# VACUOUS CHEWING MOVEMENTS AND VARIABILITY IN NEUROPEPTIDE AND DOPAMINE RECEPTOR EXPRESSION IN THE DIRECT AND INDIRECT STRIATAL EFFERENT PATHWAYS

by

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# Vacuous Chewing Movements and Variability in Neuropeptide and Dopamine Receptor Expression in the Direct and Indirect Striatal Efferent Pathways

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts at George Mason University

By

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Bachelor of Science
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## DEDICATION

This is dedicated to my parents, John and Mildred Blanchard, my girlfriend, Wen Jin, and our son, Bryce Nolan Blanchard.

## ACKNOWLEDGEMENTS

I am grateful for the support provided by my friends and family, especially Adriana Falco. I also grateful to my committee, Dr. Susan Bachus, Dr. Robert Smith, and Dr. Craig McDonald for their support and guidance.

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## ABSTRACT

VACUOUS CHEWING MOVEMENTS AND VARIABILITY IN NEUROPEPTIDE AND DOPAMINE RECEPTOR EXPRESSION IN THE DIRECT AND INDIRECT STRIATAL EFFERENT PATHWAYS

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George Mason University, 2011

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Tardive dyskinesia is a serious and limiting side effect in long-term neuroleptic treatment that is still poorly understood. This study examined the expression of enkephalin (ENK), dynorphin (DYN), and D1 dopamine receptor (DRD1) mRNA, utilizing *in situ* hybridization histochemistry, in a cohort of rats chronically given haloperidol (HAL) decanoate (N=43) or control (N=21) injections for 24 weeks to determine if a relationship between striatal mRNA expression and vacuous chewing movements (VCMs) in two different noise conditions could be determined. Although HAL decanoate significantly increased ENK mRNA, this increase was not correlated with VCMs. Neither a significant HAL effect nor a correlation with VCMs was found for DRD1 mRNA. While HAL decanoate did not statistically significantly increase DYN mRNA in the experimental group, a statistically significantly positive correlation was found between DYN mRNA expression and VCM rates, but only when noises were present. These

results indicate that a significant change in the direct pathway contributes to the determination of VCM activity, but only in some of the rats treated with HAL, implicating individual variation in striatal DYN mRNA as playing a critical role in VCM activity, specifically in the exacerbation of VCMs by stress.

# INTRODUCTION

The introduction of neuroleptics in the 1950's was considered a major advance in the treatment of psychosis, especially positive symptoms of schizophrenia (delusions and hallucinations) (Kane, 2006), but it became clear by the beginning of the 1960's that chronic treatment using these first generation neuroleptics, known as classical or typical antipsychotics, had serious, limiting side effects. The most common of these side effects is a movement disorder known as tardive dyskinesia (TD), an involuntary condition, largely isolated to the orofacial region (worm-like tongue movements, tongue protrusions, lip pouting, chewing movements, and facial grimacing), but also known to involve trunk and limb movements on rare occasions (Barnes, Martin, & Trauer, 1983). Beyond the obvious social stigma, TD is a painful condition that can impede speaking and eating and is associated with a significant increase mortality rate (Youssef & Waddington, 1987). Symptoms can persist for years after treatment has been discontinued and are irreversible in some subjects (Casey, 1985). The importance of TD for the treatment of schizophrenia is evident when one considers that one of the determining factors between a medication being defined as an atypical rather than as a typical antipsychotic is the ability to induce TD (Farah, 2005).

Not all individuals are equally likely to develop neuroleptic-induced TD and a variety of factors predetermine individual vulnerability. Patients with pre-existing

conditions producing structural brain pathology (i.e. reduced right basal ganglia), neuropsychological deficits, diabetes mellitus, a history of extrapyramidal symptoms, and severe mental illness, such as unipolar depression and schizophrenia, have a higher prevalence rate for TD (Andreassen & Jørgensen, 2000). The most significant factor in developing TD is age. Some studies concerning typical antipyschotics have measured a mean prevalence TD rate of 20-25% in young subjects (Jeste & Wyatt, 1981) but found a prevalence rate greater than 50% in individuals over 50 years old. It should be noted that TD rates should be viewed with caution because, as per Apotenc Inc's package insert for HAL decanoate (2004), HAL can suppress the signs and symptoms of TD. Interestingly, the TD rate for the neuroleptic-naive elderly population is 5%, suggesting that age-associated changes in the brain may not only provide insight into the condition, but that typical antipsychotics might promote and/or exacerbate these changes (Leon, 2007).

**Basal Ganglia Involvement in TD**

The underlying pathology explaining TD remains unclear, but the evidence strongly suggests a dysfunction of the basal ganglia. The basal ganglia are composed of the dorsal (or neo-) striatum (the caudate nucleus and the putamen) and the ventral striatum (containing the nucleus accumbens and the olfactory tubercle), the globus pallidus, the substantia nigra (pars compacta and pars reticulata), and the subthalamic nucleus (Groves, 1983). The dorsal striatum is the largest component of the basal ganglia and integrates glutamatergic input from the cortex, primarily from the premotor and the motor cortex, and dopaminergic (DA) inputs from the substantia nigra, primarily the pars compacta, and gives rise to two distinct efferent GABA projections (the direct and

indirect pathways) (Alexander, 1986). Although the two pathways are critical in control of movement, the striatum is also involved in a wide variety of nonmotor functions, including motivation and memory (Bartels & Themelis, 1983).

The two pathways modulate movement in an opposing manner. The direct pathway contains the DYN-expressing neurons that are modulated by the D1 dopamine receptors (DRD1) and promote movement by disinhibiting the thalamus (Xu et al., 1994). The indirect pathway is the more complicated path and contains the ENK-expressing neurons that are modulated by D2 dopamine receptors (DRD2), and inhibits movement by tonically inhibiting the thalamus (Rivera et al., 2002). While the release of DA activates both receptor types, it stimulates the direct pathway and inhibits the indirect pathway, functionally breaking the tie between the glutamate stimulation of both pathways (Graybiel, 1990). Unlike the DRD1, which are primarily present on the postsynaptic neurons in the striatum, the DRD2 are also present presynaptically and play an important role in modulating DA via a negative feedback loop (Clark & White, 1987).

The rate at which neuroleptics induce TD is associated with their ability to bind as an antagonist to the DRD2. HAL, an antipsychotic with a high DRD2 affinity, induces the highest rate of TD when compared to clozapine, an atypical antipsychotic with a low DRD2 affinity, and risperidone, which has an affinity and TD rate between haloperidol and clozapine (Turrone, Remington, & Nobrega, 2002). The rate at which risperidone induces TD is dose dependent, indicating that DRD2 occupancy increases with higher doses, leading to higher rates of TD. PET scans have shown that, while a minimum DRD2 occupancy rate of 65% is required to induce a therapeutic effect, an occupancy

rate of 85% is associated with TD (Kapur, Zipursky, Jones, Remington, & Houle, 2000). This occupancy rate may account for advanced age being such a critical pre-existing condition, because it has been shown that DRD2 decreases signicantly with age in rats, which would indicate that a lower dose would be required to reach this critical 85% occupancy rate (Petkov, Petkov, & Stancheva, 1988).

The literature involving DA and DRD2 alterations in both human and rat subjects is consistent (reviewed in the Theories of TD section of this paper), but alterations in DRD1 are not. No changes have been noted in DRD1 density in some acute treatment studies (Fox, Mansour, & Watson, 1994), and no change was found after chronic treatment (Petersen, Finsen, Andreassen, Zimmer, & Jørgensen, 2000). The studies that did indicate increases in DRD1 density must be viewed with caution because the researchers defined increases in antagonists binding DRD1 as evidence of increased density, but increases in DRD1 binding affinities could be a potential confound in this line of thought (Sasaki, Kennedy, & Nobrega, 1998). DRD1 agonists have been shown to promote vacuous chewing movements (VCM), the animal model of TD, while DRD1 antagonists have also been shown to inhibit the activity (Ohno, Ishida-Tokuda, Ishibashi, & Nakamura, 1997), suggesting that the direct pathway does play a role in the activity.

**Opioid Involvement in VCM's**

The opioid peptides ENK and DYN, and their three receptors (mu, delta, kappa), contribute a multi-functional role in TD and VCM research. First, they allow researchers to segregate the two pathways, because DYN is only present DRD1 neurons and ENK is only present in DRD2 neurons. The neurons co-expressing both of these opioid peptides,

a very small subset of the striatum, express both DRD1 and DRD2 (Li et al., 1990). This is an invaluable tool, given that the two pathways are not compartmentalized in the striatum. ENK and DYN contribute a second but equally important role because they are reactive to DA-induced activity of each of the pathways. It has been suggested they play a role in tonically inhibiting DA-induced activity (Dourmap, Michael-Titus, & Costentin, 1992).

The literature supporting elevations in ENK in the VCM literature is extensive. Andreassen et al. (2000) did find a inverse correlation between the number of neurons expressing elevated ENK and VCM activity, but no other research has found a direct relationship between VCMs and ENK. Decreases in DA-induced activity in the indirect pathway, as seen in rats given chronic HAL or 6-hydroxydopamine (6-OHDA) induced lesions of the substantia nigra, lead to increases in ENK levels in the striatum (Tang, Costa, & Schwartz, 1983), which are reversed with DRD2 agonists (Gerfen et al., 1990), but the increase in ENK levels does not occur until DA depletion reaches approximately 90% (Li et al., 1990). Interestingly, the increases in ENK do not occur immediately after 6-OHDA lesions but can be delayed by weeks (Steiner & Gerfen, 1998) and were positively correlated with a recovery from 6-OHDA induced-hypokinesia, suggesting that ENK contributes to a compensatory mechanism. A delayed increase in ENK levels in response to HAL treatments was not found in the VCM literature. Egan et al. (1996) did find a significant increase in ENK mRNA one hour and twenty-four hours after injections of HAL, when compared to controls, but it should be noted that most research has found a delay in neuroleptic induced-TD and HAL-induced VCM presentation. The elevations

that Egan found might not have reached a threshold that would be associated with VCM activity.

The research regarding DYN in the VCM literature is not as extensive as that for ENK. Most researchers found that chronic HAL did not alter DYN expression (Nylander & Terenius, 1986, Truijillo, Day & Akil, 1990). Egan et al. (1994) and Meredith et al. (2000) did find elevations in DYN mRNA associated with HAL-induced VCMs after chronic treatment. Quirion (1985) found that chronic HAL led to elevations in DYN, but his work did not include behavior. Recent research into dyskinesia models has shown clear connections between elevated DYN and increased L-DOPA induced dyskinesia (Hanrieder, Ljungdahl, Fälth, Mammo, Bergquist &, Andersson, 2011). Stimulation by a D1 agonist led to increased DYN mRNA levels in 6-OHDA lesioned rats (Steiner & Gerfen, 1998) as well as DYN peptides in non-lesioned rats (Sivam, 1989). Interestingly, one study that looked at both ENK and DYN in 6-OHDA lesioned rats that were given the DYN receptor agonist SKF 38393 found significant elevations in both ENK and DYN mRNAs (Carta et al., 2008). Finally, the use of U50,488H, an agonist for the kappa opioid receptor that has been found to stimulate increases in DYN, was found to significantly decrease 3H-DA uptake in both dorsal striatum and the nucleus accumbens, suggesting that it may contribute to an increase in DA sensitivity (Das, Rogers, & Michael-Titus, 1994).

**Theories Regarding TD**

The oldest theory to explain TD is the DA receptor hypersensitivity hypothesis which postulates that chronic blockage of DRD2 leads to an increase in DRD2 receptor

affinity. Chronic HAL has been shown to decrease DA release in the striatum (Blaha & Lane, 1987) and upregulate DRD2 in rats (Vasconcelos, Nascimento, Nogueira, Vieira, Sousa, Fonteles, et al., 2003), possibly in response to decreased striatal DA transporter expression and to the decreased levels of extracellular striatal DA (Saldañaa, Bonastrea, Aguilara, & Marin, 2007). Research in rats has shown that an age-related reduction in DRD2 concentrations is also associated with an increase in DA binding affinity to DRD2. Clinical studies have shown that TD symptoms can be attenuated with increases in the dose of the antipsychotics, theoretically blocking more of the overly sensitive D2 receptors, while a decrease in the dose, or providing medications that lead to increases in DA (amphetamines, L-DOPA), leads to an exacerbation of TD symptoms (Egan, Apud, & Wyatt, 1997).

This theory has lost popularity in recent years, due in large part to its weaknesses (Galili-Mosberg et al., 2000). The research centering on genetic polymorphisms of the DRD2 that might explain hypersensitivity has been inconclusive (Tsai, North, West & Poole, 2010). There is no direct evidence correlating DA receptor densities with the number of dyskinetic movements in either human subjects (Kornhuber et al., 1989) or in rats (Knable et al., 1994). TD activity persists even when DA receptor density has returned to normal (Blin et al., 1989).

The second leading hypothesis of TD, the neuronal degeneration hypothesis, has gained popularity in TD literature in recent years. Brain imaging studies have revealed a number of abnormalities associated with TD, including enlargement of the striatum (Gur, Maany, Mozley, Swanson, Bilker, & Gur, 1998), decreased basal nucleus density rate

(Ueyama, et al., 1993), smaller caudate nuclei (Mion, Andreasen, Arndt, Swayze, & Cohen, 1991), and brain atrophy (McCreadie, Thara, Padmavati, Srinivasan, & Jaipurkar, 2002). Animal studies have shown a decrease in striatal neurons expressing ENK (Andreassen, Finsen, Østergaard, Sørensen, J, West, & Jørgensen, 1999), and reduced numbers of substantia nigra neurons in rats with antipsychotic induced VCM's. One study found that rats with the lowest number of nerve cells in the substantia nigra pars compacta had the highest VCM rates (Andreassen, Ferrante, Aamo, Beal, & Jørgensen, 2003).

A number of different mechanisms leading to neuronal damage have been postulated. Excessive glutamate activity is known to induce excitotoxicity (Yi & Hazell, 2006). In addition, it has been shown that chronic HAL promotes glutamate release in the striatum (Andreassen, Meshul, Moore, & Jørgensen, 2001). Chronic HAL has been shown to increase the expression of TNF-α and NF-κB, two protein markers for apoptosis (Bishnoi, Chopra, & Kulkarni, 2008). A prominent feature of TD is free radical damage of the lipid membranes and typical antipsychotics increase DA turnover, leading to an increase in reactive oxygen species. HAL's metabolite is the quaternary pyridinium HPP+, which is a strong inhibitor of the electron transport chain's complex I, leading to an increase in free radicals (Balijepalli, Boyd, & Ravindranath, 1999). Human subjects treated with chronic HAL have shown decreases in catalase and superoxide dismutase in the cerebrospinal fluid, enzymes known to decrease lipid peroxidation (Lohr & Bracha, 1988). HAL has been shown to deplete glutathione, a protein which is involved in DNA repair and protein synthesis, in addition to control of free radicals (Yokoyama, et al.,

1998). A recent double-blind study found that the antioxidants vitamins E and B6, and piracetam reduced the severity of TD symptoms, possibly through the reduction of free radicals (Lerner, et al., 2007).

This hypothesis does have a number of weaknesses. Neuronal degeneration is a prominent feature of Parkinson's Disease, but oral dyskinesias are not normally present in non-treated human subjects (Ondo, 2011) or its animal model (Steiner & Gerfen, 1998). Brain damage has been shown to promote both HAL-induced TD (Andreassen & Jorgensen, 2000) and VCM activity (Andreassen et al., 1999) but it has not been shown that brain damage alone can replicate oral dyskinesia. A reduction in dendritic spines appears to contribute more to VCM activity than neuronal death (Meredith et al., 2000).

Given the extent of research regarding TD and VCM's, the lack of a single, centralized theory illustrates the complexity of the subject and also reflects inconsistencies in the methodology. Most studies define chronic treatment in rats as 21 days, but Egan et al. (1996) found that challenging rats with HAL after a 21 day treatment did not suppress VCM activity, while rats treated for 30 weeks did show suppression of VCM activities when challenged with HAL, indicating that a much longer treatment is needed to produce a viable model of TD. The inconsistent use of washout periods also carries the potential for producing inconsistent results. A cohort that received HAL i.p. treatments for 30 days had continued increases in DRD2 seven days after the final dose but these increases were not evident in animals sacrificed 15 days after the final treatment (Vasconcelos et al., 2003). Finally, it has been shown that stress can increase VCM activity (Glenthøja & Hemmingsen, 1991) and TD activity

(Baldessarini & Tarsy, 1980) but few papers actually address how the researchers avoided this potential confound.

**Specific Aims of this Study**

1. To determine whether variability in striatal expression of DRD1, ENK, or DYN mRNA is correlated with VCM activity.
2. To determine whether these mRNAs are correlated with striatal DRD2 mRNA previously measured in this cohort.
3. To determine whether relationships between mRNAs and VCMs differ when VCMs are measured during a noisy or a quiet environment.

# METHODS

## Material to be Utilized from a Previous Study

This study is a continuation of previous study (Nealon, et al., 2002) in which a cohort of 64 male, hooded Long Evan rats were treated at three week intervals for twenty-four weeks with either intramuscular injections of HAL (28.5 mg/ml/kg, N=44) suspended in sesame oil, or vehicle (sesame oil; N=21). All animals were provided free access to food and water, and were provided twelve hours of light each day. One HAL rat died prior to the end of the experiment.

VCMs were counted for two minute samples weekly for each subject by an observer blind to treatment group. It was noted that VCMs were affected by noises that occurred during observations and the decision was made to control for noise. At the end of the HAL treatment, rats were observed in both a noisy environment (jingling of keys and taped Celtic music played at approximately 70 decibels) and quiet environments (the soft hum of the air conditioner), for two minute intervals, for four days for each condition. VCM values were averaged across the noisy and across the quiet trials.

At the end of twenty-four weeks, the rats were sacrificed via decapitation, and their brains were immediately removed, frozen in powdered dry ice, and stored at -70°C. Residual HAL was measured in cerebellum by high pressure liquid chromatography (courtesy of Manickam Aravagiri, Psychopharmacology Unit, Veterans Administration

Greater Los Angeles Healthcare System, University of California at Los Angeles, Los Angeles, CA). Brains were subsequently cryostat-sectioned coronally to a thickness of 16μ for *in situ* hybridization, and two brain sections per slide were mounted onto gelatin subbed slides.

DRD2 expression was measured using *in situ* hybridization histochemistry according to the method described by Young (1992). A 48 base long oligonucleotide probe complementary to the mRNA for DRD2 was labeled with an $^{35}$SdATP tail. Slides containing the striatum were exposed to probe, incubated, and then run through a series of washes to remove any nonspecific binding. The labeled tissue and $^{14}$C standards (ARC, Inc., St. Louis, MO) were then placed in a film cassette containing Biomax film for two weeks, and then developed. Autoradiographic images from the films were scanned, calibrated using the $^{14}$C standards, and analyzed using NIH Image (Rasband, NIMH).

**Present Study**

Using the same *in situ* histochemistry procedure used for DRD2 mRNA, probes for DRD1, ENK, and DYN mRNAs were obtained, labeled, and exposed to tissue sections containing striatum, and analyzed using NIH image. Each probe was 48 oligonucleotides long. The only deviation from the DRD2 procedure was length of time in the cassette, which was four days for ENK, and six weeks for DRD1 and DYN.

Measurements were taken in four areas of the neostriatum using NIH image: the dorsal lateral (DL), the dorsal medial (DM), the ventral lateral (VL), and the ventral medial (VM) neostriatum. In addition, measurements were also taken in the nucleus

accumbens (NA), which was further subdivided into core and shell components in the ENK and DRD2 images, but was not in the DRD1 and DYN, where expression was homogeneous in the NA.

All data in this study were first determined to be approximately normally distributed by examination of Q-Q plots before statistics were performed, and all statistical work was performed using SPSS. An Independent Samples *t*-test was used to determine if the means of the control group differed significantly from the means of the HAL rats for each of the targets. Correlations were then calculated to determine whether there was an association between the levels of mRNA and VCM rates in either the control or HAL group. All statistics utilized two-tailed tests of significance unless otherwise noted.

Egan et al. (1994) found a significant increase in DYN mRNA expression when they subdivided the HAL group. To examine whether a comparable increase in DYN mRNA occurred in this study, the HAL group was subdivided into a high VCM (hVCM) and a low VCM group, and independent t-tests were utilized to determine if there was a significant difference change in DYN mRNA expression in the hVCM group when compared to the controls. Individuals were placed in the hVCM group if they received HAL and had a VCM rate in the noisy condition that was greater than the median.

# RESULTS

## Previous Study

The behavioral data were obtained from the previous study (Nealon, et al., 2002. The mean VCM's obtained can be seen in Figure 1. By two-way ANOVA, a significant effect was found for the HAL treatment effect ($F(1, 62) = 12.21$, $p < .001$), and for the environmental stress condition (noise)
($F( 1,62) = 11.70$, $p < .001$), but not for an interaction effect ($F(1, 62) = 1.68$, $p< .01$), in the production of VCM activity. Post-hoc Independent Samples *t*-tests revealed that VCMs were significantly increased in HAL rats, relative to vehicle controls, in both the quiet ($t(62) = -2.61$, $p =.01$) and noisy ($t(62) = -2.57$, $p =.01$) conditions. For HAL rats in the noisy environment, the mean VCM production was significantly elevated when compared to the non-noisy environment ($p < .001$ by post-hoc LSD test). However, VCM's did not differ between these conditions for the vehicle rats ($p= .20$ by post-hoc LSD test).

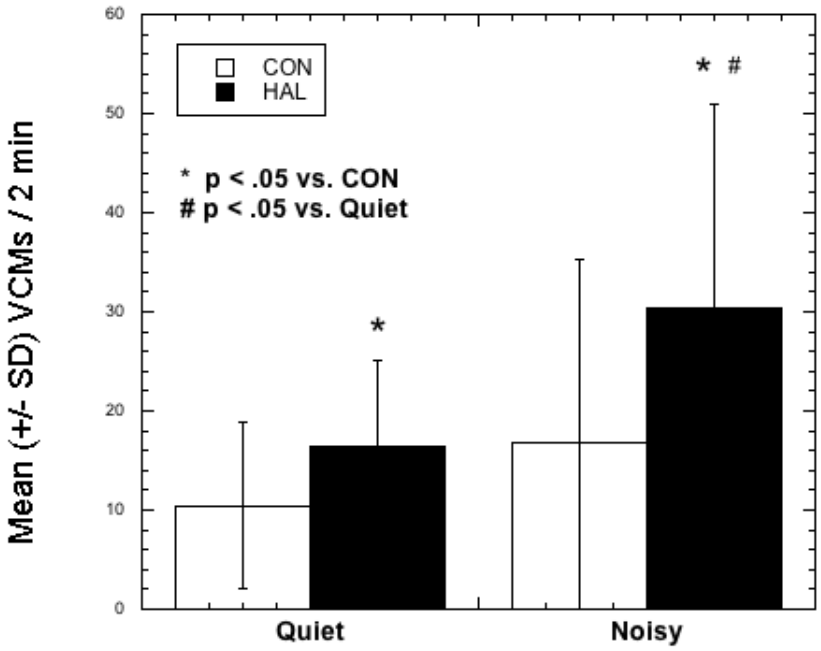



Figure 1. VCM means (± SD) for controls and HAL-treated rats during quiet and noisy conditions.

Residual HAL levels in the cerebellum were found to be not be significantly correlated with VCMs among the HAL-treated rats.

**HAL's effect on DRD2 mRNA expression**

HAL was found to significantly increase DRD2 mRNA in all four areas of the neostriatum (see Table 1) (DL ($t(62) = -3.952$, $p < .001$), DM ($t(62) = -4.787$, $p < .001$), VL ($t(62) = -5.369$, $p < .001$ ), VM ($t(62) = -4.295$, $p < .001$ )), and the NA core ($t(62) = -2.236$, $p < .029$) but not in the NA shell (see Table 1).

Table 1
Independent Samples *t*-tests for increases in DRD2 mRNA caused by HAL

| Source | df | *t* | *p* |
|---|---|---|---|
| DL | 62 | -3.952 | **.001**** |
| DM | 62 | -4.787 | **.001**** |
| VL | 62 | -5.369 | **.001**** |
| VM | 62 | -4.295 | **.001**** |
| NA core | 62 | -2.236 | **.029*** |
| NA lateral shell | 62 | -.991 | .326 |
| NA medial shell | 62 | -.471 | .639 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

Residual HAL levels were not found to be significantly correlated with DRD2 mRNA in any region (see Table 2).

Table 2
Correlations between DRD2 mRNA and residual HAL

| Source | *r* | *p* |
|---|---|---|
| DM | .088 | .574 |
| DL | .082 | .602 |
| VL | .120 | .444 |
| VM | .186 | .234 |
| NA core | .000 | .999 |
| NA lateral shell | .037 | .813 |
| NA medial shell | -8.000 | 0.96 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

Correlations were calculated to examine whether a relationship exists between VCM rates during both the quiet and the noisy conditions and DRD2 expression. No significant correlations were found in the controls (see Table 3) or the HAL-treated rats (see Table 4).

Table 3
Correlations between DRD2 mRNA and VCM rates for controls

| Quiet | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.058 | .804 |
| DM | -.341 | .131 |
| VL | -.077 | .739 |
| VM | -.108 | .640 |
| NA core | -.084 | .718 |
| NA lateral | -.070 | .770 |
| NA medial | .168 | .467 |
| Noisy | | |
| Source | *r* | *p* |
| DL | -.083 | .722 |
| DM | -.175 | .449 |
| VL | -.024 | .919 |
| VM | -.016 | .945 |
| NA core | .052 | .824 |
| NA lateral | -.068 | .776 |
| NA medial | .052 | .823 |

Note. N=21, two-tailed test,* p<.05, ** p<.01.

Table 4
Correlations between DRD2 mRNA and VCM rates for HAL group

| Quiet condition | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.105 | .503 |
| DM | -.114 | .466 |
| VL | -.122 | .437 |
| VM | -.134 | .392 |
| NA core | -.059 | .705 |
| NA lateral | -.035 | .823 |
| NA medial | .057 | .716 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | .200 | .199 |
| DM | .060 | .701 |
| VL | .030 | .851 |
| VM | .023 | .885 |
| NA core | .046 | .769 |
| NA lateral | .041 | .794 |
| NA medial | .059 | .722 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

**Present Study**

Representative images for each of the probes are shown in Figure 2.

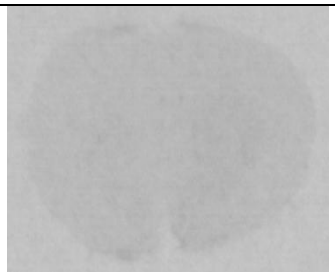
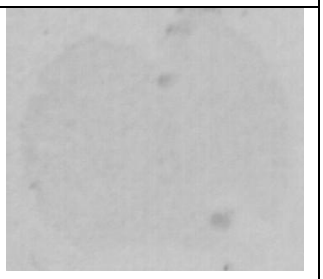
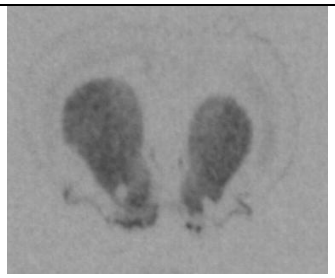
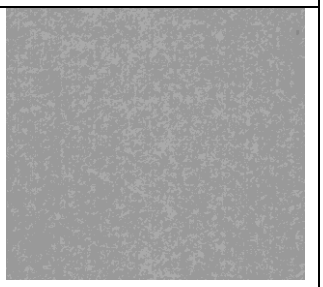
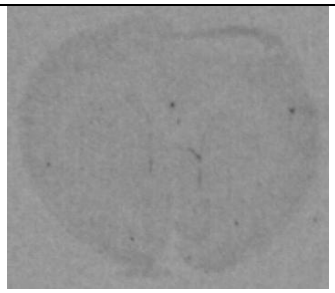
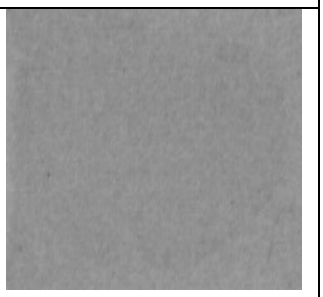
| | Antisense | Missense |
|---|---|---|
| DRD1 |  |  |
| ENK |  |  |
| DYN |  |  |

Figure 2. Representative images for each of the probes.

**HAL's effect on DRD1 mRNA expression**

Independent Samples *t*-tests found no significant differences in the group mean between the controls and the HAL group (see Table 5).

Table 5
Independent Samples *t*-tests for increases in DRD1 mRNA caused by HAL

| Source | df | *t* | *p* |
|---|---|---|---|
| DL | 62 | 1.500 | .139 |
| DM | 62 | .932 | .355 |
| VL | 62 | .617 | .539 |
| VM | 62 | 1.767 | .082 |
| NA | 62 | .634 | .529 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

No significant correlations between residual HAL levels and DRD1 mRNA were found in the neostriatum or the NA (see Table 6).

Table 6
Correlations between DRD1 mRNA and residual HAL

| Source | *r* | *p* |
|---|---|---|
| DL | -.248 | .108 |
| DM | -.180 | .247 |
| VL | .108 | .490 |
| VM | -.110 | .484 |
| NA | -.168 | .282 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

No significant correlations were found between DRD1 mRNA and VCM's among either the controls (see Table 7) or the HAL group (see Table 8).

Table 7
Correlations between DRD1 mRNA and VCM rates for controls

| Quiet condition | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.092 | .692 |
| DM | -.255 | .265 |
| VL | -.251 | .273 |
| VM | -.008 | .997 |
| NA | -.041 | .860 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | -.194 | .399 |
| DM | -.220 | .337 |
| VL | -.222 | .924 |
| VM | -.060 | .795 |
| NA | -.044 | .850 |

Note. N=21, two-tailed test,* p<.05, ** p<.01.

Table 8
Correlations between DRD1 mRNA and VCM rates for HAL group

| Quiet condition | | |
|---|---|---|
| Source | *r* | *p* |
| DL | .183 | .240 |
| DM | .043 | .784 |
| VL | .215 | .165 |
| VM | .147 | .346 |
| NA | .210 | .177 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | -.065 | .678 |
| DM | -.172 | .271 |
| VL | -.093 | .554 |
| VM | -.153 | .328 |
| NA | -.185 | .235 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

**Relationship between ENK mRNA expression and DRD2 mRNA expression**

In the control group, significant positive correlations were found between ENK mRNA and DRD2 mRNA in the VL ($r$ = .468, $p$< .032), and VM neostriatum ($r$ = .575, $p$< .006), NA core ($r$ = .764, $p$< .000), and NA shell ($r$ = .487, $p$< .029), but not the in the DM or the DL neostriatum (see Table 9 and Figure 3). In the HAL group, no significant correlation was found in the neostriatum, but a significant positive correlation between ENK and DRD2 mRNAs was found in both the NA core ($r$ = .621, $p$< .000) and NA shell ($r$ = .615, $p$< .000) (see Table 1 and Figure 4).

Table 9
Correlation between ENK mRNA and DRD2 mRNA in the controls

| Source | *r* | *p* |
|---|---|---|
| DL | .296 | .192 |
| DM | .406 | .068 |
| VL | .468 | **.032*** |
| VM | .575 | **.006**** |
| NA core | .764 | **.000**** |
| NA shell | .487 | **.029*** |

Note. N=43, two-tailed test, * p<.05, ** p<.01.

Table 10
Correlation between ENK mRNA and DRD2 mRNA in the HAL group

| Source | *R* | *p* |
|---|---|---|
| DL | .241 | .119 |
| DM | .269 | .081 |
| VL | .119 | .447 |
| VM | .153 | .326 |
| NA core | .621 | **.000**** |
| NA shell | .615 | **.000**** |

Note. N=64, two-tailed test, * p< .05, ** p<.01.

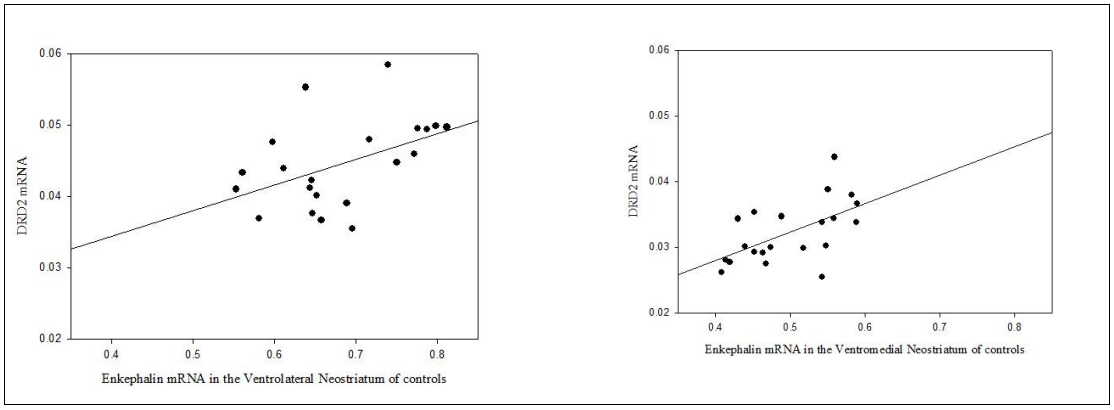



Figure 3. Correlations between ENK mRNA and DRD2 mRNA in both the VL and the VM neostriatum of the controls.

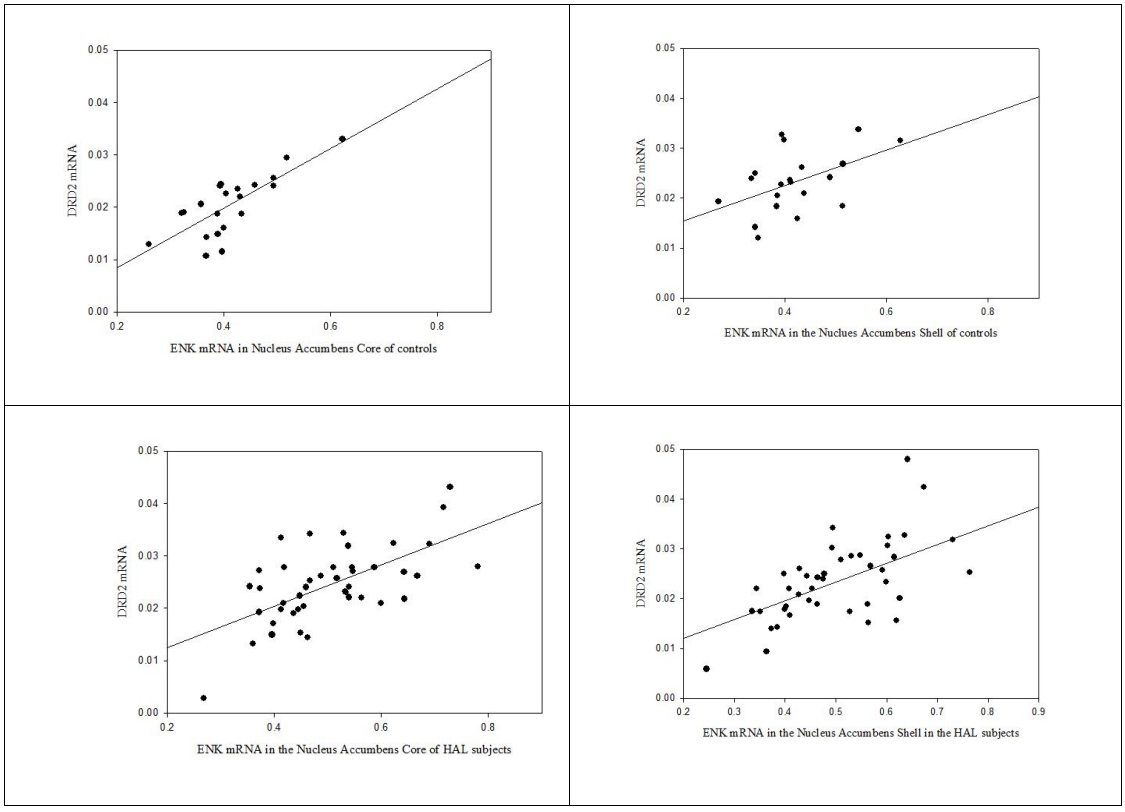


Figure 4. Correlations between ENK mRNA and DRD2 mRNA.

**HAL's effect on ENK mRNA expression**

HAL was found to significantly increase DRD2 mRNA in all four areas of the neostriatum (DM ($t(62) = -12.546$, $p < .001$), DL ($t(62) = -11.372$, $p < .001$), VL ($t(62) = -11.976$, $p < .001$), VM ($t(62) = -10.928$, $p < .001$)), and in the NA core ($t(62) = -3.400$, $p < .001$) and NA shell ($t(62) = -3.076$, $p < .003$) (see Table 11 and Figure 5) .

Table 11
Independent Samples *t*-tests for increases in ENK mRNA caused by HAL

| Source | df | *t* | *p* |
|---|---|---|---|
| DL | 62 | -12.546 | **.001**** |
| DM | 62 | -11.372 | **.001**** |
| VL | 62 | -11.976 | **.001**** |
| VM | 62 | -10.928 | **.001**** |
| NA core | 62 | -3.400 | **.001**** |
| NA shell | 62 | -3.076 | **.003**** |

Note. N=43, two-tailed test.,* p<.05, ** p<.01.

Significant positive correlations were found between ENK mRNA and residual HAL levels in all four areas of the neostriatum (see Table 12), utilizing a one-tailed test (DL ($r$ = .289, $p$< .030), DM ($r$ = .285, $p$< .032), VL ($r$ = .311, $p$< .021), VM ($r$ = .299, $p$< .026) (see Figure 2). Significance was not found in either region of the NA.

Table 12
Correlations between ENK mRNA and residual HAL

| Source | *R* | *p* |
|---|---|---|
| DL | .289 | **.030*** |
| DM | .285 | **.032*** |
| VL | .311 | **.021*** |
| VM | .299 | **.026*** |
| NA core | .165 | .144 |
| NA shell | .145 | .177 |

Note. N=43, one-tailed test,* p<.05, ** p<.01.

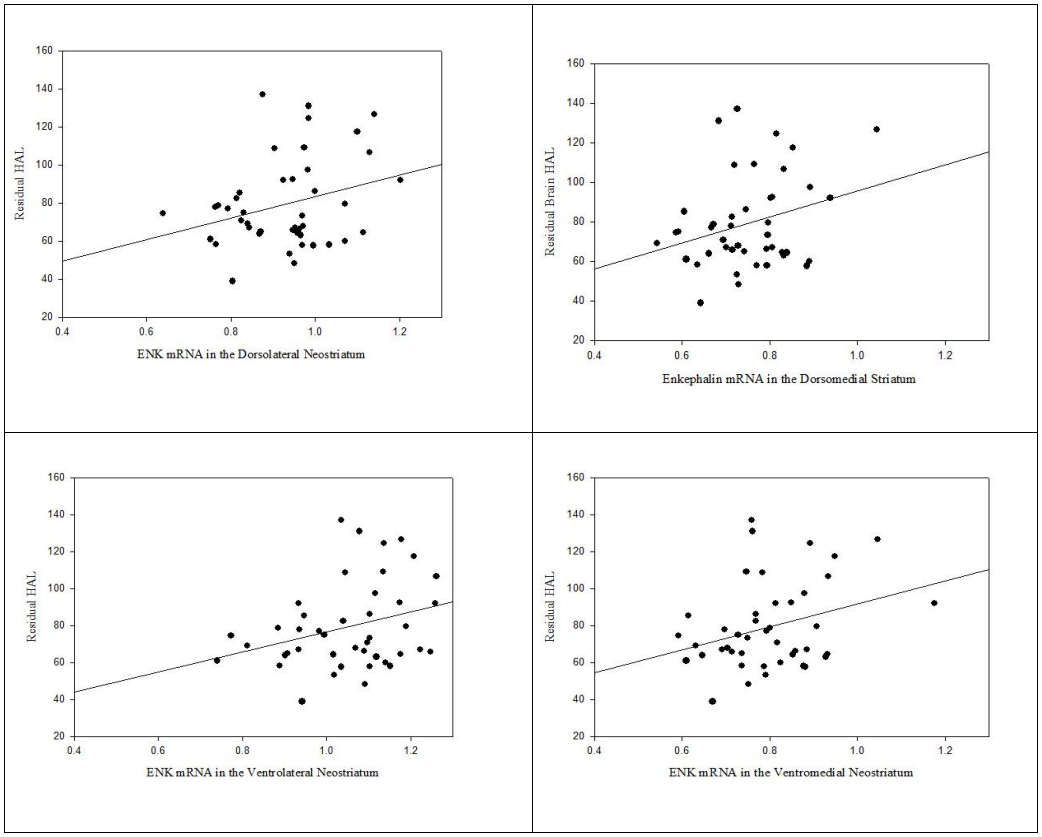


Figure 5. Correlations between ENK and residual HAL.

No significant correlations were found between ENK mRNA and VCM activity, measured during either the quiet condition or the noisy condition, in either the controls (see Table 13) or the HAL-treated rats (see Table 14).

Table 13
Correlations between ENK mRNA and VCM rates for controls

| Quiet | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.309 | .174 |
| DM | -.367 | .102 |
| VL | -.145 | .530 |
| VM | -.376 | .093 |
| NA core | .128 | .581 |
| NA shell | .116 | .616 |
| Noisy | | |
| Source | *r* | *p* |
| DL | .135 | .560 |
| DM | .032 | .891 |
| VL | .194 | .399 |
| VM | .058 | .803 |
| NA core | .005 | .983 |
| NA shell | .116 | .615 |

Note. N=21, two-tailed test,* p<.05, ** p<.01.

Table 14
Correlations between ENK mRNA and VCM rates for HAL group

| Quiet condition | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.030 | .846 |
| DM | .026 | .867 |
| VL | .016 | .920 |
| VM | .039 | .849 |
| NA core | -.024 | .881 |
| NA shell | .032 | .838 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | .182 | .243 |
| DM | .224 | .149 |
| VL | .076 | .628 |
| VM | .027 | .865 |
| NA core | .136 | .386 |
| NA shell | -.001 | .995 |

Note. N=43, two-tailed test,* p<.05, ** p<.01.

**HAL's effect on DYN mRNA expression**

No significant effect of HAL on DYN mRNA was shown by Independent Samples *t*-tests (see Table 15).

Table 15
Independent Samples *t*-tests for increases in DYN mRNA caused by HAL

| Source | df | *t* | *p* |
|---|---|---|---|
| DL | 62 | -1.175 | .244 |
| DM | 62 | -.706 | .483 |
| VL | 62 | -.927 | .358 |
| VM | 62 | -.996 | .323 |
| NA | 62 | -1.229 | .224 |

Note. N=41, two-tailed test,* p<.05, ** p<.01.

A significant negative correlation was found between DYN mRNA and residual HAL levels in the DL ($r$ = -.440, $p$< .004), the DM ($r$ = -.372, p< .017) and the VM ($r$ = -.329, $p$< .036) neostriatum and in the NA ($r$ = -.349, $p$< .025), but not in the VL neostriatum (see Table 16 and Figure 6).

Table 16
Correlations between DYN mRNA and residual HAL

| Source | *r* | *p* |
|---|---|---|
| DL | -.440 | **.004**** |
| DL | -.372 | **.017*** |
| VL | -.250 | .116 |
| VM | -.329 | **.036*** |
| NA | -0.35 | **.025*** |

Note. N=41, two-tailed test,* p<.05, ** p<.01.

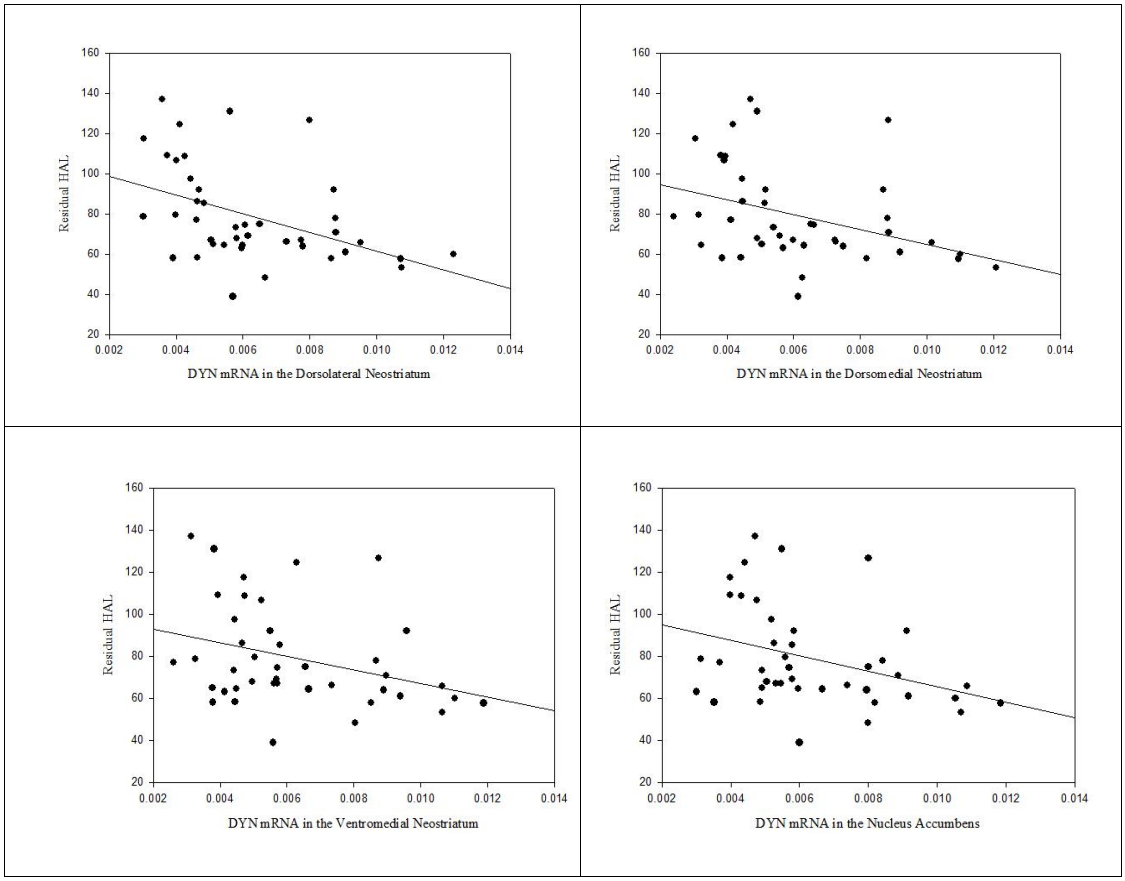


Figure 6. Correlations between DYN mRNA and residual HAL.

No significant correlation between VCM activity measured during the quiet condition and DYN mRNA was found in either group. A significant positive correlation was obtained between VCM activity measured during the noisy condition in the HAL rats and DYN mRNA in all four neostriatal regions (DL ($r$ = .420, $p$< .006), DM ($r$ = .361, $p$< .021), VL ($r$ = .355, $p$< .023), VM ($r$ = .383, $p$< .014)), and in the NA ($r$ = .339, $p$< .030)) (see Table 17 and Figure 6), but no significant correlation was found in the control subjects (see Table 17).

Table 17
Correlations between DYN mRNA and VCM rates for controls

| Quiet condition | | |
|---|---|---|
| Source | *r* | *p* |
| DL | -.104 | .654 |
| DM | -.057 | .807 |
| VL | -.082 | .725 |
| VM | -.105 | .649 |
| NA | -.082 | .723 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | .285 | .211 |
| DM | .215 | .349 |
| VL | .242 | .291 |
| VM | .236 | .303 |
| NA | .144 | .534 |

Note. N=21, two-tailed test,* p<.05, ** p<.01.

Table 18
Correlations between DYN mRNA and VCM rates for HAL group

| Quiet condition | | |
|---|---|---|
| Source | r | p |
| DL | -.157 | .327 |
| DM | -.194 | .224 |
| VL | -.210 | .187 |
| VM | -.170 | .287 |
| NA | -.206 | .197 |
| Noisy condition | | |
| Source | *r* | *p* |
| DL | .323 | **.040*** |
| DM | .307 | **.051*** |
| VL | .325 | **.038*** |
| VM | .359 | **.021*** |
| NA | .310 | **.049*** |

Note. N=41, two-tailed test,* $p \leq .05$.

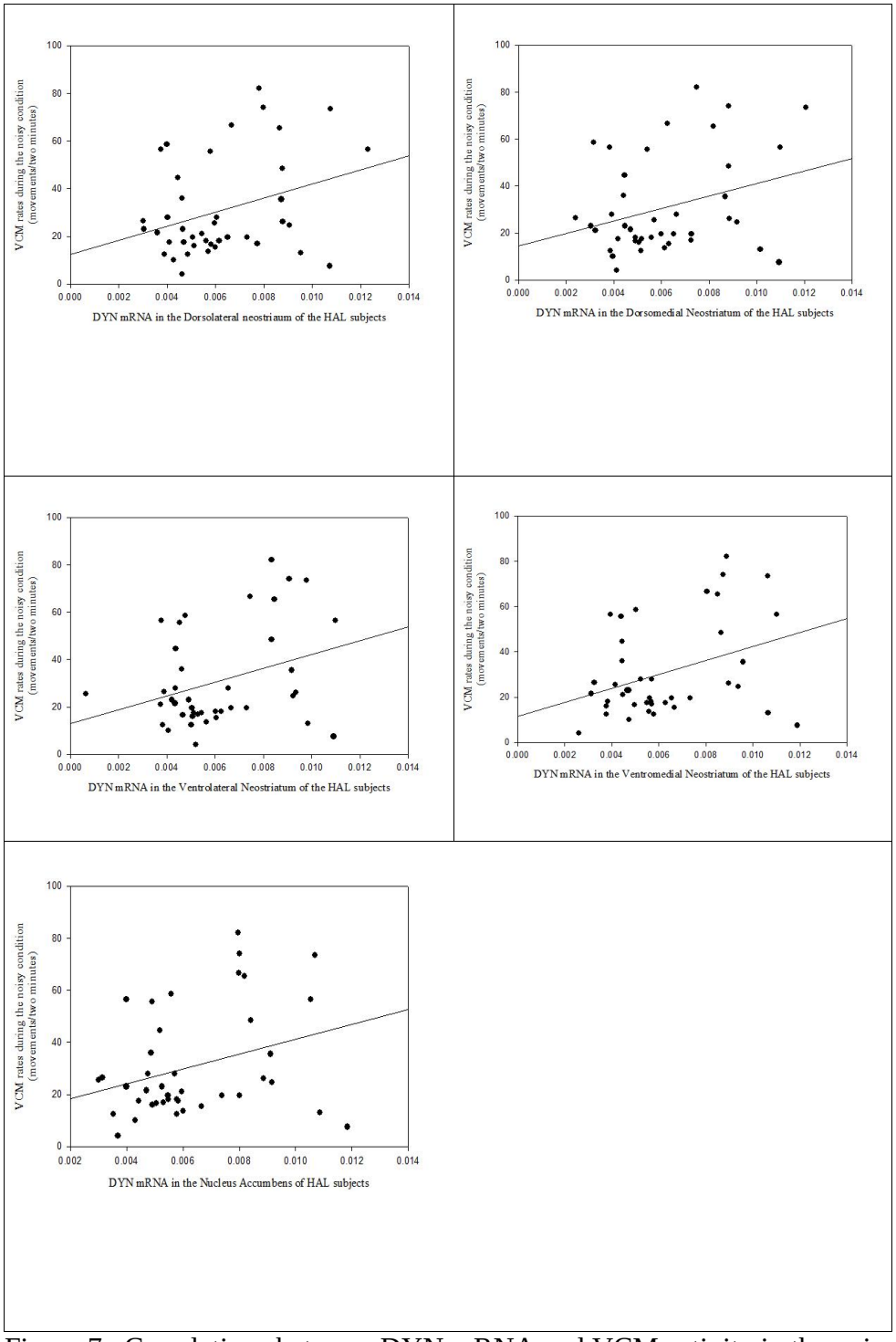



Figure 7. Correlations between DYN mRNA and VCM activity in the noisy condition for the HAL subjects.

HAL was found to significantly increase DYN mRNA in the DL ($t(40) = -2.477$, $p < .018$), the VM ($t(40) = -2.389$, $p < .022$) neostriatum, and in the NA ($t(40) = -2.213$, $p < .033$) but not in the (DM ($t(40) = -1.819$, $p < .076$) nor in the VL ($t(40) = -1.996$, $p < .053$) neostriatum of the hVCM group (see table 19).

Table 19
Independent Samples *t*-tests for increases in DYN mRNA caused by HAL in the hVCM group

| Source | df | *t* | *P* |
|---|---|---|---|
| DL | 40 | -2.477 | .009* |
| DM | 40 | -1.819 | .038* |
| VL | 40 | -1.996 | .027* |
| VM | 40 | -2.389 | .011* |
| NA | 40 | -2.213 | . 017* |

Note. N=40, one-tailed test,* p<.05, ** p<.01.

**Relationship between DYN mRNA expression and DRD1 mRNA expression**

A significant correlation between DYN expression and DRD1 expression was not found in either the control or the HAL groups (see Tables 19 and 20).

Table 19
Correlation between DYN mRNA and DRD1 mRNA in the controls

| Source | *r* | *p* |
|---|---|---|
| DL | -.036 | .876 |
| DM | -.059 | .798 |
| VL | -.048 | .835 |
| VM | -.074 | .749 |
| NA | .216 | .347 |

Note. N=21, two-tailed test, * p<.05, ** p<.01.

Table 20
Correlation between DYN mRNA and DRD1 mRNA in the HAL group

| Source | *r* | *p* |
|---|---|---|
| DL | -.135 | .399 |
| DM | -.142 | .375 |
| VL | -.135 | .399 |
| VM | -.168 | .293 |
| NA | -.125 | .436 |

Note. N=41, two-tailed test, * p<.05, ** p<.01.

# DISCUSSION

To the best of our knowledge, this is the first study to examine how different noise levels alter the correlations between VCM activity and regional striatal neuropeptide and dopamine receptor mRNA expression in a HAL model that is truly chronic and does not utilize a washout period. This study utilized correlations in addition to *t*-tests, which allowed us to determine how individual differences in striatal mRNAs between subjects were related to VCM activity during a stressful (noisy) and a non-stressful (quiet) condition. The two different noise level conditions allowed us to determine how a stressor (noise) may differentially influence the relationships between regional mRNA expression and activity under the two conditions.

## Alterations in the Indirect Pathway

This study found a highly significant increase in DRD2 mRNA expression in the HAL group in all striatal regions and a significant increase in the NA core but not the NA shell, consistent with the earlier literature. However, the non-significant correlations between DRD2 mRNA and VCMs, in both groups, strongly indicate that, while blockage of DRD2 leads to alterations in the HAL group, there is no direct relationship between the amount of DRD2 expression and VCM activity, and that other changes are responsible for modulating VCM activity. While changes in the direct pathway could be a necessary condition for the inductions of VCMs, they are evidently not sufficient to

determine whether VCMs develop.

No significant correlation between DRD2 mRNA and residual HAL was found. This result indicates that HAL had a strong, homogeneous effect on the striatal indirect pathway in all the HAL subjects, and that individual differences in residual HAL levels contributed little to DRD2 expression. This lack of variation suggests that the alterations in DRD2 expression stabilized prior to the end of our study. Egan et al. (1994), utilizing a similar model, also found a significant increase in DRD2 after a six month washout period, suggesting that the stabilizing of expression at the end of this regimen may be a permanent result.

A highly significant increase in striatal ENK mRNA expression was found in the HAL group in all striatal regions and in both areas of the NA, which is consistent with prior research (Egan et al., 1996). Furthermore, the increases in ENK mRNA in all striatal regions were correlated with increases in residual HAL levels, though only by a one-tailed test. These results again describe a homogeneous effect such that, although HAL produced a strong group effect, differences in residual HAL levels were rather weakly driving differences in expression. Again, however, this effect was not correlated with VCMs, consistent with the idea that striatal ENK mRNA elevation could be necessary, but not sufficient, to produce VCMs, also in agreement with the finding by Egan et al. (1996) that striatal ENK mRNA elevation precedes VCM emergence.

These results seem somewhat contradictory to those of Andreassen et al. (1999), who found an inverse correlation between VCM activity and the number of neurons expressing elevated levels of ENK in a chronic HAL model. We did not perform cell

counting as part of this study, so it is possible that the subjects with the elevations in ENK mRNA expression, who also had elevated HAL-induced VCM activity, might have had fewer cells expressing ENK, but doing so in an overly robust manner.

Consistent with what would be expected due to their co-localization, in the control group, a significant positive correlation was found between DRD2 mRNA and ENK mRNA in all striatal regions, except DL, and both NA areas. In the HAL group, a significant positive correlation was found between DRD2 mRNA expression and ENK mRNA expression, but only in the NA. These results indicate that, in the neostriatum, the relationship between DRD2 expression and ENK expression is altered by HAL, and it also suggests that different pathways are involved in modulating ENK and DRD2 expression. In the NA, this relationship was maintained in both the control and the HAL subjects.

**Alterations in the Direct Pathway**

No significant HAL effects were found for DRD1 mRNA, nor was there any significant association between DRD1 mRNA expression and VCM activity in either the control or the HAL group. Nor was any significant difference found in DYN mRNA expression between the control group and the HAL group, a finding consistent with earlier studies which failed to find elevated DYN mRNA after chronic HAL (Nylander & Terenius, 1986; Truijillo, Day & Akil, 1990), even though our study utilized a higher dose of HAL, since the literature suggests a dopamine depletion of at least 80% is critical in the dysregulation of DYN mRNA expression. Our results indicated that, while differences in the direct pathway do not occur in all rats treated with chronic HAL,

individual differences in response of the direct pathway to HAL may still be implicated in determining vulnerability to VCMs.

Of the three previous studies that noted an increase with DYN mRNA expression in response to HAL treatments, Quirion (1985) did not examine behavior and Meredith et al. (2000) primarily examined synaptic connections in the NA. Only Egan et al. (1994) examined HAL-induced changes in DYN mRNA expression in relation to VCM activity. They utilized a similar model, in that they subjected the cohort to 24 weeks of treatment of HAL decanoate, given in three week intervals, measured residual HAL levels in the cerebellum, and examined alterations in expression of DRD1, DRD2, ENK, and DYN mRNA. They reported elevations in DYN expression in the HAL group, but the increase was only in the subgroup still expressing VCM's after a six month washout period, which is in fact consistent with our finding that striatal DYN mRNA was elevated in the rats which developed VCMs. Our significant HAL-induced increases in DRD2 and ENK mRNA, but not in DRD1 mRNA, are consistent with Egan's study.

The Egan et al. (1994) study was different from ours in several important respects. First, they utilized a six month washout period, which we did not, so our study better represents the conditions present during and at the end of chronic HAL treatment. Egan et al. also only utilized ANOVA's to determine significant group differences, while we used both Independent Samples *t*-tests, to measure group changes, and correlations, to explore how differences between the subjects were related to VCM activity. Egan also utilized Sprague-Dawley rats, which have been shown to have lower and less variable VCM rates than the hooded Long Evan rats we utilized (Tamminga, Dale, Goodman,

Kaneda & Kaneda, 1990). Finally, we utilized a noisy condition, in addition to a quiet condition, to exacerbate VCM activity, producing an expanded range of activity, allowing us to better determine the condition that best reveals relationships between VCMs and alterations in striatal neuropeptides.

Significant negative correlations were found between residual HAL levels and DYN mRNA in both areas of the NA and in all striatal regions, except for the VL. However, significant positive correlations were found between VCM activity in the noisy condition and DYN expression in all four striatal regions and in the NA among the HAL subjects. Results found in rats with dopamine depletion induced by 6-OHDA lesions, where an increase in DYN was associated with dykinetic movements (Hanrieder, et al., 2010), are in agreement with this study. The lack of a significant treatment effect may be due to a differential effect across the subjects, in which the subjects with altered levels of DYN mRNA are the same individuals who develop altered VCM rates.

The specificity of the correlations between DYN mRNA and VCM activity, during the noisy, but not the quiet condition, indicate that the role that variability in striatal DYN expression contributes in the determination of development of VCMs appears to be involved in the exacerbation of VCMs by stress, rather than the baseline HAL-induced VCMs. This observation is consistent with the literature implicating striatal DYN in the mediation of response to stress (see, e.g., Zhou et al., 2002; McLaughlin, Marton-Popovic, & Chavkin, 2003; Beardsley, Howard, Shelton, & Carroll, 2005; McLaughlin, Li, Valdez, Chavkin, & Chavkin, 2006; Bruchas, et al., 2007; Land et al., 2008).

## Conclusion

This study examined how mRNAs for striatal DA receptors and neuropeptides are associated with chronic HAL-induced VCM rates under different ambient conditions. Consistent with most published studies, significant changes in the indirect pathway, the pathway responsible for inhibiting movement, were found. However, these changes were not related to variability in development of VCMs among the HAL-treated rats. Conversely, it was also determined that individual differences in striatal DYN expression appear to parallel vulnerability for developing VCMs, even though HAL did not significantly alter striatal DYN mRNA expression, specifically implicating the direct pathway in mediating variability in susceptibility to VCMs, and by analogy, TD. This study also found that DYN mRNA expression is associated with VCM activity specifically during the noisy condition, suggesting that stress contributes an important role in that relationship. Shifts in correlations between DRD2 and ENK mRNAs, within the neostriatum, between the control and HAL-treated groups, also suggest that chronic HAL exposure causes dysregulation of the normal coordination of these proteins. These results add to similar findings that both regionally specific mRNAs, and VCMs, are differentially affected by HAL across individuals in a parallel fashion, implicating these regional substrates in the determination of vulnerability to HAL-induced TD.

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## CURRICULUM VITAE

Charles J. Blanchard grew up in northern Virginia on a small farm. After serving in the Middle East with the 382nd Field Hospital during the Gulf War, he completed a Bachelor of Arts in English in 1998 and a Bachelor of Science in Biology in 2002.