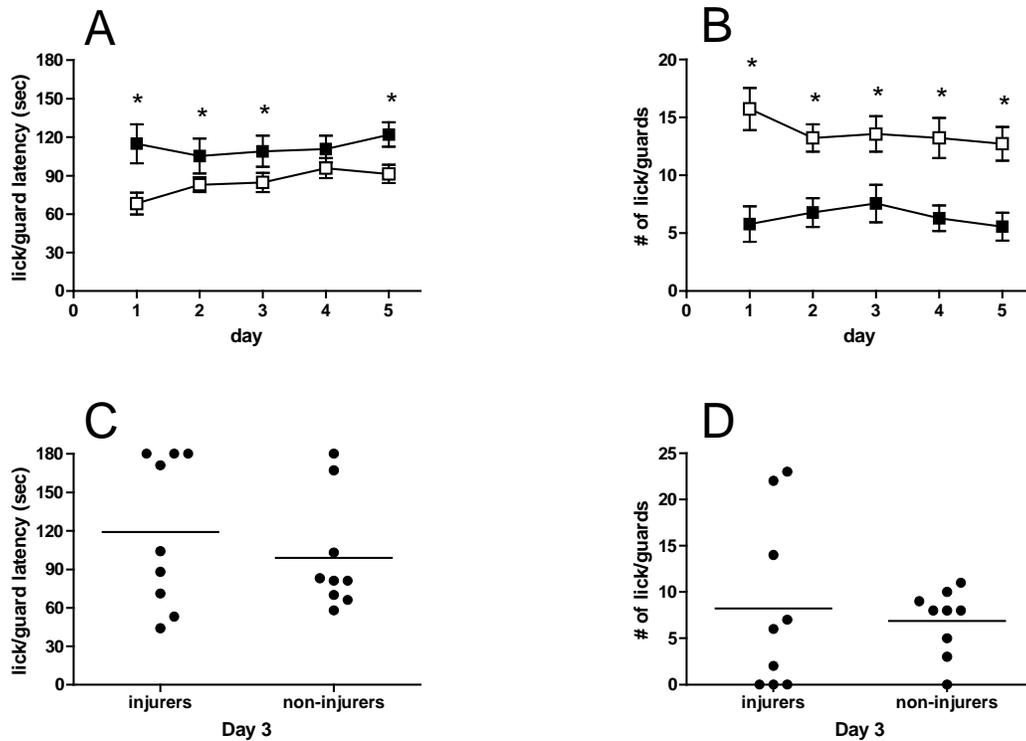


Figure 2-4. Effects of pemoline- and vehicle-treatment on hot plate behaviors. Lick/guard latency (A) and frequency (B) stayed relatively stable across days of pemoline and vehicle administration. Pemoline-treated rats had higher pain thresholds and exhibited fewer pain-related behaviors compared to vehicle-treated rats. On day 3 of pemoline administration there were no differences in lick/guard latency (C) or frequency (D) between pemoline-treated injurers or non-injurers. All values are expressed as group means \pm SEM. Significant differences between pemoline- and vehicle-treated rats are depicted with an asterisk.

Discussion

In this experiment we evaluated the effects of pemoline on pain thresholds and pain-related behaviors using the hot plate test. Although all pemoline-treated rats exhibited self-injury during the later days of the experiment, when we analyzed pain responsiveness on a day that half of the rats exhibited injury and half did not, we saw no differences in lick/guard latency and frequency. Furthermore, pain responsiveness did

not change across days, whereas self-injurious behaviors did. On average, the pemoline-treated rats had longer latencies and lower numbers of lick/guard behaviors, which suggests that pemoline-treated rats have reduced pain sensitivity. This is redolent of findings from some (but not all) clinical syndromes associated with self-injury.

These data suggest that reduced pain sensitivity may be a requisite condition for the vulnerability to develop self-injury, but that other neurobiological conditions are necessary for the induction of self-injury. Reduced pain sensitivity has been found in other animal models of self-injury. In the muscimol model where injections into the SNr cause some rats to self-injure, the self-injurious rats failed to show any pain responsiveness on a 55°C hot plate. Rats that did not self-injure after injection of muscimol in the SNr did react to the painful stimulus of the hot plate. Furthermore, baclofen injected into the SNr also decreased pain sensitivity but did not induce self-injury (Frye et al., 1986). This suggests that decreased pain sensitivity is necessary for the induction of muscimol-induced self-injury, but that other neurobiological changes (induced by muscimol, but not by baclofen) are also required. Additionally, amphetamine-induced self-injury is associated with increased pain thresholds, though like pemoline-treated rats, there were no differences in lick/guard latency in amphetamine-treated self-injurers and non-injurers (Jain and Sharma, 2002). These findings indicate that decreased pain sensitivity may be a contributing factor to a vulnerability to develop pemoline-induced self-injury, but that additional neurobiological changes may also be required for the induction of self-injury.

CHAPTER 3 EFFECTS OF REPEATED STRESS ON PEMOLINE-INDUCED SELF-INJURIOUS BEHAVIOR

Background

Stress and novelty appear to play important roles in mediating the expression of self-injurious behavior; however these roles have not received much empirical study. A survey of parents of males with Lesch-Nyhan syndrome reported that self-injury was exacerbated by the presence of new people or the stress of an illness (Anderson and Ernst, 1994). Additionally, a recent study of nonhuman primates found that the stress of relocation to a novel environment with unfamiliar neighbor animals increased the self-injury of rhesus monkeys with a prior history of social deprivation and self-wounding, but had no self-injury-inducing effects on monkeys without a history of self-wounding (Davenport et al., 2008). Furthermore, assays of stress hormones, ACTH and cortisol, reveal that they are dysregulated in clinical populations in which self-injury is a phenotypic trait (Curin et al., 2003; Hall et al., 2008; Hessler et al., 2002; Sandman et al., 2008; Symons et al., 2003b; Verhoeven et al., 1999), however the direction of the dysregulation (i.e. findings of increases or decreases) has been inconsistent. These findings from human and nonhuman primate self-injurers suggest that the neurobiological response to stress may induce a self-injurious episode in those with a dysregulated HPA axis and a vulnerability to self-injure.

Using the pemoline model of self-injury, we examined the effect of repeated social/emotional stress on the expression and severity of pemoline-induced self-injurious behavior. Repeated social defeat stress alters basal levels of corticosterone (Covington and Miczek, 2001), thereby inducing similar endocrine dysfunction that has been reported in some self-injurers. In light of this, we investigated the effect of

repeated social defeat stress on the expression of pemoline-induced self-injury. We also analyzed the levels of AMPA receptors, neurotensin receptors, and the organic cation transporter 3 (OCT3) to evaluate potential neurotransmitter systems that may mediate an exacerbation of pemoline-induced self-injury by repeated social defeat stress.

Methods

Animals

Twenty six male LE rats weighing 150-175g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in a climate controlled vivarium with a 12h/12h light/dark schedule (lights on at 6:00 a.m.). Twenty two of these were the experimental rats and were used as the “intruders” (n=11) and handled controls (n=11; see Social Defeat section). Four additional rats were used as vehicle controls. They were neither used as intruders nor handled controls. Standard laboratory rat chow (Lab Diet 5001) and tap water were available *ad libitum*. These rats were pair-housed in standard polycarbonate cages (43 cm x 21.5 cm x 25.5 cm) during 6 days of acclimation to the housing facility and throughout either the social defeat or handling regimens. The vehicle control rats were left pair-housed and undisturbed until the injection protocol began. An additional six male LE rats weighing 300-325g and an additional six female LE rats weighing 200-225g were pair-housed in standard polycarbonate cages with rats of their same sex for seven days. These male rats were later used as “residents” (see Social Defeat section). Male and female pairs were housed together (after the males were vasectomized) in a separate housing room from the intruders with opposite reverse 12h/12h light/dark schedule (lights off at 6:00 a.m.). All the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were pre-approved by the Institutional Animal Care and Use

Committee at the University of Florida. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgery

The male resident rats were vasectomized under ketamine-xylazine anesthesia (ketamine, 62.5 mg/kg; xylazine, 12.5 mg/kg; i.p.) and Ketorolac analgesia (2 mg/kg, s.c.). Supplemental surgical anesthesia (isoflurane) was administered as necessary. Each anesthetized rat was shaved from the rostral edge of the scrotal area to the caudal abdomen. A midline incision was made rostral to the penis, the vas deferens was isolated with forceps, and a 0.5 cm section was removed from each duct using a miniature cautery utensil. The internal incision was sutured with absorbable 4-0 Ethilon monofilament vicryl suture (Ethicon Inc.) and the external incision was closed with 9 mm stainless steel wound clips (World Precision Instruments Inc.) which were removed seven days after surgery.

Social Defeat Procedure

At least two weeks prior to the onset of the social defeat sessions the vasectomized male rats were pair-housed with cycling female rats. Social defeat sessions were run during the resident rat's dark schedule starting at approximately 7:00 a.m. At the beginning of each social defeat session each female resident was removed from her home cage and placed in a similar cage nearby. Ten minutes after removal of the female resident, an intruder rat was placed into the home cage that the male and female resident rats shared. Each male resident was trained to exhibit dominance behavior. The resident male and intruder rat were allowed to interact for five minutes or until the intruder displayed a submissive, defeated posture three times. An intruder rat was considered to be defeated when it lay motionless in a supine posture, with the

resident male rat on top of it, for a period of at least two seconds. Following this direct interaction phase, each intruder was removed from the home cage of the resident, placed in to a separate 10 cm x 15 cm x 10 cm double-layered wire mesh cage and returned to the home cage of the resident male rat. This indirect interaction phase allowed the intruder rat to be out of direct contact of the male resident rat but still able to experience visual, auditory, and olfactory interactions with the resident male. The intruder was maintained in the wire mesh cage until 10 min had elapsed from the start of the direct interaction phase. After the entire 10 min interaction had concluded both the female resident rat and the intruder were returned to their respective home cages. The intruder rats were put through the social defeat procedure each day for twelve days, seeing each resident male twice in that period, six days apart. Non-defeated control rats were handled for 2 min each day for 12 days.

Drugs

Pemoline (2-amino-5-phenyl-1,3-oxazol-4-one; Spectrum Chemicals, New Brunswick, New Jersey) was suspended at a concentration of 50 mg/ml in peanut oil. In order to get the pemoline into suspension, the solution was kept stirring overnight.

Ketamine/xylazine, isoflurane, and ketorolac tromethamine were purchased from Henry Schein Inc. (Melville, New York).

Drug Treatment

Following the 12 day social defeat regimen or the equivalent days for the handling control rats, the rats were weighed and injected with pemoline at 150 mg/kg (s.c.) each day for five days. These injections were administered at the nape of the neck and either flank on a rotating basis. For protein expression controls, four rats were administered peanut oil vehicle for five days.

Assays of Self-Injury and Stereotypy

The rats were visually inspected each time they were injected and then again every evening, and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Any rat with an open lesion was immediately euthanized.

Still images of the injured tissue were taken from the videotapes, and MCID software (Imaging Research Inc., St. Catherines, ON, Canada) was used to draw outlines around the injured tissue, and to calculate the area of injury in mm². Additionally, night-vision cameras were focused on the cages of the stress and handled rats (one camera per cage), and 5-minute time samples were recorded once every three hours over each entire day. The duration of self-injurious oral contact and stereotypy were quantified during each videotaped interval by a trained observer. Self-injurious oral contact was defined as all oral contact that stayed fixed on any one body part for longer than 2 seconds. This was differentiated from grooming, which is oral contact with any part of the body that continues to move from site to site on the body (e.g. oral contact with the forepaws, that then moves up each forelimb and continues to the ventrum, and the contact is not sustained at any spot on the body for longer than 2 seconds). The stereotypy measure is a compilation of the duration of stereotyped bob/lick, wherein the rat either bobbed its head in a stereotyped manner or licked the side or floor of the cage, and the duration of stereotyped digging, sniffing, or burrowing through the bedding. The duration (in seconds) of self-injurious oral contact and stereotypy were each summed over each day (i.e. from the eight video samples) and

divided by the total number of seconds recorded, 2400 seconds. For example, the duration measures on day 1 cover the percent of time the rat was exhibiting that behavior during the recording periods between the first pemoline injection and just prior to the second injection, thereby covering the entire 24 hours following the first pemoline injection. A second blind observer scored the duration of self-injurious oral contact and stereotyped behavior for a sample of the recordings (one day of recordings for each rat) and inter-observer reliability was evaluated by Pearson correlation.

Twenty four hours after the last pemoline or vehicle injection, the rats were rapidly decapitated. Brains were immediately removed, frozen in 2-methylbutane kept on dry ice at -40°C , and stored at -80°C .

Protein Analyses

The frozen brains were cut into 1mm slices in the coronal plane using a stainless steel rat brain matrix (Braintree Scientific, MA). Micropunches were taken from the core and shell of the nucleus accumbens and stored at -80°C . The methods for protein crosslinking and western blot analyses were adapted from Nelson et al. (2009). Punches were incubated with protein crosslinking reagent bis(sulfosuccinimidyl) suberate (BS^3) at 2uM in artificial extracellular fluid for 30 min at 4°C with gentle agitation. Chemical crosslinking of proteins in intact cells allows for analysis of surface expressed proteins (which form high molecular weight aggregates when crosslinked, as opposed to the intracellular proteins that are not accessible to the cross-linking reagent). Crosslinking was terminated by quenching the reactions with 500ul of 300uM glycine for 10 min at 4°C . Tubes containing the cross-linking reactions were centrifuged at 20,800g for 2 min at 4°C . Supernatant was discarded and the protein pellet was sonicated in 25ul of ice cold lysis buffer containing protease and phosphatase inhibitors.

The tubes were again centrifuged at 20,800g for 2 min at 4°C. Supernatant was separated and the total protein content was assayed using the Lowry method (Total Protein Kit, Sigma).

Equal amounts of protein samples were loaded on to 3-8% Tris Acetate gels and run under reduced conditions. Proteins were then transferred onto polyvinylidene fluoride membranes. Membranes were incubated with primary antibody at 4°C. The following primary antibodies were used: polyclonal rabbit anti-GluR1 (1:500; Millipore), polyclonal rabbit anti-GluR2 (1:1000; Millipore), polyclonal rabbit anti-NTS1 (1:500; Abcam), polyclonal rabbit anti-OCT3 (1:1000; Abcam). Membranes were exposed to secondary antibody (horseradish peroxidase-linked anti-rabbit IgG, 1:5000; Cell Signaling) for 1 hr at room temperature. Proteins were detected using the chemiluminescence method and analyzed by densitometry using MCID Image Analysis software. Stripped membranes were re-probed for b-actin (rabbit polyclonal, 1:2000; Cell Signaling) and secondary antibodies. Protein expression levels for GluR1, GluR2, NTS2, and OCT3 are normalized to b-actin protein levels.

Statistical Analyses

Differences between the stressed group and the unstressed group in duration of self-injurious oral contact, size of the injured tissue, and duration of stereotypy were each evaluated using RM-ANOVA. Proteins were analyzed according to their localization: surface, intracellular, surface + intracellular (S+I), and surface/intracellular. Differences between the vehicle group, the pemoline + stress group, and the pemoline + no stress group in these measures were each analyzed by one-way ANOVAs. Between-groups differences in all dependent measures were treated as statistically

reliable when the p-values were less than 0.05. All significant effects were further analyzed with pre-planned Fisher's LSD post-tests.

Six rats were euthanized before the end of the experiment (four stressed rats and two control rats) because they had open lesions. In these cases, the missing behavioral and tissue injury data were replaced by repeating the final score that was attained for these dependent measures through the end of the experiment. This strategy was used to avoid the potential that the group means would underestimate the area of tissue damage and self-injurious oral contact scores, and to avoid the potential that the group means would over- or under-estimate the stereotypy scores when the most severe self-injurers were terminated. Brains were collected from the rats that were euthanized early and their tissues are included in the protein expression analyses.

Results

Repeated social defeat stress did not affect the incidence of pemoline-induced self-injury (Fig. 1A). The dose of pemoline used (150mg/kg/day) caused all the non-stressed rats and all the stressed rats to show some sign of self-injury. The severity of the injuries, however, was significantly increased by repeated social defeat stress. Specifically, the rats that were repeatedly stressed showed significantly larger areas of tissue damage than the handled control rats did (Fig. 1B). The RM-ANOVA revealed a significant main effect of time ($F_{(10,210)} = 39.91$, $p < 0.01$) and a significant stress x time interaction ($F_{(10,210)} = 2.095$, $p < 0.05$), wherein rats first displayed injured tissue around day 2 or 3, the size of injured tissue began to asymptote on day 4 or 5, and the size of injured tissue was greater in the stressed rats. There were no significant differences in the amount of time spent engaging in self-injurious oral contact between stressed and unstressed rats (Fig. 1C). There was a significant main effect of time ($F_{(4,84)} = 46.47$, $p <$

0.01) as all rats began to show self-injurious oral contact on day 2, which peaked on day 3, and continued throughout the experiment, however there was no significant main effect of stress nor a stress x time interaction effect. Although the statistical test was not significant, the stressed rats did appear to engage in more oral contact on days 4 and 5 when the rates of oral contact of the non-stressed rats begin to decline. There were also no differences in the duration of other pemoline-induced stereotypies (Fig. 1D). The RM-ANOVA revealed a significant main effect of time ($F_{(4,84)} = 14.35, p < 0.01$), as all rats showed moderate amounts of stereotypy on days 1 and 2, which lessened on days 3-5 as self-injurious oral contact duration increased. No significant main effect of stress or a stress x time interaction effect was found for stereotypy duration. Inter-observer reliability, as determined by Pearson correlation, was $r = 0.9647$ for the duration of oral contact and $r = 0.9306$ for the duration of stereotypy.

The significant effect of stress on the severity of pemoline-induced self-injurious behavior was not a result of altered neurotransmitter receptor or transporter expression or localization in the nucleus accumbens. We analyzed levels of two glutamatergic AMPA receptor subunits, GluR1 (Fig. 3-2A,B) and GluR2 (Fig. 3-2C,D), a neurotensin receptor (NTS1; Fig. 3-2D,E), and the OCT3 (Fig. 3-2F,G). We found that repeated social defeat stress, which exacerbated the pemoline-induced self-injury, did not change expression or localization of GluR1, GluR2, NTS1, or OCT3 in the nucleus accumbens. Total expression (i.e. surface + intracellular levels) of NTS1 was the only effect to approach significance ($p = 0.087$), and a t-test comparing the vehicle group and the pemoline + no stress group revealed a significant effect ($t_{(13)} = 2.412, p < 0.05$).

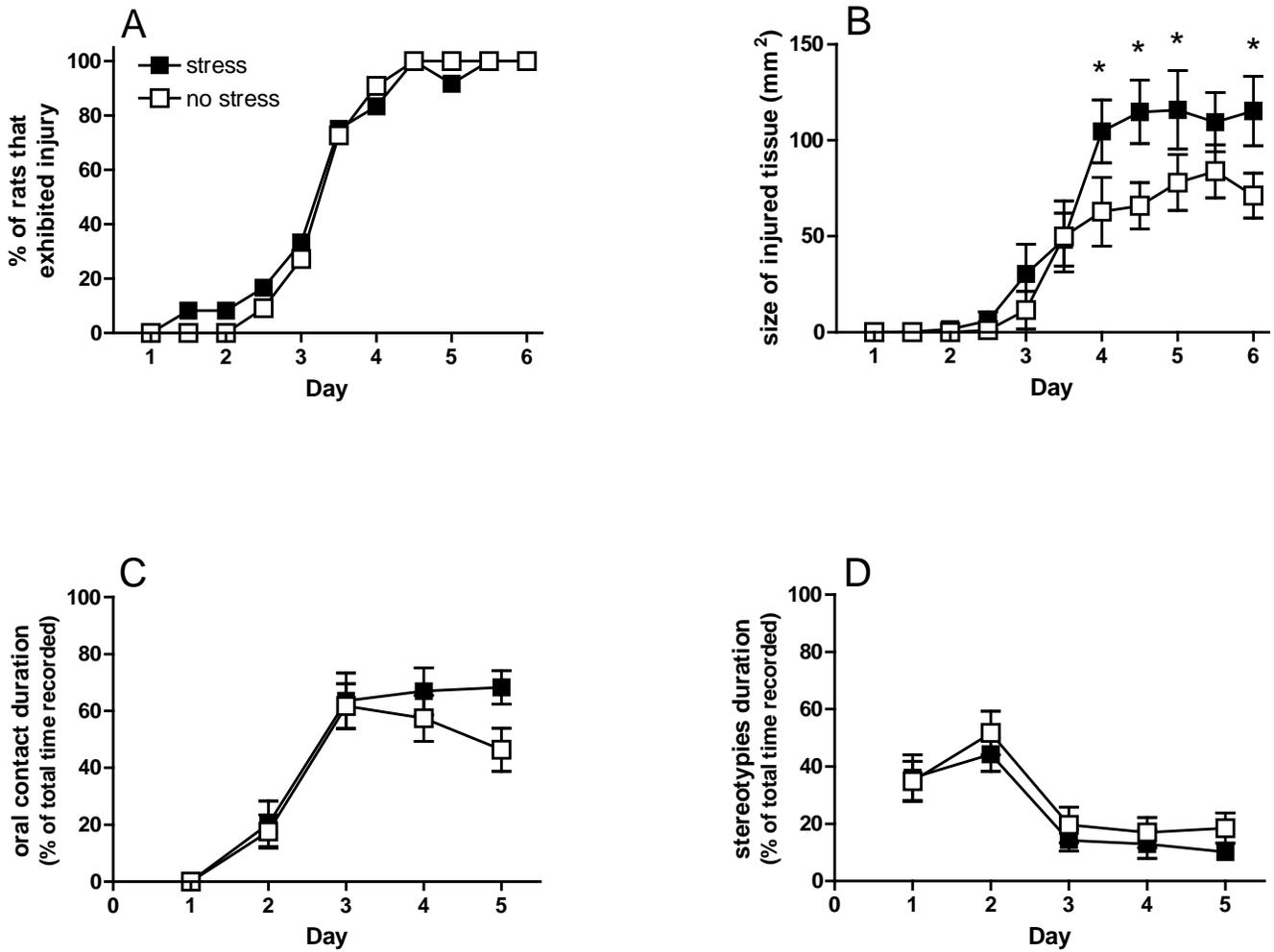


Figure 3-1. Effects of repeated social defeat stress on pemoline-induced self-injury and stereotypy. All rats exhibited pemoline-induced self-injury (A), however the rats with a history of repeated social defeat stress had larger areas of tissue damage (B). Repeated social defeat stress did not have any significant effect on pemoline-induced oral stereotypy (C) or whole body stereotypies (D). All values are expressed as group means \pm SEM. Significant differences between stressed and non-stressed rats are depicted with an asterisk.

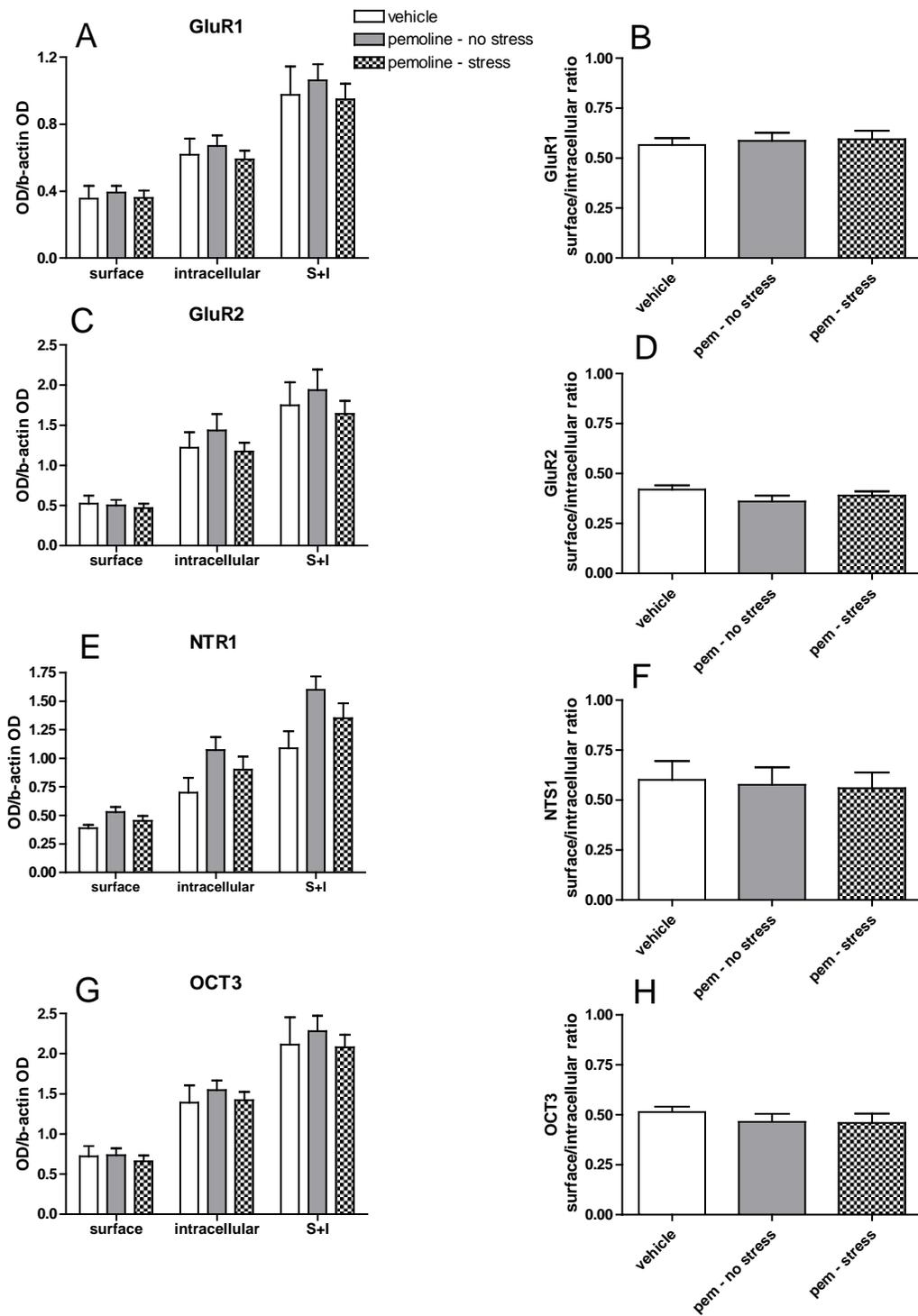


Figure 3-2. Effects of pemoline treatment and repeated stress on surface (S) and intracellular (I) levels of GluR1, GluR2, NTS1, and OCT3 in the nucleus accumbens. There were no between-groups differences in measures of surface levels, intracellular levels, total expression levels (S+I), or localization

ratios (S/I) of the AMPA receptor subunits, GluR1 (A,B) and GluR2 (C,D), NTS1 (E,F), or OCT3 (G,H). All values are expressed as group means \pm SEM.

Discussion

In this experiment we found that the dose of pemoline used caused every rat to exhibit some pemoline-induced self-injury. This incidence measure does not address the component characteristics of pemoline-induced self-biting, which were evaluated by our oral contact duration and size of injured tissue measures. Those assessments revealed that repeated social defeat stress did not increase the amount of self-injurious oral contact but did significantly increase the severity of the self-biting, which resulted in larger areas of injured tissue. The oral contact duration results (i.e. the amount of oral stereotypy) are consistent with our other finding that social defeat stress had no impact on the expression of whole body stereotypies like head bobbing and burrowing. These results suggest that the history of repeated stress increases the severity of pemoline-induced self-injurious behavior, which is consistent with reports that stressors can exacerbate self-injury in humans (Anderson and Ernst, 1994).

We utilized social defeat as our stressor because it is a potent, non-habituating, processive stress that offers clear parallels to the psychosocial stressors that humans experience each day (Huhman, 2006; Tornatzky and Miczek, 1994). Additionally, repeated social defeat alters the normal functioning of the HPA axis, which is responsible for the physiological responses to stress. Specifically, levels of the stress hormone corticosterone is dysregulated after repeated social defeat (Covington and Miczek, 2001) and this is consistent with HPA axis dysfunction in the patient populations that exhibit self-injurious behavior (Curin et al., 2003; Hall et al., 2008; Hessel et al., 2002; Sandman et al., 2008; Symons et al., 2003b; Verhoeven et al., 1999).

Behavioral sensitization has been described in relation to several psychostimulants and involves increased behavioral responsiveness with repeated injection of drug (Pierce and Kalivas, 1997). Pemoline-induced self-injury fits the criteria of the behavioral sensitization phenomenon because the initial injections of pemoline do not induce self-injurious behavior, but repeated injections result in the expression of self-injury after days of treatment. The induction and maintenance of pemoline-induced self-injury appear to be caused in part by glutamate-mediated neuroplastic changes (Muehlmann and Devine, 2008), and similar changes appear to also be responsible for behavioral sensitization triggered by cocaine and amphetamine (Wolf, 1998). Furthermore, social defeat stress cross sensitizes with cocaine and amphetamine (Covington and Miczek, 2001; Dietz et al., 2008). Our finding that stress potentiates pemoline-induced self-injury suggests that social defeat stress also cross sensitizes with pemoline, as measured by self-injury severity. Stress (Kalivas and Duffy, 1995; Rouge-Pont et al., 1993) and psychostimulants similar to pemoline (Rouge-Pont et al., 1995) cause increases in dopamine release in the nucleus accumbens, and stress (Campioni et al., 2009) and psychostimulants induce glutamate-mediated plasticity (Wolf et al., 2004) in the nucleus accumbens. This suggests that the effects on dopaminergic and glutamatergic signaling in the nucleus accumbens may mediate the cross sensitization between psychostimulants and stress (Pacchioni et al., 2007).

We investigated the expression levels of several proteins within the nucleus accumbens to evaluate their role in the potentiation of pemoline-induced self-injury by social defeat stress. We found no differences in either surface or intracellular levels of the AMPA receptor subunits, GluR1 or GluR2, in comparisons between vehicle-treated

rats, pemoline-treated non-stressed rats, and pemoline-treated stressed rats. These negative results are consistent with other behavioral sensitization experiments wherein one day after the last repeated cocaine injection there were no changes in surface or intracellular levels of GluR1 or GluR2 in the nucleus accumbens (Boudreau and Wolf, 2005; Boudreau et al., 2007). If AMPA receptor subunit expression and localization are pursued in this model in the future, it is recommended that the nucleus accumbens be separated into shell and core regions before analysis, as these subregions differ in their expression of dopamine receptors and neuropeptides (Meredith, 1999), and may reveal differential changes in response to pemoline and/or stress.

Neurotensin is a neuromodulatory peptide found throughout the brain. Neurotensin increases the release of dopamine in the nucleus accumbens, through actions in the VTA (Kalivas and Duffy, 1990) and by activating presynaptic receptors in the nucleus accumbens terminal regions that inhibit dopaminergic autoreceptor functioning (Fawaz et al., 2009). Furthermore, following stress, neurotensin levels are elevated in the VTA (Deutch et al., 1987) and neurotensin binding sites in the nucleus accumbens are increased (Xing et al., 1998). For these reasons we evaluated whether the exacerbation of pemoline-induced self-injury may be a result of changes in neurotensin neurotransmission associated with changes in NTS1 expression or localization. Our statistical tests found no significant change in NTS1 levels between vehicle controls, pemoline-treated stressed and pemoline-treated non-stressed rats. However, a t-test comparison of the vehicle controls and pemoline-treated non-stressed rats revealed a significant difference in total NTS1 expression. This suggests that changes in

neurotensin neurotransmission and receptor expression may be induced upon pemoline administration.

Finally, we evaluated the expression and localization of a monoamine transporter called OCT3. The OCT3 is a low affinity, high capacity transporter of monoamines, including dopamine, which is highly expressed throughout the striatum and nucleus accumbens (Amphoux et al., 2006; Gasser et al., 2009). There is evidence that OCT3s aid in transport of psychostimulants into the brain and partially regulate the development of behavioral sensitization and drug-induced dopamine release. More specifically, rats that develop methamphetamine-induced behavioral sensitization have lower expression of OCT3 (Kitaichi et al., 2003) and reducing OCT3 function through antisense administration or with genetic manipulations increases methamphetamine-induced elevations in dopamine levels in the nucleus accumbens and methamphetamine- and cocaine-induced locomotion (Kitaichi et al., 2005; Nakayama et al., 2007; Vialou et al., 2008). Additionally, OCT3 transport of monoamines is blocked by the stress hormone corticosterone (Gasser et al., 2006). In regards to the current experiment, we hypothesized that the exacerbation of pemoline-induced self-injury may be a result of increased dopamine release in the nucleus accumbens resulting from the cumulative effects of repeated stress and pemoline administration. Moreover, both social defeat stress and pemoline administration are associated with elevations in corticosterone levels (Covington and Miczek, 2001; Kies and Devine, 2004). Though not directly measured in this experiment, the potential concomitant rise in circulating corticosterone following repeated social defeat stress and pemoline administration might further increase dopamine levels in the nucleus accumbens by blocking transport by OCT3.

This convergence of dopamine and corticosterone involvement in pemoline-induced self-injury, and the localization of OCT3 in the nucleus accumbens (the brain region responsible for the expression of behavioral sensitization) made OCT3 an interesting target of investigation. However, we found no change in expression or localization of OCT3 in our comparisons of vehicle- and pemoline-treated groups, or pemoline-treated stressed and non-stressed groups. This suggests that changes in OCT3 expression or function are not responsible for the potentiation of pemoline-induced self-injury by repeated social defeat stress.

CHAPTER 4 INDIVIDUAL DIFFERENCES IN VULNERABILITY FOR PEMOLINE-INDUCED SELF- INJURIOUS BEHAVIOR IS PREDICTED BY STRESS RESPONSIVENESS

Background

The prevalence of self-injurious behavior differs among the disorders in which self-injurious behavior is expressed. All, or nearly all, individuals with Lesch-Nyhan syndrome self-injure (Anderson and Ernst, 1994). The incidence of self-injurious behavior in Prader-Willi syndrome is estimated around 50-80% (Hiraiwa et al., 2007; Symons et al., 1999), and the incidence of self-injury in autism is roughly 50% (Baghdadli et al., 2003). Taken together, in the disorders that exhibit symptom variability (e.g. Prader-Willi syndrome, autism) there is also variability in individual vulnerability to self-injure.

In Chapter 3 we described the effect of stress on the self-injury exhibited by humans and animals (e.g. Anderson and Ernst, 1994, Davenport et al., 2008). Findings from human self-injurers especially highlight the importance of responsiveness to stress in the expression of self-injurious behavior, though the potential that innate responsiveness to stressful stimuli may predispose individuals toward self-injury has not been explored. A very interesting rodent model of stress responsiveness elucidated individual differences in neuronal, hormonal, and behavioral responses to stress. In this model, rats that exhibit high rates of locomotor reactivity, or exploratory behavior, in a novel environment are compared to rats that exhibit low rates of locomotor reactivity. The rats that show more exploratory behavior in a novel environment are said to be high responder (or HR) rats and the rats that show less exploratory behavior in the novel environment are termed low responder (or LR) rats. The HR rats have increased and prolonged corticosterone release after a mild stress (Kabbaj et al., 2000), they show

less anxiety-like behavior in tests of anxiety (Dellu et al., 1996; Kabbaj et al., 2000), and they differ in expression levels of many gene products that regulate the HPA axis, or stress pathway, as compared to the LR rats (Kabbaj et al., 2000). Early studies of these rats also revealed that HR rats were more likely to self-administer low doses of amphetamine, which suggested that the HR rats were more vulnerable to developing drug abuse (Piazza et al., 1989).

In the initial characterization of the pemoline model (Kies and Devine, 2004) our lab described a dose-response analysis wherein incidence of pemoline-induced self-injury was related to dose. At moderate doses of pemoline some failed to show any pemoline-induced self-injury, while other rats injured quite severely. In this study we investigated the potential that individual differences in stress responsiveness predict individual vulnerability to develop self-injurious behavior in the pemoline model.

Methods

Animals

Eighteen male LE rats weighing 150-175g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in a climate controlled vivarium with a 12h/12h light/dark schedule (lights on at 7:00a.m.). Standard laboratory rat chow (Lab Diet 5001) and tap water were available *ad libitum*. The rats were pair-housed in standard polycarbonate cages (43 cm x 21.5 cm x 25.5 cm) during 6 days of acclimation to the housing facility and for eight days following circular corridor screening. The rats were then singly-housed in identical polycarbonate cages upon initiation of pemoline treatment. All of the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were pre-approved by the Institutional Animal Care and Use Committee at the University of Florida.

Drug

Pemoline (Spectrum Chemicals, New Brunswick, New Jersey) was suspended at a concentration of 50 mg/ml in warm peanut oil (held at approximately 36°C), with constant stirring.

Stress Responsiveness Screening

The novel environment of a circular corridor was used to screen for locomotor responsiveness to novelty. Circular corridors were constructed using a small plastic cylinder placed inside of a larger plastic cylinder (forming the inner and outer walls of the corridor, respectively). Both cylinders were washed with 4% bleach and standard bedding was placed on the path (7 cm wide, 44 cm outer diameter) of the circular corridor. The lights in the experimentation room were turned off and dim illumination was provided by small lamps placed at the floor beside each circular corridor. Rats were placed in this novel environment between one to two hours after lights on (i.e. between 8 and 9 a.m.). Video cameras secured to the ceiling were used to record locomotor behavior. Locomotion was scored for the first 60 minutes that the rats were in the circular corridor. A trained observer quantified locomotion by dividing the video of the corridor into quadrants. One count was recorded each time the rat crossed a line, but no further counts from that line were made until the rat crossed another line. Rats were determined to be either HRs or LRs based on whether their locomotor scores were above or below, respectively, the median of the distribution of a large sample of rats (n=336). The overall median of the entire population of rats screened in our laboratory is 223 line crossings.

Drug Treatment

An experimenter blind to the HR or LR designation of the rats weighed and injected each rat with pemoline at 150 mg/kg (s.c.) each day for ten days. These injections were administered at the nape of the neck and either flank on a rotating basis.

Assays of Self-Injury, Stereotypy, and Related Behaviors

The rats were visually inspected each time they were injected and then again every evening, and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Any rat with an open lesion was immediately euthanized.

Still images of the injured tissue were taken from the videotapes, and MCID software (Imaging Research Inc., St. Catharines, ON, Canada) was used to draw outlines around the injured tissue, and to calculate the area of injury in mm². Additionally, night-vision cameras were focused on the cages of the rats (one camera per cage), and 5-minute time samples were recorded once per hour for 8 hours each night. The duration of self-injurious oral contact, stereotypy, grooming, rearing and the amount of locomotion were quantified during each videotaped interval by a trained observer who was blind to the HR or LR designation. Self-injurious oral contact was defined as all oral contact that stayed fixed on any one body part for longer than 2 seconds. This was differentiated from grooming, which is oral contact with any part of the body that continues to move from site to site on the body (e.g. oral contact with the forepaws, that then moves up each forelimb and continues to the ventrum, and the contact is not sustained at any spot on the body for longer than 2 seconds. A second

trained but experimentally-blind observer re-scored a subset of the video-recordings to evaluate inter-observer reliability for the duration of self-injurious oral contact (one randomly-selected night of recordings for each rat).

The duration of stereotyped behaviors was also quantified from the video time samples. These behaviors included episodes of stereotyped head-bobbing, cage-licking, and digging/sniffing/burrowing through the bedding that exceeded 3 sec duration (Cromwell et al., 1999). Since individual rats exhibited substantial differences in the expression of stereotypy (e.g. some primarily exhibited head-bobbing or cage-licking, whereas others exhibited digging/sniffing/burrowing) the stereotypy scores are reported as aggregate scores that compile the total duration of all these stereotyped behaviors.

Home-cage rearing and locomotion were also scored from the video recordings. Rearing was counted each time a rat lifted both forepaws off the cage floor for at least 3 seconds. A subsequent rear was only counted after the rat resumed and maintained contact with the cage floor for at least 1 sec. Locomotion was counted by sectioning the video image of the cage into thirds (i.e. drawing lines on the television monitor dividing the cage into three equal parts along its length) and tallying the number of times the rat's forepaws crossed these lines. However, a subsequent crossing of that line was only counted after the rat crossed the other line. Thus, each locomotor count represents a minimum of 14.3 cm traversing of the cage.

On the morning of the 11th day each rat was visually inspected. After this final inspection, each rat was rapidly decapitated. Trunk blood (6 ml) was collected into chilled polypropylene tubes with 600 µl of disodium EDTA at 20 mg/ml. The tubes were centrifuged at 1000g at 4°C for 5 min. The plasma fraction was aliquotted and frozen at

-80°C. Radioimmunoassay was performed to quantify the levels of corticosterone in plasma using a kit from Diagnostic Products Corporation (Los Angeles, CA).

Additionally, after termination the thymus and adrenal glands were removed in order to assess whether pemoline administration caused differential endocrine or immune responses in the HR or LR rats.

Statistical Analyses

Between-groups differences in circular corridor line crossings, duration of self-injurious oral contact, number of injured sites, size of the injured tissue, duration of stereotypy, grooming, rearing, the amount of locomotion, and body weight were each evaluated using repeated measures analyses of variance (RM-ANOVA). Between-groups differences in all these dependent measures were treated as statistically reliable when the p-values were less than 0.05. All significant effects were further analyzed with pre-planned Fisher's least significant difference (LSD) post-tests. Between-groups differences in circular corridor totals, thymus weight, adrenal gland weight, and corticosterone levels were each analyzed by 2-tailed t-tests.

Two HR rats were euthanized before the end of the experiment because they had open lesions. In these cases, the missing data were replaced by repeating the final score that was attained for each dependent measure through the end of the experiment. This strategy was used to avoid the potential that the group means would underestimate the area of tissue damage and self-injurious oral contact scores, and to avoid the potential that the group means would over- or under-estimate the scores of the other behaviors when the most severe self-injurers were terminated.

Results

HR rats ($n = 9$) exhibited more locomotion in the circular corridor at each of the six, ten minute intervals than did the LR rats ($n = 9$; Fig. 4-1A). The RM-ANOVA revealed a significant main effect of time ($F_{(5,80)} = 68.19, p < 0.0001$), wherein all rats locomoted more at the beginning of the circular corridor screening and decreased as time passed, and a main effect of stress responsiveness ($F_{(1,80)} = 11.01, p < 0.01$). Cumulatively, HR rats had higher total rates of locomotion compared to the LR rats (Fig. 4-1B; $t(16) = 3.319, p < 0.01$).

More of the HR rats exhibited pemoline-induced self-injurious behavior compared to the LR rats (Fig. 4-2A). HR rats also spent more time self-injuring (Fig. 4-2B), had more sites of tissue injury (Fig. 4-2C), and larger total size of injured tissue (Fig. 4-2D) than did the LR rats. For the self-injurious oral contact measure, significant main effects of time ($F_{(9,144)} = 3.106, p < 0.01$) and stress responsiveness ($F_{(1,144)} = 5.354, p < 0.05$) were found as HR rats exhibited self-injurious oral contact throughout nights 3 through 10, whereas the LR rats typically did not. The HR rats also had significantly more injured sites than the LR rats did. The RM-ANOVA revealed a significant main effect of time ($F_{(10,160)} = 8.905, p < 0.01$), a significant main effect of stress responsiveness ($F_{(1,160)} = 6.805, p < 0.05$), and a significant time x stress responsiveness interaction ($F_{(10,160)} = 3.604, p < 0.001$). HR rats also displayed larger areas of tissue damage than did the LR rats. There were significant main effects of time ($F_{(10,160)} = 5.440, p < 0.0001$) and stress responsiveness ($F_{(1,160)} = 4.924, p < 0.05$) and a significant time x stress responsiveness interaction ($F_{(10,160)} = 3.147, p < 0.01$). Taken together, the HR rats exhibited a greater vulnerability to develop pemoline-induced self-injury and as a result

spent more time self-injuring and injured more tissue area and at more body sites than the LR rats did.

LR rats expressed higher rates of other pemoline-induced behaviors than HR rats did. Specifically, LR rats spent more time exhibiting pemoline-induced stereotypies than did HR rats (Fig. 4-3A). The RM-ANOVA analyses revealed a significant main effect of stress responsiveness ($F_{(1,144)} = 7.730$, $p < 0.05$), and the LSD post tests confirmed that LR rats exhibited higher rates of stereotyped behavior at the beginning and toward the end of the ten day pemoline administration period. Grooming was a low rate behavior and did not differ in duration between the HR and LR rats (Fig. 4-3B), although a significant main effect of time was found by the RM-ANOVA ($F_{(9,144)} = 2.107$, $p < 0.05$). The rate of locomotion did not differ between HR and LR rats (Fig. 4-3C). A significant main effect of time ($F_{(9,144)} = 5.048$, $p < 0.0001$) was found, wherein all rats exhibited high rates of locomotion on nights 1 and 2, and then again on nights 5 through 10. The duration of rearing behavior was not different between HR and LR rats (Fig. 4-3D), although the rates of rearing did change across days, as revealed by a significant main effect of time ($F_{(9,144)} = 5.048$, $p < 0.0001$).

The body weights of all the rats declined throughout the first four days of pemoline administration, rebounded to baseline levels by day 6, and continued to increase until the end of the experiment (Fig. 4-4A). These changes contributed to a significant main effect of time ($F_{(10,160)} = 17.83$, $p < 0.0001$) in the RM-ANOVA analysis. No significant differences in thymus (Fig. 5-4B) or adrenal gland mass (Fig. 5-4C) were found, replicating previous findings (Kies and Devine, 2004). Circulating corticosterone

levels were not different between HR and LR rats following ten days of pemoline administration (Fig. 4-4D).

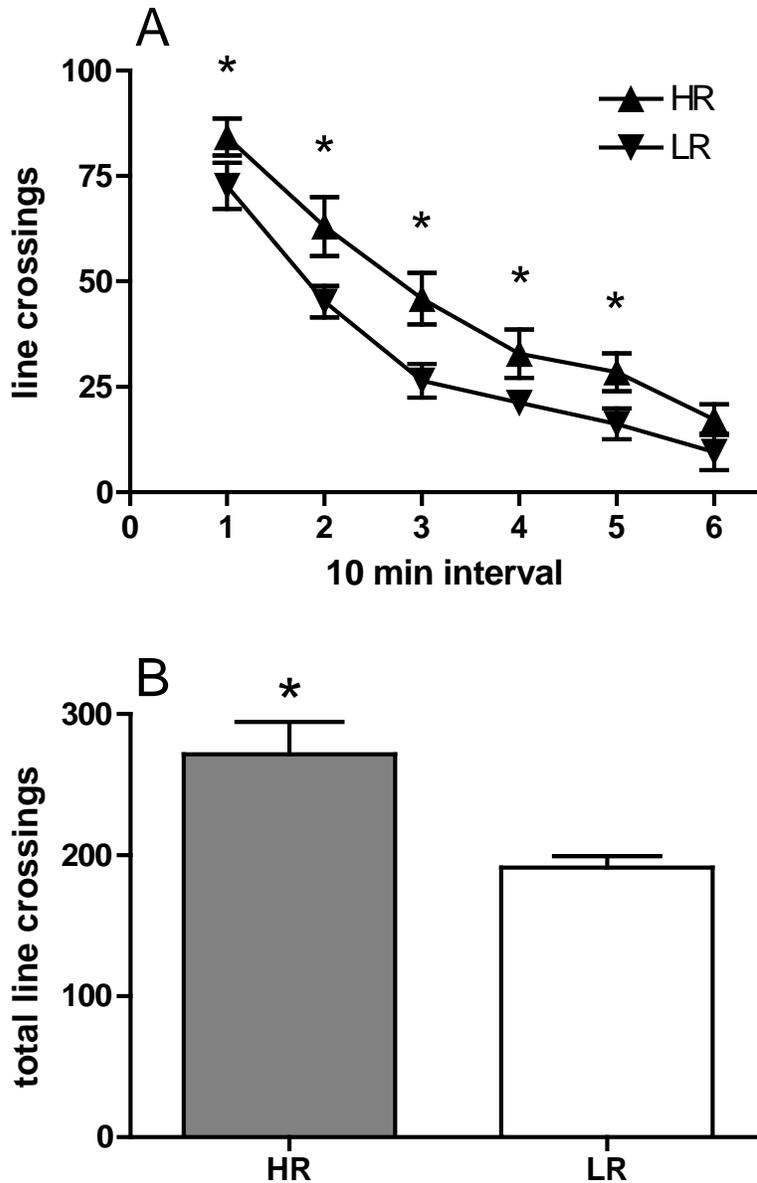


Figure 4-1. Differences between HR and LR rats in the circular corridor. HR rats exhibited more novelty-induced locomotion. Data are represented across the six, ten minute intervals (A) and as cumulative totals (B). All values expressed are group means \pm SEM. Significant differences between HR and LR rats are depicted with an asterisk.

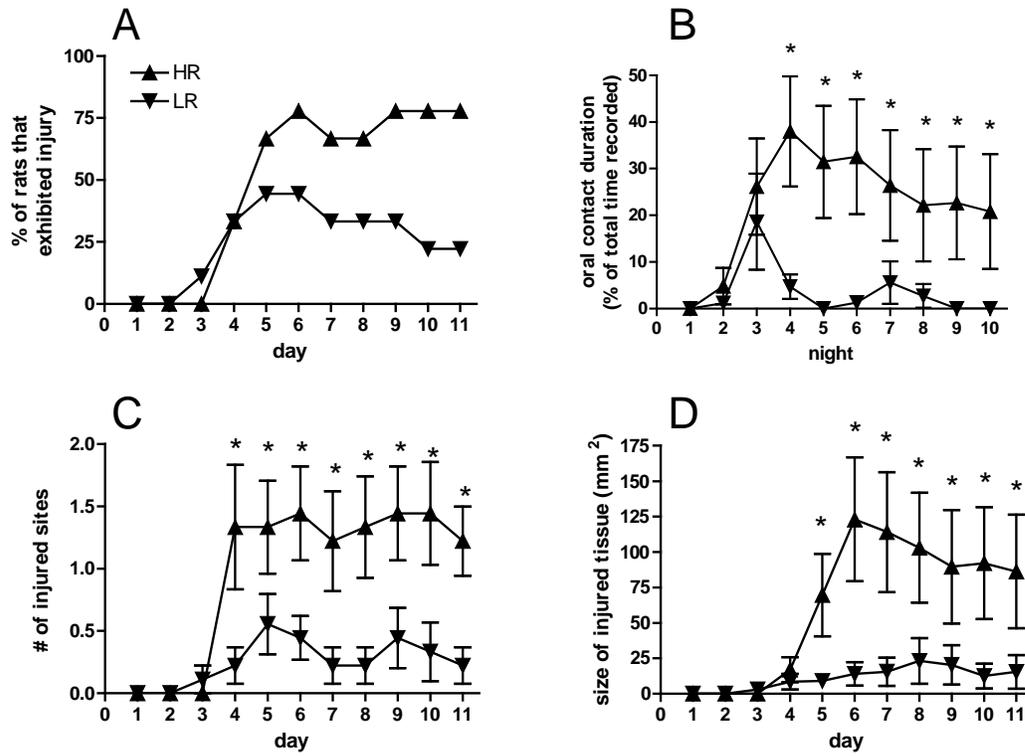


Figure 4-2. Effects of stress responsiveness on pemoline-induced self-injury. HR rats were more likely to exhibit pemoline-induced self-injury (A), spent more time injuring (B), injured more body sites (C), and had larger total area of tissue damage (D) than the low responder (LR) rats did. All values expressed are group means \pm SEM. Significant differences between HR and LR rats are depicted with an asterisk.

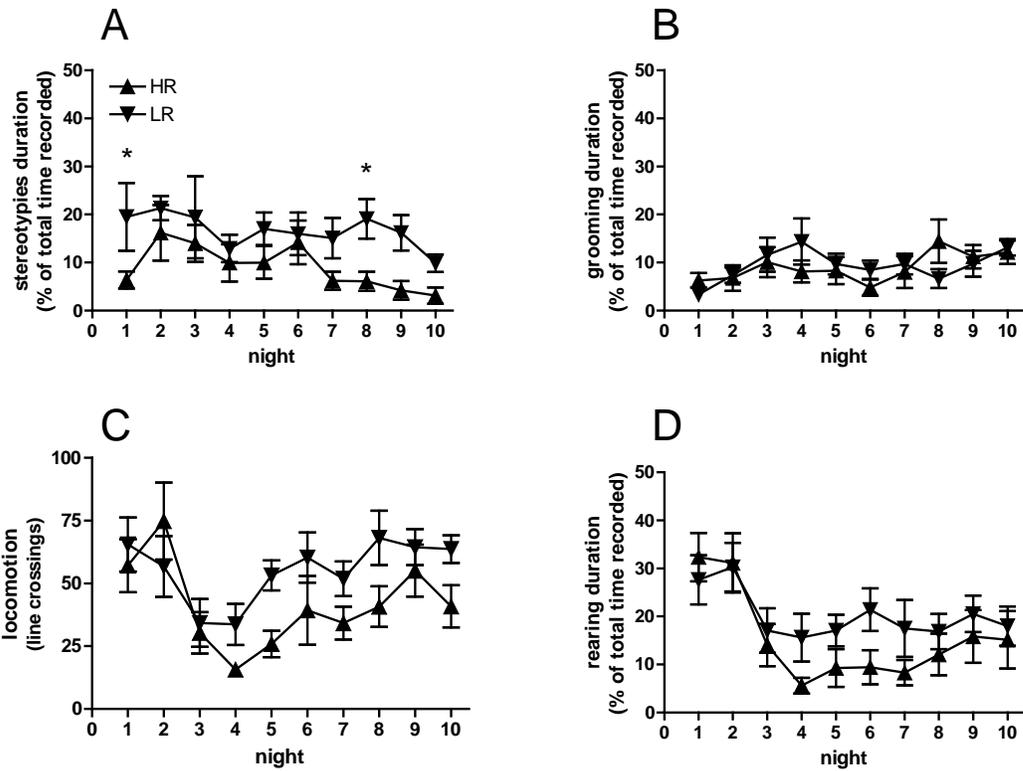


Figure 4-3. Effect of stress responsiveness on pemoline-induced stereotypy, grooming, locomotion, and rearing. LR rats exhibited more pemoline-induced stereotypy than the HR rats did (A). Duration of grooming was not different between HR and LR rats (B). LR and HR rats had equal locomotor rates (C). There were no differences between HR and LR rats on measurements of rearing duration. All values expressed are group means \pm SEM. Significant differences between HR and LR rats are depicted with an asterisk.

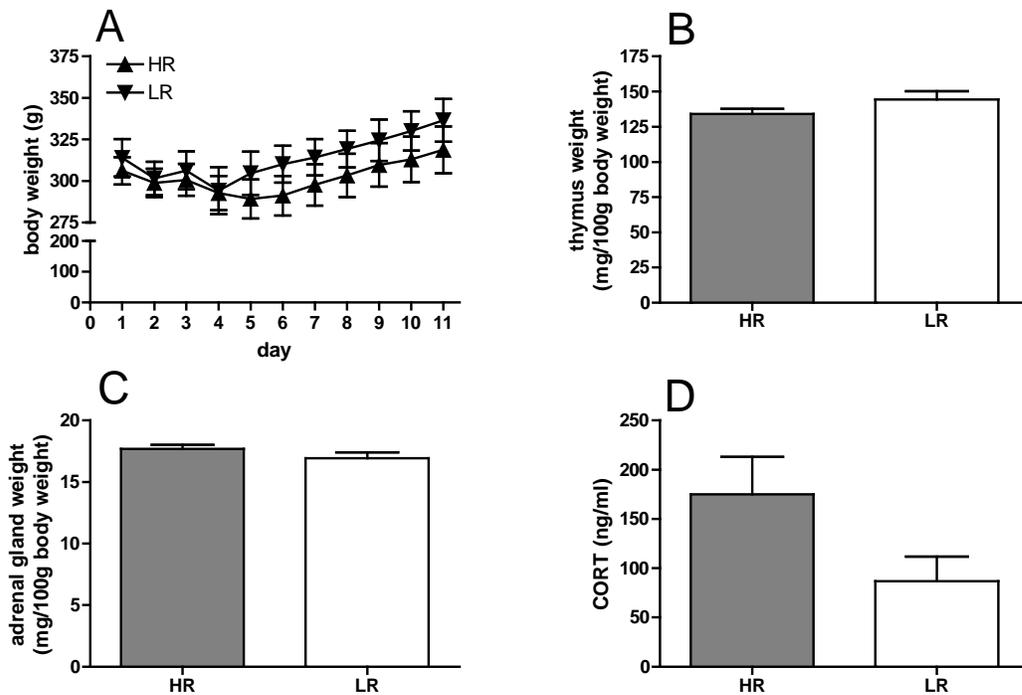


Figure 4-4. Effects of pemoline on body weight, gland mass, and corticosterone concentrations. HR and LR rats exhibited the same amount of weight loss and eventual weight gain during pemoline administration (A). There were no differences in thymus (B) or adrenal gland (C) mass between HR and LR rats. There were also no statistically significant differences in circulating corticosterone concentrations between HR and LR rats (D). All values expressed are group means \pm SEM.

Discussion

The HR/LR model has been used to elucidate differential expression of emotionality (Blanchard et al., 2009), antidepressant drug response (Jama et al., 2008), and stress-induced pathologies, including drug abuse (Piazza et al., 1989, Kabbaj et al., 2004). Since self-injurious behavior is also stress-induced or stress-responsive, we hypothesized that the individual differences in stress-responsiveness that are assayed in the HR/LR model would also predict which rats would be particularly vulnerable to pemoline-induced self-injury. We found that HR rats are more susceptible to develop pemoline-induced self-injurious behavior and in doing so exhibit longer durations of self-

injurious oral contact, more sites of injury, and larger areas of tissue damage than the LR rats do.

The body and gland weight data from the HR and LR rats confirm our previous findings that repeated pemoline administration generally does not negatively impact the health of the rats (Kies and Devine, 2004). Interestingly, we found a trend toward higher concentrations of corticosterone in the HR rats, although this did not reach statistical significance. Previous studies have shown that mild stressors cause increases in circulating corticosterone in HR rats relative to LR rats (Kabbaj et al., 2000). In light of this, there may potentially be significant differences in corticosterone levels between the HR and LR rats at the beginning of the pemoline treatment regimen that may have lessened as the repeated treatment persisted.

There are many interesting basal and drug-induced differences in dopaminergic and stress pathways between the HR and LR rats, which may explain the differential vulnerability to develop pemoline-induced self-injurious behavior. Since pemoline-induced self-injury is a product of dysregulation of dopaminergic systems (Cromwell et al., 1997), the most relevant finding is that HR rats have more basal extracellular dopamine in the nucleus accumbens compared to LR rats (Hooks et al., 1992). Dopaminergic and non-dopaminergic systems may be responsible for this differential dopaminergic tone. First, there is less tyrosine hydroxylase protein in the VTA of the HR rats (Lucas et al., 1998). Less tyrosine hydroxylase in this dopaminergic cell body region leads to less dopamine release from somatodendritic regions of the neuron, which causes less autoreceptor activation and precipitates increased dopamine release from axonal regions (i.e. the nucleus accumbens; White and Wang, 1984). Furthermore,

there is less dopamine D₂ receptor binding in the nucleus accumbens of the HR rats (Hooks et al., 1994), which further reduces autoreceptor activation and allows dopamine release to continue. Non-dopaminergic mechanisms may also be responsible for the increase in dopamine release in the nucleus accumbens of HR rats. Lower levels of cholecystokinin mRNA has been found in the substantia nigra and VTA of HR rats compared to those of LR rats (Lucas et al., 1998, Ballaz et al., 2008). Cholecystokinin in these brain regions exerts inhibitory control over dopamine release in dopaminergic projection nuclei (Xie et al., 2001). Additionally, more preproenkephalin mRNA has been found in the nucleus accumbens of HR rats compared to LR rats (Lucas et al., 1998). Nucleus accumbens to VTA enkephalinergic projections exert excitatory control over dopamine release (Gysling and Wang, 1983; Kalivas, 1993).

Basal differences in dopaminergic tone between the HR and LR rats cause differential responsiveness to psychostimulant drugs. HR rats show behavioral sensitization to low doses amphetamine, whereas LR rats do not (Dietz et al., 2005; Hooks et al., 1991a). Moreover, HR rats have an increased locomotor response to acute cocaine injection, which is associated with increased dopamine release from that cocaine administration (Hooks et al., 1991b). Our finding that HR rats are more vulnerable to develop pemoline-induced self-injury is consistent with our previous work, which suggested that pemoline-induced self-injurious behavior is also a form of behavioral sensitization that is produced through glutamate-mediated neuroplastic changes (Muehlmann and Devine, 2008).

There are also very interesting differences between HR and LR rats in the stress pathway, or HPA axis. HR rats have more corticotropin releasing hormone (CRH)

mRNA in the paraventricular nucleus of the hypothalamus compared to LR rats (Kabbaj et al., 2000). Increased CRH mRNA causes increased HPA axis activation. CRH release from the PVN causes release of ACTH from the anterior pituitary, which then stimulates release of corticosterone from the adrenal gland. Corticosterone binding to glucocorticoid receptors in the brain causes several biological reactions, the most important being negative feedback to the PVN from the hippocampus to reduce HPA activation (Herman and Cullinan, 1997). HR rats have less glucocorticoid receptor mRNA in the CA1 region of the hippocampus, which results in less negative feedback (Kabbaj et al., 2000). In summary, HR rats have increased activation and less negative feedback of the HPA axis than LR rats do. This biological variation causes increased corticosterone release in the HR rats after stress (Kabbaj et al., 2000). Corticosterone produces dopamine release in the nucleus accumbens (Piazza and LeMoal, 1996) and HR rats have increased dopamine release in the nucleus accumbens than LR rats do after stress (Dellu et al., 1996, Rouge-Pont et al., 1998) or after corticosterone injections (Piazza and LeMoal, 1996).

In summary, these findings indicate that rats with higher dopaminergic and HPA axis tone are more vulnerable to develop pemoline-induced self-injurious behavior. Very little attention has been given to dopaminergic and stress interactions in clinical populations, however using an animal model with individual vulnerability may help to elucidate specific sites of interaction between dopaminergic and stress systems that confer heightened vulnerability to develop self-injury. These results suggest that we can begin to use the HR/LR model in order to determine the basis for the genetic or epigenetic predisposition to develop pemoline-induced self-injurious behavior by

comparing HR and LR rats. Furthermore, we can also use the pemoline model to determine which pemoline-induced cellular changes are specifically responsible for causing the induction of self-injury by comparing pemoline-treated, non-injurious LR rats and pemoline-treated, self-injurious HR rats. These comparisons will undoubtedly help to unveil the currently unknown biological bases of this greatly destructive behavior disorder.

CHAPTER 5
CHARACTERIZATION OF DRUG TITERS, MONOAMINES, MONOAMINE
METABOLITES, AND AMINO ACIDS IN THE PEMOLINE MODEL OF SELF-
INJURIOUS BEHAVIOR

Background

Only a limited number of studies have investigated the effect of pemoline on catecholaminergic systems; most report some efficacy of pemoline to block reuptake of dopamine (Fuller et al., 1978) and norepinephrine (Molina and Orsingher, 1981) and to induce dopamine (Fuller et al., 1978) and norepinephrine (Gilbert et al., 1978) release. No study to date has characterized changes in the dynamics of monoamine neurotransmission in relation to the induction and maintenance of pemoline-induced self-injurious behavior. Furthermore, the role of pemoline titers in relation to induction and maintenance of self-injurious behavior with repeated pemoline administration has also not been investigated. To this end we analyzed monoamine, monoamine metabolite, and amino acids levels in different brain regions across days of repeated vehicle or pemoline administration. We also measured plasma and brain pemoline levels throughout five days of repeated pemoline treatment. Finally, as a follow up to our investigations of the role of stress in pemoline-induced self-injurious behavior, we measured the changes in corticosterone levels across days of repeated pemoline or vehicle injections.

Methods

Animals

Sixty four LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in a climate controlled vivarium with a 12h/12h light/dark schedule (lights on at 7:00a.m.). Standard laboratory rat chow (Lab Diet 5001) and tap

water were available *ad libitum*. The rats were pair-housed in standard polycarbonate cages (43 cm x 21.5 cm x 25.5 cm) during six days of acclimation to the housing facility. All of the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were pre-approved by the Institutional Animal Care and Use Committee at the University of Florida.

Drug

Pemoline (2-amino-5-phenyl-1,3-oxazol-4-one; Spectrum Chemicals, New Brunswick, New Jersey) was suspended at a concentration of 50 mg/ml in peanut oil. In order to get the pemoline into fine suspension, it was kept stirring overnight.

Drug and Vehicle Treatment

Following six days of habituation to the housing facility, the rats were separated and singly housed in cages identical to the cages they were habituated in. Each morning (approximately 8 am) the rats were weighed and injected subcutaneously with either pemoline at 150 mg/kg (n=32) or peanut oil vehicle (n=32). These injections were administered at the nape of the neck and either flank on a rotating basis.

Assays of Self-Injury

The rats were visually inspected each time they were injected and immediately before they were decapitated and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Still images of the injured tissue were taken from the videotapes, and MCID software (Imaging Research Inc., St. Catharines, ON, Canada) was used to draw outlines around the injured tissue, and to calculate the area of injury in mm².

Night-vision cameras were focused on the cages of the rats (one camera per cage), and 5-minute time samples were recorded once every three hours over the entire day. The duration of self-injurious oral contact was quantified during each videotaped interval by a trained observer. Self-injurious oral contact was defined as all oral contact that stayed fixed on any one body part for longer than 2 seconds. This was differentiated from grooming, which is oral contact with any part of the body that continues to move from site to site on the body (e.g. oral contact with the forepaws, that then moves up each forelimb and continues to the ventrum, and the contact is not sustained at any spot on the body for longer than 2 seconds). The duration (in seconds) of oral contact behavior was summed over the entire day (i.e. from the eight video samples) and divided by the total number of seconds recorded, 2400 seconds. For example, the duration measures on day 1 cover the percent of time the rat was exhibiting oral contact during the recording periods between the first injection and just prior to the second injection, thereby covering the entire 24 hours following the first injection.

Decapitation Schedule

Four pemoline-treated rats and four vehicle-treated rats were decapitated at each of the following eight time points (Table 5-1): two hours after the first injection, 12 hours after the first injection, 24 hours after the first injection (i.e. day 2), 24 hours after the second injection (i.e. day 3), 24 hours after the third injection (i.e. day 4), 12 hours after the fourth injection (i.e. middle of day 4), 24 hours after the fourth injection (i.e. day 5), and 24 hours after the fifth injection (i.e. day 6).

Group name on graphs	Experimental day the rats were decapitated	Number of injections received	Hours since last injection
1	1	1	2
1.5	1	1	12
2	2	1	24
3	3	2	24
4	4	3	24
4.5	4	4	12
5	5	4	24
6	6	5	24

Table 5-1. Decapitation schedule for pemoline- and vehicle-treated rats.

Sample Collection

The rats were rapidly decapitated and 5ml of truck blood was collected in chilled tubes containing 500ul disodium ethylenediaminetetraacetic acid (EDTA; 20ug/ul). Plasma was separated by centrifugation at 1000g for 5 min at 4°C. Brains were immediately removed and dissected on ice in to the following regions: septum, striatum, hippocampus, amygdala with overlying temporal cortex, cortex (from frontal, parietal and occipital lobes), thalamus, and ventral tegmentum (including VTA and SN). The dissection technique was adapted from Glowinski and Iversen (1966). Plasma and brain regions were frozen on dry ice and stored at -80°C until processed.

High Pressure Liquid Chromatography (HPLC) System

Our HPLC system consisted of a Waters 510 pump and a Machery-Nagel analytical column (125 x 4 mm, C18, 120A pore, 5um particle), which was protected by a guard column. An ultraviolet detector was used for pemoline analyses. Electrochemical detection was used for monoamine, metabolite, and amino acid analyses. For this configuration we added a guard cell, a dual electrode analytical cell, and a Coulochem electrochemical detector.

Pemoline Titer Analyses

The protocol for pemoline analyses was adapted from Aoyama et al. (1988). The mobile phase was 80:20 distilled water: acetonitrile, pH 5 adjusted with 15uM phosphoric acid, filtered, degassed, and pumped at a flow rate of 0.7 ml/min.

Plasma pemoline extraction

Plasma (0.1ml) was added to 0.5ml 1M carbonate buffer (pH 10) and 4ml methylene chloride. This solution was vortexed for 10 min, followed by centrifugation at 1680g for 5 min. The upper aqueous phase was removed and the organic phase was transferred to a fresh tube containing 1 ml of methylene chloride containing 1ug/ml of the internal standard, 5-methyl-5-phenylhydantoin. This solution was dried down in a Speed Vac overnight. The next morning 50ul of mobile phase was added to the tube, heated at 70°C for 30 min, centrifuged at 1000g for 2 min, sonicated for 5 min, and centrifuged again at 1000g for 2 min. 40ul of sample was then immediately injected in to the HPLC system.

Brain pemoline extraction

Brain regions were weighed and sonicated in 2 volumes (using the formula, 1mg = 0.84ul) 0.05M ice cold perchloric acid. Whole homogenate (0.1ml) was transferred to a fresh tube and extracted like the plasma was. After the mobile phase was added to the dried down pemoline pellet, sonicated, and centrifuged at 1000g, the supernatant was transferred to a fresh tube and centrifuged at 15,300g for 5 min to remove any remaining particulates from the sample. 40ul of sample was injected in to the HPLC system.

Pemoline standards

Pemoline standards were made up in methanol (10, 20, 50, 200, 1000, 2000, 4000, and 10000 ng/ml). Samples (0.1ml) of each were dried down in the Speed Vac. Either 0.1ml blank plasma or 0.1ml blank brain homogenate was added to the dried down standard and extracted as described above.

Ultraviolet detection

The ultraviolet detector was set at 215nm for pemoline and internal standard detection.

Corticosterone Radioimmunoassay

The corticosterone radioimmunoassay was purchased from Diagnostic Products Corporation.

Monoamine Analyses

The protocol used for measuring brain monoamines (norepinephrine (NE), dopamine (DA), and serotonin (5-HT) and their metabolites (HVA, DOPAC, and 5-HIAA) was adapted from Saito et al. (1992). Mobile phase consisted of 0.02M sodium acetate, 12.5mM citric acid, 10% (v/v) methanol, 0.042% heptanesulfonic acid, and 0.01mM disodium EDTA, pH 2.9. The mobile phase was filtered through 0.45um filter, degassed, and pumped at 2.0 ml/min.

Brain homogenate samples prepared for the pemoline analyses were centrifuged at 12,000g for 15 min at 4°C. Supernatant was removed, added to 0.45um filter, and centrifuged at 20,000g for 10 min at 4°C. 40ul of this filtered supernatant was injected in to the HPLC system.

Monoamine and metabolite standards

Monoamine and metabolite standards (3.91, 7.8, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/ml) were made in 0.1M perchloric acid containing 0.1mM disodium EDTA. Standards were stored at -80°C.

Electrochemical detection of monoamines and metabolites

For monoamine and metabolite analyses the guard cell was held at +550mV, cell 1 of the analytic cell was held at -100mV, 10nA, 1V output, and cell 2 was held at +450mV, 5uA for all analytes except for serotonin, which was analyzed at 2uA, with 1V output and a 5sec filter. For amino acids analyses, the guard cell was held at +700mV, cell 1 was held at +400mV, and cell 2 was held at +600mV.

Amino Acid Analyses

The protocol for analyzing brain amino acid concentrations (glutamate and GABA) was adapted from Murai et al. (1992). The mobile phase consisted of 0.05M NaH_2PO_4 , 10% methanol, 9% tetrahydrofuran, and 0.1mM disodium EDTA, pH 6.0. The mobile phase was filtered, degassed, and pumped at 1.2ml/min.

Amino acid derivatization

In order to be analyzed by HPLC, amino acids were derivatized using o-phthalaldehyde (OPA) and beta-mercaptoethanol. The prepared OPA/beta-mercaptoethanol stock solution was diluted each day of HPLC analyses. The stock solution was made with 20mg of OPA, dissolved in 1ml of ethanol. 30ul of beta-mercaptoethanol and 9ml of 0.1M sodium tetraborate (pH 9.1) was added. OPA/beta-mercaptoethanol stock solution was stored at 4°C. Each day 1ml of stock solution was diluted with 9ml of 0.1M sodium tetraborate to make the working solution.

Ninety seconds before injection in to the HPLC system, 5ul of brain supernatant sample and 45ul of the OPA/beta-mercaptoethanol working solution were combined and vortexed. 40ul of this sample was injected in to the HPLC system.

Amino acid standards

Stock amino acid standards (1mg/ml) were prepared in 50% methanol and stored at 4°C for one month. Working standards (3.91, 7.8, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/ml) were diluted in 0.05M perchloric acid. Working standards were stored at 4°C for five days.

Electrochemical detection of amino acids

For amino acids analyses, the guard cell was held at +700mV, cell 1 was held at +400mV, and cell 2 was held at +600mV.

Protein Content Analyses

Protein content was analyzed by the Bradford method for each of the dissected brain regions.

Pemoline, Monoamine, Metabolite, and Amino Acid Concentration Analyses

Area under the peak for each analyte was measured using Logger Pro software. For pemoline analyses this area measurement was divided by that of the internal standard. Concentration values were interpolated using the standard curve produced for each analyte and each sample type (e.g. plasma, brain regions). Concentration values were then corrected for protein content of each sample. The biosynthesis ratios of the monoamine were analyzed by calculating DA+DOPAC, DA+HVA, 5-HT+5-HIAA. The turnover ratios of the monoamines were analyzed by calculating DOPAC/DA, HVA/DA, 5-HIAA/5-HT (Commissiong, 1985; Miura et al., 2002).

Statistical Analyses

Measures of self-injury (size of injured tissue and duration of oral contact) were analyzed by two-way ANOVA. For pemoline analyses, between-groups comparisons of measures from rats killed at the two times on days 1 and 4 were analyzed by t-test. For an analysis of plasma pemoline across days 2-6 a one-way ANOVA was used. For comparisons between striatum and cortex pemoline levels, a two-way ANOVA was used with Bonferroni post tests. For the corticosterone analyses, day 1 levels were compared between groups using a two-way ANOVA; a two-way ANOVA was also used for comparisons between pemoline- and vehicle-treated groups across days 2 through 6; and a t-test was used to compare corticosterone levels between the pemoline and vehicle groups for the one time point 12 hours after the fourth injection. Monoamine, metabolite, and amino acid levels were analyzed by 2-way ANOVAs, as were the biosynthesis and turnover ratios

Results

Pemoline-Induced Self-Injurious Behavior

Some of the pemoline-treated rats began to exhibit self-injury on day 3 of the experiment. All rats that received 5 daily injections of 150mg/kg/day pemoline exhibited self-injurious behavior (Fig. 5-1A), which is consistent with our previous studies (Chapters 2 and 3). The size of injured tissue (Fig. 5-1C) and oral contact duration (Fig. 5-1E) measures are slightly lower than the measures from our previous experiments, but this is most likely caused by the small numbers of rats that were included at each time point. Two-way ANOVAs revealed significant main effects of drug ($F_{(1,36)} = 13.64$, $p < 0.001$), time ($F_{(5,36)} = 4.812$, $p < 0.01$), and a significant drug x time interaction ($F_{(5,36)} = 4.812$, $p < 0.01$) for size of injured tissue and for oral contact duration (drug: $F_{(1,36)} =$

29.47, $p < 0.0001$; time: $F_{(5,36)} = 5.193$, $p < 0.01$; drug x time interaction: $F_{(5,36)} = 5.193$, $p < 0.01$).

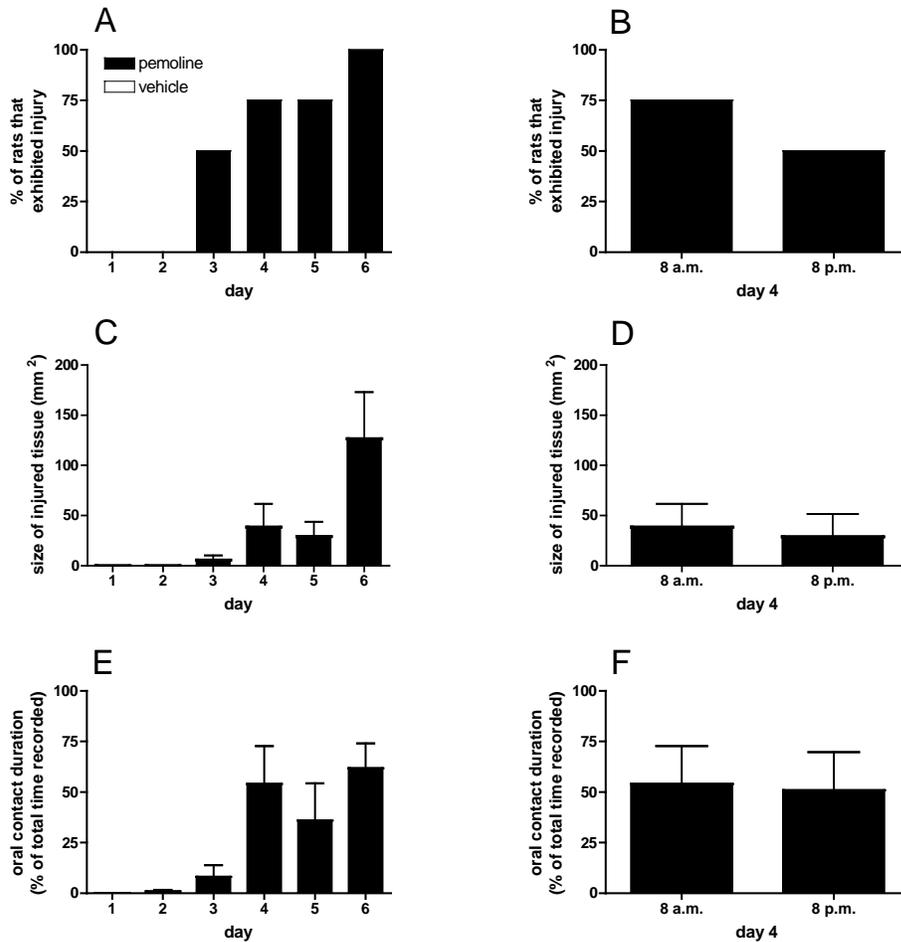


Figure 5-1. Pemoline induced self-injurious behavior. Discontinuous data of the incidence (A,B), size (C,D), and duration (E,F) of self-injury in the rats decapitated on the respective day is represented on the x-axis. Data from the group of rats that were terminated 12 hours after the 4th injection (i.e. at 8 p.m.) are shown separately (B, D, F). Data from rats killed at 8 a.m. on day 4 are included in graphs B, D, F for comparison. Data points represent the data for all rats that were terminated in the experiment on the day represented on the x-axis. All values are expressed as group means \pm SEM.

Pemoline Analyses

No chromatogram peaks were seen at the time pemoline was to be resolved from the column in any of the analyses of vehicle-treated rats, therefore all scores from

vehicle-treated rats are zero. Plasma and brain pemoline titers followed a similar pattern across days (Fig. 5-2B,E). Anecdotally, both plasma and brain pemoline levels were higher two hours after the first injection than they were twelve hours post injection (Fig. 5-2A,D), though neither analyses (t-test in plasma comparisons, two-way ANOVA in comparisons of brain regions) reached statistical significance. By 24 hours following the first injection (the day 2 time point) pemoline was still present in plasma and brain. At this time the rats that continued in the experiment received another pemoline injection, meaning that pemoline is still on board when the rats receive their next pemoline injection. This remains true for the injections given after the second, third, and fourth injections as well (Fig. 5-2B,E). A one-way ANOVA revealed significant differences in pemoline levels between the groups killed on days 2 through 6 ($F_{(4,19)} = 12.79$, $p < 0.0001$), which indicates a significant change in plasma pemoline levels across days. A two-way ANOVA revealed a significant main effect of time in the brain pemoline analyses ($F_{(4,29)} = 7.612$, $p < 0.001$). There were no significant effects found for region or region x time interaction. For analyses 12 hours after the fourth injection (Fig. 5-2C,F), only the pemoline levels in the cortex were significantly greater 12 hours after injection, compared to 24 hours after the previous injection.

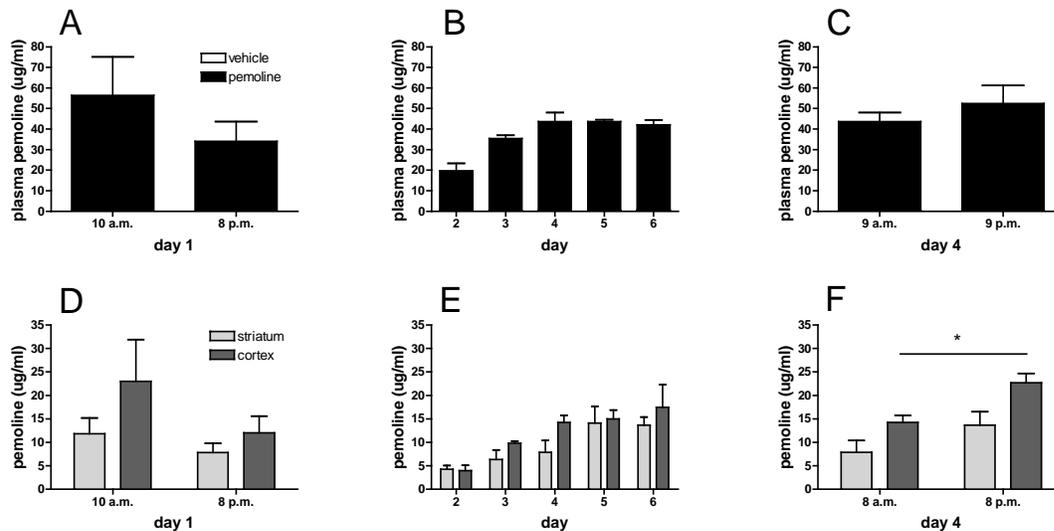


Figure 5-2. Plasma and brain pemoline concentrations across days of pemoline administration. Plasma pemoline levels change drastically over the course of the day following the first pemoline injection (A), and titers remain high as repeated daily pemoline administrations continue (B). This is also the case for striatal and cortical pemoline concentrations (D,E). Twelve hours following the fourth injection, pemoline levels are elevated in plasma (C), striatum (F), and cortex (F), though differences from pre-injection on day 4 only reach statistical significance in the cortex measures. No pemoline-associated peaks were seen on the chromatograms from vehicle-treated rats. All values are expressed as group means \pm SEM. Significant differences are depicted with an asterisk.

Corticosterone Analysis

Two hours following the first injection (the 10 a.m. time point on day 1), corticosterone levels were significantly elevated in pemoline-treated rats compared to those of vehicle-treated rats (Fig. 5-3A). Conversely, near the zenith of the circadian cycle on day 1 (the 8 p.m. time point), corticosterone levels were blunted in the pemoline-treated rats, while the vehicle-treated rats showed the normal corticosterone peak (Fig. 5-3A). A two-way ANOVA for day 1 corticosterone levels revealed a significant main effect of time ($F_{(1,12)} = 5.671, p < 0.05$) and a significant drug \times time interaction ($F_{(1,12)} = 5.583, p < 0.05$), but no significant main effect of drug. This

suggests that the on-going elevation of corticosterone by pemoline caused a blunting of the diurnal corticosterone rhythm on day 1. At most morning time points analyzed, pemoline-treated rats had higher circulating corticosterone concentrations relative to those of vehicle-treated rats (Fig. 5-3B). This was revealed by the two-way ANOVA which found a significant drug effect ($F_{(1,30)} = 8.179$, $p < 0.01$), but there were no significant main effects of time or drug x time interaction, and Bonferroni posttests did not reveal a significant difference between the pemoline and vehicle groups at any particular time point. There was also no significant difference of corticosterone levels between the pemoline and vehicle groups 12 hours following the fourth injection (Fig. 5-3C), which suggests that the normal circadian rhythmicity of corticosterone may be restored in the pemoline-treated group by this time.

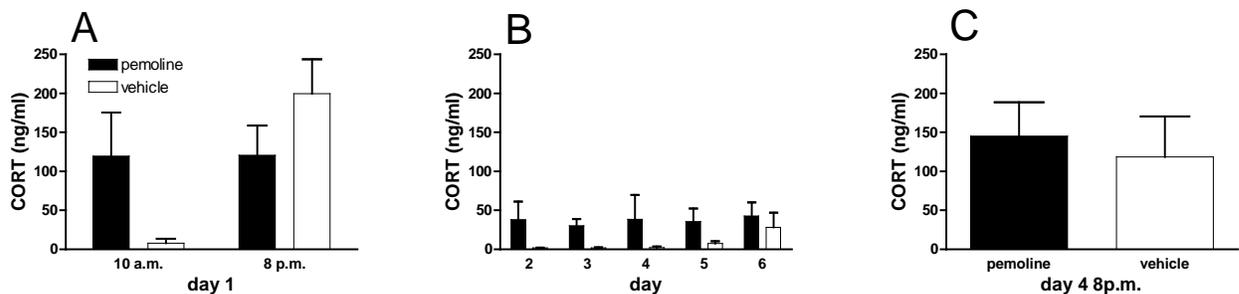


Figure 5-3. Effects of pemoline or vehicle injections on plasma corticosterone. Two hours following the first injection, corticosterone levels are elevated in the pemoline-treated rats, though these levels are later blunted during the peak of the daily circadian cycle (A). Circulating corticosterone levels at the nadir of the daily cycle are also slightly raised in pemoline-treated rats, compared to vehicle-treated rats (B). Circadian rhythmicity is re-established by the fourth day of injections as there are no longer differences between peak corticosterone levels in pemoline- and vehicle-treated rats. All values are expressed as group means \pm SEM.

Monoamine, Metabolite, and Amino Acid Analyses

Data from the groups that were killed 12 hours after the injections on days 1 and 4 were left out of the graphs and analyses for the monoamine, metabolite, and amino acid measures. To focus specifically on stable pemoline-induced changes in these measures, we focused on the samples that were taken at approximately the same time each morning (8-10am).

Striatum

The striatum consists mostly of GABAergic medium spiny neurons that receive dopaminergic and glutamatergic inputs. In this experiment we found that as pemoline-induced self-injury developed across days, intracellular dopamine levels in the striatum began to deplete ($F_{(1,33)} = 4.623$, $p < 0.05$; Fig. 5-4A) and the extracellular dopamine metabolite, HVA, increased ($F_{(1,33)} = 25.77$, $p < 0.0001$; Fig. 5-4B). There were no changes in the levels of the intracellular dopamine metabolite, DOPAC (Fig. 5-4C). These results suggest that pemoline causes significant increases in extracellular dopamine in the striatum, either by blocking reuptake, increasing release, or both, which is subsequently metabolized. Pemoline did not have any significant effect on dopamine synthesis (i.e. measures of DA+HVA and DA+DOPAC; Fig. 5-5A,B), but turnover of extracellular dopamine was significantly enhanced (HVA/DA, $F_{(1,33)} = 44.38$, $p < 0.0001$; Fig. 5-5C), whereas no changes in intracellular dopamine turnover were found (DOPAC/DA; Fig. 5-5D). We also found some reductions in serotonin levels (Fig. 5-4D), though these did not reach statistical significance. Striatal 5-HIAA was higher in pemoline-treated rats starting on day 2 and continuing throughout the experiment ($F_{(1,33)} = 27.77$, $p < 0.0001$; Fig. 5-4E). The synthesis of striatal serotonin was not different between the pemoline- and vehicle-treated groups (Fig. 5-5E), though serotonergic

turnover was significantly increased in the pemoline-treated group ($F_{(1,33)} = 92.31$, $p < 0.0001$; Fig. 5-5F). Amino acids analyses revealed no differences in striatal glutamate levels between pemoline- and vehicle-treated rats (Fig. 5-6A), but significant increases in GABA levels were found ($F_{(1,35)} = 7.312$, $p < 0.05$, Fig. 5-6B).

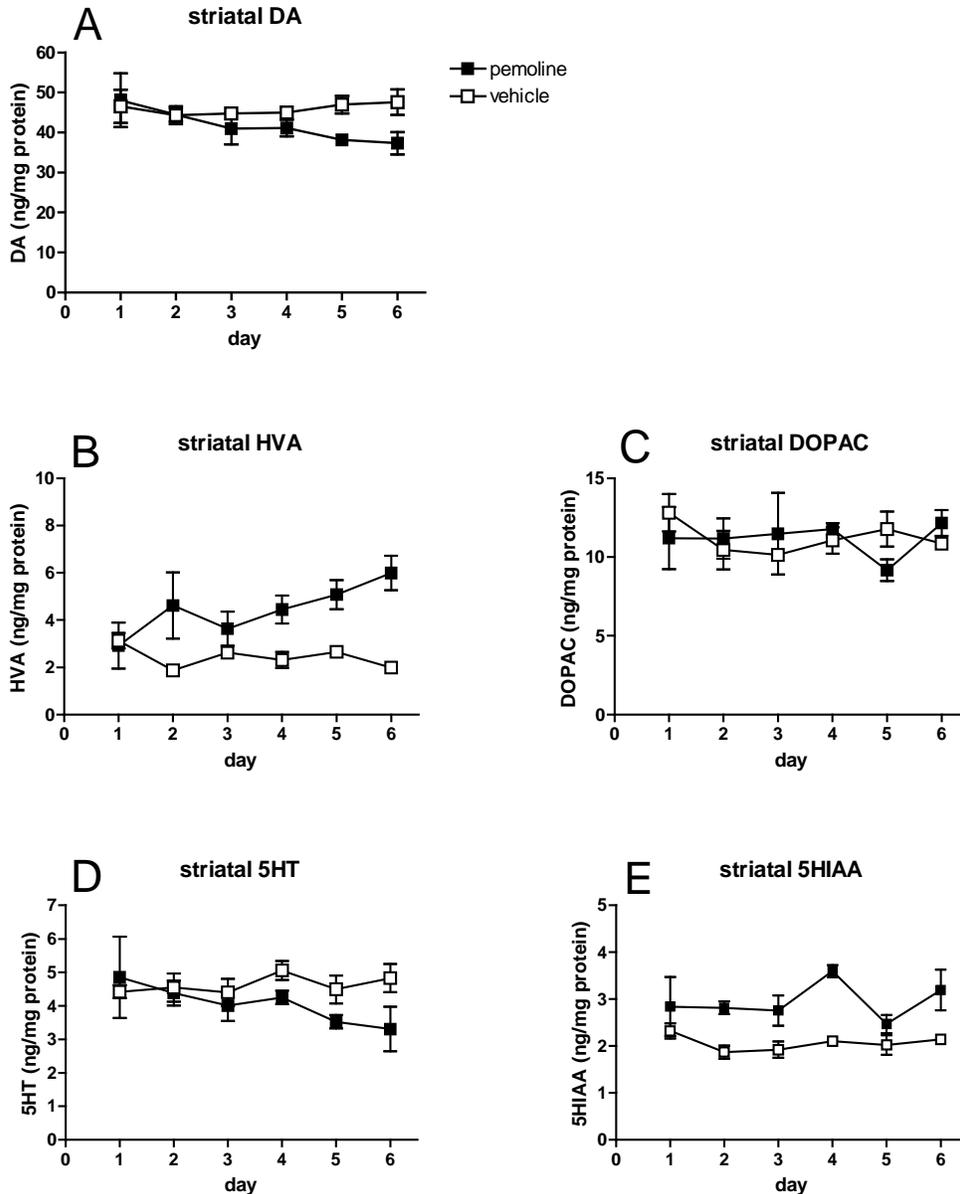


Figure 5-4. Effects of pemoline or vehicle injections on striatal monoamine and metabolite levels. Intracellular DA levels were reduced (A) as the extracellular

metabolite of DA, HVA, was increased (B) following repeated pemoline injections. No changes in the intracellular DA metabolite, DOPAC (C), or in 5-HT levels were found. Levels of the 5-HT metabolite (5-HIAA) was also significantly elevated (E) in pemoline-treated rats, compared to those of vehicle-treated rats. All values are expressed as group means \pm SEM.

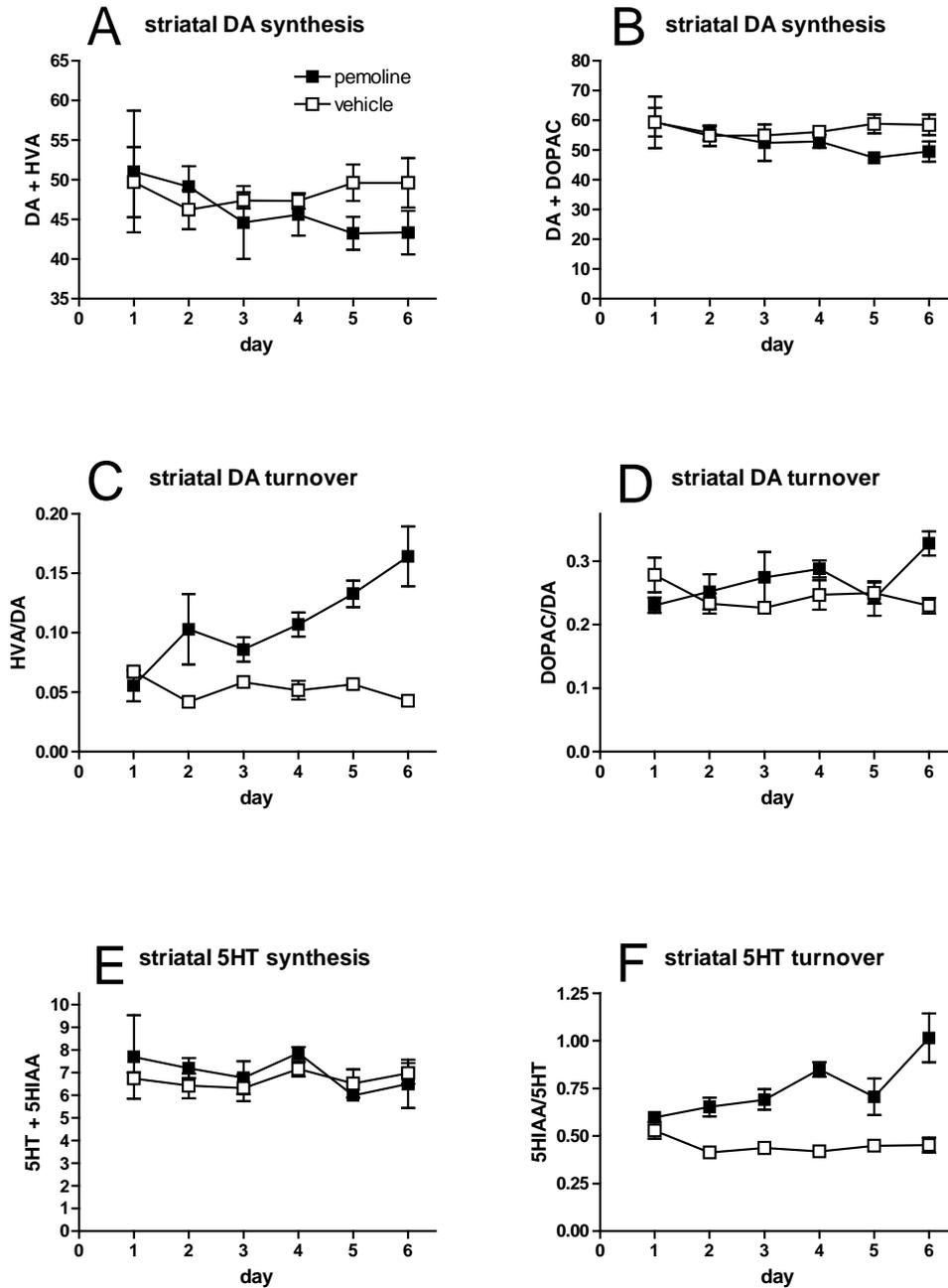


Figure 5-5. Effects of pemoline or vehicle injections on striatal monoamine synthesis and turnover ratios. There were no differences in dopamine synthesis rates in

pemoline- or vehicle-treated rats (A,B). Dopamine turnover, as quantified by HVA/DA, was significantly increased in pemoline-treated rats (C), whereas DOPAC/DA was not (D). Serotonin synthesis was not different between pemoline- and vehicle-treated groups (E), however turnover of 5-HT was increased in pemoline-treated rats (F). All values are expressed as group means \pm SEM.

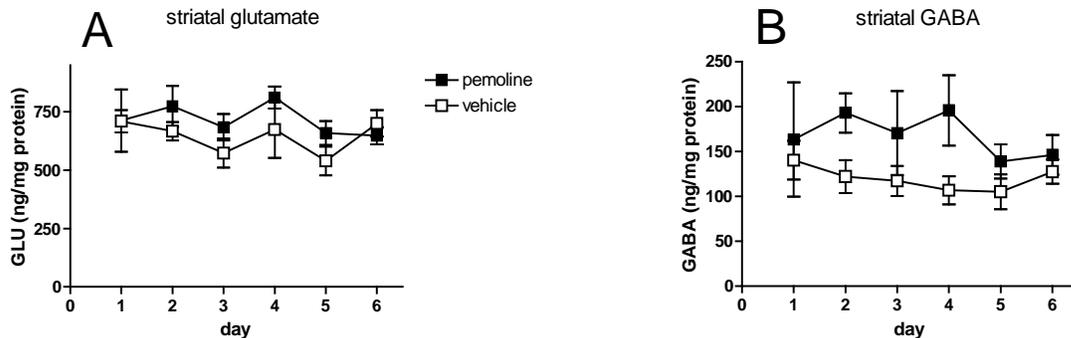


Figure 5-6. Effects of pemoline or vehicle injections on striatal amino acid levels. No differences in glutamate concentrations were found between pemoline- and vehicle-treated rats (A), however pemoline-treated rats had significantly elevated GABA concentrations compared to those of vehicle-treated rats (B). All values are expressed as group means \pm SEM.

Ventral tegmentum

We analyzed the monoamine and amino acid concentrations in the ventral tegmentum because it contains the cell bodies (in the VTA and SN) for the two main dopaminergic projections, the nigrostriatal and mesolimbic pathways. These nuclei also contain GABAergic cell bodies and receive glutamatergic projections. Our analyses revealed no significant changes in ventral tegmental norepinephrine (Fig. 5-7A), dopamine (Fig. 5-7B), serotonin (Fig. 5-7E), glutamate (Fig. 5-9A), or GABA (Fig. 5-9B) levels in pemoline-treated rats as compared to those of vehicle-treated rats. There were also no significant changes in the dopamine metabolite concentrations (HVA, Fig. 5-7C; DOPAC, Fig. 5-7D) in pemoline-treated rats, and no differences in any measure of dopamine synthesis (Fig. 5-8A,B) or turnover (Fig. 5-8C,D). 5-HIAA levels were

significantly elevated in pemoline-treated rats, compared to vehicle-treated rats ($F_{(1,36)} = 20.87, p < 0.0001$; Fig. 5-7F) and this corresponded to significant increases in serotonin synthesis ($F_{(1,36)} = 8.669, p < 0.01$; Fig. 5-8E) and turnover ratios ($F_{(1,36)} = 76.28, p < 0.0001$; Fig. 5-8F).

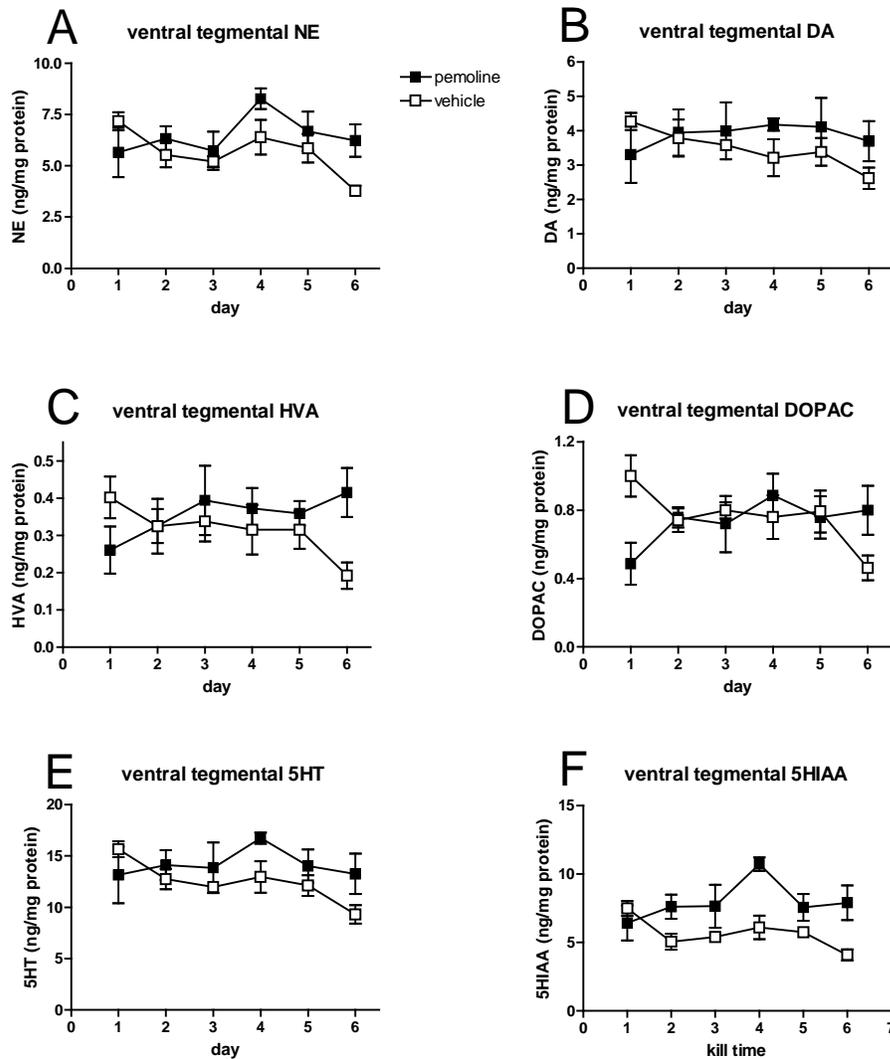


Figure 5-7. Effects of pemoline or vehicle injections on ventral tegmental monoamine and metabolite levels. There were no pemoline-associated changes in ventral tegmental monoamine concentrations, including NE (A), DA (B), or 5-HT (E). There were also no differences in dopamine metabolites, HVA (C) or DOPAC (D). Ventral tegmental 5-HIAA, however, was significantly higher in pemoline-

versus vehicle-treated rats (F). All values are expressed as group means \pm SEM.

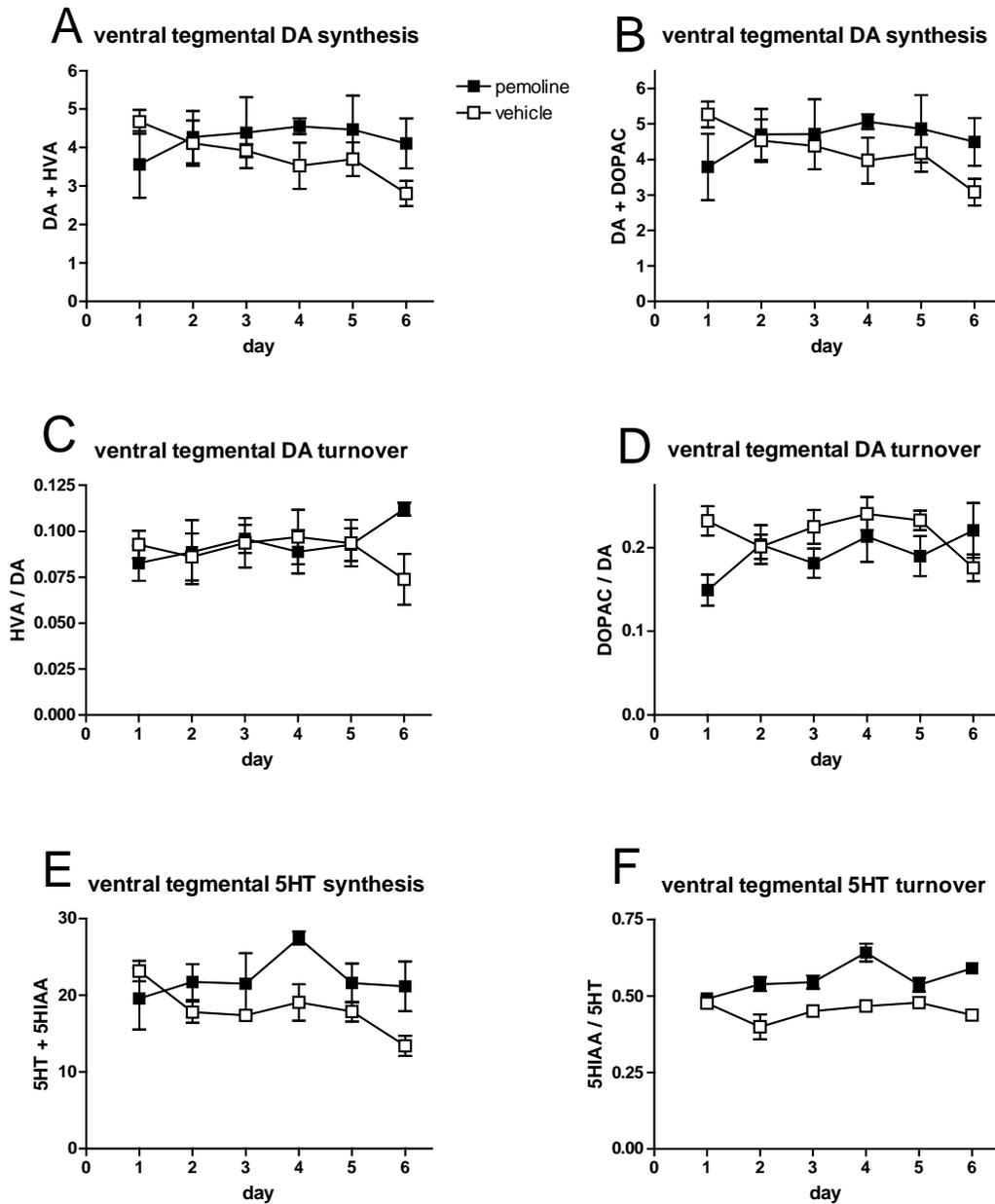


Figure 5-8. Effects of pemoline or vehicle injections on ventral tegmental monoamine synthesis and turnover ratios. Pemoline did not significantly alter dopamine synthesis (A,B) or turnover (C,D). 5-HT synthesis and turnover ratios, however, were significantly increased in pemoline-treated rats compared to the ratios in vehicle-treated rats. All values are expressed as group means \pm SEM.

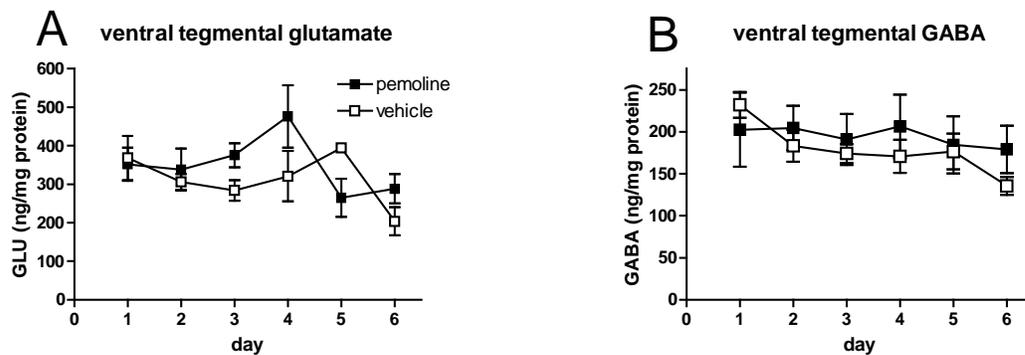


Figure 5-9. Effects of pemoline or vehicle injections on ventral tegmental amino acid levels. No differences in either glutamate (A) or GABA (B) concentrations were found between pemoline- and vehicle-treated rats. All values are expressed as group means \pm SEM.

Cortices

Cortical regions receive inputs from the dopaminergic mesocortical pathway, as well as from GABAergic and glutamatergic projections. The cortex also contains the glutamatergic cell body regions that project to limbic and basal ganglia structures. For these reasons we analyzed the cortical content of monoamines and amino acids. We found no differences between pemoline- and vehicle-treated rats in measures of cortical dopamine (Fig. 5-10A), serotonin (Fig. 5-10D), glutamate (Fig. 5-12A), or GABA (Fig. 5-12B). There were also no changes in DOPAC (Fig. 5-10C) or 5-HIAA (Fig. 5-10E) levels in the cortical samples from pemoline-treated rats. Cortical HVA was significantly higher in pemoline-treated rats ($F_{(1, 36)} = 11.98$, $p < 0.01$; Fig. 5-10B), which contributed to a significant difference in cortical HVA/DA turnover ratio ($F_{(1,36)} = 21.05$, $p < 0.0001$; Fig. 5-11C). There were no differences in dopamine (Fig. 5-11A,B) or serotonin (Fig. 5-11E) synthesis ratios, or in DOPAC/DA (Fig. 5-11D) or 5-HIAA/5-HT (Fig. 5-11F) turnover ratios.

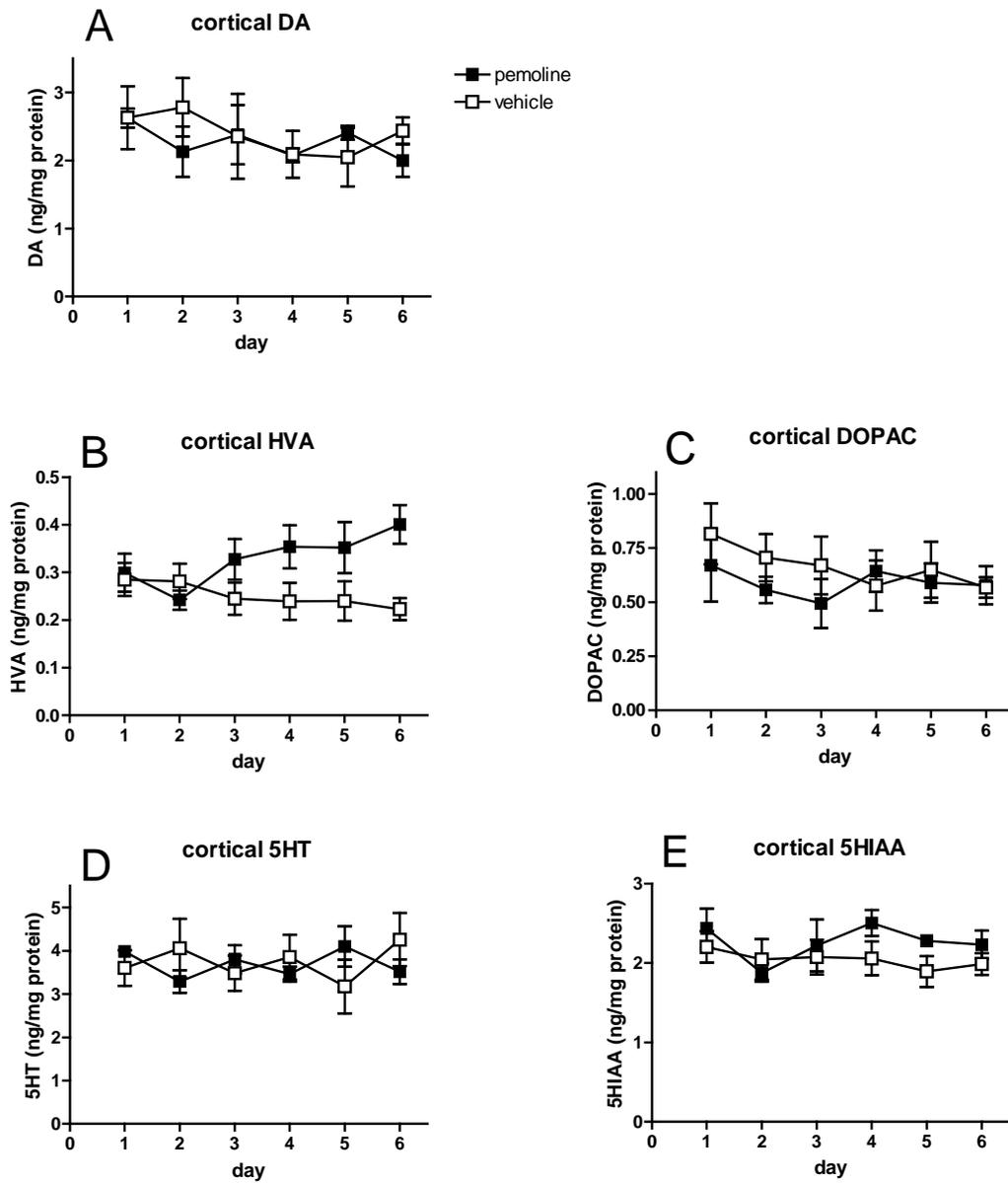


Figure 5-10. Effects of pemoline or vehicle injections on cortical monoamine and metabolite levels. There were no pemoline-associated changes in cortical DA (A) or 5-HT (D) concentrations. Cortical HVA levels were increased in pemoline-treated rats, compared to those of vehicle-treated rats (B). No significant differences in DOPAC (C) or 5-HIAA (E) were found. All values are expressed as group means \pm SEM.

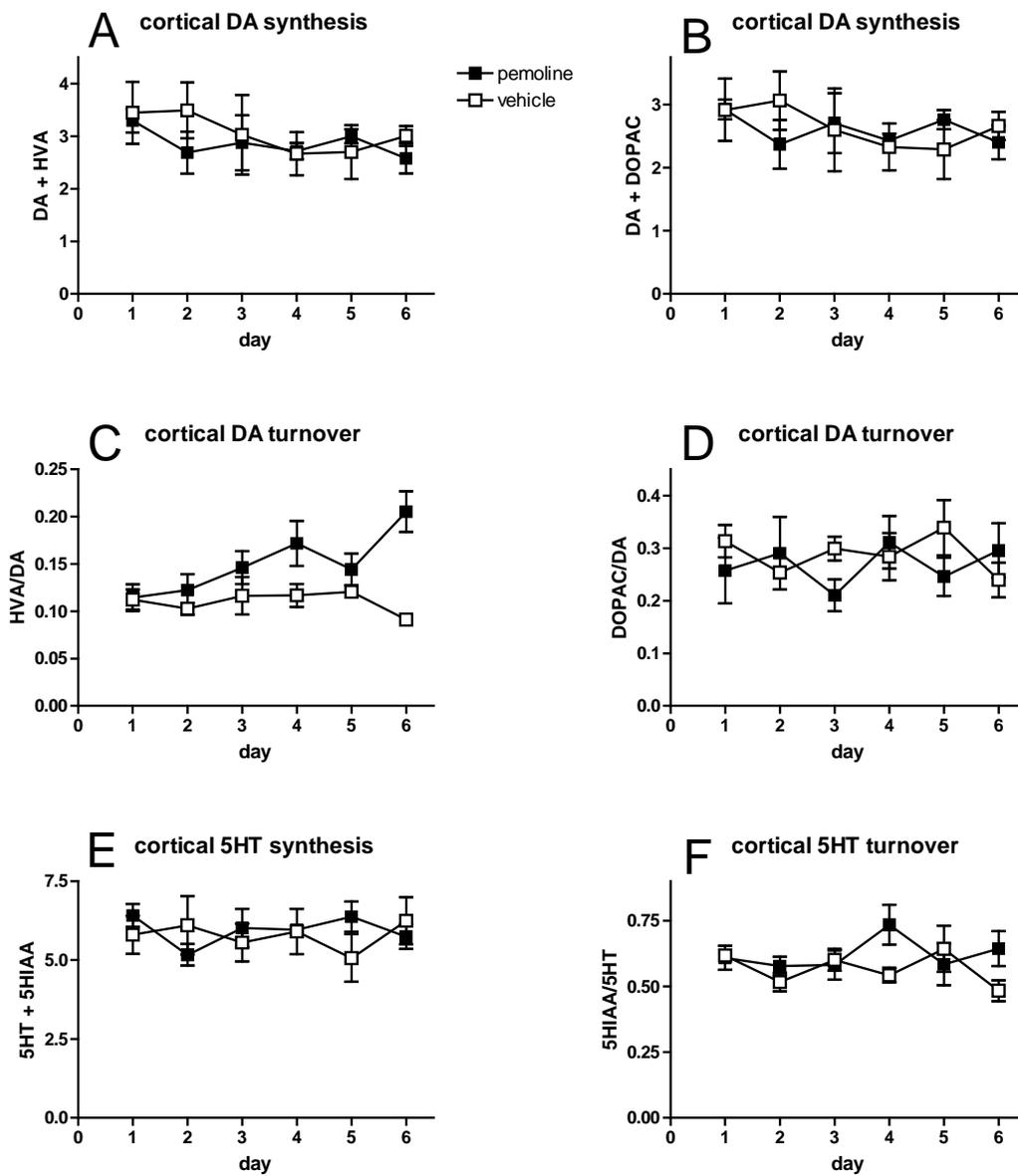


Figure 5-11. Effects of pemoline or vehicle injections on cortical monoamine synthesis and turnover ratios. There were no differences in dopamine synthesis rates between pemoline- or vehicle-treated rats (A,B). Dopamine turnover, as quantified by HVA/DA, was significantly increased in pemoline-treated rats (C), whereas DOPAC/DA was not (D). Neither 5-HT synthesis (E) nor turnover (F) was significantly different between pemoline- and vehicle-treated groups. All values are expressed as group means \pm SEM.

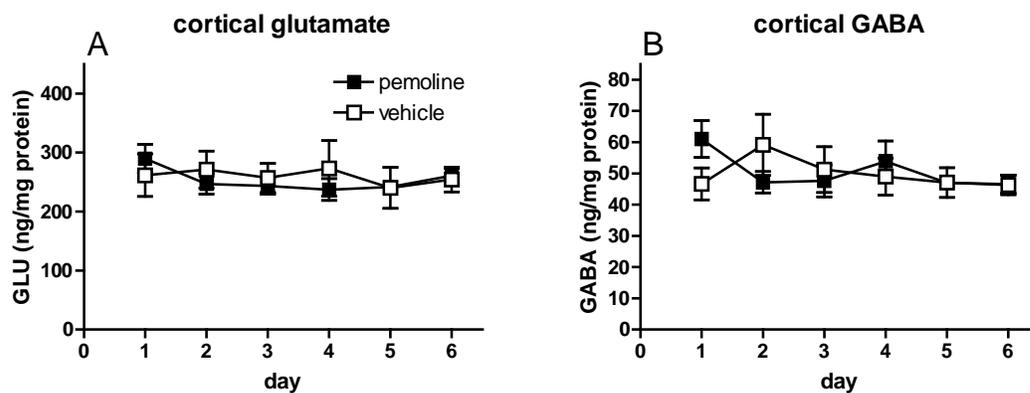


Figure 5-12. Effects of pemoline or vehicle injections on cortical amino acid levels. No differences in either glutamate (A) or GABA (B) concentrations were found between pemoline- and vehicle-treated rats. All values are expressed as group means \pm SEM.

Amygdala

The dopaminergic mesolimbic pathway projects to several limbic sites, including the amygdala. We examined dopamine, DOPAC, serotonin, and 5-HIAA levels in the amygdalar samples to determine pemoline's effects on this projection area of the mesolimbic pathway (HVA levels in this brain region were consistently near our lower detection limit, so they are not presented here). The amygdala also contains and receives glutamatergic and GABAergic projections, though because of technical difficulties, we were unable to analyze amygdalar samples for amino acid concentrations. In these analyses we found no significant differences between pemoline- and vehicle-treated rats in measures of amygdalar dopamine (Fig. 5-13A), DOPAC (Fig. 5-13B), or serotonin (Fig. 5-13C). Pemoline-treated rats, however, did have higher 5-HIAA levels, though this effect appears transient, only occurring on days 3 and 4 ($F_{(1,36)} = 9.765$, $p < 0.01$; Fig. 5-13D). We found no changes in dopamine synthesis (Fig. 5-14A) or turnover (Fig. 5-14B) ratios in pemoline-treated rats. Serotonin synthesis rates (Fig. 5-14C) were

also not different between groups, whereas serotonin turnover was significantly elevated in pemoline-treated rats, but mostly only on days 3 and 4 ($F(1,36) = 26.11$, $p < 0.0001$; Fig. 5-14D).

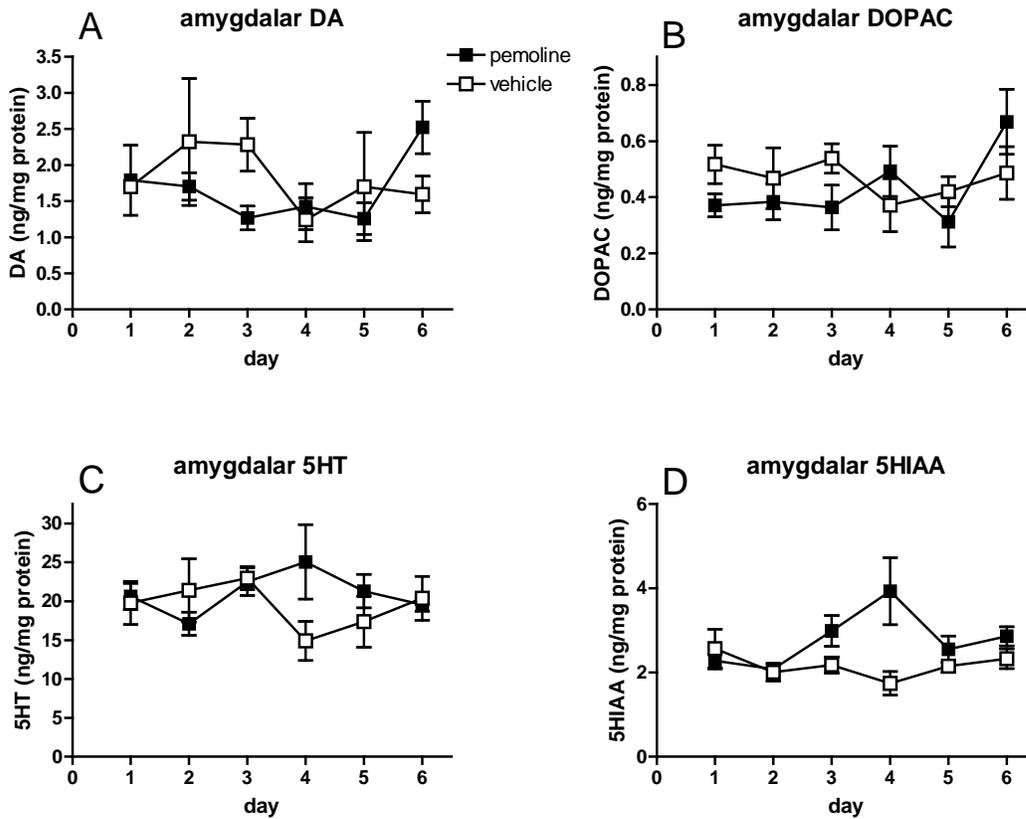


Figure 5-13. Effects of pemoline or vehicle injections on amygdalar monoamine and metabolite levels. There were no pemoline-associated changes in amygdalar DA (A), DOPAC (B), or 5-HT (C) concentrations. 5-HIAA concentrations were significantly elevated in pemoline-treated rats as compared to those of vehicle-treated rats. All values are expressed as group means \pm SEM.

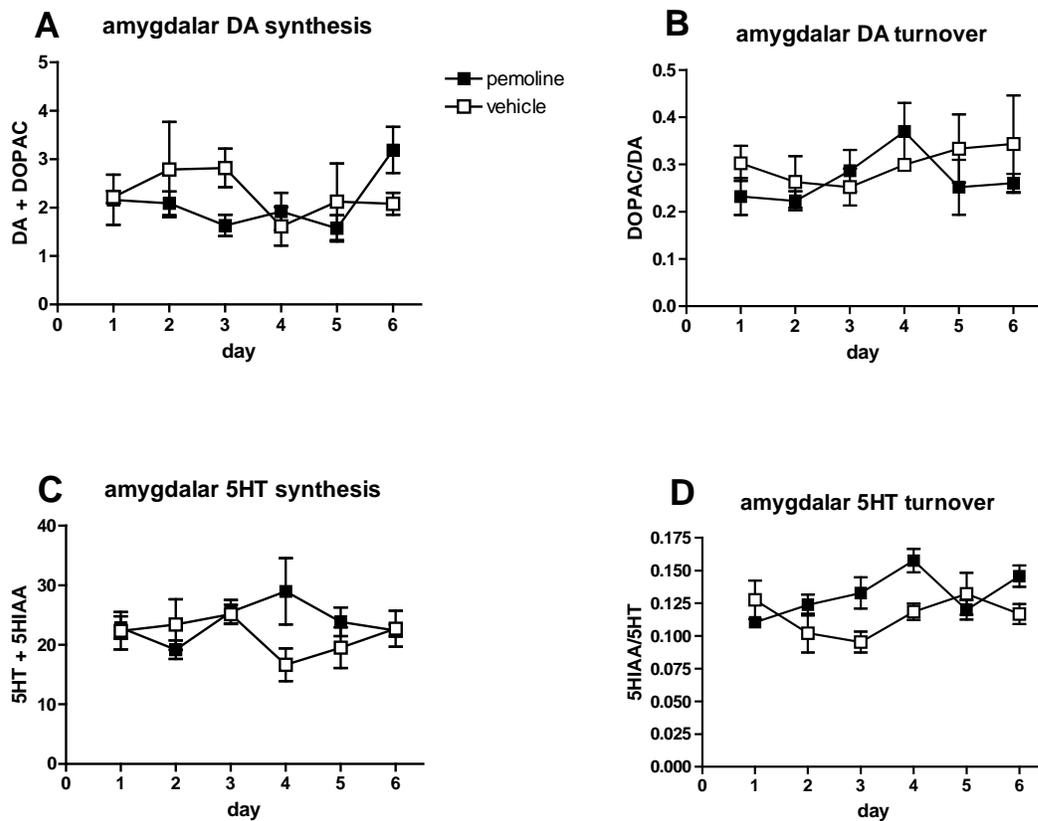


Figure 5-14. Effects of pemoline or vehicle injections on amygdalar monoamine synthesis and turnover ratios. There were no differences in dopamine synthesis (A) or turnover (B) rates between pemoline- or vehicle-treated rats. 5-HT synthesis was also not changed by pemoline treatment (C). 5-HT turnover, however, was significantly higher at most time points in pemoline-treated rats versus vehicle-treated rats (D). All values are expressed as group means \pm SEM.

Hippocampus

We were interested in studying the effects of pemoline on the neurochemistry of the hippocampus for several reasons. The hippocampus receives dopaminergic, serotonergic, and glutamatergic inputs from midbrain nuclei, and there is considerable evidence suggesting an important role for these neurotransmitters in self-injurious behavior. Furthermore, our lab previously found changes in cellular metabolism (as assayed by cytochrome oxidase immunohistochemistry) in hippocampal regions in

pemoline-treated rats (data not published). Unfortunately, we were unable to measure dopamine and its metabolites in the hippocampus with our HPLC and detection settings. Serotonin levels were unchanged in hippocampal samples from pemoline-treated rats (Fig. 5-15A), while 5-HIAA concentrations rose significantly across days of pemoline administration ($F_{(1,36)} = 15.91, p < 0.001$; Fig. 5-15B). These results contributed to significantly higher serotonin turnover ratios ($F_{(1,36)} = 26.11, p < 0.0001$; Fig. 5-16B), but no significant changes in serotonin synthesis (Fig. 5-16A). We also found no changes in hippocampal glutamate (Fig. 5-17A) or GABA (Fig. 5-17B) in pemoline-treated rats.

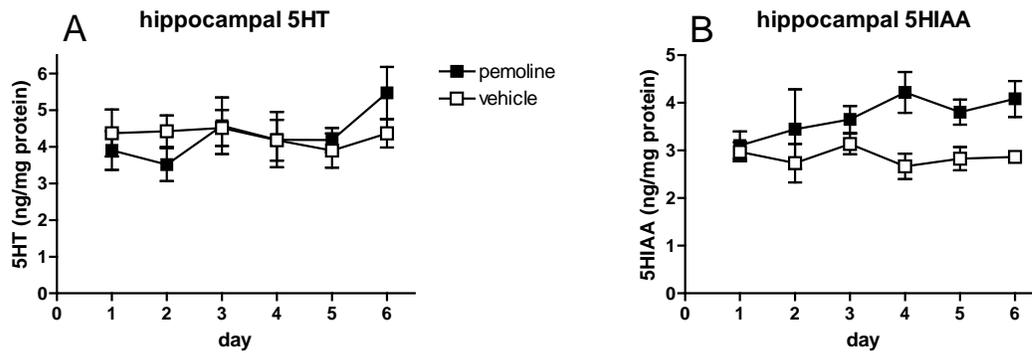


Figure 5-15. Effects of pemoline or vehicle injections on hippocampal 5-HT and 5-HIAA. There were no pemoline-associated changes in hippocampal 5-HT (A), whereas hippocampal 5-HIAA was significantly increased in pemoline-treated rats (B). All values are expressed as group means \pm SEM.

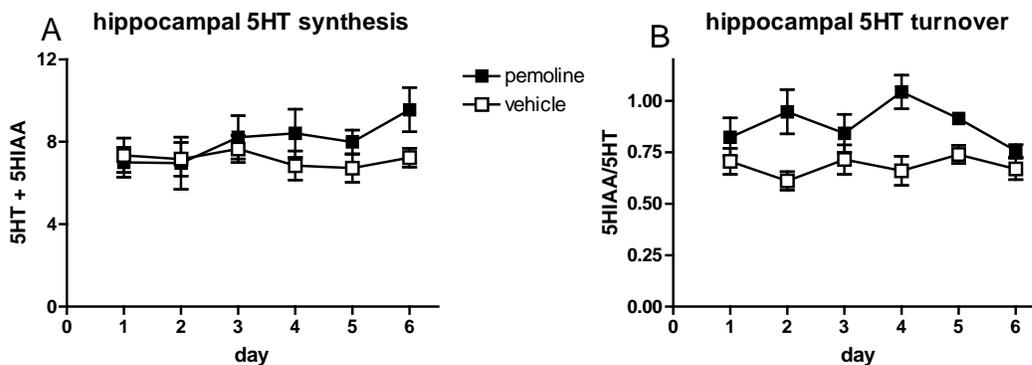


Figure 5-16. Effects of pemoline or vehicle injections on hippocampal 5-HT synthesis and turnover ratios. Pemoline did not significantly alter serotonin synthesis ratios (A), but did significantly enhance 5-HT turnover (B). All values are expressed as group means \pm SEM.

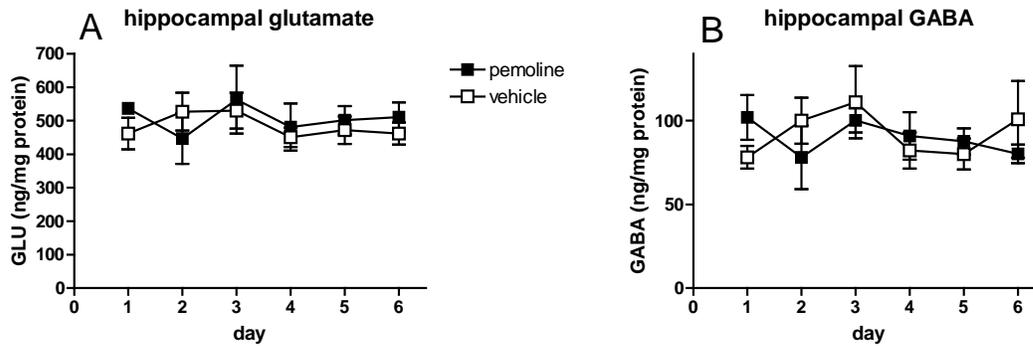


Figure 5-17. Effects of pemoline or vehicle injections on hippocampal amino acid levels. No differences in either glutamate (A) or GABA (B) concentrations were found between pemoline- and vehicle-treated rats. All values are expressed as group means \pm SEM.

Septum

The septum is another limbic brain structure that is innervated by the dopaminergic mesolimbic pathway. It was also an area in which our previous investigation found significant reduction in cellular metabolism with repeated pemoline administration (data not published). We analyzed norepinephrine (Fig. 5-18A), dopamine (Fig. 5-18B), serotonin (Fig. 5-18E), and metabolites (Fig. 5-18C,D,F) in the septum of pemoline- and vehicle-treated rats and found no significant differences in any measure. There were also no differences found in dopamine and serotonin synthesis or turnover ratios (Fig. 5-19A-F). There was not enough septal sample to also run amino acid analyses.

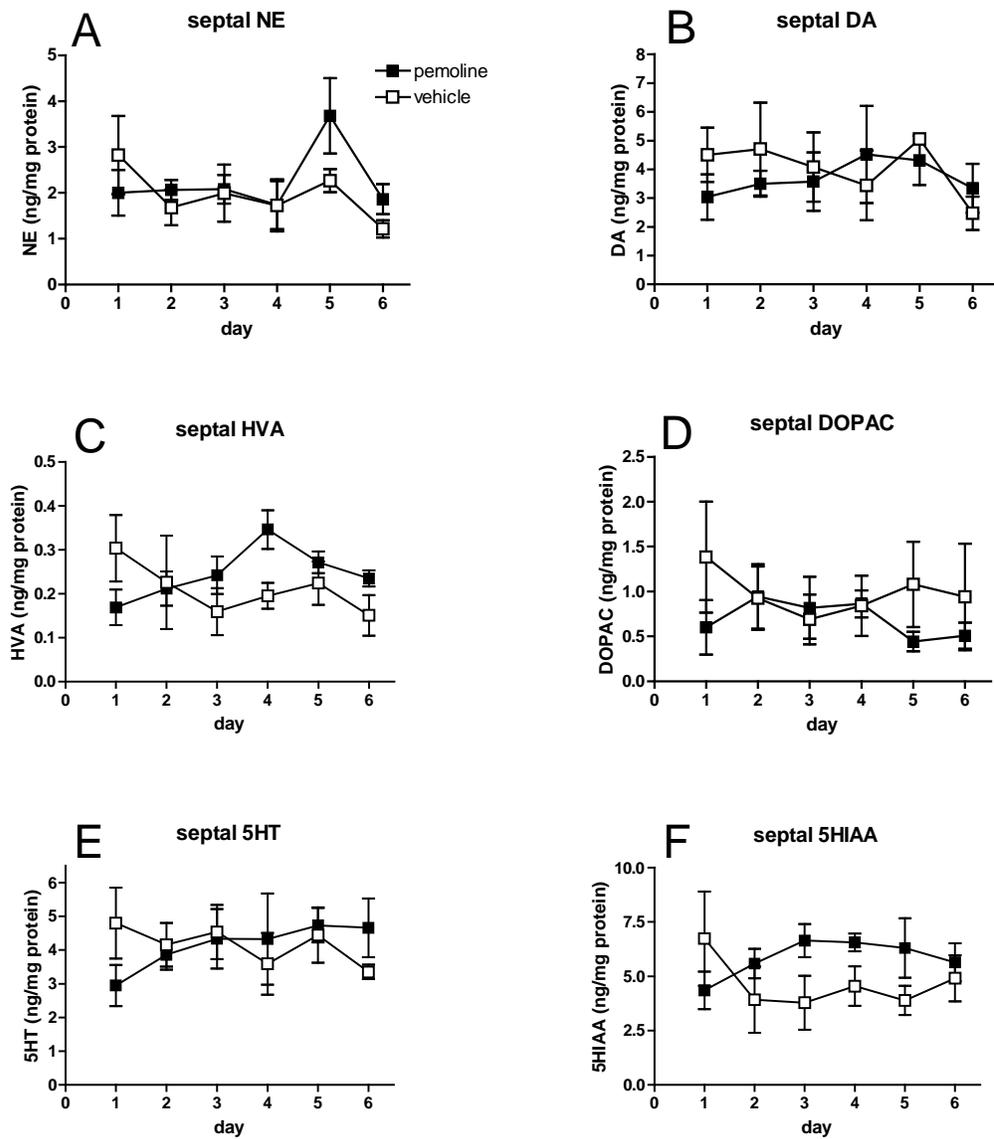


Figure 5-18. Effects of pemoline or vehicle injections on septal monoamine and metabolite levels. There were no pemoline-associated changes in septal NE (A) DA (B), or 5-HT (E) concentrations. There were also no significant differences in HVA (C), DOPAC (D), or 5-HIAA (F) between pemoline- and vehicle-treated rats. All values are expressed as group means \pm SEM.

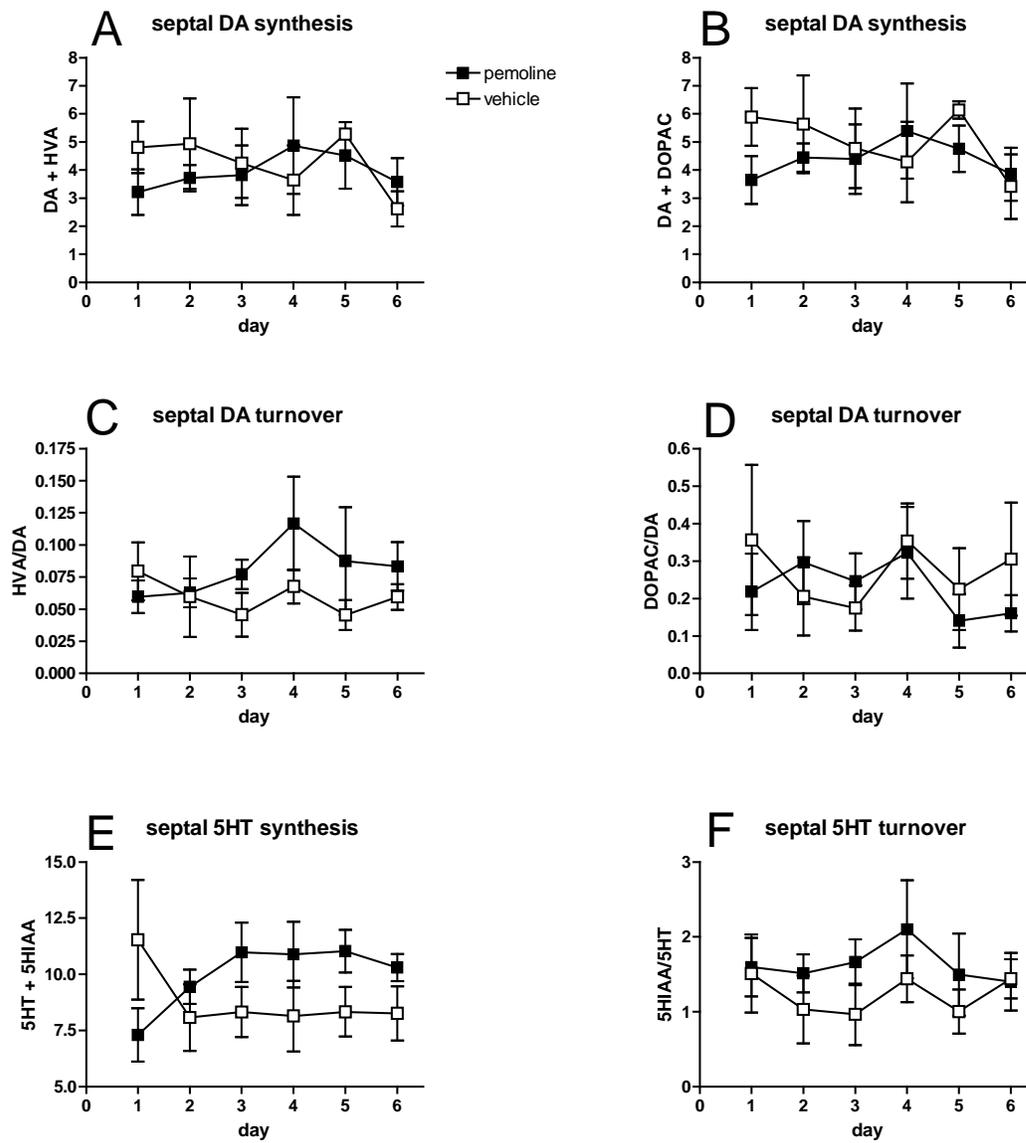


Figure 5-19. Effects of pemoline or vehicle injections on septal monoamine synthesis and turnover ratios. Pemoline did not significantly alter dopamine (A,B,C,D) or 5-HT (E,F) synthesis or turnover ratios. All values are expressed as group means \pm SEM.

The significant drug effects from this experiment are summarized in Table 5-2.

	Striatum	Ventral tegmentum	Cortices	Amygdala	Hippocampus	Septum
DA	*	n.s.	n.s.	n.s.	n/a	n.s.
HVA	***	n.s.	**	n/a	n/a	n.s.
DOPAC	n.s.	n.s.	n.s.	n.s.	n/a	n.s.
DA synthesis DA + HVA	n.s.	n.s.	n.s.	n/a	n/a	n.s.
DA synthesis DA + DOPAC	n.s.	n.s.	n.s.	n.s.	n/a	n.s.
DA turnover HVA/DA	***	n.s.	***	n/a	n/a	n.s.
DA turnover DOPAC/DA	n.s.	n.s.	n.s.	n.s.	n/a	n.s.
5-HT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
5-HIAA	***	***	n.s.	**	**	n.s.
5-HT synthesis 5-HT + 5-HIAA	n.s.	**	n.s.	n.s.	n.s.	n.s.
5-HT turnover 5-HIAA/5-HT	***	***	n.s.	***	***	n.s.
NE	n/a	n.s.	n/a	n/a	n/a	n.s.
GLU	n.s.	n.s.	n.s.	n/a	n.s.	n/a
GABA	*	n.s.	n.s.	n/a	n.s.	n/a

Table 5-2. Summary of significant effects of pemoline on regional concentrations of monoamines, metabolites, amino acids, and of monoamine synthesis and turnover ratios. Acronyms and symbols: n.s., not significant; n/a, not analyzed; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

Discussion

This study was designed to document pemoline titers in the brain and plasma, to examine potential changes in corticosterone circadian regulation, and to give a global impression of changes in monoamine and amino acid neurotransmission during repeated administration of pemoline. We harvested samples three times within 24 hours of the first injection, then 24 hours after each subsequent injection. We also took samples 12 hours after the fourth injection to assess whether changes during the first day of pemoline administration remained consistent during the subsequent injections.

The data from the pemoline titer analyses reveal that pemoline is present in both plasma and brain throughout the six day experimental protocol. As seen from the

samples taken 24 hours after each injection (Fig. 5-2B), pemoline is never fully cleared from the plasma or brain when the subsequent injection is given. This long-lasting bioavailability of pemoline is likely due to suspension of pemoline in peanut oil and its depot delivery under the skin. Without knowing the pharmacodynamics of pemoline or its dopamine transporter occupancy thresholds, it is difficult to deduce whether pemoline administration in this model constitutes intermittent or continuous stimulation of the majority of pre-synaptic transporter target(s) or the pre- or post-synaptic receptors consequently activated by enhanced neurotransmission. For most stimulants, cellular and behavioral responsiveness differs depending on whether stimulation is intermittent versus continuous (Post, 1980). The increased incidence, duration, and severity of pemoline-induced self-injurious behavior across days of pemoline administration suggest that this altered behavioral responsivity exemplifies behavioral sensitization, though the role for pemoline accumulation in the expression of pemoline-induced self-injury cannot be ruled out.

Analyses of corticosterone levels using radioimmunoassay indicate very low levels of corticosterone in the samples collected from vehicle-treated rats in the morning hours (8-10am, roughly 1 to 3 hours after lights on). This is consistent with the normal circadian rhythm of corticosterone, which reaches its nadir at the beginning of the inactive period (Seggie et al., 1985). In pemoline-treated rats, corticosterone levels were elevated at most morning testing times, the greatest of which was revealed two hours after the first pemoline injection. Corticosterone levels were also elevated 24 hours after the first, second, third, and fourth injections, which suggests that pemoline causes marked changes to the circulating corticosterone levels throughout the day. The

consequence of repeated pemoline injection does not appear to be additive across days, which may indicate that the elevation in corticosterone may not be a primary contributor to the development of pemoline-induced self-injury. Though our results from other experiments (e.g. chapters 3 and 4) indicate that stress, and perhaps corticosterone and its intracellular actions, may play a modulatory role in the expression of pemoline-induced self-injurious behavior. Other psychostimulants also cause an elevation in circulating corticosterone (Moldow and Fischman, 1987), and blocking this by adrenalectomy or pharmacological challenge, reduces the psychostimulant-induced dopamine release and behavioral sensitization (Barrot et al., 2000; De Vries et al., 1996, Rivet et al., 1989). These results suggest that blocking the corticosterone elevation after pemoline injection may also reduce pemoline-induced self-injurious behavior.

Disregulation of dopaminergic systems has been the most common finding in studies regarding the neurobiological basis of human (Brucke et al., 1987; Ernst et al., 1996; Lloyd et al., 1981; Muller-Vahl et al., 2000; Rassin et al., 1982; Riederer et al., 1986; Saito et al., 1999; Singer et al., 1991; Wenk, 1995; Wolf et al., 1996; Wong et al., 1996; Wong et al., 1997) and animal (Criswell et al., 1992; Cromwell et al., 1997; Kasim and Jinnah, 2003; Lewis et al., 1990; Martin et al., 1991; Mueller and Nyhan, 1982; Shishido et al., 2000; Sivam, 1989) self-injurious behavior. In this experiment we found the significant changes in intracellular dopamine concentrations were only in the striatum. Reduction of intracellular dopamine corresponds with elevations in pemoline titers in the striatum and the induction of pemoline-induced self-injury. This dopamine effect is consistent with clinical findings of dopaminergic depletion in the striatum.

Future analyses of the neurobiological basis of self-injury should continue to focus on the striatum and the direct and indirect basal ganglia pathways within it.

Intracellular dopamine depletion has also been used as a preliminary indicator of dopamine terminal loss (Cadet et al., 1994). We did not directly study the status of the striatal dopaminergic terminals (analyses of this would include assays of tyrosine hydroxylase levels and autoradiographic assays of DAT number and binding characteristics), however loss of dopaminergic terminals in the caudate and putamen have been reported in Lesch-Nyhan and Rett patients (Wong et al., 1996; Wong et al., 1998), as well as several other animal models of self-injurious behavior (Martin et al., 1991; Kita et al., 2000).

A corresponding rise in HVA levels were also found in the striatum. Dopamine is metabolized into HVA by two distinct enzymatic pathways (Kopin, 1985). The first utilizes catechol-O-methyl transferase (COMT), a nonneuronal and/or extracellular enzyme, to convert dopamine into 3-methoxytyramine (3MT), then monoamine oxidase (MAO) converts 3MT to HVA. MAO is an intracellular enzyme located on the membrane of mitochondria (Westlund et al., 1993). The other pathway uses MAO to convert dopamine to DOPAC, which is converted to HVA by COMT. The lack of differences in DOPAC levels between pemoline- and vehicle-treated rats suggests that the increase in HVA is a result of higher levels of extracellular dopamine metabolized by the nonneuronal and/or extracellular COMT in the pemoline-treated rats. This finding should be confirmed using microdialysis of the striatum, but is consistent with the action of pemoline at the dopamine transporter.

GABA levels in the striatum were elevated in pemoline-treated rats, in comparison to vehicle-treated rats. This finding, and the role of striatal GABAergic functioning in pemoline-induced self-injury, are difficult to interpret for several reasons. First, the differences in GABA levels between pemoline-treated and vehicle-treated rats are most striking on days 2-4, which does not correspond to the days in which pemoline-induced self-injurious behavior was most severe. Second, there is considerable variability in the GABA measurements in the pemoline-treated rats, which may indicate these differences represent changes in only a few samples. Third, both the direct and indirect pathway cells of the striatum are GABAergic, so this analysis does not give any insight into how the release of GABA in either pathway may change with pemoline administration. Although difficult to interpret, these results further support our rationale for focusing on striatal function in the pemoline model.

Finally, significant changes in 5-HIAA were found in striatum, VTA, amygdala, and hippocampus, which contributed to significant differences in serotonin turnover. Given that there are no significant increases in 5-HIAA when serotonin transporters are blocked by SSRIs (Chertkow et al., 2007) or when they are knocked down in genetic models (Fox et al., 2008), suggests that pemoline's main effects on the serotonin system is not through its actions on the serotonin transporter, but that the changes in serotonin turnover (as measured by the 5HIAA/5HT ratio, Karstaedt et al., 1994) may be a result of potential indirect actions and modulation by dopaminergic systems (Sivam, 1995). These increases in 5-HIAA levels in pemoline-treated rats are also consistent with increased 5-HIAA levels in brain (Lloyd et al., 1981) and CSF (Jankovic et al., 1988) of Lesch-Nyhan patients.

This project had a very wide focus and was designed to identify the time course and the most critical regions of the brain for pemoline-induced changes. We were also interested in pemoline levels in plasma and brain and the effect of pemoline on circulating corticosterone levels. Results from this experiment suggest that our six day experimental protocol is sufficient for studying changes in pemoline-induced behaviors and neurochemistry. These results also justify our continued concentration on striatal functioning in pemoline-induced self-injurious behavior.

CHAPTER 6 NEUROTENSIN PLAYS A MODULATORY ROLE IN PEMOLINE-INDUCED SELF- INJURIOUS BEHAVIOR

Background

The neurobiological basis of pemoline-induced self-injurious behavior appears to be mediated by several neurochemical systems within the cortico-basal ganglia pathway. Our previous work suggests that dopaminergic signaling is increased in the striatum (which leads to a depletion of intracellular dopamine; Chapter 4) and that glutamatergic neurotransmission is required for the induction of pemoline-induced self-injurious behavior (Muehlmann et al., 2008). We have also found that a history of stressful experience worsens the severity of pemoline-induced self-injury (Chapter 3) and that pemoline causes modest increases in circulating corticosterone levels (Chapter 4). Additionally, pain sensitivity is diminished in pemoline-treated rats (Chapter 2). Studying how these separate neurochemical systems interact, and finding pharmacological tools that modulate these systems, may significantly improve our understanding of the neurobiological basis of self-injury and its treatment.

Neurotensin is a modulatory neurotransmitter that binds to three different receptors. The two G-protein coupled receptors, NTS1 and NTS2, predominantly control the central nervous system effects of neurotensin. NTS1 is a high affinity receptor localized in high abundance in the VTA, substantia nigra, striatum, and nucleus accumbens, as well as other limbic and cortical areas (Binder et al., 2001), and is most closely co-localized with dopamine and glutamate systems. NTS2 is a low affinity receptor found mainly in cortical and limbic regions, as well as pain-related areas like the periaqueductal gray and superior colliculus (Asselin et al., 2001). The NTS3 is a single transmembrane domain receptor that is not associated with a G-protein. It is also

found in many cortical and limbic regions, though the functioning of this receptor in the CNS when neurotensin is bound is not well understood (Sarret et al., 2003).

Neurotensin strongly co-localizes with dopaminergic pathways. Neurotensin stimulates dopaminergic neurons in the cell body regions of these pathways, the VTA and SNc (Myers and Lee, 1983; Binder et al., 2001). Neurotensin receptors (namely NTS1) complex with both pre- and post-synaptic D2 receptors in the striatum and nucleus accumbens (Antonelli et al., 2007; Boudin et al., 1996; Delle Donna et al., 1996; Fuxe et al., 1992) and neurotensin release in these axon terminal regions causes D2 autoreceptor inhibition and increased dopamine release (Fawaz et al., 2009). Consequently, neurotensin administration increases dopamine metabolite concentrations in these dopaminergic projection areas (Widerlov et al., 1982). Postsynaptically, neurotensin activation of NTS1 also antagonizes D2 receptor function, but in addition stimulates the phospholipase 3/IP3/calcium mobilization pathway (Yin et al., 2008).

Neurotensin receptors are also located on glutamatergic terminals of corticostriatal neurons (Goedert et al., 1984). The activation of these pre-synaptic neurotensin receptors increases glutamate release (Ferraro et al., 1995; Ferraro et al., 2000). Stimulation of the postsynaptic neurotensin receptors of the striatum amplifies the signaling of glutamate's NMDA receptor (Antonelli et al., 2004). Additionally, glutamate agonists increase neurotensin release, suggesting significant cross-talk between these two neurochemical systems (Radke et al., 2001).

Reciprocal relationships have also been identified between neurotensin and the HPA axis. Stressors, such as footshock, initiate the circulation of corticosterone as well

as increase neurotensin content in the VTA (Deutsch et al., 1987; Kilts et al., 1992). The promoter region of the neurotensin/neuromedin N gene contains a glucocorticoid response element (Harrison et al., 1995), which suggests a relationship between stress pathway activation and neurotensin/neuromedin N gene transcription and neurotensinergic tone. This suggests that an increase in neurotensin content in the VTA may be due to increased synthesis, instead of decreased release. Moreover, intracerebroventricular injections of neurotensin increase circulating corticosterone concentrations (Gudelsky et al., 1989) and NTS1 receptor antagonism blocks diurnal elevations as well as restraint stress-induced corticosterone release (Rowe et al., 1997).

Neurotensin is also involved in pain processing; specifically, non-opioid mediated forms of analgesia. Intracisternal injection of neurotensin significantly increases the latency to respond to a hot plate, and decreases writhing in the acetic acid test (Clineschmidt & McGuffin, 1977). These effects are not blocked by the opioid antagonist, naloxone (Clineschmidt et al., 1979). Non-opioid mediated analgesia is also responsible for stress-induced antinociception that occurs with high intensity stressors (Grisel et al., 1993). Neurotensin knockout mice do not exhibit stress-induced analgesia (Gui et al., 2004), and the NTS2 most likely mediates this form of analgesia, considering NTS2 knockouts show lower stress-induced corticosterone release and decreased stress-induced analgesia (Lafrance et al., 2010).

The localization of the neurotensin systems, which closely parallel the dysregulated systems involved in pemoline-induced self-injurious behavior, suggests that neurotensin may enhance the progressive dysregulation of the dopaminergic and glutamatergic systems that we think are involved in the etiology of SIB. To evaluate this

possibility, we compared neurotensin levels in brain regions containing dopaminergic cell bodies and terminal fields of pemoline- and vehicle-treated rats. We also evaluated the effects of a neurotensin agonist and antagonist on pemoline-induced self-injury and their effects on the concentrations of presynaptic monoamines and metabolites.

Methods

This project consisted of three experiments. The first experiment assessed neurotensin concentrations in the striatum and tegmentum in pemoline- and vehicle-treated rats following six daily injections. The second experiment evaluated the efficacy of a NTS1 antagonist (SR48692) to reduce pemoline-induced self-injury and its effects on striatal monoamine and metabolite concentrations. The third experiment tested the effects of a NTS1 agonist (PD149163) on pemoline-induced self-injury and striatal monoamines and metabolites.

Experiment 1: Neurotensin Concentrations in Vehicle- and Pemoline-Treated Rats

Animals

Twenty male LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed in a climate controlled vivarium with a 12h/12h light/dark schedule (lights on at 7:00 a.m.). Standard laboratory rat chow (Lab Diet 5001) and tap water were available *ad libitum*. The rats were pair-housed in standard polycarbonate cages (43 cm x 21.5 cm x 25.5 cm) during six days of acclimation to the housing facility. All of the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were pre-approved by the Institutional Animal Care and Use Committee at the University of Florida. One rat was excluded from the analyses because it exhibited substantial denuding on its forepaws when administered vehicle.

Drug

Pemoline (2-amino-5-phenyl-1,3-oxazol-4-one; Spectrum Chemicals, New Brunswick, New Jersey) was suspended at a concentration of 50 mg/ml in peanut oil. The pemoline was left stirring in peanut oil over night in order to achieve an even suspension.

Drug treatment

Following six days of acclimation, stress responsiveness was assessed as described in Chapter 5. HR and LR rats were balanced between the vehicle and pemoline treatment groups. The rats were weighed and injected with peanut oil vehicle (n=9) or pemoline (n=10) at 150 mg/kg (s.c.) each day for five days. These injections were administered at the nape of the neck and either flank on a rotating basis.

Assay of self-injury

The rats were visually inspected each time they were injected, and immediately before they were decapitated, and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Still images of the injured tissue were taken from the videotapes, and MCID software (Imaging Research Inc., St. Catharines, ON, Canada) was used to draw outlines around the injured tissue, and to calculate the area of injury in mm².

Neurotensin radioimmunoassay

The rats were rapidly decapitated 24 hours after the last injection. Brains were immediately removed and the striatum and ventral tegmentum were dissected, frozen on dry ice, and stored at -80°C until processed. The neurotensin radioimmunoassay

was purchased from Phoenix Pharmaceuticals, Inc (Burlingame, CA) and performed according to their instructions.

Statistical analyses

Between-group differences in size of injured tissue were evaluated using RM-ANOVA. Two-tailed t-tests were used to analyze between-group differences in neurotensin content in the striatum and tegmentum.

Experiment 2: Effects of NTS1 Antagonist, SR48692, on Pemoline-Induced Self-Injurious Behavior

Animals

Thirty three male LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed identically to the ones described for Experiment 1.

Drugs

Pemoline was prepared as described in Experiment 1. SR48692, a NTS1 antagonist was provided by the National Institute on Mental Health Chemical Synthesis and Drug Supply Program. SR48692 was suspended in 0.9% saline with 0.1% Tween 80.

Drug treatments

The rats were weighed and injected with either peanut oil vehicle or pemoline (150 mg/kg s.c.) at approximately 8:00 a.m. on each of five consecutive days. These injections were administered at the nape of the neck and either flank on a rotating basis. The rats were also injected twice daily with either 0.1% Tween 80 vehicle or SR48692 (1.0 mg/kg s.c.). These injections were administered at approximately 8:00 a.m. (immediately after the pemoline injection) and approximately 6:00 p.m and also rotated

between the nape of the neck and either flank, always administering the SR48692 or Tween 80 vehicle injections at a different site from the pemoline or peanut oil injections on any given day. The drug and vehicle groups were as follows: pemoline + SR48692 (n=11), pemoline + 0.1% Tween 80 (n=10), peanut oil + SR48692 (n=6), and peanut oil + 0.1% Tween 80 (n=6). Rats were prescreened for stress responsiveness and balanced across groups.

Assays of self-injury

The rats were visually inspected each time they were injected and immediately before they were decapitated and the inspections were videotaped. During these inspections, each rat was held in front of a video camera and the head, forepaws, hindpaws, ventrum and tail were displayed. The presence or absence of tissue injury (denuded skin, erythema, edema, or open lesion) was noted for each rat. Still images of the injured tissue were taken from the videotapes, and MCID software (Imaging Research Inc., St. Catharines, ON, Canada) was used to draw outlines around the injured tissue, and to calculate the area of injury in mm².

Night-vision cameras were focused on the cages of the rats (one camera per cage), and 5-minute time samples were recorded once every three hours over the entire day. The duration of self-injurious oral contact was quantified during each videotaped interval by a trained observer. Self-injurious oral contact was defined as all oral contact that stayed fixed on any one body part for longer than 2 seconds. This was differentiated from grooming, which is oral contact with any part of the body that continues to move from site to site on the body (e.g. oral contact with the forepaws, that then moves up each forelimb and continues to the ventrum, and the contact is not sustained at any spot on the body for longer than 2 seconds). The duration (in seconds)

of oral contact behavior was summed over the entire day (i.e. from the eight video samples) and divided by the total number of seconds recorded, 2400 seconds.

Monoamine analyses

Monoamine concentrations, metabolite concentrations, and protein content were analyzed as described in Chapter 5, using the same HPLC system and electrochemical detector. A fresh set of monoamine and metabolite standards were made and concentration values were interpolated using that new standard curve. Concentration values were then corrected for protein content of each sample. The biosynthesis ratios of the monoamine were analyzed by calculating DA+DOPAC, DA+HVA, 5-HT+5HIAA; turnover ratios of the monoamines were analyzed by calculating DOPAC/DA, HVA/DA, 5-HIAA/5-HT (Commissiong, 1985; Miura et al., 2002).

Statistical analyses

Between-groups differences in size of injured tissue and duration of self-injurious oral contact were each evaluated using RM-ANOVA. Monoamines, metabolites, monoamine synthesis ratios, and monoamine turnover ratios were analyzed by one-way ANOVAs. Between-groups differences in all dependent measures were treated as statistically reliable when the p-values were less than 0.05. Pre-planned Fisher's LSD post-tests were used to further analyze between-groups differences between pemoline + vehicle and pemoline + SR48692 groups, when RM-ANOVAs revealed a significant effect.

Experiment 3: Effects of NTS1 Agonist, PD149163, on Pemoline-Induced Self-Injurious Behavior

Animals

Twenty six male LE rats weighing 200-225g upon delivery (Charles River Laboratories, Raleigh, NC) were housed identically to the ones described for Experiments 1 and 2.

Drugs

Pemoline was prepared as described in Experiment 1. PD149163, a NTS1 agonist was provided by the National Institute on Mental Health Chemical Synthesis and Drug Supply Program. PD149163 was dissolved in saline.

Drug treatments

The rats were weighed and injected with either peanut oil vehicle or pemoline (150 mg/kg s.c.) at approximately 8:00 a.m. on each of five consecutive days. These injections were administered at the nape of the neck and either flank on a rotating basis. The rats were also injected twice daily with either saline or PD149163 (0.01 mg/kg i.p.). These injections were administered at approximately 8:00 a.m. (immediately after the pemoline injection) and approximately 6:00 p.m. The drug and vehicle groups were as follows: pemoline + PD149163 (n=8), pemoline + saline (n=8), peanut oil + PD149163 (n=5), and peanut oil + saline (n=5). Rats were prescreened for stress responsiveness and balanced across groups.

Assays of self-injury, monoamines, metabolites, and protein content

Measures of size of injured tissue, self-injurious oral contact, monoamines, metabolites, and protein content were as described above for Experiment 2.

Statistical analyses

Between-groups differences were analyzed as described above for Experiment 2.

Results

Experiment 1

Rats administered five daily injections of pemoline at 150mg/kg/day exhibited significant self-injury. There were significant main effects of drug ($F_{(1,170)} = 37.58$, $p < 0.0001$), time ($F_{(10,170)} = 19.93$, $p < 0.0001$), and a significant drug x time interaction ($F_{(10,170)} = 19.91$, $p < 0.0001$; Fig. 6-1A). Significantly higher concentrations of neurotensin were found in the striatum of pemoline-treated rats as compared to vehicle-treated controls using RIA (Fig. 6-1B; $t(17) = 2.385$, $p < 0.05$). There were no significant differences in neurotensin levels in the tegmentum of pemoline- and vehicle-treated rats (Fig. 6-1C).

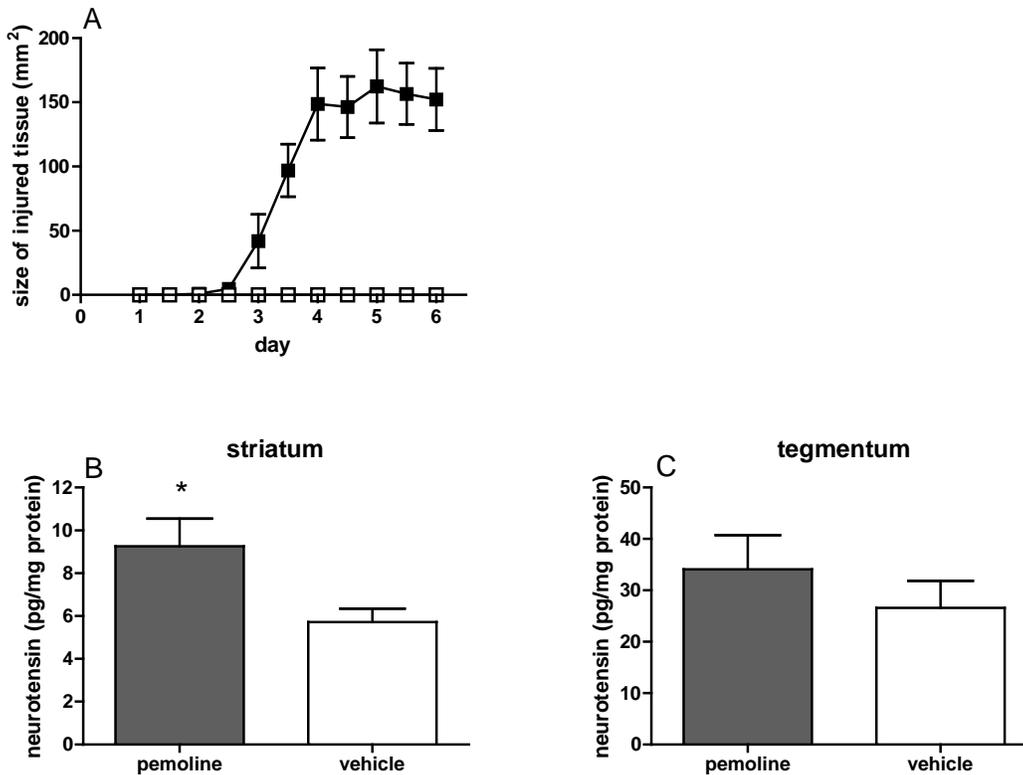


Figure 6-1. Pemoline-induced self-injury and neurotensin concentrations in discrete brain regions. Five daily pemoline injections produced self-induced injury that peaked on day 4 and stayed at an asymptote until the end of the experiment on day 6 (A). Neurotensin levels within the striatum were significantly elevated after five daily pemoline injections (B), but were not significantly changed in the tegmentum (C), as compared to neurotensin levels of vehicle-treated rats. All values are expressed as group means \pm SEM.

Experiment 2

The NTS1 antagonist, SR48692, significantly reduced the severity of pemoline-induced self-injurious behavior, as measured by size of injured tissue and self-injurious oral contact (Fig. 6-2A,B). For the size of injured tissue measure, the RM-ANOVA revealed significant main effects of drug ($F_{(3,290)} = 6.536$; $p < 0.01$) and time ($F_{(10,290)} = 11.26$, $p < 0.0001$), and a significant drug x time interaction effect ($F_{(30,290)} = 4.875$, $p < 0.0001$). For the self-injurious oral contact measure, an analysis using RM-ANOVA also found significant main effects of drug ($F_{(3,116)} = 30.54$, $p < 0.0001$) and time ($F_{(4,116)} =$

26.87, $p < 0.0001$), as well as a significant drug x time interaction effect ($F_{(12,116)} = 9.770$, $p < 0.0001$).

There were very few significant between-groups differences in the measures of monoamines, metabolites, monoamine biosynthesis ratios, and monoamine turnover ratios. The one-way ANOVA did not find any significant differences between groups for dopamine concentrations ($F_{(3,29)} = 2.375$, $p = 0.091$); however, striatal dopamine content was lower in the pemoline + vehicle group, compared to the peanut oil + vehicle group ($t_{(14)} = 2.247$, $p < 0.05$), which is similar to our findings of dopamine depletion in Chapter 5 (Fig. 6-3A). Interestingly, the pemoline + SR48692 group had similar dopamine concentrations to that of the vehicle groups, but the overall ANOVA was not significant. Levels of HVA did differ between the groups as revealed by the one-way ANOVA ($F_{(3,29)} = 9.764$, $p = 0.0001$). Both pemoline-treated groups had significantly higher levels of HVA compared to those of the vehicle-treated groups, and LSD posttests revealed that the pemoline + SR48692 groups had even higher levels of HVA than did the pemoline + vehicle group (Fig. 6-3B). There were no significant between-groups differences for DOPAC levels (Fig. 6-3C). Significant between groups differences were found for serotonin levels ($F_{(3,29)} = 3.305$, $p < 0.05$). Serotonin levels in the pemoline + vehicle group were lower than the peanut oil + vehicle group, which is consistent with our findings in Chapter 5. Furthermore, LSD posttests revealed a significant difference between the pemoline + vehicle group and the pemoline + SR48692 group, wherein the serotonin concentrations of the latter group were significantly higher and more similar to the peanut oil control groups (Fig. 6-3D). There were no significant between-groups differences for 5HIAA levels, which is inconsistent with our findings from Chapter 5 (Fig.

6-3E), although the trend in these data does match the trend in the Chapter 5 data, where 5HIAA content was higher in the pemoline-treated rats than in the vehicle controls.

Pemoline and SR48692 administration had more significant effects on monoamine turnover rates, than they had on monoamine biosynthesis ratios. Neither measure of dopamine biosynthesis, DA+HVA or DA+DOPAC, reached statistical significance using one-way ANOVA; though in both measures the pemoline + SR48692 group more closely resembled the peanut oil controls groups than they did the pemoline + vehicle group. One-way ANOVAs revealed significant differences in both measures of dopamine turnover, HVA/DA ($F_{(3,29)} = 33.60, p < 0.001$) and DOPAC/DA ($F_{(3,29)} = 5.968, p < 0.01$), though these differences were between pemoline-treated groups and the peanut oil-treated groups, with no significant effect of SR48692 administration. Our HPLC analysis in Chapter 4 also found a significant difference in HVA/DA in pemoline versus vehicle, though DOPAC/DA was not significant in this analysis. The data on day 6 of that experiment clearly show that the DOPAC/DA measures are higher in the pemoline group, compared to that of the vehicle group, whereas they are not different at any other time point. The monoamine measures in this experiment are only taken on day 6. This suggests that effects on dopamine turnover, which involve intracellular dopamine metabolism into DOPAC, may only occur after several days of pemoline administration. One-way ANOVAs did not find statistical differences in the measures of serotonin biosynthesis or turnover, though both measures approached significance ($p = 0.057$ and $p = 0.066$, respectively). The lack of differences in the measure of serotonin biosynthesis is consistent with our previous findings (see Chapter 4), though

the measure of serotonin turnover ratio was significantly higher in pemoline-treated rats in our previous experiment. In this experiment, serotonin turnover ratios were higher in pemoline + vehicle rats than they were in peanut oil + vehicle rats, and pemoline + SR48692 rats were intermediate between the two groups.

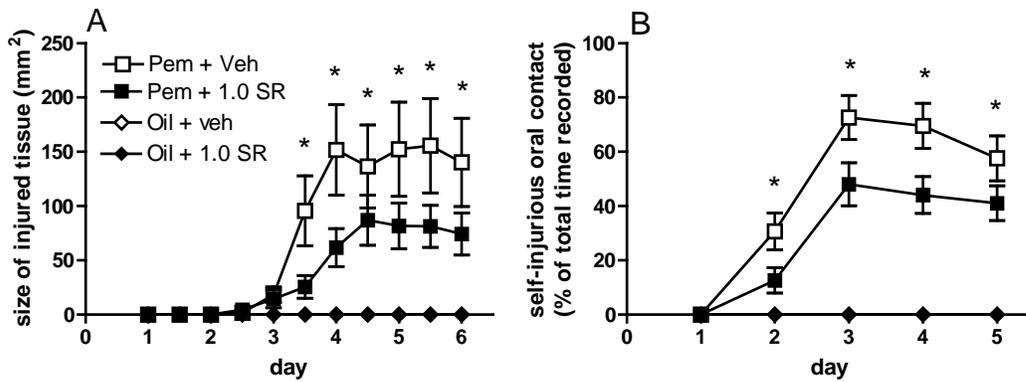


Figure 6-2. Effects of the NTS1 antagonist, SR48692, on pemoline-induced self-injurious behavior. SR48692 significantly lessened the size of injured tissue and time spent self-injuring in pemoline-treated rats.

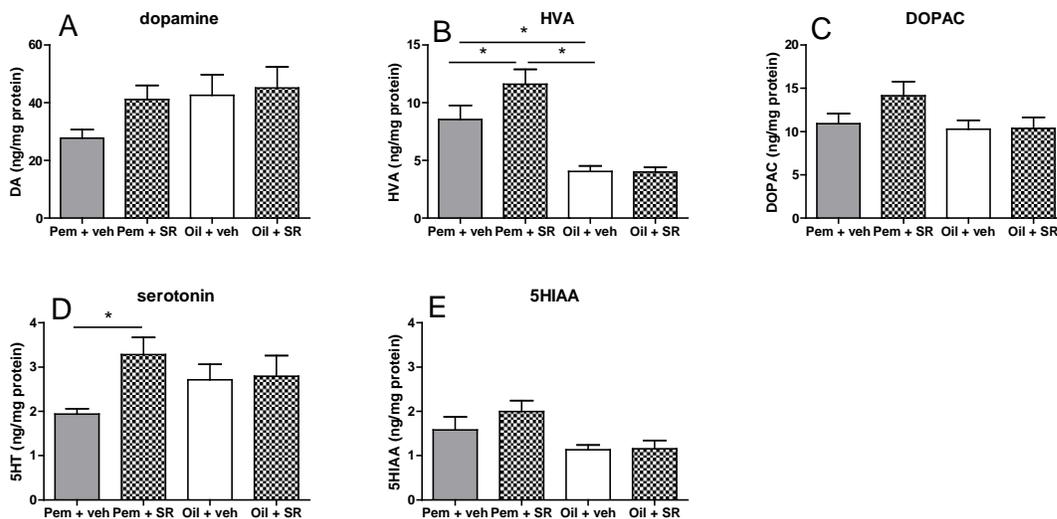


Figure 6-3. Effects of pemoline and SR48692 on striatal monoamines and metabolites. No significant between-groups differences were found for measures of dopamine (A), DOPAC (C), or 5HIAA (E). Levels of HVA were significantly

increased in both pemoline-treated groups, and SR48692 further enhanced HVA levels relative to the pemoline + vehicle group (B). Serotonin levels were also different between groups (D). The pemoline + vehicle group had significantly lower serotonin levels, compared to the pemoline + SR48692 group, which more closely resembled those of the peanut oil control groups.

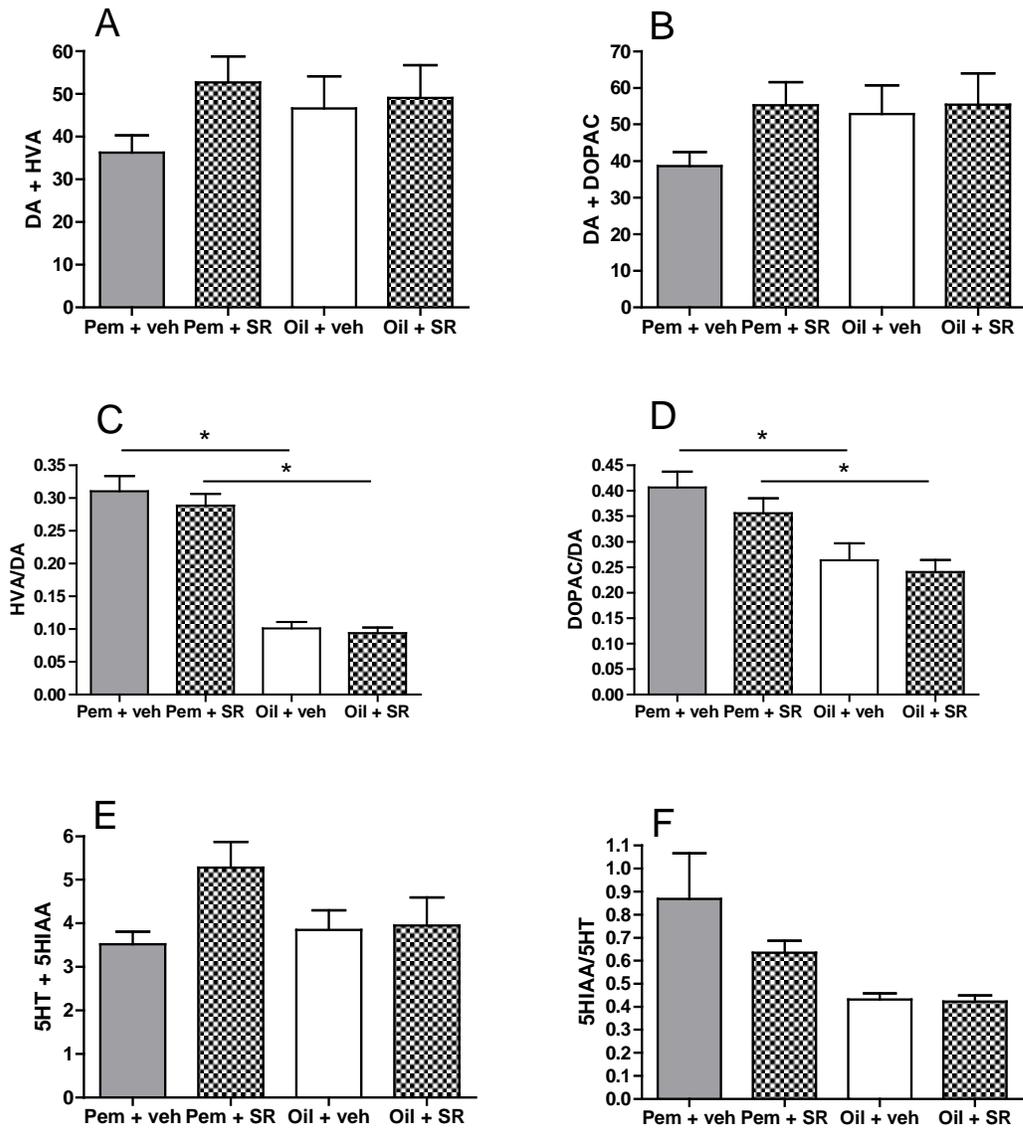


Figure 6-4. Effects of pemoline and SR48692 on striatal monoamine biosynthesis and turnover ratios. There were no significant between-groups differences on measures of monoamine biosyntheses, DA+HVA (A), DA+DOPAC (B), or 5HT+5HIAA (E). Significant between-groups differences were found for both measures of dopamine turnover ratios, HVA/DA (C) and DOPAC/DA (D), though these differences were between pemoline and peanut oil groups, and

not due to SR48692-induced effects. No between-groups differences were found for serotonin turnover ratio, 5HIAA/5HT (F).

Experiment 3

The NTS1 agonist, PD149163, significantly increased the size of injured tissue and the duration of self-injurious oral contact in pemoline treated rats (Fig. 6-5A, B). For the size of injured tissue measure, a RM-ANOVA revealed a significant effect of time ($F_{(10,220)} = 6.124$, $p < 0.0001$) and a significant drug x time interaction ($F_{(30,220)} = 2.280$, $p < 0.001$), as pemoline + PD149163-treated rats began to exhibit pemoline-induced self-injury earlier and consistently showed greater injury size throughout the experiment. For the duration of self-injurious oral contact measure, significant effects of drug ($F_{(3,88)} = 23.71$, $p < 0.0001$), time ($F_{(4,88)} = 16.47$, $p < 0.0001$), and drug x time interaction ($F_{(12,88)} = 6.312$, $p < 0.0001$) were found. Pemoline + PD149163-treated rats were the first to exhibit considerable amounts of self-injurious oral contact, and rates stayed elevated compared to pemoline + vehicle-treated rats on days 2-4. Durations of self-injurious oral contact were no different between these groups on day 5.

Pemoline and PD149163 caused significant changes in monoamine metabolites, without significantly affecting dopamine (Fig. 6-6A) or serotonin (Fig. 6-6D) levels. Striatal dopamine contents were lower in pemoline + vehicle-treated rats, compared to those of peanut oil + vehicle-treated rats ($t_{(11)} = 3.274$, $p < 0.01$), which is consistent with our findings from Experiment 2 and Chapter 5. The pemoline + PD149163 group had dopamine contents intermediate between these two groups, which is similar to the effects we found in pemoline + SR48692 rats. There were no between-groups differences in serotonin levels, which is also consistent with our findings from Chapter 4. Significant between-groups differences in HVA levels were revealed by one-way

ANOVA (Fig. 6-6B). Both pemoline-treated groups had higher concentrations of HVA than the peanut oil-treated groups did ($F_{(3,22)} = 11.57$, $p < 0.0001$), and the pemoline + PD149163 group had even greater HVA levels than the pemoline + saline group. These results are also consistent with our findings from Experiment 2 and Chapter 4. DOPAC levels were also significantly different between groups ($F_{(3,22)} = 3.894$, $p < 0.05$; Fig. 6-6C), however the pemoline + PD149163 group was the only group different from the other drug-treated groups; the DOPAC levels in this group were significantly elevated. One-way ANOVA revealed significant differences in 5HIAA levels, though this effect was due to a consistent elevation in 5HIAA levels in the pemoline-treated groups compared to those of the peanut oil-treated control groups (Fig. 6-6E). This effect is also consistent with our findings in Chapter 4.

Also similar to the results of Experiment 2, PD149163 administration did not have significant effects on monoamine biosynthesis ratios, but did significantly alter all measures of monoamine turnover rates. Both measures of dopamine biosynthesis, DA+HVA and DA+DOPAC, failed to show between-groups differences (Fig. 6-7A, B). The measure of serotonin biosynthesis, 5HIAA+5HT, was also not different between groups (Fig. 6-7E). One-way ANOVAs revealed significant between-groups differences for both measures of dopamine turnover rates, HVA/DA ($F_{(3,25)} = 25.90$, $p < 0.0001$) and DOPAC/DA ($F_{(3,25)} = 7.334$, $p < 0.01$), though these differences were each influenced by the effects of pemoline, as both pemoline-treated groups had higher dopamine turnover rates than the peanut oil control groups did. There were no differences in either measure between pemoline + saline and pemoline + PD149163 groups. A similar effect was found in the serotonin turnover measure, 5HIAA/5HT, wherein a significant

between-groups difference was found ($F_{(3,25)} = 9.331, p < 0.001$; Fig. 6-7F), though these differences were guided mainly by the pemoline-treated groups, and not by PD149163 administration.

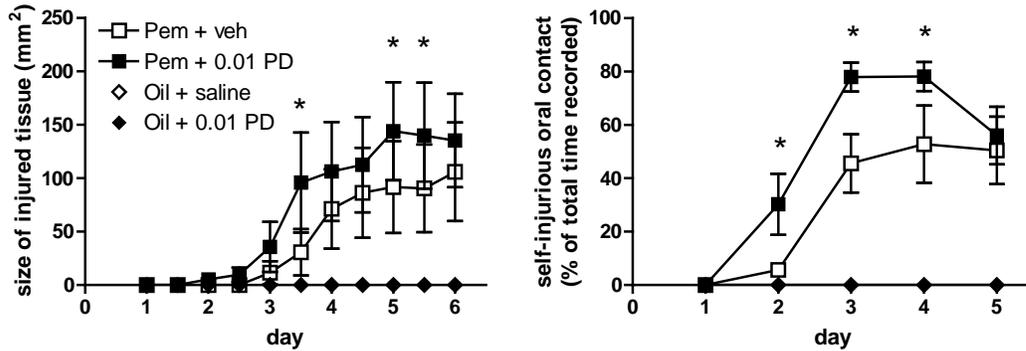


Figure 6-5. Effects of the NTS1 agonist, PD149163, on pemoline-induced self-injurious behavior. PD149163 significantly increased the size of injured tissue and time spent self-injuring in pemoline-treated rats.

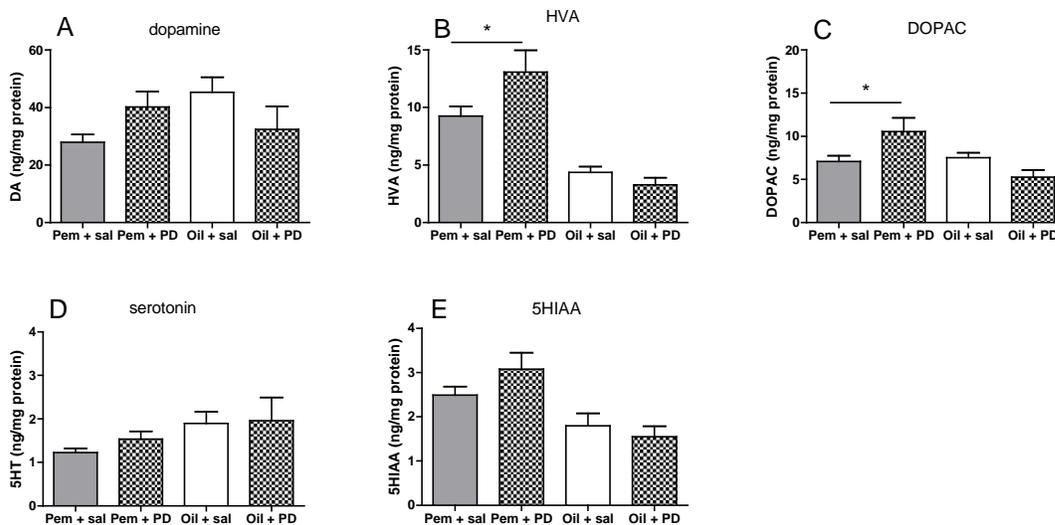


Figure 6-6. Effects of pemoline and PD149163 on striatal monoamines and metabolites. No significant between-groups differences were found for measures of dopamine (A), serotonin (D), or 5HIAA (E). Levels of HVA were significantly increased in both pemoline-treated groups, and PD149163 further enhanced HVA levels relative to the pemoline + saline group (B). DOPAC levels were not different between pemoline + saline and peanut oil +

saline (C). PD149163 increased DOPAC levels in the pemoline + PD149163 relative to the pemoline + saline group.

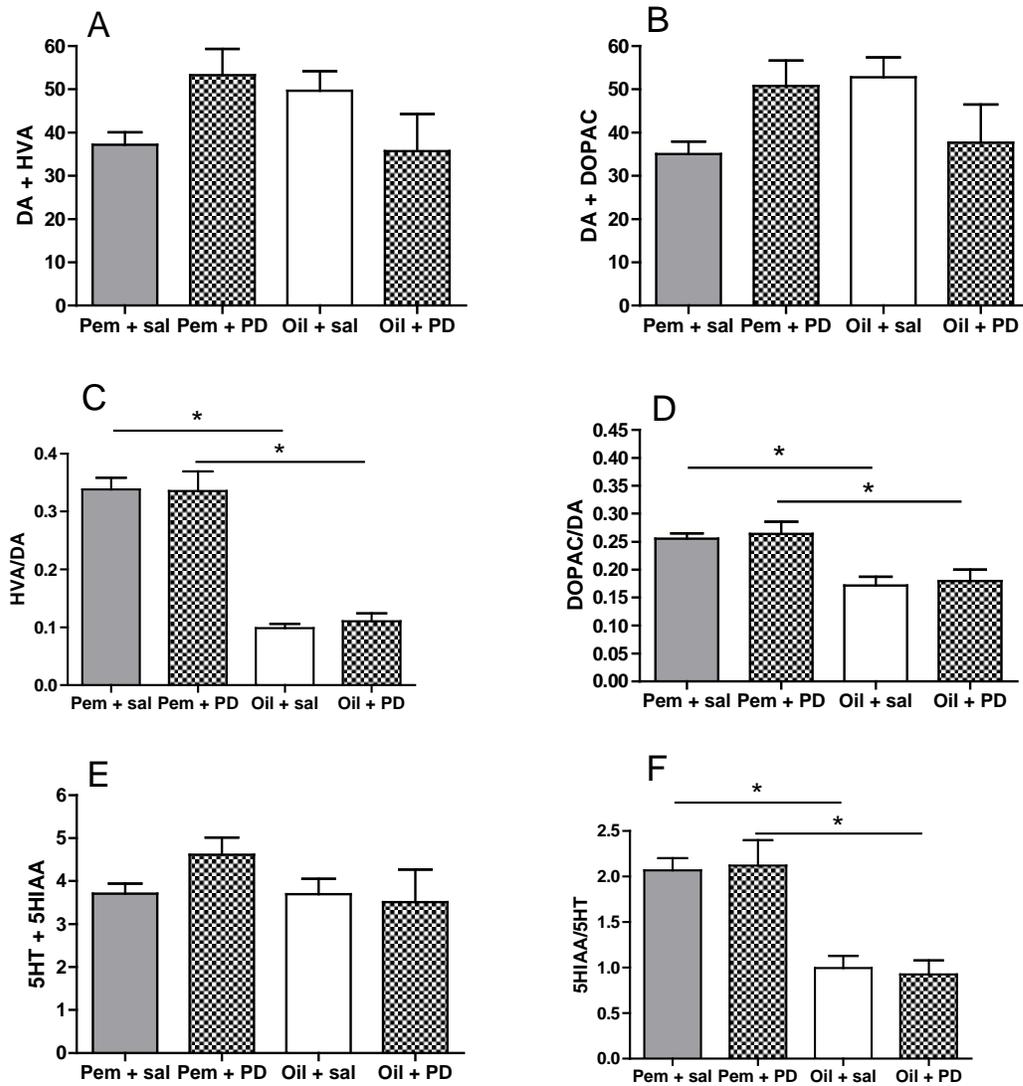


Figure 6-7. Effects of pemoline and PD149163 on striatal monoamine biosynthesis and turnover ratios. There were no significant between-groups differences on measures of monoamine biosyntheses, DA+HVA (A), DA+DOPAC (B), or 5HT+5HIAA (E). Significant between-groups differences were found for both measures of dopamine turnover ratios, HVA/DA (C) and DOPAC/DA (D), though these differences were between pemoline and peanut oil groups, and not due to PD149163-induced effects. A similar pattern of between-groups differences were also found for serotonin turnover ratio, 5HIAA/5HT (F).

Discussion

In this study we found that repeated pemoline administration increases neurotensin content in dopaminergic axon terminal regions and that an NTS1 antagonist reduces pemoline-induced self-injury, and conversely, an NTS1 agonist potentiates pemoline-induced self-injury. Taken together these results suggest that there is an increase in synthesis and release of neurotensin in the striatum and that by reducing the stimulation of NTS1 receptors we can reduce the severity of pemoline-induced self-injurious behavior. Findings of pemoline-induced increases in neurotensinergic signaling fit well with our previous data. Neurotensin signaling is associated with increased dopamine (Fawaz et al., 2009) and glutamate release (Ferraro et al., 1995; Ferraro et al., 2000), heightened HPA axis activation (Gudelsky et al., 1989), and reduced pain sensitivity (Clineschmidt & McGuffin, 1977). These effects are all consistent with our previous characterization of the effects of repeated pemoline and their role in producing self-injurious behaviors.

We focused our investigations on presynaptic monoamine and metabolite concentrations because of our earlier findings of intracellular dopamine depletion and increased HVA levels in pemoline-treated rats. Additionally, NTS1 receptors are predominantly located on presynaptic dopaminergic and glutamatergic axon terminals. Interestingly, although the NTS1 antagonist and agonist had opposite effects on pemoline-induced self-injury, they caused remarkably similar effects on presynaptic measures of monoamines and their metabolite concentrations. This suggests that the converse effects that these neurotensinergic drugs had on pemoline-induced self-injury were perhaps mediated by postsynaptic mechanisms that were not evaluated in this study. Another potential explanation for the lack of differences in presynaptic markers of

monoamine function is the internalization of NTS1 receptors upon agonist activation.

With agonist bound to NTS1 receptors, the complex rapidly internalizes, the receptor is marked for degradation, and the cell desensitizes (Mazella and Vincent, 2006; Souzae, 2001; Souzae and Forgez, 2006; Vandenbulcke et al., 2000). This may have the same biological effect of repeated antagonist administration, and perhaps explains the similar presynaptic effects of both the NTS1 agonist and antagonist.

The effects of pemoline on striatal neurotensin content are similar to those found with other psychostimulants. Cocaine and methamphetamine each significantly increase neurotensin-like immunoreactivity in the striatum (Hanson et al., 1992). The increased neurotensin content in the striatum upon methamphetamine exposure is blocked by administration of the NMDA antagonist, MK801 (Hanson et al., 1992). Pemoline-induced self-injury is significantly reduced by MK801 (Muehlmann and Devine, 2008), which suggests that this effect may be partially due to an attenuation of pemoline-induced increase of neurotensin levels in the striatum.

Contrary to this hypothesis, however, are the effects of neuroleptics, such as haloperidol, pimozide, and risperidone. These drugs each significantly decrease measures of pemoline-induced self-injurious behavior (Muehlmann et al., 2008; Mueller and Nyhan, 1982) but also increase neurotensin content in the striatum (Govoni et al., 1980; Kinkead et al., 2000). Studies of these neuroleptics on striatal neurotensin content have not been completed in stimulant administered animals, so it is difficult to assess what effects they might have on neurotensin levels in stimulant-induced dysregulated neurons of the striatum.

Although we were unable to identify the particular mechanism that mediates the significant effects of the NTS1 antagonist and agonist to decrease and increase, respectively, pemoline-induced self-injurious behavior, these data suggest that neurotensin antagonists may offer pharmacotherapeutic efficacy to human self-injurers. Interestingly, a recent report showed significantly higher levels of serum neurotensin in children with autism (Angelidou et al., 2010). This report did not include rates of self-injurious or other repetitive behaviors, but suggests that an NTS1 antagonist, as opposed to an NTS1 agonist, may be an effective treatment for autistic-like traits.

CHAPTER 7 GENERAL DISCUSSION

Self-injurious behavior is a devastating behavior disorder associated with several neurodevelopmental, neurological, and psychiatric syndromes. Our understanding of the genetic basis of vulnerability and the neurobiological mechanisms responsible for the expression of self-injury has only improved marginally since the study of self-injurious behavior began. The development of relevant animal models is critical for the advancement of self-injury research. Using these tools, researchers are now beginning to understand the neurobiological basis of self-injury and can begin to elucidate genetic mechanisms that confer vulnerability and to develop logical drug treatments.

The pemoline model is an especially useful tool for these examinations. Work in our laboratory has begun to demonstrate the validity of the pemoline model to represent human self-injurious behavior. The pemoline model offers face, construct, and predictive validity, which are essential for any relevant model of human pathology.

There are also many other characteristics of the pemoline model that make it a compelling animal model of self-injurious behavior. We propose that these characteristics, which are specific to the pemoline model, will contribute significantly to the advancements in the field of self-injurious behavior research. Firstly, the development of pemoline-induced behaviors is highly patterned. Over days of pemoline administration, the pemoline-induced behaviors evolve from high rates of locomotion (day 1), to the onset of stereotyped, repetitive behaviors (e.g. head bobbing, burrowing through bedding on day 2), to high rates of self-biting behavior (days 3 and 4; as demonstrated in Chapter 2). This predictable time course allows for evaluation of the neurobiological basis of these different behaviors. Understanding the subtle differences

in neurochemistry or structural biology that are associated with these different behaviors will advance our understanding of the ways in which stereotypy may be related to self-injury and more broadly, how the brain controls behavior.

Secondly, our finding that glutamate-mediated neuroplasticity is involved in the development of pemoline-induced self-injury (Muehlmann and Devine, 2008) highlights an interesting research opportunity for studying the behavioral-based theories of self-injurious behavior in an animal model. Over the past few decades, neuroscientists have begun to elucidate long term brain changes that are responsible for learning and memory and the pairing of operant contingencies (McKee et al., 2010). A preponderance of evidence suggests that these involve glutamate-based changes (though acetylcholine and serotonin probably also contribute) and are mediated by the specific properties of the glutamate receptors (Peng et al., 2011). No animal model to date has been able to incorporate the principles of the behavioral perspective of self-injury, and as described in the Introduction, behavior analysts are the most productive researchers of human self-injury. Therefore, the pemoline model is well situated to bring together behavioral and neurobiological theories of self-injury to create a more cohesive understanding of the mediators of self-injurious behavior.

Thirdly, a predominant role for dopamine in human and pemoline-induced self-injury has been documented. This point of convergence supports our contention that the pemoline model has construct validity, but also offers a useful parallel between pemoline-induced self-injury and other stimulant (i.e. indirect dopamine agonist)-induced behaviors. Our work suggests that the development of pemoline-induced self-injury is a behaviorally sensitized response to the stimulant properties of pemoline.

Researchers in the area of drug abuse have been studying the neurobiological basis of behavioral sensitization and its role in drug taking, drug craving, and relapse for several decades now. Following the research path of this area of study may allow the investigation of self-injurious behavior to progress much faster than it would without this parallel line of research.

Another special characteristic of the pemoline model that makes it a useful model of human self-injury is the individual differences in vulnerability that are exhibited in the pemoline-treated rats. These differences allow us to analyze the general effects of pemoline on the brain (in pemoline versus vehicle comparisons), as well as the self-injury specific effects of pemoline on the brain (in comparisons of pemoline-treated self-injurious versus pemoline-treated non-injurious rats). These different comparisons will allow researchers to identify self-injury related brain dysfunction more specifically, which may lead to quicker advancements in our understanding of the neurobiological basis of self-injury. Furthermore, we've identified a behavioral correlate to the vulnerability to develop pemoline-induced self-injury (stress responsiveness in a novel environment), so we can use this information to analyze genetic vulnerability to develop pemoline-induced self-injury by comparing non-pemoline-treated HR and LR rats. Research on behavioral pathologies associated with neurodevelopmental disorders highlights the importance of early intervention for success in reducing problem behaviors and the ability to identify vulnerable populations early through genetic screening or behavioral testing of novelty responsiveness may enhance the probability of successful therapy.

Lastly, our laboratory has recently found that pemoline-induced self-injury can also be established in mice. There are many genetic research tools that have been best

established in mice (e.g. gene knockouts and knock-in mice). The addition of these research tools to our analysis of the pemoline model will undoubtedly enhance our understanding of self-injurious behavior.

Overall, these projects have significantly increased our confidence in the utility of pemoline to model human self-injury. They have also improved our understanding of the neurobiological basis of self-injurious behavior and potential drug therapies to reduce this maladaptive behavior in individuals with neurodevelopmental and perhaps even neurological, and psychiatric conditions.

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