ANTI-PSYCHOTIC DRUG INDUCED TARDIVE DYSKINESIA

ANTI-PSYCHOTIC DRUG INDUCED TARDIVE DYSKINESIA: A ROLE FOR THE ANTI-APOPTOTIC MOLECULE CURCUMIN

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TITLE: Anti-psychotic drug induced tardive dyskinesia: A role for the anti-apoptotic molecule curcumin in the prevention of an animal model of human tardive dyskinesia

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ABSTRACT

The use of anti-psychotic drugs to treat schizophrenia can result in the development of movement disorders including tardive dyskinesia (TD), characterized by abnormal oro-facial movements and occasionally movements of the trunk and limbs. The most widely accepted model of TD is the anti-psychotic drug induced vacuous chewing movement (VCM). While the mechanism of induction of TD remains unclear, there are two prevailing hypothesis: oxidative stress and dopamine supersensitivity. The currently available anti-psychotic drugs antagonize the dopamine D2 receptor and this can result in excessive dopamine accumulation and oxidation which has been shown to cause striatal neurodegeneration. This is the oxidative stress hypothesis. The dopamine supersensitivity hypothesis proposes that anti-psychotic drug treatment causes an up-regulation of high affinity dopamine D2 receptors to compensate for D2 receptor antagonism. The widely acclaimed anti-oxidant curcumin, an extract of turmeric, has been additionally demonstrated to affect dopamine levels. Thus, the goal of this study was to investigate curcumin's potential to prevent haloperidol-induced behavioural and biochemical abnormalities. Four groups of rats were utilized: control; haloperidol; curcumin and curcumin plus haloperidol. Rats were administered curcumin at 200mg/kg daily in jello, followed by daily intra-peritoneal injections of haloperidol at 2mg/kg. VCMs, catalepsy and locomotor activity were assessed. Animals were sacrificed and tissues removed for qPCR, immunoblot, receptor binding and UPLC assessments. At day14 there was a significant increase in VCMs and catalepsy following haloperidol treatment, which was prevented by concurrent curcumin treatment. Interestingly, curcumin did not alter

locomotor activity. Curcumin was demonstrated to increase the expression of the anti-apoptotic molecule, Bcl2-like protein 1 (BclXL) and counter-intuitively to increase striatal dopamine D2 receptors. These investigations provide support for the potential effectiveness of curcumin in the prevention of TD and provide further insight into the complex pathophysiology of this devastating iatrogenic disorder.

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I dedicate this work to my family, friends and colleagues who have made this journey unforgettable.

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ABBREVIATIONS

AADC, aromatic amino acid decarboxyase

ADH, aldehyde dehydrogenase

AIF, apoptosis inducing factor

AIMS, Abnormal Involuntary Movements Scale

BclXL, Bcl2-like protein 1

BH_4, tetrahydrobiopterin

cAMP, cyclic-AMP

CAF, central animal facility

CNS,central nervous system

COMT, Catechol-O-methyltransferase

CREB, cAMP-response element binding protein

Cyt c, cytochrome c

DARPP-32, 32kDa dopamine and cAMP-regulated phosphoprotein

DAT, dopamine transporter

DDC, L-DOPA decarboxylase

DISCUS, Dyskinesia Identification System: Condensed User Scale

DIABLO, direct inhibitor of apoptosis (IAP)-binding protein with low pI

DOPAC, 3,4-dihydroxyphenylacetic acid

DOPAL, 3,4-dihydroxyphenylacetaldehyde

DSM IV, Diagnostic and statistical manual of mental disorders IV

ECL, enzymatic chemiluminescence

EDTA, ethylenediaminetetraacetic acid

EndoG, endonuclease G

GFAP, glial fibrillary acidic protein

GTP, guanosine triphosphate

GTPCH, guanosine triphosphate cyclohydrolase

H_2O_2, hydrogen peroxide

HVA, homovanillic acid

IAP proteins, inhibitor of apoptosis proteins

LC/MS, Liquid Chromatography/Mass Spectrometry

L-DOPA, L-dihydroxyphenylalanine

MAO, monoamine oxidase

NA, nucleus accumbens

NF, neurofilament

O_2•, superoxide radicals

OH•, hydroxyl radicals

Omi/HtrA2, high-temperature-requirement protein A2

PKA, protein kinase A

qPCR, quantitative real-time polymerase chain reaction

SMAC, second mitochondria-derived activator of caspases

SN, substantia nigra

SNpc, substantia nigra pars compacta

SNpr, substantia nigra pars reticulata

SQ•, semiquinone radicals

STN, sub-thalamic nucleus

TD, tardive dyskinesia

TH, tyrosine hydroxylase

UPLC, Ultra performance liquid chromatography

VCM, vacuous chewing movement

VMAT2, vesicular monoamine transferase 2

VTA, ventral tegmental area

1. INTRODUCTION

Tardive dyskinesia is a disfiguring and debilitating iatrogenic disorder that has impeded the treatment of mental health disorders since the advent of anti-psychotic drugs in the 1950's (Lopez-Munoz et al., 2005; Jasovic-Gasic et al., 2012; Wolf et al., 1993; Natesan & Kapur, 2012). While it is primarily associated with anti-psychotic drug use, it has also been demonstrated to be an important side effect that can result from drugs used to treat other direct or indirect dopamine-related disorders. Tardive dyskinesia can result from treatment with anti-Parkinson's drugs, anti-epileptic drugs, anxiolytic drugs, anti-depressant drugs, anti-emetics and anti-cholinergics amongst others (Bhidayasiri & Boonyawairoj, 2011; Lerner & Miodownik, 2011). This diversity of causative agents renders tardive dyskinesia a significant risk to a major portion of the 1 million Ontarians and 450 million individuals worldwide that currently suffer from mental health diseases (Ontario Brain Institute, 2013; World Health Organization, 2010). The ensuing investigations present a novel method for the prevention of tardive dyskinesia and provide insight into the underlying pathophysiology of this disorder.

1.1. HISTORY

1.1.1. An introduction to anti-psychotic drugs

Over half a century ago, in an effort to produce an effective anti-histamine Paul Charpentier discovered chlorpromazine (a derivative of phenothiazine) at the French company Rhône Poulenc (Reviewed by Shen, 1999). Chlorpromazine was demonstrated by Simone Courvosier, also at Rhône Poulenc, to induce a state of indifference and decreased responsiveness in animal models (Kapur & Mamo, 2003; Courvoisier, 1956; Shen, 1999). It was this effect of chloropromazine that led Henri-Marie Laborit to consider this drug for the potentiation of the effects of surgical-anesthetics and to discuss the potential of this drug with his psychiatric colleagues; Pierre Hamon in Val de Grace Military Hospital as well as Pierre Deniker and Jean Delay at Sainte-Anne Hospital in Paris (Paulson, 2005; Kapur & Mamo, 2003; Natesan & Kapur, 2012; Shen, 1999). Subsequently, Bernard P, a patient of Hamon, was one of the first individuals to receive chlorpromazine as a treatment for mania and chlorpromazine became the first neuroleptic (Kapur & Mamo, 2003). Around the same time that chlorpromazine was making an impression in Europe, Reserpine was starting to be used in North America for the treatment of psychoses amongst other things (Shen, 1999; Kapur & Mamo, 2003; Kapur & Mamo, 2003). The term neuroleptic was not well embraced in North America and these drugs were instead termed tranquilizers and later became known as anti-psychotic drugs (Wolf et al., 1993). In 1958, haloperidol, a butyrophenone, was released by Haase and Janssen in Belgium. Haloperidol, another influential anti-psychotic drug, was

demonstrated to produce an even more potent therapeutic effect in the prevention of hallucinations and delusions than its predecessors (Singh & Kay, 1975).

The advent of first generation (typical) anti-psychotic drug use in the 1950's marked a transition in the clinical realization that mental illness had underlying biochemical etiologies. It was no longer the deviance of a person that underlay his psychiatric challenges but potentially there were pathophysiological changes in the brain that could lead to these behavioural and psychological correlates.

1.1.2. Extra-pyramidal side effects: An introduction to tardive dyskinesia

Unfortunately, just two years (1954) after the clinical release of chlorpromazine and reserpine, anti-psychotic drugs were realized to have their limitations (Wolf et al., 1993; Faurbye et al., 1964; Paulson, 2005). These drugs caused "extra-pyramidal" side effect which included symptoms of Parkinsonianism, characterized by tremor, bradykinesia, stiffness, imbalance and rigidity; as well as akathesia or an inner restlessness (Haddad & Dursun, 2008; Faurbye et al., 1964; Wolf et al., 1993). Additional disfiguring symptoms, including the development of abnormal oro-facial movements and occasionally abnormal movements of the trunk and limbs, defined the final and arguably most severe side effect of this first generation of drugs (Faurbye et al., 1964). In 1963 this side effect was named tardive dyskinesia (Faurbye et al., 1964; Fernandez & Friedman, 2003; Marsalek, 2000; Shen, 1999).

"During congress a dispute arose between those who saw tardive dyskinesia as a serious and frequent adverse effect of neuroleptics [anti-psychotic drugs] *and those who argued that dyskinesias were simply symptoms of schizophrenia, especially of the catatonic type"* (Oldrich Vinar, 2010)

Initially, it was difficult to conceive that drugs which affected cognition might also have implications in movement (Lerner & Miodownik, 2011). The clinical assessments were complicated by the non-iatrogenic abnormalities in movement that could have occurred as a consequence of the psychoses or other factors. In particular, the spontaneous dyskinesia's that occurred in Schizophrenia, also coined *parakinetic catatonia* by Kraepelin in 1919, were frequently argued to be the movements that were being observed in these individuals (Lerner & Miodownik, 2011). These spontaneous dyskinesia's occurred in 5-10% of individuals diagnosed with schizophrenia at that time and were more frequent in the elderly (Lerner & Miodownik, 2011; Faurbye et al., 1964). Yet, in 1957 Schönecker et al. and in 1959 Sigwald et al. were amongst the first to challenge this thought and to describe tardive dyskinesia as a consequence of anti-psychotic drug treatment (Faurbye et al., 1964; Paulson, 2005; Lerner & Miodownik, 2011). They characterized the involuntary oro-facial movements that would occur following chronic anti-psychotic administration (Faurbye et al., 1964; Paulson, 2005; Lerner & Miodownik, 2011). By 1963, Faurbye's paper defined and distinguished tardive dyskinesia from spontaneous dyskinesia's and other anti-psychotic drug-induced effects. Importantly, Faurbye described additional symptoms of tremor, rigidity and that

symptoms could disappear during attention tasks which were not observed in the non-iatrogenic spontaneous dyskinesia's (Faurbye et al., 1964). He further observed that the incidence of these movement disorders were much higher than would be anticipated with spontaneous dyskinesia's alone (Faurbye et al., 1964). Thus, supporting the hypothesis of that time that tardive dyskinesia was an unfortunate side-effect of anti-psychotic drug treatment.

However, if these movements were indeed consequences of the anti-psychotic drugs then the question arose of whether their presence was a necessary phenomenon that was indicative of the therapeutic effect of the drug (Faurbye et al., 1964). Cole and Clyde in 1961 (referenced by Faurbye) renounced this possibility due to another characteristic feature of tardive dyskinesia (Faurbye et al., 1964; Cole & Clyde, 1961). Only a portion of the population administered anti-psychotic drugs would develop these movements, meanwhile a portion of the population that experienced the therapeutic effect remained free of movement disorders (Faurbye et al., 1964).

1.1.3. A second generation of anti-psychotic drugs

The widespread acknowledgement that psychoses had biological underpinnings and that the current medications presented severe side effects promoted drug development in the field of psychiatry. The motivations were manifold, there was obvious financial profit but an additional challenge existed. If tardive dyskinesia was a side effect of drug treatment then it may be possible to un-couple the psychological therapeutic effect from the motor abnormalities.

In 1958, Stille at Wander Pharmaceuticals in Switzerland discovered and developed Clozapine (Hippius, 1999). This second generation of anti-psychotic drugs, termed atypical, brought hope for a treatment free of tardive dyskinesia. By the 1990's this drug was marketed in North America, but has since presented with a number of new and old challenges (Li et al., 2009; Volavka & Citrome, 2009). There was a reduced, but not eliminated, incidence of tardive dyskinesia that was observed with this drug and there were additional and often more detrimental side effects including an increased risk of agranulocytosis (acute leukopenia), hypotension, seizures, weight gain as well as abnormal glucose and lipid metabolism (Klawans, Jr. & Rubovits, 1972; Blin et al., 1989; Li et al., 2009). Consequently, typical anti-psychotic drugs continue to be used in the clinic and tardive dyskinesia remains a significant challenge to the medical community today.

1.2. TARDIVE DYSKINESIA

1.2.1. Characterization

Tardive is derived from the French word *tardif* which means "late". Dyskinesia is composed of the prefix dys meaning "disturbed" and kinesia is derived from the Greek word *kinein* meaning "to move" (Bhidayasiri & Boonyawairoj, 2011; Lerner & Miodownik, 2011). Collectively, the root words "late, disturbed, movement" accurately defines the major characteristics of tardive dyskinesia. It is an iatrogenic disorder that occurs following chronic anti-psychotic drug treatment that is characterized by abnormal hyperkinetic involuntary movement of the oro-facial, lingual and buccal regions (Bhidayasiri & Boonyawairoj, 2011; Lerner & Miodownik, 2011). Additional choriform (jerky and involuntary) and athetoid (writhing and twisting) movements of the trunk and limbs can also occur (Faurbye et al., 1964).

The phenotype of tardive dyskinesia include vermicular tongue movements that can protrude in and out of the mouth (Bhidayasiri & Boonyawairoj, 2011). Chewing, grinding and grimacing as well as pursing, smacking and sucking of lips, can also occur. These movements are involuntary and physically disfiguring and can become limiting as the disorder progresses interrupting speech and food intake. Individuals suffering from tardive dyskinesia can experience dental challenges, in particular with the maintenance of dentures which can be regularly displaced or broken due to the movements. The facial abnormalities that are characteristic of tardive dyskinesia include blinking and movements of the eyebrows (Bhidayasiri & Boonyawairoj, 2011; Haddad & Dursun, 2008; Gardos et al., 1987).

When the effect extends beyond the oro-facial region there may be involuntary laryngeal changes and consequent vocalization (Bhidayasiri & Boonyawairoj, 2011). Breathing can be affected, as well as the flexion and extension of shoulders, fingers, wrists, hips, knees, ankles and toes (Bhidayasiri & Boonyawairoj, 2011). These effects are challenging in social settings, but can also interrupt daily life and personal care. These symptoms are rarely present during sleep and can be halted during attention tasks, however most commonly individuals affected by tardive dyskinesia are not aware of the presence of their symptoms or their ability to voluntarily modulate symptoms (Bhidayasiri & Boonyawairoj, 2011).

1.2.2. Diagnosis

Schooler and Kane have developed three main criteria for the diagnosis of tardive dyskinesia. Firstly, drug exposure must have occurred for a minimum of 3 months. Abnormal movements should be observed in one or more region of the body and finally, there should not be another cause for a movement abnormality (Schooler & Kane, 1982; Bhidayasiri & Boonyawairoj, 2011). The Diagnostic and statistical manual of mental disorders IV, DSM IV, requirements are similar to Schooler and Kane's with the exception that exposure can be just one or more months in individuals who are considered elderly (older than 60 years) (Bhidayasiri & Boonyawairoj, 2011). The dyskinetic phenotype rarely appears sooner than 3 months of treatment and most commonly will not become apparent until 12-24 months of treatment (Bhidayasiri & Boonyawairoj, 2011).

Symptoms can also develop following abrupt discontinuation of treatment (Tranter & Healy, 1998).

A number of rating scales have been developed for the assessment and characterization of tardive dyskinesia in humans. These assessments include: Abnormal Involuntary Movements Scale (AIMS), Extra-pyramidal Symptoms Rating Scale, Tardive Dyskinesia Rating Scale, St. Hans Rating Scale and Dyskinesia Identification System: Condensed User Scale (DISCUS) (Lane et al., 1985; Gharabawi et al., 2005; Kalachnik & Sprague, 1993; Simpson & Singh, 1988; Chouinard & Margolese, 2005; Ringo et al., 1996). Assessments are recommended to be performed consistently; at the same time of day, at a constant standard of activity level and position and following the same interval of drug treatment.

1.2.3. Incidence, Prevalance and Risk for tardive dyskinesia

The published epidemiological assessments for the incidence and prevalence of tardive dyskinesia have varied extensively. Differences in the sample populations being assessed, the heterogeneity of treatment regimens, high drop-out rates, short study duration as well as variations in the selection criteria and the assessment tools used to identify and define tardive dyskinesia have influenced the currently established incidence and prevalence ranges (Correll & Schenk, 2008).

The incidence of tardive dyskinesia currently ranges from 0.7-68.0% with persistence rates of 82% (Novick et al., 2010; Glazer et al., 1993; Jeste et al., 1999; Blumberger et al.,

2013; Merrill et al., 2013). The prevalence of tardive dyskinesia has ranged from 1.2-42.0% (Woerner et al., 1998; Kane, 2004; DeLong & Wichmann, 2007; Gardos et al., 1994). There are a number of important factors that must be considered when interpreting these ranges and attempting to decipher on what end of the spectrum an individual might reside in terms of susceptibility to tardive dyskinesia. Individuals who are female, elderly, are of ethnic (non-Caucasian) decent, have diabetes, history of alcohol abuse, or are being treated for psychoses or schizophrenia are more vulnerable to tardive dyskinesia (Merrill et al., 2013; Jeste et al., 1995; Morgenstern & Glazer, 1993; Tenback et al., 2009). The choice of drug, high doses and longer durations of treatment can render individuals more susceptible for the development of tardive dyskinesia (Morgenstern & Glazer, 1993; Bhidayasiri & Boonyawairoj, 2011; Glazer et al., 1993; de Leon, 2007; Jeste et al., 1995). Finally, the appearance of early extra-pyramidal symptoms has been suggested to be indicative of the potential for the development of tardive dyskinesia (Bhidayasiri & Boonyawairoj, 2011; Merrill et al., 2013).

Early investigations, comparing the relative effectiveness of first and second generation anti-psychotic drugs on the incidence of tardive dyskinesia and treatment of psychoses, were widely marketed in favor of the newer second generation drugs (Tandon et al., 2008). However, the CATIE and CUtLASS investigations demonstrated that the thearapeutic effectiveness of first and second generation anti-psychotic drugs were not significantly different for the treatment of psychoses in terms of compliance, effectiveness or quality of life (Naber & Lambert, 2009; Tandon et al., 2008). Similarly the claims that the atypical treatments were “free of extra-pyramidal side effects”, and in

particular tardive dyskinesia, have since been refuted (Li et al., 2009; Tandon et al., 2008). More recently these newer generation anti-psychotic drugs have been widely acknowledged to still pose the threat of tardive dyskinesia albeit at a lower risk (Li et al., 2009). The use of a second generation anti-psychotic drug may as such render one on the lower end of the incidence range for the development of tardive dyskinesia but does not preclude the development of tardive dyskinesia altogether (Correll et al., 2004; Tarsy et al., 2011; Correll & Schenk, 2008).

1.2.4. Treatments of tardive dyskinsia in humans

The current clinical and innovative research approaches that attempt to address the challenge of tardive dyskinesia are primarily preventative in nature. In an attempt to minimize the risk of developing tardive dyskinesia clinically conservative use of anti-psychotic drugs, regular re-assessments of drug dose and duration and avoiding co-treatments particularly with anti-cholinergic drugs is implemented. Symptoms are carefully monitored predominantly by the use of AIMS testing.

Since tardive dyskinesia is a complex disorder, once developed, treatment or reversal of the dyskinetic phenotype remains a challenge (Jeste & Wyatt, 1979). The chances of successful therapy are however increased in lower risk populations (risk was previously outlined in ***section 1.2.1***) (Jeste & Wyatt, 1979). Reassessment of anti-psychotic drug choice (i.e. switching the anti-psychotic drug) and dose, and a tapering of the current medications until full cessation of treatment can facilitate some therapeutic effect (switch

to aripiprozole (Osorio et al., 2010). However, full cessation of the drug treatment is not usually a feasible option when confronting psychoses. If tapering of the anti-psychotic drug does not facilitate sufficient mitigation of symptoms, then clozapine, risperidone, or quetiapine can be added to the drug regimen. If this is insufficient tetrabenzanine, reserpine, or vitamin E can be added (Louza & Bassitt, 2005; Bai et al., 2005; Spindler et al., 2013). In the event that none of these treatments are therapeutic, deep brain stimulation that is targeted to the globus pallidus internal has been proposed as a final option (Spindler et al., 2013). Other drugs that have demonstrated some efficacy in relieving tardive dyskinesia symptoms include Vitamin B6, Piracetam, Amantadine, Clonazepam, Levetiracetam, Buspirone, Melatonin, ginkgo biloba, omega 3, branched-chain amino acids and Donepezil (Emsley et al., 2004; Howland, 2011; Emsley et al., 2006; Zhang et al., 2011; Caroff et al., 2001; Moss et al., 1993; Shamir et al., 2001; Richardson et al., 2003; Bobruff et al., 1981; Angus et al., 1997; Woods et al., 2008; Lerner et al., 2001; Ondo et al., 1999; Lieberman et al., 1991).

While these drugs demonstrate some efficacy in mitigating dyskinesia, in most cases this effect has not been demonstrated to be a permanent solution. On the contrary with many of these atypical switches the dyskinetic phenotype is hidden rather than eliminated. Once individuals are taken off these drugs the dyskinetic phenotypes resume (Louza & Bassitt, 2005; Chouinard, 1995; Bai et al., 2005). Additionally, the therapeutic effect has been suggested to be occurring at the expense of the development of Parkinsonianism (Faurbye et al., 1964; Damier et al., 2007).

1.2.5. Complexity of the disease: Important pieces to the puzzle

Tardive dyskinesia is a complex side effect of anti-psychotic drug treatment, which perplexed clinicians in the 1970's and continues to elude us today. There are a number of important features of this disorder that make a complete understanding of its pathophysiology particularly challenging.

Tardive dyskinesia only appears following chronic drug exposure. Once developed it is predominantly irreversible. However, symptoms only develop in a portion (0.7-68.0%) of the population of individuals administered anti-psychotic drugs (Novick et al., 2010; Glazer et al., 1993; Jeste et al., 1999; Blumberger et al., 2013; Merrill et al., 2013). Consequently anti-psychotic drugs are necessary but not on their own sufficient to cause tardive dyskinesia. Interestingly, this population division is observed in animal models also, even populations of genetically identical animals. Thus, the additional contributors to the development of tardive dyskinesia remain elusive. Symptoms are stereotyped within any one patient and very similar between individuals also. Suggesting a similar motor brain region is implicated in all incidences. Symptoms disappear during sleep and during attention tasks (Lerner & Miodownik, 2011; Jus et al., 1972). Thus, the dyskinetic phenotype can be halted for a time which can complicate the clinical assessments for the development of tardive dyskinesia (Faurbye et al., 1964). As was previously alluded to, it was this unique feature which facilitated the early distinctions between tardive dyskinesia from the "spontaneous" dyskinesias observed in un-medicated psychotic populations (Paulson, 2005; Faurbye et al., 1964; Lerner & Miodownik, 2011). Finally, abrupt discontinuation of anti-psychotic drug treatment can result in the development of

symptoms. Counter-intuitively, in some cases placing the individuals back on the anti-psychotic drug can mitigate or reverse symptoms (Lerner & Miodownik, 2011). However the usefulness of this approach to therapy continues to be debated. The possibility that there can be irreversible brain damage from chronic treatment makes the option of continued treatment as a therapeutic option for tardive dyskinesia less desirable. It might offer only a short term remedy with more devastating long term risks.

1.3. ANTI-PSYCHOTIC DRUGS AND THE DOPAMINE D2 RECEPTOR

In the 1970's the dopamine hypothesis of schizophrenia was proposed as an attempt to address the biochemical changes that occur in psychoses and the therapeutic mechanisms of anti-psychotic drugs (Howes & Kapur, 2009). It was identified that the relative effectiveness of typical anti-psychotic drugs was proportional to their affinity for the dopamine D2 receptor (Creese et al., 1976; Seeman & Lee, 1975). These preliminary findings provoked associations between psychoses and the D2 receptor. It was later acknowledged that dopamine levels were significantly increased in individuals who had schizophrenia or psychoses and that anti-psychotic drugs antagonized the dopamine D2 receptor blocking the effects of native agonist, dopamine (Farde et al., 1989; Mackay et al., 1982). Further, 65-80% occupancy of the D2 receptor by anti-psychotic drugs was optimal for a therapeutic action beyond which side effects, including tardive dyskinesia, become increasingly apparent (De et al., 2011; Madras, 2013; Nord & Farde, 2011). However, dopamine occupancy is a physiological change that remains a challenge to regulate clinically particularly with chronic treatment (De et al., 2011; Nord & Farde, 2011).

Atypical, second generation, anti-psychotic drugs have also been demonstrated to exert their therapeutic effects by interaction with the dopamine D2 receptor. However, they exhibit a lower specificity than the first generation typical anti-psychotic drugs, binding also to the serotonin receptor amongst others (Meltzer & Massey, 2011; Meltzer et al., 2003). It is this distinction in specificity coupled with a faster on-off binding that mitigates the extent of antagonism of the dopamine D2 receptor by atypical anti-psychotic

drugs relative to the typical anti-psychotic drugs. This attenuated D2 receptor binding that is observed with atypical anti-psychotic drugs is thought to account for the decreased risk of tardive dyskinesia.

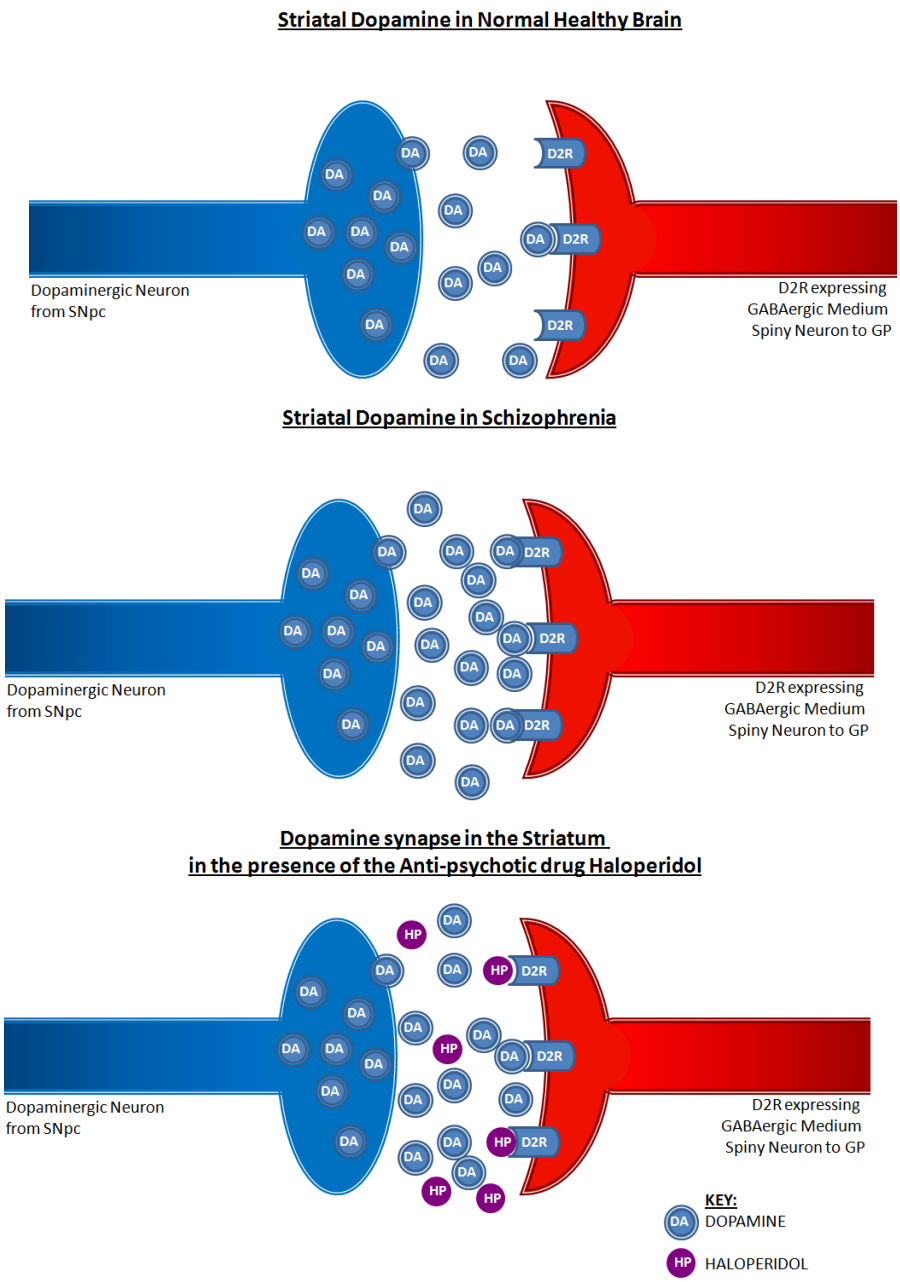


Figure 1. The pathophysiology of Schizophrenia and the mechanism of action of anti-psychotic drugs. In schizophrenia there is an increased release of dopamine in the synaptic cleft causing over-stimulation of the post-synaptic cell. Anti-psychotic drugs antagonize the dopamine D2 receptor blocking D2 receptor stimulation by the native ligand dopamine and the consequential down-stream effect.

1.3.1. An introduction to Dopamine

Arvid Carlsson discovered the neurotransmitter dopamine in Sweden in the 1960's and along with Eric Kandel and Paul Greengard went on to win the Nobel prize in medicine for this discovery in the year 2000 (Iversen & Iversen, 2007; Bjorklund & Dunnett, 2007). This prize was awarded for forty years of work on a molecule that revolutionized the field of psychopharmacology (Iversen & Iversen, 2007).

Dopamine is the most abundant catecholamine neurotransmitter in the brain and has now been recognized to influence many aspects of CNS and peripheral functions (Vallone et al., 2000). In the CNS dopamine affects locomotion, positive reinforcement, food intake, endocrine regulation, spatial memory and cognition (Giros et al., 1992; Goldman-Rakic, 1998; Murphy et al., 1996; Leebaw et al., 1978; Schwartz et al., 2000; Abi-Dargham et al., 2003; Jones et al., 1981; Volkow et al., 2011). In the periphery dopamine can modulate cardiovascular functions, vascular tone, catecholamine release, renal function and gastrointestinal motility (Missale et al., 1998; Dive et al., 2000; Lejeune et al., 1987; Goldberg, 1972).

1.3.1. (i) Synthesis

Dopamine synthesis involves the catalytic addition of a hydroxyl- group to the phenol ring of tyrosine to produce L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) (Daubner et al., 2011). This is the limiting step for dopamine synthesis and is regulated by guanosine triphosphate cyclohydrolase (GTPCH) which synthesizes

guanosine triphosphate (GTP) and consequently tetrahydrobiopterin (BH_4) a necessary cofactor for TH activity (Meiser et al., 2013). L-DOPA is in turn decarboxylated by aromatic amino acid decarboxyase (AADC) also known as L-DOPA decarboxylase (DDC) to produce dopamine (Daubner et al., 2011). AADC uses vitamin B6 as a cofactor in this step of the reaction (Meiser et al., 2013). Further manipulations of dopamine result in the production of the other catecholamine neurotransmitters: norepinephrine and epinephrine as is illustrated in **Figure 2** (Daubner et al., 2011). Once synthesized, dopamine is sequestered into vesicles by vesicular monoamine transferase 2 (VMAT2), which facilitates storage at a low pH (Bisaglia et al., 2013). Excitation of dopaminergic neurons can trigger the release of dopamine into the synaptic cleft wherein dopamine is the natural agonist for the dopamine family of receptors (discussed in ***section 1.3.1 (ii)***) (**Figure 3**).

1.3.1. (ii) Metabolism

Once the necessary chemical communication is complete the synaptic levels of dopamine must be cleared to prevent constant stimulation or over-stimulation of the post-synaptic cell. There are two mechanisms by which this is accomplished as is illustrated in **Figure 3**. Most synaptic dopamine reuptake occurs in the pre-synaptic dopaminergic cell via the dopamine transporter, DAT (Giros et al., 1992; Cass et al., 1993; Nirenberg et al., 1996). Once in the cell dopamine can be repackaged for future utilization. Excess cytosolic dopamine is metabolized by monoamine oxidase (MAO) to hydrogen peroxide (H_2O_2) and the 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Meiser et al., 2013).

DOPAL is finally oxidized by aldehyde dehydrogenase (ADH) into 3,4-dihydroxyphenylacetic acid (DOPAC) (Meiser et al., 2013; Bisaglia et al., 2013). Alternatively, excess dopamine can be up taken by glial cells or astrocytes and similarly metabolized by MAO and ADH to DOPAC (Meiser et al., 2013). Catechol-O-methyltransferase (COMT) is present predominantly in glial cells and can additionally metabolize DOPAC to produce homovanillic acid (HVA) (Meiser et al., 2013). MAO exists as two isoenzymes MAO-A and MAO-B that are most commonly located on the outer mitochondrial membrane (Shih et al., 1999). MAO-A is present in both the neuronal and glial cell populations, while MAO-B only functions in glial cells and astrocytes (Shih et al., 1999). Interestingly, MAO-B is the predominant isoform in humans and MAO-A is the predominant form in rats (Meiser et al., 2013).

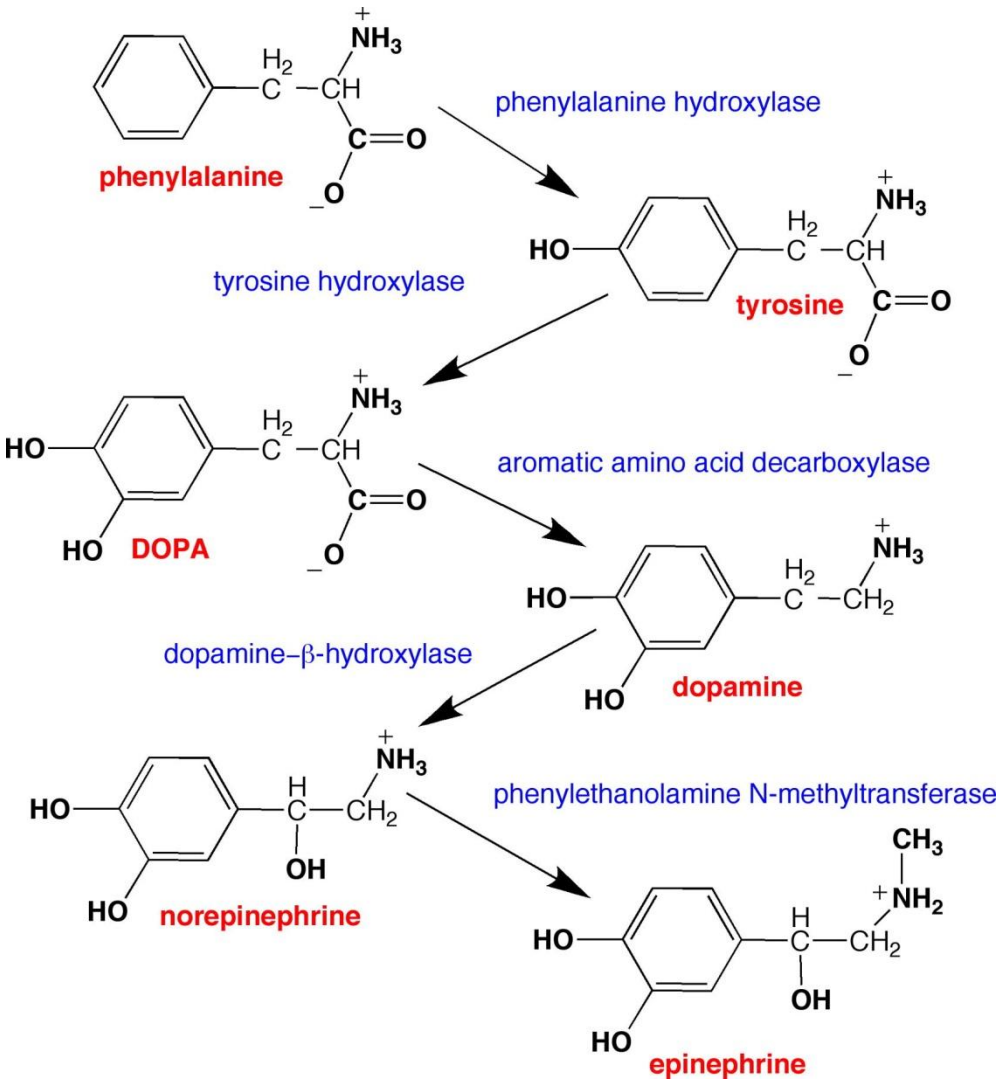


Figure 2. Catecholamine synthesis. This diagram is figure 1, published by (Daubner et al., 2011)

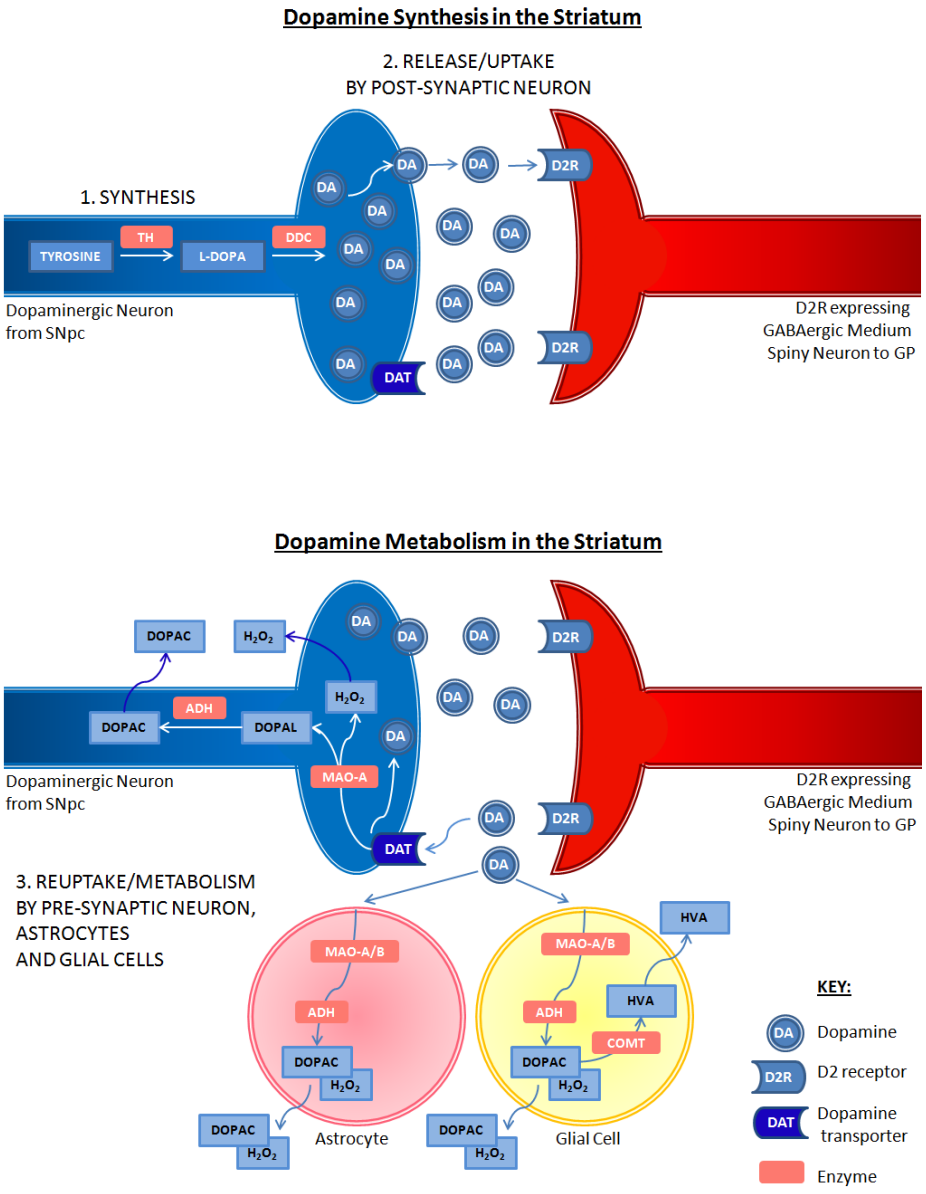


Figure 3. Dopamine synthesis and metabolism. Illustration of a synapse in the striatum wherein a dopaminergic afferent from the Substantia Nigra Pars Compacta (SNpc) (blue) synapse's unto a D2 receptor expressing medium spiny GABAergic efferent neuron (red). Extensively modified from figure 1 published by (Youdim et al., 2006).

1.3.2. The Dopamine Receptors

"*While anti-psychotics originally helped to discover dopamine receptors, the five cloned dopamine receptors are now facilitating the discovery of selective anti-psychotic and antiparkinson drugs*" (Seeman & Van Tol, 1994)

The dopamine receptors were first identified in the 1970's when it was determined that dopamine could have an effect on adenyl cyclase activity (Missale et al., 1998; Spano et al., 1978; Kebabian & Calne, 1979). P. F. Spano's laboratory (1978) were amongst the first to determine that there were at least two families of dopamine receptors the D1 and D2 which could be differentiated based on their capacity to positively or negatively modulate the activity of adenyl cyclase (Garau et al., 1978). Spano further identified that anti-psychotic drugs could interrupt this effect. The introduction of molecular cloning techniques facilitated further distinctions between the dopamine receptors and today five dopamine receptors are commonly recognized; D1, D2, D3, D4 and D5 (Missale et al., 1998; Sibley & Monsma, Jr., 1992).

The D1 family of receptors include the D1 and D5 receptors. Upon stimulation of a D1 family receptor, the stimulatory G-protein, $G\alpha_{s/olf}$, translocations to and activates adenyl cyclase which in turn catalyzes the conversion of ATP into cyclic-AMP (cAMP) (Missale et al., 1998; Beaulieu & Gainetdinov, 2011). cAMP in turn activates protein kinase A (PKA) and thus D1 stimulation indirectly modulates the targets of PKA including the ion channels, the cAMP-response element binding protein (CREB),

ionotropic glutamate receptors (AMPA and NMDA) and the 32kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) (Missale et al., 1998; Beaulieu & Gainetdinov, 2011). The D2 family of receptors includes the D2, D3 and D4 receptors which upon stimulation activates the inhibitory G-protein, $G\alpha_{i/o}$, to inhibit adenyl cyclase activity and therein prevent cAMP production (Beaulieu & Gainetdinov, 2011).

Molecular cloning techniques further distinguished two splice variants of the dopamine D2 receptor, producing the subtypes $D2_S$ and $D2_L$. $D2_L$ has 29 more amino acids in its third cytoplasmic loop. It is this intracellular loop that has been suggested to be vital in G-protein coupling, indicating a distinction in function of the two D2 receptors (Malek et al., 1993). $D2_S$ is considered predominantly a pre-synaptic auto-receptor responsible for the detection and regulation of synaptic dopamine levels (Beaulieu & Gainetdinov, 2011; Missale et al., 1998). The $D2_L$ is post-synaptic in the striatum facilitating down-stream signaling (Beaulieu & Gainetdinov, 2011; Missale et al., 1998) (**Figure 4**).

While preliminary distinctions between the dopamine receptors were made via observations of dopamine's effect on adenyl cyclase activity, there are currently numerous other downstream effects of dopamine receptor signalling that are recognized. Some of the signalling pathways of the dopamine receptors include the effects of the β and γ subunits (which also couple to the dopamine receptors), the effects on calcium signalling, and potassium channels, modulation of arachidonic acid synthesis and other indirect effects. For the purpose of this work, these downstream effects are considered

beyond the scope of relevance for our investigations (Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

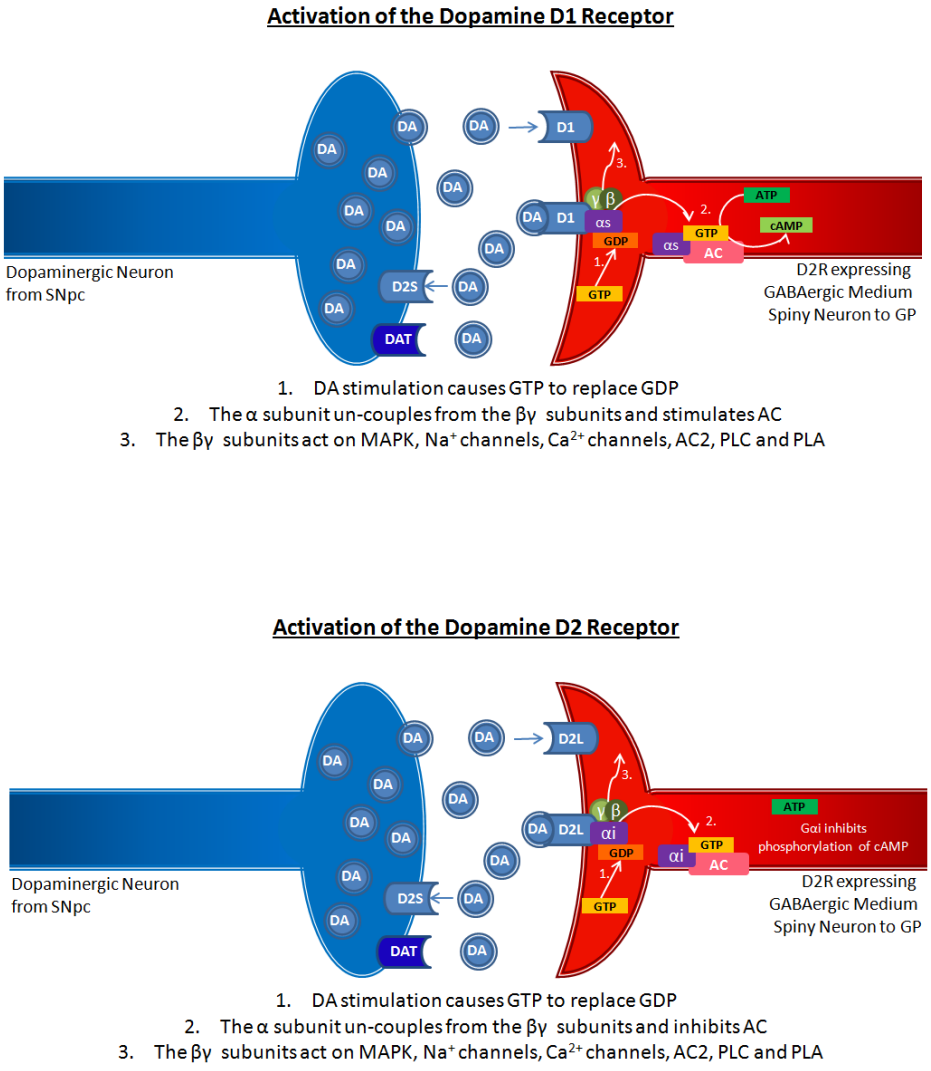


Figure 4. Dopamine D1 Receptor and Dopamine D2 receptor down-stream signaling. The D1 and D2 families of receptors were originally distinguished by their effects on adenyl cyclase (AC). D1 family of receptors stimulate AC while D2 receptors inhibit AC.

1.3.3. The Dopaminergic pathways in the Brain

There are four recognized dopaminergic pathways in the brain. The nigro-striatal pathway projects from the substantia nigra pars compacta (SNpc) to the striatum and has been demonstrated to modulate movement (Vallone et al., 2000). The mesolimbic pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens (NA), olfactory tubercle and parts of the limbic system and has been demonstrated to influence motivational systems (Vallone et al., 2000). The mesocortical pathway projects from the VTA to the cortex and is involved in learning and memory (Vallone et al., 2000). Finally the tuberinfundibular pathway projects from the hypothalamus to the pituitary gland where it inhibits prolactin release (Vallone et al., 2000). The pathway that has been primarily investigated to account for the pathophysiology of tardive dyskinesia is the nigrostriatal pathway which exists as part of the basal ganglia system, discussed in ***section 1.3.4***.

1.4. THE BASAL GANGLIA

The basal ganglia is composed of a collection of nuclei that that, along with the cerebellum, contribute to the modulation of the amplitude, tone and velocity of motion (Sanders & Gillig, 2012). Importantly, these brain regions also hold important roles in the modulation of cognition and emotion (Sanders & Gillig, 2012). As such antagonism of receptors in the basal ganglia by anti-psychotic drugs might account for both the therapeutic contribution that addresses the cognitive deficits of schizophrenia and psychoses as well as the detrimental motor abnormalities.

1.4.1. Anatomical distinctions

The corpus striatum is composed of the neostriatum and paleostriatum. The neostriatum is in turn made up of the dorsal striatum (caudate nucleus and the putamen) and the ventral striatum (NA, nucleus accumbens). The paleostriatum or globus pallidus is in turn composed of the dorsal (GPi, internal and GPe, external segments) and ventral regions. The substantia nigra (SN) is composed of two segments the pars compacta (SNpc) and pars reticulata (SNpr). Finally the sub-thalamic nucleus (STN) and occasionally the ventral tegmental area (VTA) are considered contributing regions to the basal ganglia circuit. The descending motor neurons from the corticospinal (pyramidal tract), corticorubrospinal, corticoreticulospinal, and corticobulbar pathways compose the terminating portion of the basal ganglia.

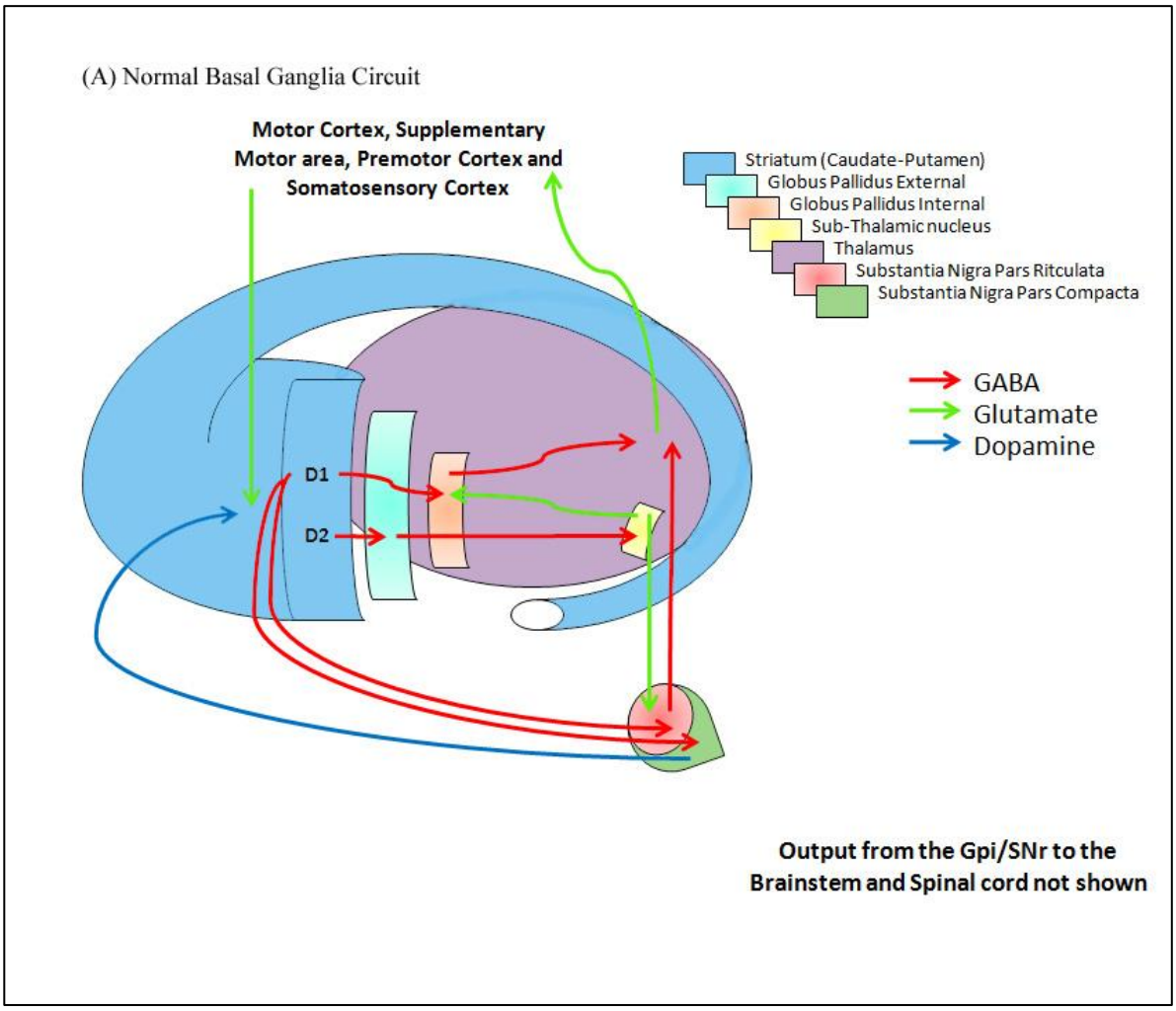


Figure 5. Normal basal ganglia circuit. Figure 1 published by (Sookram et al., 2011).

1.4.2. Functional distinctions

The cortex is considered the starting and ending point of the basal ganglia circuit with the final output descending from this region. Meanwhile, the striatum is a centre for integration. The medium spiny neurons, which constitute 90-95% of the neurons in the striatum, receive glutamatergic input from the cortex, cholinergic input from inter-neurons and dopaminergic input from the SNpc (Nicola et al., 2000; Surmeier et al., 2007; Yan et al., 2001; Chang & Kitai, 1985). Glutamate and acetylcholine neurotransmitter levels are detected by medium spiny neurons that express both glutamate receptors (mGluR1,5, mGluR2,3, AMPA and NMDA) and muscarinic acetyl-choline receptors (M2 and M4). These neurons additionally express either predominantly dopamine D1 receptors (and substance P) or D2 receptors (and enkephalin) (Surmeier et al., 1996) (**Figure 6**). Substance P and enkephalin are neuropeptides which are commonly utilized as markers of the two populations of neurons (Surmeier et al., 1996). The D1 expressing and D2 expressing medium spiny neurons have in turn distinguished the two major circuitry pathways of the basal ganglia; the indirect and direct circuits. These circuits culminate to account for the stimulation or inhibition of the motor cortices.

There have been recent suggestions, based on real time polymerase chain reaction assessments, that a sub-population of medium spiny neurons may co-express the D1 receptors, D2 receptors, substance P and enkephalin (Nicola et al., 2000; Surmeier et al., 1996). The exact influence of this groups of cells on our interpretations of the direct and indirect circuits remains undetermined, but this challenge and the plausible existence of branching and feedback loops within our systems reflects the over-simplification of these

circuits (Nicola et al., 2000). However, these circuits still provide a foundation from which more complex assessments can be made.

1.4.2. (i) Direct circuit

The D1 expressing medium spiny neurons release GABA directly in the GPi, SNpc and SNpr. GABAergic projections from the GPi and SNpr to the thalamus synapse unto glutamatergic projections to the motor cortical regions as was similarly described for the indirect circuit. Stimulation of the SNpc likely modulates a feedback loop for dopaminergic efferents from the SNpc to the striatum.

Stimulation of the direct circuit causes inhibition of the GABA output from the GPi to the thalamus. This loss of inhibition to the thalamus facilitates increased glutmatergic output from the thalamus to the motor cortices and the promotion of movement.

1.4.2. (ii) Indirect circuit

The D2 expressing medium spiny neurons release GABA in the GPe. GABAergic projections from the GPe to the STN synapse unto glutamatergic efferents to the GPi and SNpr. GABAergic projections from the GPi and SNpr to the thalamus synapse unto glutamatergic projections to the motor cortical regions.

Stimulation of the indirect circuit (increased GABAergic output from the striatum), inhibits the GABAergic output to the STN and consequently increased glutamatergic

stimulation of the GPi and SNpr. This in turn increases the GABAergic inhibition of the thalamus, decresing its glutamatergic output to the motor cortices. Thus, stimulation of the indirect circuit from the striatum can inhibit movement. It is important to consider that while the indirect circuit's output of medium spiney neurons express the dopamine D2 receptor predominantly which is inhibitory on neuronal firing, here we are considering the stimulation of the circuit in terms of the neuronal firing and not specifically dopamine receptor stimulation.

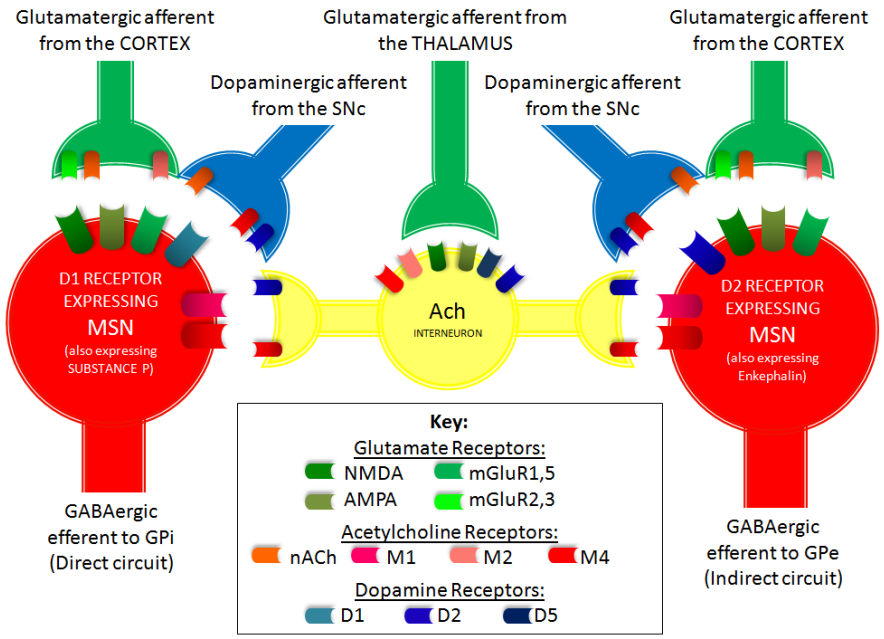


Figure 6. The expression of receptors in the striatum. A convergence of activity from the cortex, the Substantia Nigra Pars Compacta (SNpc) and the Striatum itself. Medium spiney neurons compose 90-95% of the striatal neuronal population. Acetylcholine interneurons compose 2% of the neuronal population and function to modulate the balance between the direct and indirect circuits. Acetylcholine release and stimulation of M1 receptors increase the excitability of the medium spiney neuron especially in the indirect pathway. M4 receptor stimulation by acetylcholine increases the excitability of the direct pathway. nAch receptor stimulation can facilitate dopamine release. Thus, acetylecholine is an important neurotransmitter to be considered when investigating TD and disorders with imbalances between the direct and indirect basal ganglia output. The GABA interneurons were not included in this figure for simplicity; however they compose up to 4% of the neuronal population and can express either paralbumin, calretinin or somatostatin.

1.5. THE PATHOPHYSIOLOGY OF TARDIVE DYSKINESIA

Tardive dyskinesia is considered an "extra-pyramidal side effect", so called because it was originally hypothesized that it resulted from changes in a parallel tract of neurons outside of the corticospinal tract (pyramidal connections) that run from the cortex to the brainstem and the spinal cord. More recently it has been suggested that this association is a misnomer and that these tracts do not form a parallel system but a circuit in humans which overlaps and encompasses the basal ganglia (Sanders & Gillig, 2012; Loonen & Ivanova, 2013). These tracts are considered inseparable from the pyramidal tracts in both function and anatomy (Loonen & Ivanova, 2013; Sanders & Gillig, 2012). Today the basal ganglia, rather than an extra-pyramidal system, define the brain regions that are most commonly attributed as the primary culprits in the pathophysiology of tardive dyskinesia. Imbalances between the direct and indirect circuits are the most consistently agreed upon etiology which unifies all of the historically identified hypotheses which have attempted to explain tardive dyskinesia (**Figures 8** and **9**).

1.5.1. The dopamine supersensitivity hypothesis

The dopamine supersensitivity hypothesis of tardive dyskinesia was first proposed in the 1970's in an attempt to account for the pathophysiology of tardive dyskinesia (Meltzer et al., 1976). By this time it was determined that anti-psychotic drugs were antagonists of the dopamine D2 receptor and that their administration could lead to an up-regulation of dopamine D2 receptors in the striatum (Silvestri et al., 2000; See et al., 1990; Laruelle et al., 1999). This up-regulation of dopamine D2 receptors in the striatum of the basal ganglia circuitry could potentially account for the symptomology of tardive dyskinesia (**Figure 9**).

1.5.1. (i) The hypothesis

The dopamine D2 receptor is an inhibitory receptor, thus increases in D2 receptor number and the sensitivity of these receptors would result in a decreased GABAergic output from the striatum. This would dysregulate the balance of the direct and indirect systems in the basal ganglia, and may account for the motor disturbances that characterize tardive dyskinesia. While this can account for some of the features of tardive dyskinesia including the worsening of symptoms after removal of the anti-psychotic drug as well as the alleviation of symptoms with increasing dosage of the anti-psychotic drug, the speed at which receptors are regulated to and from the surface of neurons does not correlate well with the chronic nature of tardive dyskinesia (Wolfarth & Ossowska, 1989; Vital et al., 1997).

1.5.2. The oxidative stress hypothesis

Around the same time that the dopamine supersensitivity hypothesis was being proposed it was also realized that chronic anti-psychotic drug administration could cause neurodegeneration in the striatum and substantia nigra (Andreassen et al., 2000; Christensen et al., 1970; Christensen et al., 1970). This effect was observed in both humans and animals that were administered these drugs (Andreassen et al., 2000; Christensen et al., 1970; Christensen et al., 1970). The concept of neurodegeneration offered a more comprehensive explanation for the chronic nature of tardive dyskinesia and could account for the same abnormalities in basal ganglia circuitry that would be anticipated from dopamine supersensitivity, as is illustrated in **Figure 9**.

1.5.2. (i) The hypothesis

Chronic antagonism of the dopamine D2 receptor can cause an up-regulation of dopamine synthesis and the accumulation of dopamine in the synaptic cleft. This effect is anticipated in regions of the brain that are highly populated with the dopamine D2 receptor such as the striatum (Moghaddam & Bunney, 1990). Since the brain naturally exhibits heightened levels of oxygen, relative to the rest of the body, there are optimal conditions within the brain for oxidation to occur (Erecinska & Silver, 2001). Excess dopamine has been proposed to be easily oxidized to reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide (O_2•), hydroxyl (OH•) and semiquinone (SQ•) radicals, via enzymatic oxidation, auto-oxidation or the fenton reaction as is illustrated in **Figure 7** (Rogoza et al., 2004; Skoblenick et al., 2006; Hastings et al., 1996; Erecinska & Silver, 2001). Furthermore, it has been demonstrated that increasing oxidative stress can

stimulate mitochondrial membrane permeability and apoptosis predominantly by the apoptosis inducing factor (AIF) pathway discussed in ***section 1.6*** (Skoblenick et al., 2006) This may account for a loss of local neuronal cell populations such as the dopamine D2 receptor expressing medium spiny neurons. Thus, it was hypothesized that decreased inhibition from the striatopallidal pathway will culminate, via the STN and GPi, to decreased inhibition in the thalamus from the GPi and SNr (**Figure 9**). Consequently, there will be increased stimulation to the cortex that can account for the hyperactivity which is characteristic of tardive dyskinesia (**Figure 9**). The proposed circuitry changes are in correlation with the observations of Mitchell et al. (Mitchell et al., 1992). Their investigations characterized 2-deoxyglucose changes in cebus monkeys and determined that following chronic anti-psychotic drug administration there was decreased 2-deoxyglucose uptake in the thalamus and GPi reflecting decreased GABA inhibition to the thalamus (Mitchell et al., 1992).

Finally, a number of studies have proposed the possibility of region specific neurodegeneration in tardive dyskinesia, which is limited to the ventrolateral striatum (Kelley et al., 1989; Salamone et al., 1990; Mittler et al., 1994). According to the topographical arrangement of the striatum this region is particularly involved in oro-facial control correlating well with the tardive dyskinesia phenotype (Kelley et al., 1989; Salamone et al., 1990; Mittler et al., 1994).

Collectively, this hypothesis provides a strong argument that addresses the chronic nature of tardive dyskinesia. It can also account for the irreversible feature of tardive

dyskinesia and might account for the greater susceptibility in the elderly wherein the natural aging process underlies a heightened state of susceptibility to oxidative stress. Yet, the oxidative stress hypothesis is not without its shortcomings. The prospect of neurodegeneration does not account for the amelioration of symptoms with increasing dosage of anti-psychotic drug and it only partially addresses the specificity of neurodegeneration. While it is plausible that neurodegeneration is more likely in regions of high dopamine D2 receptor populations this association has not been proven.

1.5.2. (ii) The direct basal ganglia circuit

While the implications of the direct basal ganglia circuit have not been as extensively investigated, Madsen *et al* proposed that D1 agonists can induce tardive dyskinetic symptoms (Madsen et al., 2011). This study further suggests a role for the CB1 receptor in modulating this effect. While the impact of the dopamine D1 receptor is a valuable factor to be considered in the assessment of tardive dyskinesia, it is beyond the scope of these investigations and as such will not be addressed further herein.

Despite the limitations of our current understanding of tardive dyskinesia it is likely that oxidative stress and dopamine supersensitivity will provide significant building blocks toward a more holistic understanding of tardive dyskinesia.

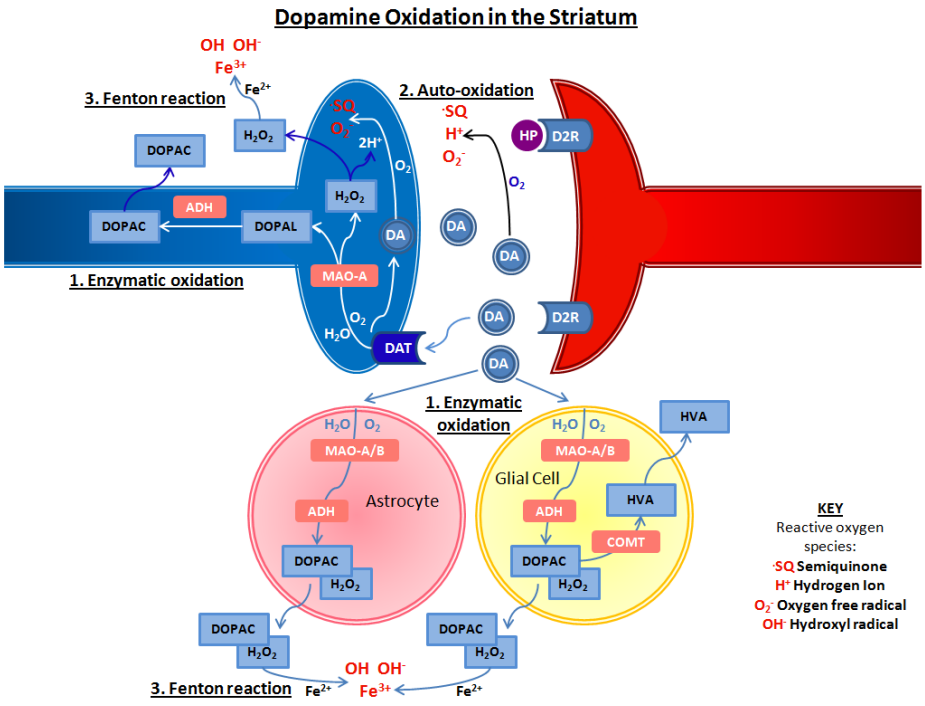


Figure 7. Mechanism's underlying the Oxidation of Dopamine. Dopamine oxidation in the striatum can occur by enzymatic oxidation involving monoamine oxidase (MAO), auto-oxidation, and/or the Fenton reaction wherein Iron (Fe^{2+}) facilitates the production of hydroxyl radicals (OH^-) from hydrogen peroxide (H_2O_2) (Rogoza et al., 2004). These reactive oxygen species are detrimental to cell survival and can act as the initiators for apoptosis to occur.

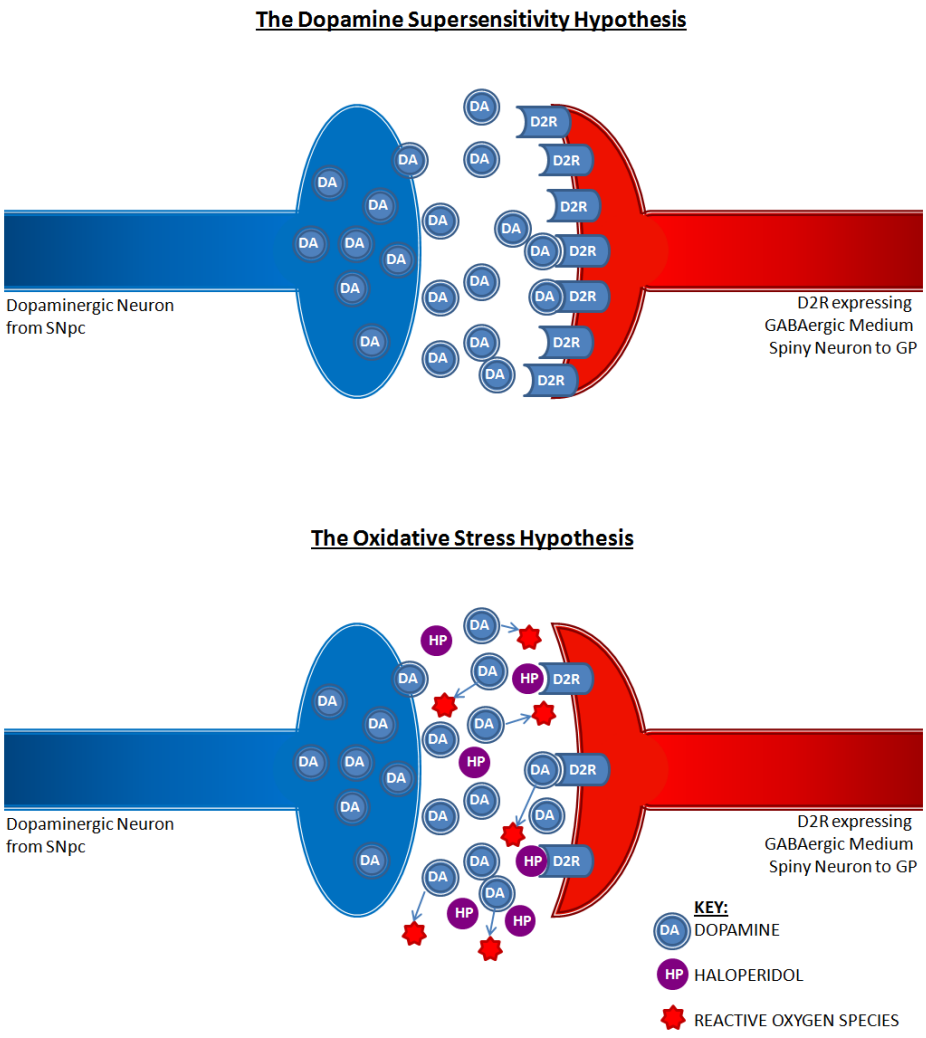


Figure 8. The Dopamine Supersensitivity and Oxidative Stress hypotheses of Tardive dyskinesia**.** The dopamine supersensitivity hypothesis proposes an over-expression of the D2 receptor as the underlying mechanism which accounts for the development of tardive dyskinesia. Alternatively, the oxidative stress hypothesis implicates dopamine accumulation and oxidation to reactive oxygen species and consequential neurodegeneration as the primary pathophysiology of tardive dyskinesia.

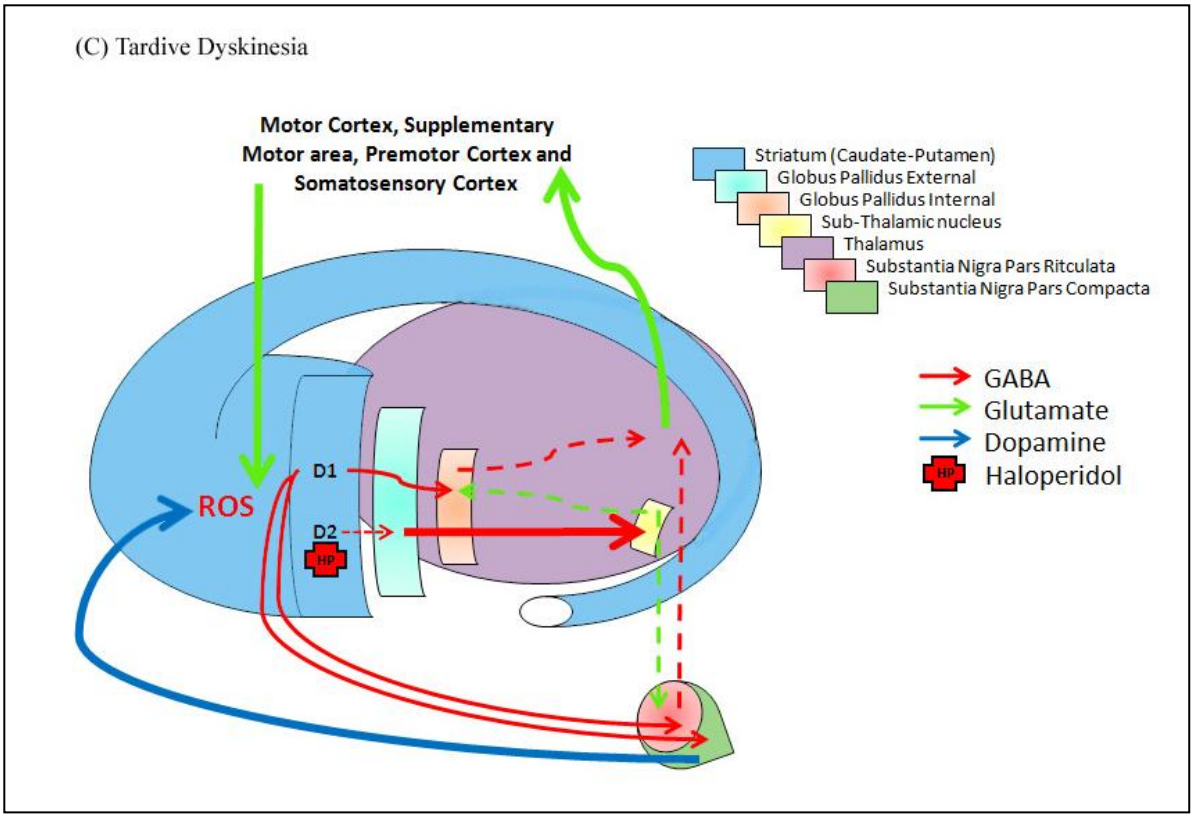


Figure 9. Hypothesized changes in the basal ganglia circuitry that would account for the symptoms of tardive dyskinesia. Thickened lines indicate increased transmission, dashed lines indicate decreased transmission. Both oxidative stress and neurodegeneration in the striatum and D2 receptor over-expression could underlie a decreased GABAergic output from the striatum to the GPe. Decreased inhibition of the GABAergic output to the Subthalamic Nucleus (STN) would cause too much inhibition of the STN. This excess inhibition of STN would in turn cause decreased glutamatergic output to the GPi and Substantia Nigra Pars Compacta (SNpc) and thus decresed GABAergic output to the thalamus. Collectively, this chain of events would lead to increased glutamatergic output to the motor cortices account for the hyper-motor state in tardive dyskinesia. Figure 1 published by (Sookram et al., 2011).

1.6. APOPTOSIS: A Mechanism of Neurodegeneration in Tardive Dyskinesia

While the exact etiology of tardive dyskinesia remains unknown the oxidative stress hypothesis implicates neurodegeneration wherein the primary mechanism of cell loss involves the apoptotic rather than the necrotic cell death pathway (Noh et al., 2000). Kerr, Wyllie, and Currie in 1972 were amongst the first to describe the DNA aggregation and degradation followed phagocytosis that was unique from necrotic cell death (Kerr et al., 1972). More recent assessments of apoptosis describe the characteristic blebbing and expression of phosphatidylserine which are extracellular signals to macrophages and microglia for the phagocytosis of the cell (Elmore, 2007; Mattson, 2000) (**Figure 10**). Additional cell shrinkage occurs, which is in contrast to that seen in necrosis wherein there is cell swelling (Elmore, 2007; Mattson, 2000).

(i) Two pathways of Apoptosis

There are two main apoptotic pathways the extrinsic (which can include the perforin/granzyme pathway) and the intrinsic. Both of these pathways converge unto the same final effector: caspase 3. The extrinsic pathway is primarily stimulated by the binding of death ligands (such as FasL) to the Fas, CD95, TNF receptor, and/or TRAIL receptor which in turn activates the specific initiator caspases 8 and 10 (Johnstone et al., 2002; Riedl & Shi, 2004). The perforin/granzyme pathway is stimulated by T-cell cytotoxicity (Elmore, 2007). However it is the intrinsic pathway that is more commonly attributed to neurodegenerative disease since this pathway is stimulated by non-receptor mediated stimuli such as hypoxia, hyperthermia, cellular stressors including free radicals, viral infections, radiation direct DNA damage, toxins and others (Elmore, 2007;

Johnstone et al., 2002). Other negative regulators such as loss of survival support from growth factors, cytokines and hormones can also function as stimulations for apoptosis via the intrinsic pathway (Elmore, 2007).

(ii) The Apoptotic Mechanism

The initiation phase of anti-psychotic drug-induced apoptotic cell death occurs via a signal that may be oxidative stress, gluatamate accumulation and/or toxic metabolites of the drugs themselves such as the pyridinium metabolite of Haloperidol, HPP^{+} (Bloomquist et al., 1994; Tsai et al., 1998). These signals can cause the intracellular increase in oxygen radicals and Ca^{2+} (Mattson, 2000; Elmore, 2007). Importantly an accumulation of reactive oxygen species can cause direct damage to DNA, proteins and lipids and therein signal apoptosis (Smith et al., 2013). Alternatively there can be an increase in Par-4, which stimulates the pro-apoptotic molecules Bax and Bad to cause the formation of mitochondrial permeability transition pores in the outer mitochondrial membrane (Mattson, 2000). These pores facilitate the escape of the inter-mitochondrial membrane proteins cytochrome c (cyt c), apoptosis-inducing factor (AIF), endonuclease G (EndoG), second mitochondria-derived activator of caspases (SMAC)/ direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO), and high-temperature-requirement protein A2 (Omi/HtrA2) (Riedl & Shi, 2004).

Cyt-c forms a complex with apaf-1 and caspase 9, wherein caspase 9 is activated and in turn activates the effector caspase 3. Upon activation Caspase 3 in turn stimulates DNA

degradation and the cross-linking and degradation of cytoskeletal and nuclear proteins (Danial & Korsmeyer, 2004; Elmore, 2007; Boatright & Salvesen, 2003). Alternatively, AIF translocation can occur directly from the inter-mitochondrial space to the nucleus where it causes DNA degradation and cell death (Norberg et al., 2010). SMAC/DIABLO and Omi/HtrA2 inhibit the inhibitor of apoptosis proteins (IAP proteins), and therein promote apoptosis (Yang et al., 2003; Verhagen et al., 2000).

Our laboratory has previously determined that in rats, haloperidol induced neurodegeneration occurs primarily via apoptosis and specifically AIF translocation (Skoblenick et al., 2006). If neurodegeneration is the primary pathophysiological mechanism underlying tardive dyskinesia then targeting the AIF pathway would be an effective approach to preventing this devastating behavioural outcome.

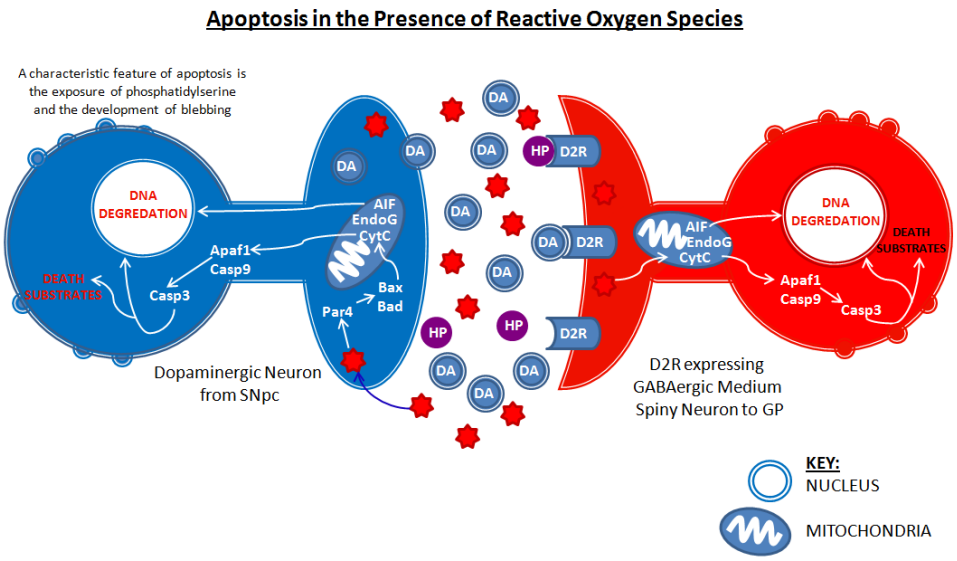


Figure 10. The pathways of Apoptosis following Haloperidol Administration. Apoptotic cell death can occur directly by the release of AIF and translocation to the nucleus where it causes DNA degredation or indirectly via caspases. Previous work from our lab has suggested cell death occurs predominantly by the former (Skoblenick et al., 2006).

1.7. AN ANIMAL MODEL OF TARDIVE DYSKINESIA

1.7.1. Validity of the Model

Anti-psychotic drug administration in rodents produces abnormal oro-facial movement's also known as vacuous chewing movements (Tamminga et al., 1990). This is the most widely acknowledged animal model of human tardive dyskinesia with well established face validity (Crowley et al., 2012). Vacuous chewing movements in rodents are very phenotypically similar to human tardive dyskinesia characterized by purposeless oro-facial chewing, tongue protrusions and grimacing (Turrone et al., 2002; Casey, 2000). Rodents however do not exhibit the abnormal limb and trunk movements that can be observed in human populations. Yet, this model is established by the same approach as the iatrogenic abnormality in humans. The vacuous chewing movement is established by chronic treatment with anti-psychotic drugs, or dopamine D2 receptor antagonists, and is exacerbated by dopamine D2 receptor agonists (Crowley et al., 2012; Glenthoj, 1993; Ellison et al., 1988; Glassman & Glassman, 1980; Rupniak et al., 1983; Lublin & Gerlach, 1988). Symptoms can worsen if the drug is abruptly discontinued and are mitigated by re-administering the anti-psychotic drug (Crowley et al., 2012; Glenthoj, 1993). Symptoms develop less frequently in populations of animals that are administered atypical anti-psychotic drugs (Crowley et al., 2012; Gao et al., 1998; See & Chapman, 1994). Interestingly, even in genetically identical animals a subset of animals will develop high vacuous chewing movements while a subset will display lower counts (McCullumsmith et al., 2003). This correlates well with the human phenotype wherein only a sub-population of individuals who are administered anti-psychotic drugs will

develop tardive dyskinesia. Finally, in correlation with the human condition Neisewander *et al.* (1994) established a dose response to anti-psychotic drug administration suggesting that not only does increasing the dose of reserpine increase the susceptibility to develop vacuous chewing movements but it can alter the onset of symptoms (Neisewander et al., 1994). Their work suggested that a high concentration of drug will cause an earlier onset of symptoms and additionally that older animals developed more severe symptoms, once again resembling the human condition (Bergamo et al., 1997; Steinpreis & Salamone, 1993). The main limitation of this model resides in our lack of understanding about the disorder itself. To date the pathophysiology of tardive dyskinesia in humans is still unknown and as such while the construct and predictive validity is partially established it is only as strong as our understanding of the disease.

1.7.2. Establishing the Model

The most established animal of choice is the rat, however the mouse, guinea pig and cebus monkey has also been characterized to develop vacuous chewing following the administration of anti-psychotic drugs (Koller, 1984; Crowley et al., 2012; Klawans, Jr. & Rubovits, 1972; Hodgson et al., 2010). Since the first generation anti-psychotic drugs induce more severe vacuous chewing movement's haloperidol and reserpine are the most commonly utilized anti-psychotic drugs for the induction of vacuous chewing movements as a model of tardive dyskinesia (Neisewander et al., 1994; Burger et al., 2004; Batool et al., 2010). The typical dosages utilized for haloperidol are 1-3mg/kg daily via intra-peritoneal injection. Reserpine on the other hand is most commonly administered every

other day sub-cutaneously for 3-30 days (Neisewander et al., 1994; Burger et al., 2004; Batool et al., 2010).

Intra-peritoneal injections however do not always produce stable vacuous chewing movements and symptom severity may fluctuate over time (Egan et al., 1996; Turrone et al., 2002). As such a number of studies have utilized haloperidol decanoate intra-muscular injections for a minimum of two months or administration in drinking water for 3 or more weeks (Egan et al., 1996). This approach to the development of a model of tardive dyskinesia can take longer to develop but mimics the human condition in that once established the phenotype is persistent and can continue even following drug discontinuation (Turrone et al., 2002).

1.8. CURCUMIN

1.8.1 An introduction to Curcumin

The traditional medicines of Asia and India have for centuries recognized turmeric as a spice with great therapeutic value. It was used to treat upset stomach, arthritis, acne, wounds, infections, inflammation, cough and numerous other ailments (Bharti et al., 2003; Singh, 2007). In 1815, curcumin (*Curcuma Longa*), the active component of turmeric, was isolated by Vogel and Pelletier (Aggarwal & Sung, 2009). In 1910, the structure of curcumin was determined in Germany by Lampe and Milobedeska who outlined its polyphenol character (**Figure 11**) (Aggarwal & Sung, 2009).

More recently, curcumin has been implicated in the treatment of a diversity of disorders and diseases including cancers, digestive disorders and diseases of the central nervous system (CNS) such as neurodegeneration including Alzheimer's, Parkinson's disease (Lin et al., 2011; Taylor & Leonard, 2011; Sookram et al., 2011). This important yellow pigment has exhibited an array of different pharmacological and mechanistic effects ranging from anti-oxidant properties to anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-fungal and anti-viral activities (Bharti et al., 2003; Chen et al., 2005; Jurenka, 2009; Rai et al., 2008; Zandi et al., 2010). The mechanisms which underlie this diversity of effects of curcumin remains an enigma that continues to be investigated.

1.8.2. Curcumin and the CNS

Curcumin's therapeutic potential for the treatment of CNS diseases has been widely acknowledged and is supported by its capacity to cross the blood brain barrier while exhibiting a very low toxicity. As such medical research investigating the usefulness of this unique molecule in the treatment of mental illness has progressed all the way to pre-clinical and clinical trials for the treatment of cancers and neurodegenerative disease (Dhillon et al., 2008). A number of trials have confirmed that curcumin can be administered at dosages of up to 12 g/day for 3 months in humans with no recorded toxicity (Carroll et al., 2011; Cheng et al., 2001; Dhillon et al., 2008). However, curcumin's greatest shortcomings are its low solubility and low bioavailability. Curcumin is relatively insoluble in water preferentially dissolving in DMSO, chloroform or ethanol (Feng & Liu, 2009). In addition to its natural hydrophobicity, curcumin is poorly absorbed across the gut mucosa with 40-75% excretion in feces (Aggarwal & Harikumar, 2009). The portion that is absorbed undergoes rapid metabolism in the liver via glucuronidation and sulfation (Aggarwal & Harikumar, 2009). Curcumin is reduced and/or conjugated to metabolically less-active products within the body and a greater portion of it undergoes rapid systemic elimination (Yang et al., 2007; Anand et al., 2007). These features of curcumin render its average oral and systemic bioavailability in physiological systems at just 1% (Yang et al., 2007; Anand et al., 2007). Thus, the focus of modern scientific investigations into the usefulness of curcumin emphasizes novel routes of administration geared towards increasing the systemic availability. The most recent innovative approaches use adjuvants to block the metabolic reactions with

curcumin (e.g piperine), enclosure curcumin in nanoparticles, or liposomes and investigate structural analogues of curcumin with increased potency, specificity, and bioavailability (Anand et al., 2007; Tsai et al., 2011; Rejinold et al., 2011). A number of these approaches have been considered for clinical trials but further research is still necessary to fully establish these techniques in clinical practice.

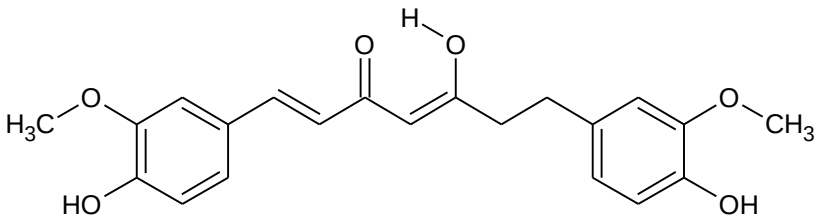


Figure 11. Structure of curcumin (1,7-bis[4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione). This molecule has two potential mechanisms by which it can function directly as an anti-oxidant or free-radical scavenger. It can exert its anti-oxidant effect through its phenolic and/or diketonic groups. It has been suggested however that it is primarily the phenolic hydroxyl groups which underlie curcumin's anti-oxidative capacity (Feng & Liu, 2009).

2. THEORETICAL RATIONALE

Curcumin has been widely characterized for its antioxidant and anti-apoptotic capacity (Bharti et al., 2003; Chen et al., 2005; Jurenka, 2009; Rai et al., 2008; Zandi et al., 2010). Furthermore, preliminary work has suggested that curcumin administration can alter dopaminergic systems (Kulkarni et al., 2008). While its exact effect on dopaminergic systems have not been clearly outlined, it has been shown to alter dopamine levels differently according to the model under investigation and thus it is important to consider curcumin's role in the vacuous chewing movement model of tardive dyskinesia. Since the hypothesized etiologies of tardive dyskinesia involve either increased oxidative stress or dopamine D2 receptor supersensitivity, a molecule that could potentially target both pathologies may prove very effective. Consequently, the biochemical profile of this molecule suggests that it has great potential in mitigating or preventing the symptoms of tardive dyskinesia. The ensuing investigations will characterize the effects of curcumin on anti-psychotic drug induced behavioural changes and delineate its role in both cell survival and dopaminergic systems.

Simplified interpretations for the assessments performed herein

While only a portion of rats treated with anti-psychotic drugs will develop tardive dyskinesia, we could not pre-distinguish this population from their peers without allowing for the development of tardive dyskinetic symptoms. Further, the oxidative stress hypothesis posits neurodegeneration as a pathophysiological mechanism for the development of this disorder, thus treatment with an anti-oxidant after the development of symptoms will be ineffective. As such our work considered the population as a whole, and defined the behavioural changes that occurred at in the collective population.

3. OBJECTIVES

The overall objective of this work is to investigate the potential of curcumin to be administered as an adjuvant to current anti-psychotic medications in preventing the animal model features of human tardive dyskinesia.

1. To characterize the induction of anti-psychotic drug induced vacuous chewing movements and catalepsy following haloperidol or haloperidol decanoate administration, and whether these are reversible by concurrent treatment with curcumin.

2. To investigate the mechanistic role of curcumin in the prevention of vacuous chewing movements according to the oxidative stress hypothesis.

3. To investigate the mechanistic role of curcumin in the prevention of dopamine supersensitivity.

4. HYPOTHESES

It is hypothesized that curcumin when administered as an adjuvant to current anti-psychotic medications will prevent the development of haloperidol induced behavioral and biochemical abnormalities in an animal model of tardive dyskinesia.

1. Haloperidol will effectively induce vacuous chewing movements, catalepsy and hypo-locomotion in rats. Concurrent curcumin administration alongside haloperidol treatment will prevent these behavioural abnormalities.

2. Curcumin administration will mitigate haloperidol induced increases in pro-apoptotic molecules and increase levels of anti-apoptotic molecules and therein function as a neuro-protective agent to prevent haloperidol induced neurodegeneration.

3. Curcumin will alter dopaminergic systems to compensate for haloperidol induced dopamine supersensitivity by down-regulation of the dopamine D2 receptor.

5. METHODOLOGY

OBJECTIVE 1

5.1.1. Experimental Set-up: Animals

Animals were housed in the central animal facility (CAF) at McMaster University. All procedures were approved by the CAF in accordance with the Guide to Care and Use of Experimental Animals (Canadian council on Animal Care, 1984, 1993). Rats were provided food and water *ad libitum*, and exposed to a 12 hour light/dark cycle at constant temperature (22^{o}C) and humidity (50%). Male Sprague Dawley rats were utilized in these investigations primarily to avoid complications due to the female estrous cycle. Furthermore this strain of rat has a well established literature defining its physiology, thus facilitating well informed biochemical interpretations.

90 Male Sprague Dawley rats, weighing 225-250g, were obtained from Charles River, Canada. Upon arrival to the CAF rats were housed individually in standard cages and were permitted one week to acclimatize to their new environment. Five cohorts of rats were prepared for the ensuing investigations (**Figure 12-16**). Cohorts 1, 3 and 4 included the following treatment groups: control (haloperidol vehicle with curcumin vehicle), haloperidol (haloperidol with curcumin vehicle), curcumin (curcumin with haloperidol vehicle) and curcumin and haloperidol treated animals. Cohort 2 was administered haloperidol decanoate or control treatment (haloperidol decanoate vehicle). Cohort 5 received curcumin or control treatment (curcumin vehicle).

All treatments were administered between 9:00am-10:30am daily. Rats were weighed daily prior to the administration of drugs. The weights of the rats were used to calculate the respective dosages of curcumin and haloperidol. All calculations for curcumin and haloperidol injections were done based on the previous days weights.

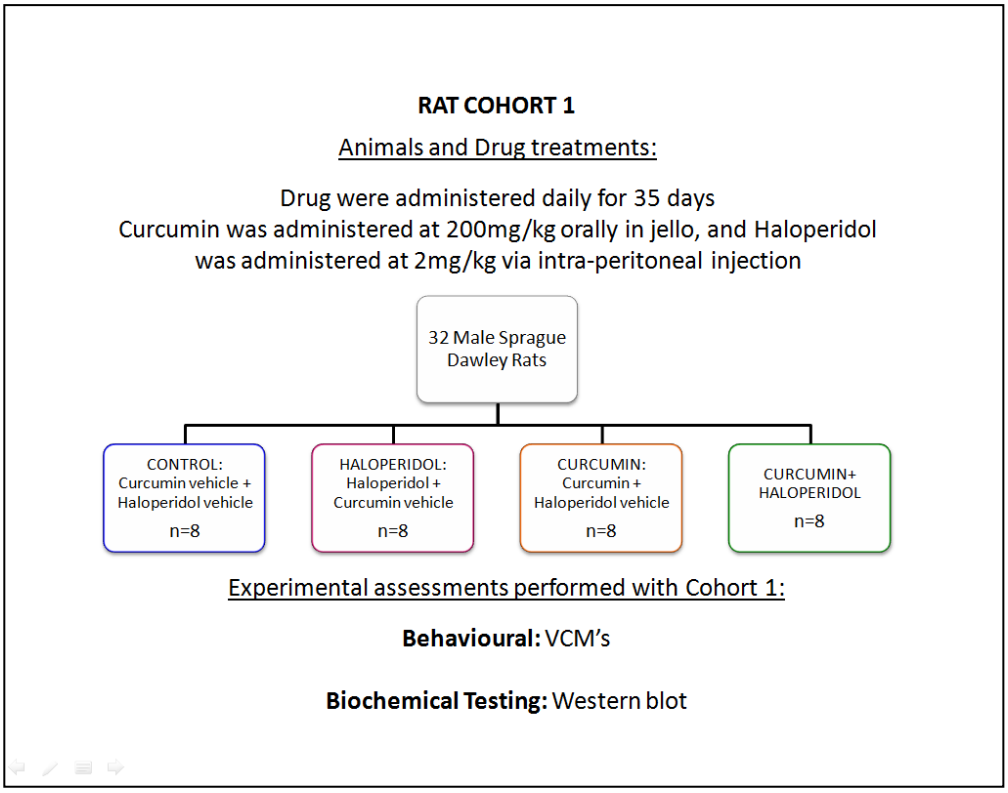


Figure 12. The experimental set-up for cohort 1 of rats

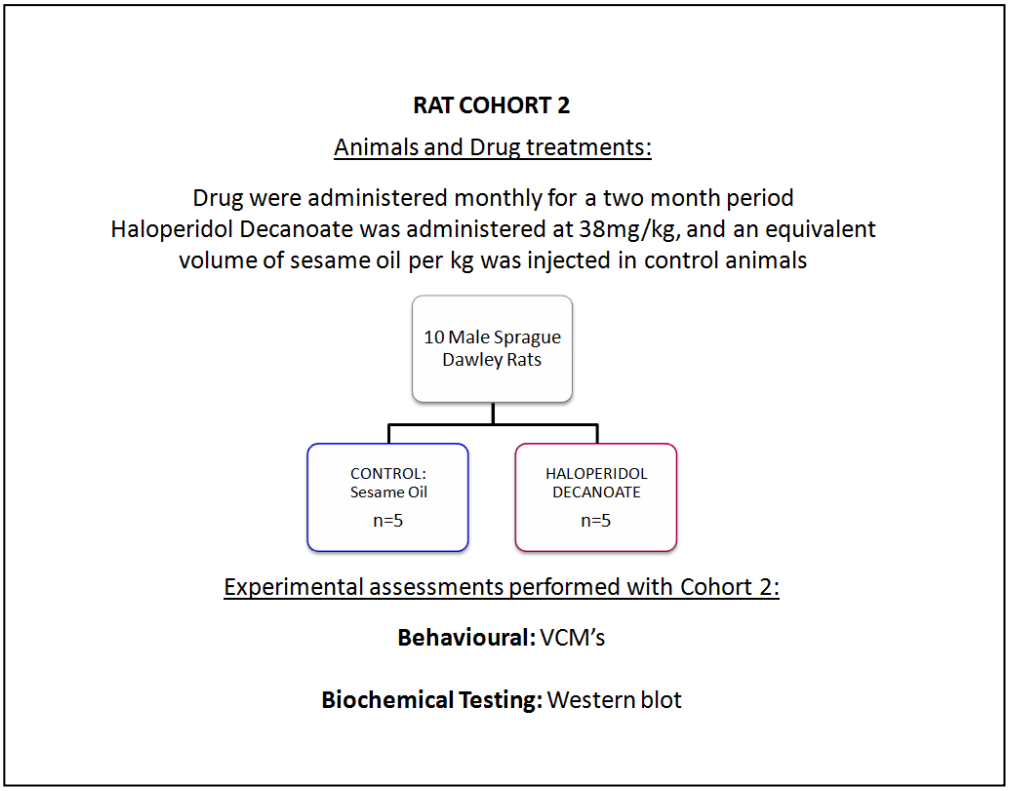


Figure 13. The experimental set-up for cohort 2 of rats

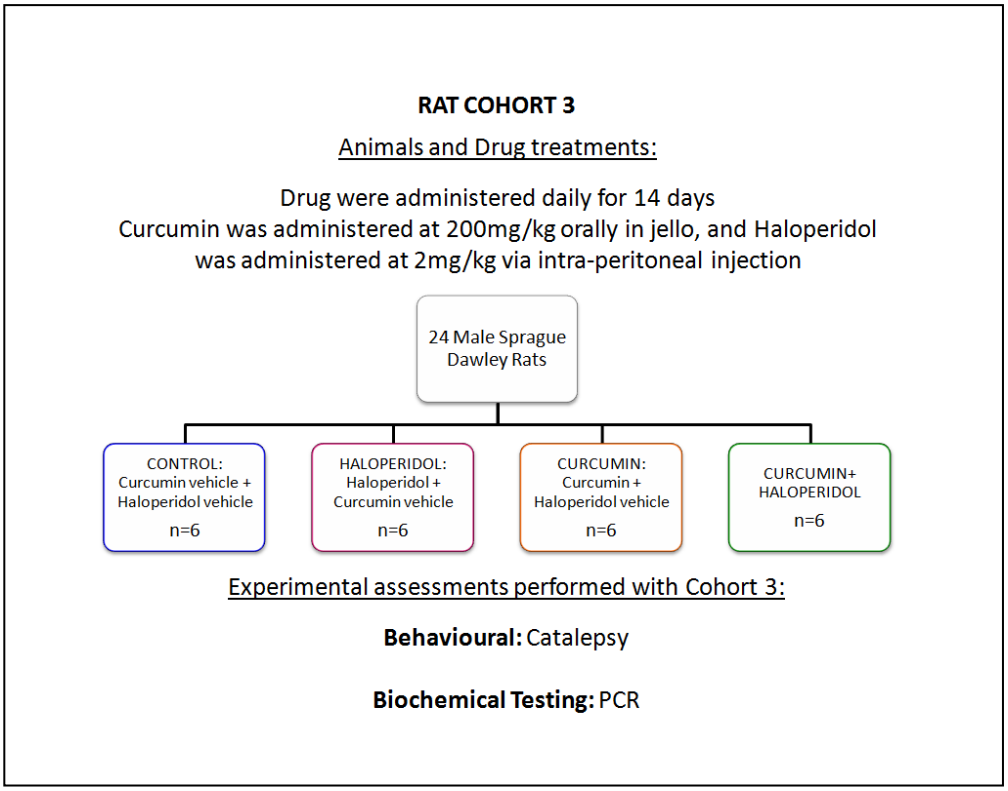


Figure 14. The experimental set-up for cohort 3 of rats

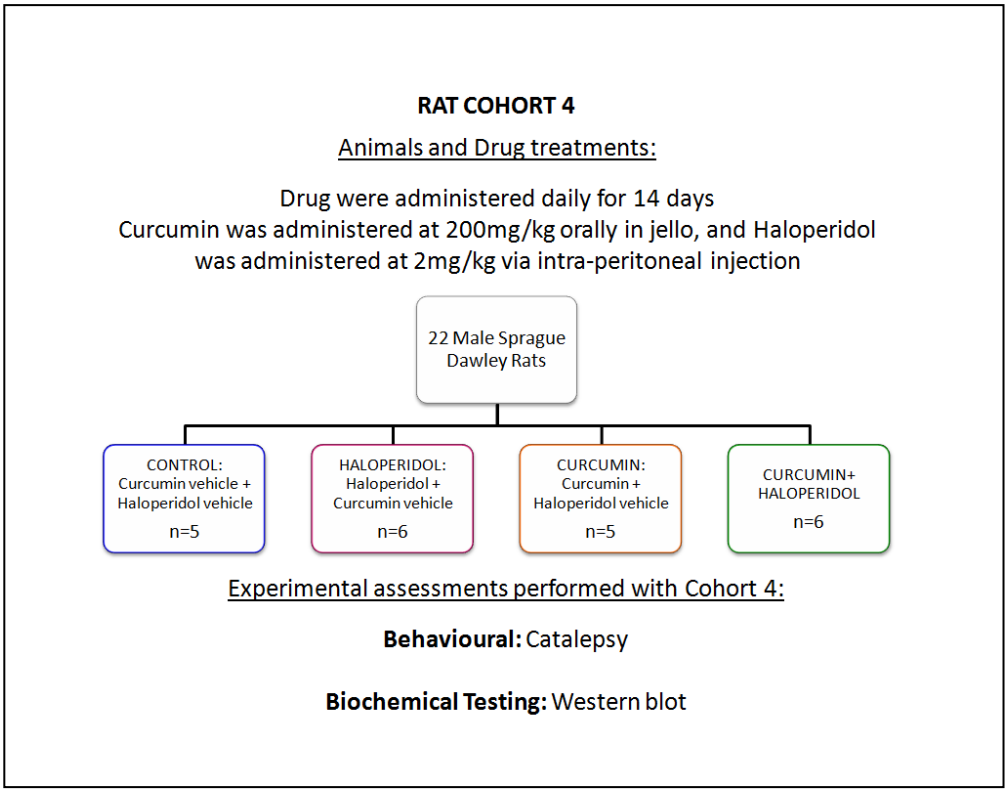


Figure 15. The experimental set-up for cohort 4 of rats

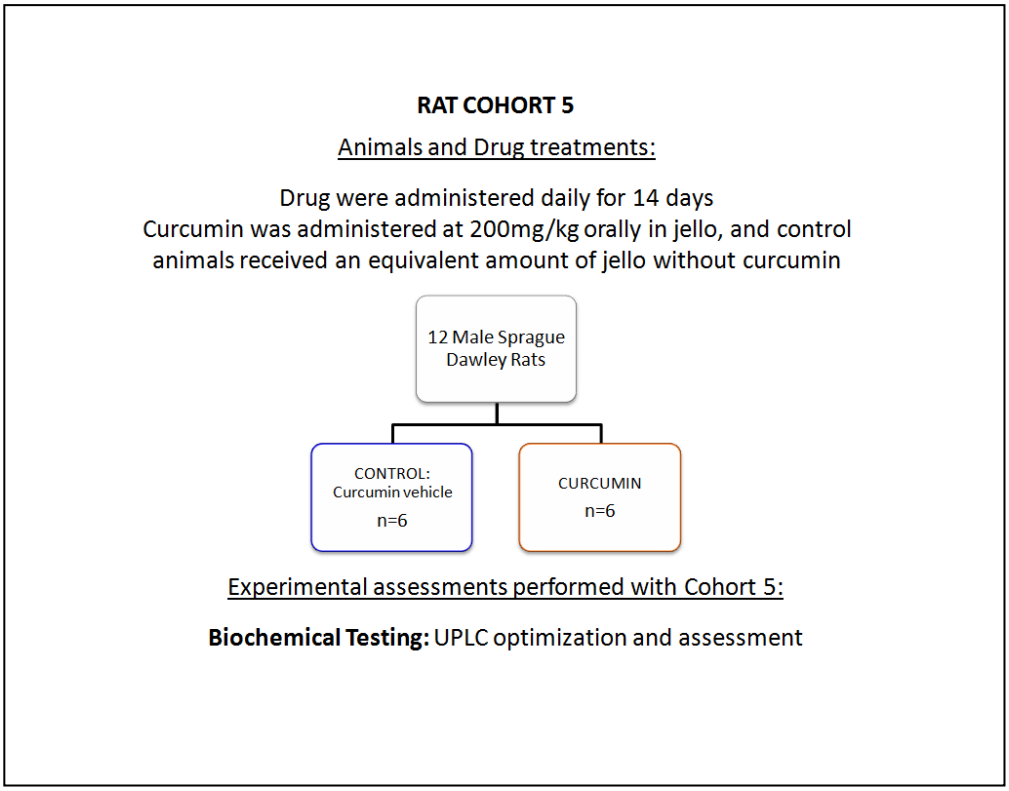


Figure 16. The experimental set-up for cohort 5 of rats

5.1.2. Experimental Set-up: Drug Treatments

5.1.2. (i) Curcumin

All rats were fed 4ml of commercially purchased cherry jell-o® (42.5g jello powder + 125ml distilled water) for three days prior to the beginning of the experiment. This was done to allow the rats to become acclimatized to the taste and to further develop their natural propensity for the drug vehicle. For two days prior to the start of the study rats were not provided any jell-o®. Curcumin (Sigma, On, Canada cat# C1386) was administered suspended in 4ml jell-o® at 200mg/kg daily. Jell-o® suspensions were used in these investigations since curcumin had low solubility in the majority of typically utilized drug vehicles, particularly at the high concentrations required for these investigations.

5.1.2. (ii) Haloperidol

Rats were administered haloperidol (chemical name: 4-[4-(p-chlorophenyl)-4-hydroxypiperidino]-4'-fluorobutyrophenone) (Sigma, On, Canada cat# H1512) dissolved in 0.15% acetic acid in 0.45% saline at 2mg/kg, via daily intra-peritoneal (i.p.) injections. This treatment regimen was previously determined in our lab to be effective for the establishment of a model of tardive dyskinesia (Rogoza et al., 2004). The side of the abdomen injected was alternated daily. Haloperidol was given following curcumin administration since the cataleptic effects induced by haloperidol made rats reluctant to eat.

5.1.2. (iii) Controls

Control treatments included the curcumin vehicle: 4ml jell-o® with no curcumin suspension and Haloperidol vehicle: 0.15% acetic acid in 0.45% saline injections.

5.1.2. (iv) Haloperidol Decanoate

Haloperidol Decanoate (chemical name: 4-[4-(p-chlorophenyl)-4-hydropiperidino]-4'-fluorobutyrophenone decanoate) is the decanoate ester of haloperidol and is available in sesame oil for intra-muscular (i.m.) injection. Haloperidol Decanoate was administered at 38mg/kg i.m. once a month for two months alongside sesame oil treated controls. n=5 rats were used for this investigation. This drug regimen was performed to consider an alternate more stable approach to inducing VCMs as a model of human tardive dyskinesia.

5.1.3. Behavioural testing

5.1.3. (i) Vacuous Chewing Movement

Chronic administration of haloperidol in rats has been established to produce vacuous chewing movements (VCM) (or abnormal oro-facial movements) that closely resemble the phenotype of tardive dyskinesia (Rogoza et al., 2004). This is the most widely accepted animal model for the investigation of tardive dyskinesia described in detail in ***section 1.7.***

For the purpose of these investigations a VCM was defined as an obvious opening and closing of the jaw, with or without protrusion of the tongue that was not directed at tasting, or grooming. Rats were randomly assigned numbers and cage cards were inverted to ensure that investigators were blinded during this assessment. Four investigators were randomly assigned to a group of rats; this was randomly reassigned on the next day of VCM counting. Animals were put into standard mouse cages and allowed to acclimatize for 5 minutes. Mouse cages were used for the test, so that there was less room for the rats to move and to ensure that their oro-facial regions were constantly within the investigators sight. The rats were then observed for 5 minutes and the number of VCMs displayed was counted. Timers were stopped for any period of time that the rat was seen to be grooming, and this was not included in the 5 minutes of a trial. VCM counting was done in four treatment groups of n=11 rats, from 8:30-9:30pm at baseline, day 7 and day 14 (Sookram et al., 2011). VCM assessments were continued weekly up to day 35 in four treatment groups of n=8 animals, and was also performed weekly on n=5 animals treated with haloperidol decanoate and n=5 controls.

5.1.3. (ii) Catalepsy

Catalepsy in rodents resembles the human symptoms of Parkinsonism, catatonic schizophrenia, and brain damage (Sanberg et al., 1988). Furthermore, the incidence of catalepsy in rodents has been established to be predictive of extra-pyramidal side effects in humans as well as the efficacy of neuroleptic activity (Sanberg, 1980; Hoffman &

Donovan, 1995). Since haloperidol induced catalepsy is widely characterized, it was important to consider the potential therapeutic effect of curcumin in preventing this rodent phenotype.

A cataleptic response was defined by the state of indifference, wherein rats were reluctant to move unless provoked and often leaned to one side of the cage. Additionally the rat's hair stood on end and their digits appeared more spread in this condition. The cataleptic responses in this investigation were quantified using the bar test on n=5 rats per treatment group. The apparatus utilized included a steel cylindrical bar, with a diameter of 1cm, mounted at 10cm from a wooden base. A towel was placed over the wooden base since rats were aversive to the wood texture. Rats were exposed to the bar test once, two days prior to the beginning of treatments to establish baseline. Both of the rat's front paws were placed on the bar and the catalepsy score was defined as the time taken to remove one or more paw's from the bar up to a maximum of two minutes. Each rat was tested three times with 30 minutes rest in between assessments. This test was performed one hour after the administration of drugs (i.e. 10am-12pm) on day 7 and day 14. Catalepsy was analysed as total time on the bar (Chiu & Mishra, 1980; Sanberg et al., 1988; Ferre et al., 1990; Hoffman & Donovan, 1995; Sanberg, 1980).

5.1.3. (iii) Locomotion

Antipsychotic drug treatment has been demonstrated to modulate locomotor activity as a reflection of dopamine D2 receptor antagonism or agonism, such that antagonism causes hypo-locomotor activity, and agonism causes hyper-locomotor activity (Storey et al., 1995).

Locomotor measurements were done using AccuScan computerized cages (AccuScan Instruments, Columbus, OH, USA). The rats were placed into clear chambers (50cm x 50cm) and allowed 5 minutes to acclimatize. The total distance travelled by the rat was recorded for 180 minutes. The first 30 minutes was taken as habituation. Locomotor tests were started at 5pm and run during the rats' dark cycle (all measurements were done in the dark). Locomotor measurements were done on days 5/6, days 12/13 and days 27/28. Due to limitations in the numbers of chambers (8 chambers available, 3 hours per test) animals were tested over the course of two days and measurements were analyzed to make sure that there was no significant difference between the two days of measurements. n=6 rats per treatment group was used for this assessment (Dyck et al., 2009).

OBJECTIVE 2

Biochemical Assessments in Rat Tissues

5.2.1. Tissue Preparation

The five cohorts of rats from Objective 1 were sacrificed and utilized for the ensuing biochemical investigations as outlined in **Figure 12-16**.

5.2.1. (i) CSF extraction

CSF was extracted from n=2 control animals and n=3 rats that received curcumin from cohort 4 (**Figure 15**). Animals were anesthetized using stock solutions prepared with a 3:2:3 ratio of Xylazine:Ketamine:water to a final injection concentration of 10mg/kg Xylazine and 75mg/kg Ketamine. Once anesthetized, rats were mounted into a sterotaxic apparatus (David Kopf Instruments, Tujunga, CA) to ensure brains were held steady. A sagittal incision was made (cranial to caudal) through the skin over the posterior skull extending past the top of the spinal cord. The subcutaneous tissues were scraped back, revealing the cistern magna. The head was tilted forward in the apparatus so to more clearly reveal the cistern magna for ease of access. The dura mata was punctured using a fire polished capillary tube and CSF collected by suction and capillary action. CSF was immediately frozen on dry ice then stored at -80^{o}C until use.

5.2.1. (ii) Sacrifice and Dissection

Rats were anesthetized using gaseous Isofluorane (Pharmeceutical partners of Canada Inc.) and quickly decapitated. The brain was sectioned using a brain mould into 4mm sections and the following brain regions were removed on ice: striatum, medial pre-

frontal cortex, nucleus accumbens, hippocampus and cerebellum. Both the left and right brain regions were dissected from each rat and pooled. All of the ensuing investigations consider the left and right brain regions as equal and equivalent tissues. Other body regions removed from these rats were: liver, heart, skeletal muscle, small intestine and kidneys. All regions dissected out of the animal was immediately frozen on dry ice and stored at -80^{o}C.

5.2.1. (iii) Trizol Purification

Striatal tissues obtained from rat cohort 1, 2 and 5 were Trizol purified to obtain protein and mRNA for molecular assessments. 1ml of Trizol reagent was added to striatal samples and incubated at room temperature for 5 minutes. 0.2ml of chloroform was then added to the samples and incubated for 20 minutes. Following this incubation period, the samples were spun down for 15 minutes at 12,000g at 4^{o}C. The centrifugation process allowed the distinction of three phases containing RNA, DNA and protein. The RNA phase was removed and stored in ethanol at -80^{o}C.

Protein: Protein was further purified using 1.5ml isopropyl alcohol. This allowed the protein to precipitate and 10 minutes of centrifugation at 10000g at 4^{o}C facilitated a pellet of this protein to separate out. This pellet was washed and sonicated in 0.3M guanidine hydrochloride in 95% ethanol. Following sonication the pellet was incubated at room temperature for 20minutes and then spun down at 7500g for 5 minutes. The supernatant was removed and then the pellet was washed, sonicated in 0.3M guanidine hydrochloride in 95% ethanol, then incubated and spun down as previously outlined, two more times.

Finally, the supernatant was removed and the samples allowed to air dry. Once the pellet appeared dry it was re-suspended in homogenization buffer composed of 50mM Tris and 1mM ethylenediaminetetraacetic acid (EDTA) at a pH of 7.4 with Mini-C (cOmplete, EDTA free) protease inhibitor (Roche Diagnostics, Mannheim, Germany).

RNA: RNA, separated from protein by TRIZOL was then vortexed in ethanol, and then spun down at 16000g for 15 minutes. Supernatant was discarded and pellet was allowed to air dry. Pellet was re-suspended in 30µl DEPC-treated RNase-free water, and incubated at 60^{o}C for 10 minutes. RNA was quantified using a Beckman Coulter 640 Spectrophotometer. Any residual DNA was removed using a DNase1 RNase-free kit according to their specified protocol.

5.2.1. (iv) Tissue Homogenization

Tissued from rat cohort 3 was utilized primarily for Immunoblot analyses. The striatal tissues from these animals were homogenized and sonicated in 50mM Tris and 1mM EDTA at a pH of 7.4 with mini-C protease inhibitor.

5.2.1. (v) Protein Quantification

Following Trizol purification or direct homogenization the protein concentrations of samples were determined by Bradford assay using that BioRad Protein Assay Reagent (Bio-Rad, Mississauga, On, Canada). A linear standard curve was prepared using bovine serum albumin at known protein concentrations of (0µg/µl, 2µg/µl, 4µg/µl, 6µg/µl, 8µg/µl, 10µg/µl). 5µg of our protein of interest was added to 200µl of bio-rad protein

assay solution and made up to 1ml with homogenization buffer (50mM Tris and 1mM EDTA at a pH of 7.4). This mixture was vortexed and incubated at room temperature for 10minutes. The samples were then vortexed and put into cuvettes. The optical density in each cuvette was determined in reference to the previously plotted standard curve using a CU-640 spectrophotometer (Beckman-Coulter, Mississauga, On, Canada). Based on the optical density output, the sample optical densities were calculated and utilized for the preparation of proteins for loading into Immunoblot assessments.

5.2.2. Ultra Performance Liquid chromatography

Ultra performance liquid chromatography (UPLC) was utilized to determine the levels of curcumin in striatal tissues and CSF obtained from cohorts 4 and 5. The striatal tissues were primarily used for optimization of the process (not illustrated herein), one control and one curcumin treated rat was reserved for the final run to identify the presence of curcumin the brain. Since this technique is novel and very preliminary in nature replication of this work is necessary in future investigations. Assessments of curcumin levels in the CSF of rats was done in a larger sample size n=3-4. However, the power of these investigations are still very low and as such must still be replicated for more accurate interpretations. The protocols utilized herein were optimized and modified from the UPLC assessments of curcumin levels previously performed by Cheng et al. (2010) and Marczylo et al. (2009).

Brain tissues were weighed, homogenized and sonicated in 200µL PBS. 50µL of CSF was similarly extracted with 200µL PBS. **Solvent A:** Acetone with 10% formic acid and **Solvent B** composed of solvent A with the internal standard quercetin at 10µg/µl added, were prepared and utilized for solvent extraction of curcumin from PBS suspensions. 400µl of solvent A or solvent B respectively were added to the samples and controls and vortexed. Samples were immediately kept at -20^{o}C, for 20 minutes, and then spun down at 16000g for 10 minutes. The supernatant was collected, and evaporated in the genvac vacuum evaporator. The pellet was re-suspended in 50µL of mobile phase and filtered through a wet durapore membrane filter of 0.22µm (Millipore) into vials with inserts added to accommodate a small volume (50µl) for UPLC analyses.

10µl of brain samples or 15ul of CSF samples were injected into and analyzed by the Agilent 1290 UPLC system using a Zorbax Eclipse Plus C18 2.1 x 10 mm, 1.8 µm, 1200 bar column which was maintained at 30^{o}C. Two mobile phases were utilized: **mobile phase A:** 0.05% aqueous phosphoric acid and **mobile phase B:** 100% acetonitrile. All reagents were HPLC grade. Mobile phases were filtered at 0.22µm on non-cellulose membrane filters and degassed prior to use. Mobile phase was kept at a constant ratio of 34% A and 66% B during assessment, with a flow rate of 0.3ml/min (0.2ml/min for CSF samples), and a UV detector was used to identify any molecules that would show up at 420nm. Due to the limited volume of sample that could be attained, only one injection of the sample was feasible, and each rat provided only one sample with both left and right

striata of the animal pooled. Samples were permitted to run through the column for 10 minutes before another injection was performed.

10µg and 100µg standards were prepared wherein curcumin powder was directly suspended in 200µL PBS, and sonicated as per the above procedure outlined for brain tissues. A PBS control without any curcumin or brain tissues was also prepared. Since there were numerous steps wherein molecules could have been lost during the processes of homogenization, sonication, evaporation, re-suspension and filtration, the levels of curcumin that resulted from these standards were not utilized to assess curcumin concentrations in the brain samples, but rather to determine an accurate location for a peak for curcumin.

In between injections of samples and standards, washing steps were introduced, wherein the mobile phase concentration was changed from 34% A and 66% B to 100% B for five minutes (since curcumin is more soluble in pure acetonitrile than an aqueous mixture) and then back to the 34% A and 66% B for re-equilibration before the next injection.

5.2.3. Liquid Chromatography/Mass Spectrometry

Liquid Chromatography/Mass Spectrometry (LC/MS) was performed with the assistance of Dr. Mehdi Keramane to determine the identity of the two peaks observed with curcumin standards and samples via UPLC. The curcumin powder that was

previously administered to rats and used as standards in UPLC optimization was assessed herein. Thus, this experiment further facilitated characterization of the purity of the curcumin used for oral administration.

Curcumin powder at 10μM was dissolved in 100% acetonitrile and filtered through a non-cellulose membrane filter of 0.22μm (Millipore). The mobile phase was prepared and provided by Dr. Mehdi Keramane, and consisted of 95% acetonitrile in water. Samples were separated using a Waters Sunfire C18 column with the parameters 4.6 x 50mm x 5 microns. Assessments for both positive and negative ions were performed.

5.2.4. Kinexus protein microarray

A pooled sample of striata from two curcumin treated rats and two control rats from cohort 1 (**Figure 12**) were homogenized on ice in Lysis buffer (provided by Kinexus), with 5μM Pepstatin and 1mM DTT. Samples were sonicated and then centrifuged at 90,000g for 30 minutes at 4^{o}C. A final concentration of 5μg/ml of samples was sent to Kinexus to be analyzed for changes in the expression of a number of proteins of interest. 1600 proteins were assessed by this protein microarray.

Table 1. Definitions to be used for the assessment of Kinexus result according to the Kinexus reference page.

Treated Average / Control Average (Fold Change)	The Fold Change is calculated by taking the ratio of the Treated Average to the Control Average.
%CFC	The percentage change from control is a measure of the change in normalized signal intensity averages between the Treated sample and the Control sample.
Log2 (Treated Average/Control Average)	The log-ratio of the Treated and Control Averages is used to calculate the differential signal intensities of each protein.

5.2.5. Immunoblotting

Quantitative Immunoblots were used to assess levels of BclXL, Bcl2, Bax, and AIF. The protein concentrations of the samples utilized herein were determined as outlined in ***section 5.2.1***.

10µg of proteins were prepared in 1x Sodium-dodecyl sulphate loading buffer, for each treatment. Samples were boiled for 10 minutes then spun down and loaded into a Sodium-dodecyl sulphate-polyacrylamide gel. The gel was run at 65V through 4% stacking gel and then 100V for 1.5 hours through an appropriate separating gel, dependant on the size of the protein of interest, as outlined in **Table 2**. Proteins were transferred unto the respective membrane at 100V for 1 hour in transfer buffer (12mM Tris–HCl, 96mM glycine and 20% methanol). Successful transfer was confirmed by Ponceau staining. Membranes were blocked in 5% skim milk in TBS-T (10mM Tris–HCl, pH 8.0, 150mM NaCl, 0.05% and Tween 20) for 1 hour. Membranes were incubated in primary antibody overnight on an orbital shaker.

Subsequently, two 5 minute washes and one 15 minute wash was done in TBS-T. The membranes were then appropriately incubated in secondary antibody for 1.5 hours. Two 5 minute washes and one 10 minute wash was done in TBS-T. Finally, proteins were visualized using enzymatic chemiluminescence (ECL) reagents and exposured to CL-X Posure TM film. The pixel intensity of the resulting bands was determined using Image J software (NIH Image computer software). These values were normalized with the relative

signal from β-actin or GAPDH labelling on the same membrane after stripping and re-probing of the membrane.

Membranes were stripped and re-probed using 1X solution of Reblot strong reagent (cat# 2504, Millipore, California, USA). Blocking was done in 5% skim milk two times for 15 minutes and then the membrane was incubated with a primary control antibody (either GAPDH or β-Actin) in TBS-T overnight as outlined in **Table 2**. Day 2 western was performed with the control antibody on the same blot, as was formerly outlined, to quantitatively account for loading and other errors.

Table 2. Parameters used for Immunoblot assessment

Primary Antibody	Concentration of Primary Antibody	Secondary Antibody	Concentration of Secondary Antibody	% of SDS gel	Membrane	Block	Exposure times
AIF	1:5000	Anti Rabbit	1:5000	12% gel	PVDF	Milk	1sec, 5sec, 15sec, 30sec, 1min
BclXL	1:5000	Anti Rabbit	1:5000	15% gel	PVDF	Milk	1sec, 5sec, 15sec, 30sec, 1min
Bax	1:200	Anti Rabbit	1:5000	15% gel	Nitrocellulose	Milk	1sec, 5sec, 15sec, 30sec, 1min
Bcl2	1:200	Anti Rabbit	1:5000	15% gel	Nitrocellulose	Milk	1sec, 5sec, 15sec, 30sec, 1min
D2R	1:1000	Anti Mouse	1:5000	10% gel	PVDF	Milk	1sec, 5sec, 15sec, 30sec, 1min
GAPDH	1:8000	Anti Mouse	1:5000	n/a	n/a	Milk	1sec, 5sec, 15sec, 30sec, 1min
β-ACTIN	1:5000	Anti Mouse	1:5000	n/a	n/a	Milk	1sec, 5sec, 15sec, 30sec, 1min

5.2.6. Real time reverse transcription PCR

A q-Script flex cDNA synthesis kit was used to reverse transcribe the RNA into more stable cDNA for PCR assessments. A 1:1 ratio of oligo DT and random primers (provided with the kit) was used. The RNA was incubated at 25^{o}C for 10 minutes, then 42^{o}C for 45 minutes finally 85^{o}C for 5minutes.

QuantiFAST SYBR green was used for qPCR analysis of these tissues. Samples were activated at 95 ^{o}C for 5 minutes. Cycling was achieved in two steps: 95^{o}C denaturing for 10 seconds and 60^{o}C combined annealing and extension for 30 seconds, this was repeated for 40 cycles. Purity of dissociation was detected by one cycle through: 95^{o}C for 1 minute, then 55^{o}C for 30 seconds and finally 95^{o}C for 30 seconds.

Table 3. Design of Primers for Real Time PCR

mRNA of interest	**NCBI Reference Sequence**	**Primers**
Rattus norvegicus glial fibrillary acidic protein (GFAP)	NM_017009.2	**Forward primer:** aagcaggagcacaaggatgt **Reverse primer:** gaattgggcctagcaaacaa
Rattus norvegicus neurofilament, medium polypeptide (NF)	NM_017029.1	**Forward primer:** gagatcgccgcatataggaa **Reverse primer:** gcctcgactttggtcttctg

Biochemical assessments in Cells

5.2.7. Experimental set up: SHSY-5Y Neuroblastoma and Human embryonic kidney cell culture

A SHSY-5Y neuroblastoma cell line and a human embryonic kidney (HEK) cell line was utilized to investigate the mechanistic changes caused by curcumin treatment. Both cell lines were stably transfected with the dopamine D2 receptor thus resembling the striatal systems which were to be modeled herein.

SHSY-5Y cells were grown in RPMI media, with 10% fetal bovine serum, 1mM glutamine, 100µg/ml penicillin and 100µg/ml streptomycin. HEK cells were grown in DMEM media, with 10% fetal bovine serum, 1mM glutamine, 100µg/ml penicillin and 100µg/ml streptomycin. These cells were maintained in an incubator at 37^{o}C and 5% carbon dioxide (CO_2).

5.2.8. Immunoblotting

5.2.8. (i) Cell seeding and treatment:

1x 10^6 cells were seeded into each well of a 6 well tissue culture treated plate and were allowed to adhere to the plate overnight. The following treatments were set up and incubated at 37^{o}C overnight: No treatment (media), DMSO, 10µM curcumin, 25µM curcumin, 50µM curcumin, 100µM curcumin. Drug treatments were removed and 500µL of 10x trypsin was added to each well, and incubated for 5 minutes. 2mL of media was used to neutralize the trypsin. Samples were spun down in an eppendorf tube at 2000g for

5 minutes. The supernatant was discarded, and pellet re-suspended in 1mL of media and spun down at 13,000g for 5 minutes. Supernatant was discarded and the pellet was finally re-suspended and sonicated in 100µL tris-EDTA buffer with Mini-C (cOmplete, EDTA free) protease inhibitor (Roche Diagnostics, Mannheim, Germany).

5.2.8. (ii) Immunoblotting:

BclXL was selected to be assessed here due to its identification in the kinexus protein microarray. A Bradford assay was done using Bio-Rad protein assay reagent (cat# 500-0006, Bio-Rad Laboratories, Hercules, CA, USA), as outlined in ***section 5.2.1 (v)***, to quantify protein yield from these cells prior to immunobot analysis of BclXL expression. Immunoblot assessment was performed at outlined in ***section 5.2.5.***

5.2.9. MTT cell viability assessment

5.2.9. (i) Cell seeding, and treatment:

$9x10^3$ cells were seeded per well into a 96 well plate, and allowed to adhere to the wells until 95% confluency (approximately 3-4 days). The optimal haloperidol exposure to induce at least a 75% decrease in cell viability was determined to be 100µM haloperidol overnight.

Cells were treated with increasing concentrations of curcumin dissolved in DMSO/media as well as the respective controls. Treatment groups included: no treatment,

5μM curcumin, 10μM curcumin, and 100μM curcumin, DMSO control, 100μM haloperidol, acetic acid control, 5μM curcumin and 100μM haloperidol, 10μM curcumin and 100μM haloperidol, 100μM curcumin and 100μM haloperidol, and the respective DMSO and acetic acid controls. A stock of these solutions was prepared to ensure accurate measurement of this and consistency and 100μL of stock added to each well and incubated overnight.

5.2.9. (ii) MTT assay:

All of the media treatments were removed from the respective cell cultures and 0.005g MTT dissolved in 10mL media was added to each well and incubated for 3 hours. MTT was removed and 100μL of DMSO was added to each well and incubated for 15minutes on a shaker, and the samples were read on a TECAN plate reader (McMaster BioPhotonics). The absorbance was determined as the difference between the measurement wavelength of 570nm and the reference wavelength of 690nm. The cell viability percentages were calculated as outlined in the formula below.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of Sample x 100\%}}{\text{Average absorbance of untreated controls}}$$

5.2.10. Experimental Set-up: Primary cell culture

Pregnant female C57BL/6J mice (Charles River) were utilized for this investigation. The abdomen of the E16 pregnant female mouse was cut open the uterus containing the embryos were removed and placed in CMF-HBSS (Calcium and Magnesium free Hanks buffered saline) solution. Typically 8-10 embryos were present. Embryos were detached from the placenta and brains dissected out and placed in another 100mm plate containing CMF-HBSS. The striatum was in turn isolated from the brains and placed in a 60mm plate with approximately 5ml of CMF-HBSS.

Excess CMF-HBSS was removed and dissected striata were incubated for 10minutes with 1x trypsin (diluted in neurobasal media) at 37^{o}C. Supernatant was removed after incubation and the pellet washed twice with filtered neurobasal media containing: 1% of 100x Glutamax-1, 2% of 50x B-27 media supplement, 100µg/ml penicillin and 100µg/ml streptomycin.

5.2.11. MTT cell viability assessment

5.2.11. (i) Cell seeding and treatment:

Cells were counted and seeded at 1 x 10^5 cells per well into tissue culture treated 24well plates containing 500µL of warm neurobasal media. Each mouse yeilded approximately 10-12 wells of a 24 well plate of tissues depending on the cell count. Cells received 50µL top-up of media as was needed (every 3-4 days) and allowed to grow for

two weeks prior to analyses until visually mature (i.e. establishment of developed processes).

Cells were administered **A:** 10µM curcumin, **B:** 10µM haloperidol, **C:** 10µM curcumin + 10µM haloperidol and **D:** no treatment. Analyses were performed using a percentage of the no-treatment group, and as a relative viability assessment.

5.2.11. (ii) MTT assay

To quantify the capacity of curcumin to prevent anti-psychotic drug induced cell death in primary striatal cell cultures. MTT assessments were performed at outlined in ***section 5.2.9. (ii)***.

OBJECTIVE 3

Biochemical assessments in Rat tissues

5.3.1. Immunoblotting

Dopamine D2 receptor protein expression in rat striatal tissues from cohort 3 (**Figure 14**) were quantified using immunoblotting according to the same protocols outlined in ***sections 5.2.1.(iv)*** and ***5.2.5.*** The specifications for the gels, membranes and antibodies that were used for this investigation are outlined **Table 2**.

5.3.2. Real time-reverse transcription PCR in rat striatal tissues

Real time PCR was performed to characterize changes in the dopamine D2 receptor mRNA expression in rat striatal tissues. Tissues were prepared from cohort 1, 2 and 5 at outlined in ***section 5.2.1.(iii)***.

Biochemical assessments in Cells

5.3.3. Experimental set up: SHSY-5Y Neuroblastoma and Human embryonic kidney cell culture

SHSY-5Y and HEK cell lines were cultured as outlined in ***section 5.2.7***. and utilized in these assessment.

5.3.4. Immunoblotting

5.3.4. (i) Cell seeding and treatment:

1x 10^6 cells were seeded into each well of a 6 well tissue culture treated plate and were allowed to adhere to the plate overnight. The following treatments were set up and incubated at 37^{o}C overnight: No treatment (media), DMSO and 10µM curcumin. Cells were scraped to detach from plate, then collected and spun down at 2000g for 5 minutes. The supernatant was discarded, and pellet re-suspended in 1mL of PBS and Mini-C (cOmplete, EDTA free) protease inhibitor (Roche Diagnostics, Mannheim, Germany). Samples were then disrupted and a Bradford assay performed using Bio-Rad protein assay reagent (cat# 500-0006, Bio-Rad Laboratories, Hercules, CA, USA) to quantify protein yield from these cells as outlined in ***section 5.2.1 (v)***.

5.3.4. (ii) Immunoblotting:

Immunoblotting was performed in curcumin treated SHSY-5Y cells for a two-fold assessment. Firstly, immunoblotting was utilized to confirm stable transfection of the D2 receptor in the cell line. Secondly to confirm the relative expression of D2 receptors in curcumin treated and control treated cells. This technique was repeated to identify changes in D2 receptor expression in human embryonic kidney cells similarly treated with curcumin. Immunoblot assessment was performed at outlined in ***section 5.2.5.***

5.3.5. Receptor Binding

5.3.5. (i) Cell seeding and treatment:

1x 10^6 cells were seeded into each well of a 6 well tissue culture treated plate and were allowed to adhere to the plate overnight. The following treatments were set up and incubated at 37^{o}C overnight: No treatment (media), DMSO and 10µM curcumin. Cells were scraped to detach from plate, then collected and spun down at 2000g for 5 minutes. The supernatant was discarded and pellet re-suspended in PBS and miniC without EDTA. These samples were disrupted at 15,000g.

5.3.5. (ii) Receptor Binding Assessment:

To assess the relative change in dopaminergic binding100µg of protein from our cell samples were incubated for 1 hour at 37^{o}C in 0.25µM ^{3}H NPA and assay buffer with or without 10µM supiride. 10µM supiride should saturate the receptors. Samples were filtered and radioactivity counts recorded using the Beckman Radioactivity counter.

6. RESULTS

OBJECTIVE 1

6.1.2. Animals and Drug treatment set-up

All treatments were effectively and efficiently carried out. Injections were accurate and proceeded without any challenges. Rats willingly ate jell-o®, and no visible traces of jell-o® remained in the cages during daily inspection. The selected dosage of curcumin administered was sufficiently high so to compensate for the possibility that any animal would be reluctant to eat their jell-o® for any un-anticipated reason. No such events were encountered.

6.1.3. Behavioural testing

6.1.3. (i) The effect of curcumin on anti-psychotic drug induced vacuous chewing movements

Fourteen days of haloperidol treatment induced a significantly increased incidence of VCMs in rats **Figure 17**. However, rats administered haloperidol for more than twenty-one days, did not display significantly increased VCMs beyond control levels. In spite of the acclimatization time provided, these animals were extremely cataleptic and their VCMs were not evident under the experimental conditions. However, they exhibited visibly exacerbated VCMs in their home cages that were not quantifiable by the defined experimental approach in ***section 5.1.3***. Since the cataleptic effect was likely skewing

measurable VCM results beyond day fourteen; two weeks of drug treatment was considered the optimized methodology so to avoid these biases. Thus, two weeks of intra-peritoneal injection with haloperidol was utilized for all ensuing biochemical investigations.

Concurrent curcumin treatment alongside haloperidol prevented the incidence of VCMs following fourteen days of treatment. Since the haloperidol induced VCM model was not maintained beyond fourteen days, curcumin's effect cannot be clearly delineated from the results observed beyond twenty-one days of treatments.

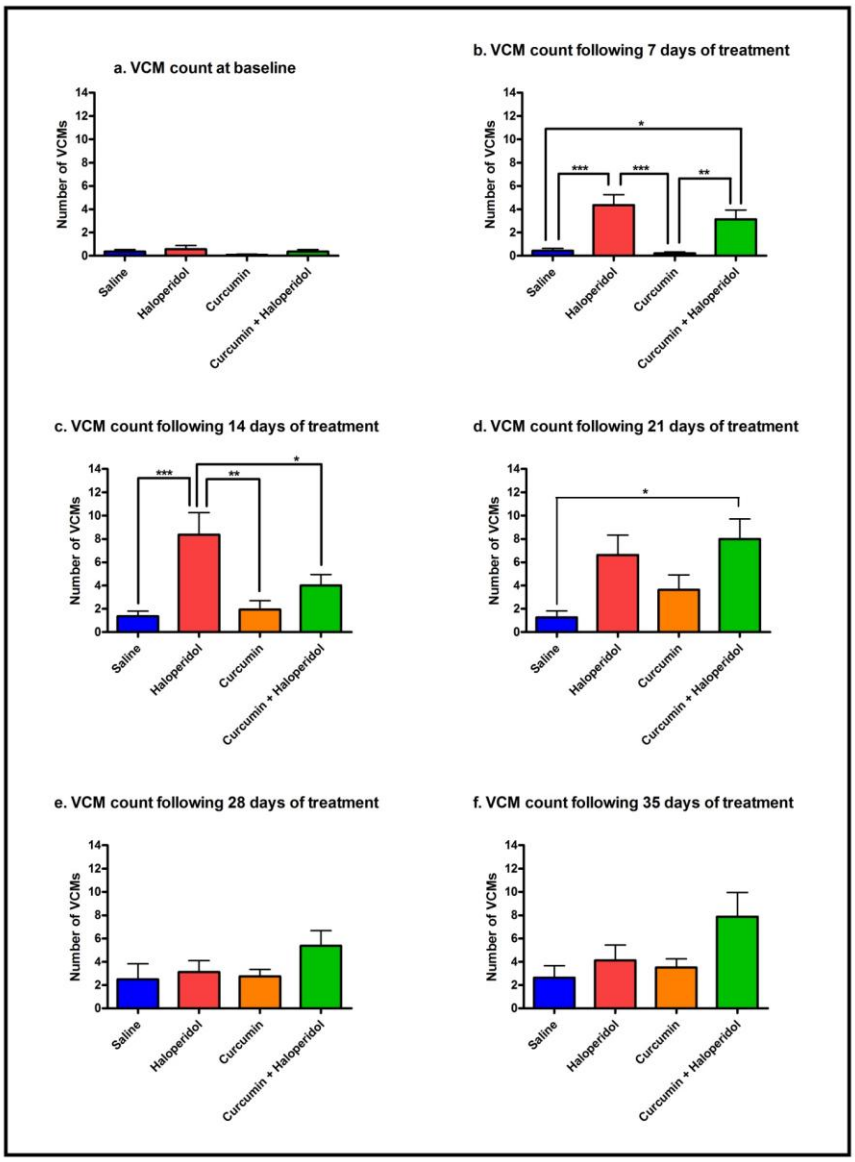


Figure 17. The effect of curcumin and haloperidol administration on the incidence of vacuous chewing movements (VCMs). The average counts of VCMs in a five minute trial in rats at **a.** baseline, **b.** day 7, **c.** day 14, **d.** day 21, **e.** day 28 and **f.** day 35. Chronic haloperidol treatment increases the incidence of VCMs after fourteen days of treatment; however this model was not maintained beyond day 21, as could be assessed by the protocols outlined in ***section 5.1.2***. Curcumin's potential therapeutic effect was observed up to day fourteen of treatment. (*p<0.05, **p<0.01, ***p<0.001)

Intramuscular Haloperidol Deconoate provides a more stable model of VCMs than i.p. injections of Haloperidol for a long term investigation

Intra-muscular injection of haloperidol decanoate (monthly) progressively induced VCMs identical in phenotype to those prepared by fourteen days of daily haloperidol intra-peritoneal injections. While, these VCMs did not fully develop in the rats until week six, which was a significantly longer time period for the development of this model relative to the intra-peritoneal correlate, once established these VCM were comparatively more stable. The limitation of this model is the length of time which is necessary for it to be established. Furthermore, most publications consider two months of intra-muscular injection a minimum. These extensive time periods for experimentation were unfeasible for our purposes but should be considered in future experiments **Figure 18**.

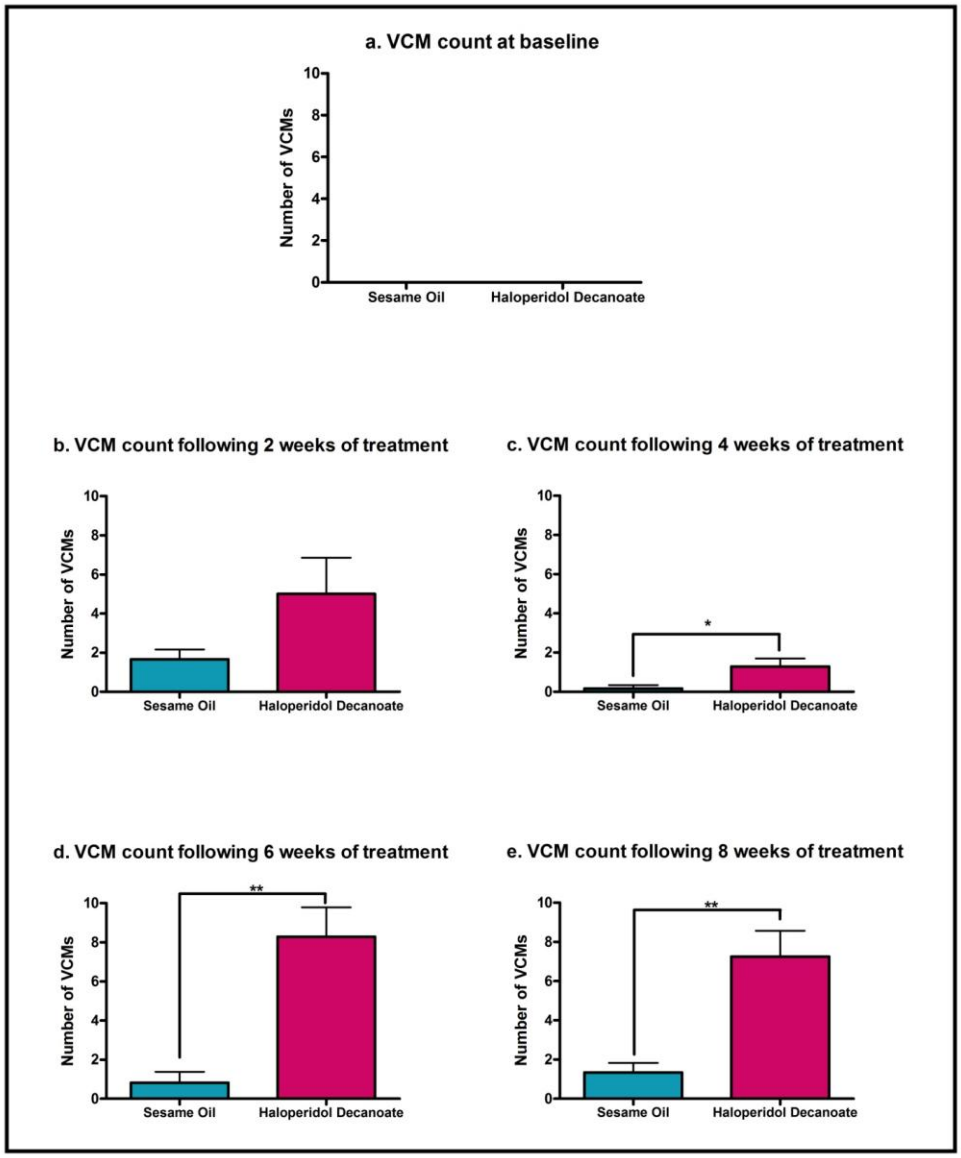


Figure 18. Incidence of vacuous chewing movements (VCMs) in rats treated with haloperidol decanoate at **a.** baseline, **b.** week 2, **c.** week 4, **d.** week 6 and **e.** week 8. VCMs are well established by week 6, and once established the VCM phenotype shows minimal fluctuations relative to the intra-peritoneal correlate. However, it is limited by the time necessary for establishment of the model. (*$p<0.05$, **$p<0.01$)

6.1.3. (ii) The effect of curcumin on anti-psychotic drug induced catalepsy

The cataleptic phenotype was immediately apparent in rats following haloperidol intra-peritoneal injection. Catalepsy was demonstrated to be significantly increased in animals treated with haloperidol as early as day seven with a more exacerbated effect observed by day fourteen. Curcumin was demonstrated to prevent the exacerbation of the cataleptic effect observed following fourteen days of haloperidol treatment, significantly preventing haloperidol induced catalepsy (**Figure 19**).

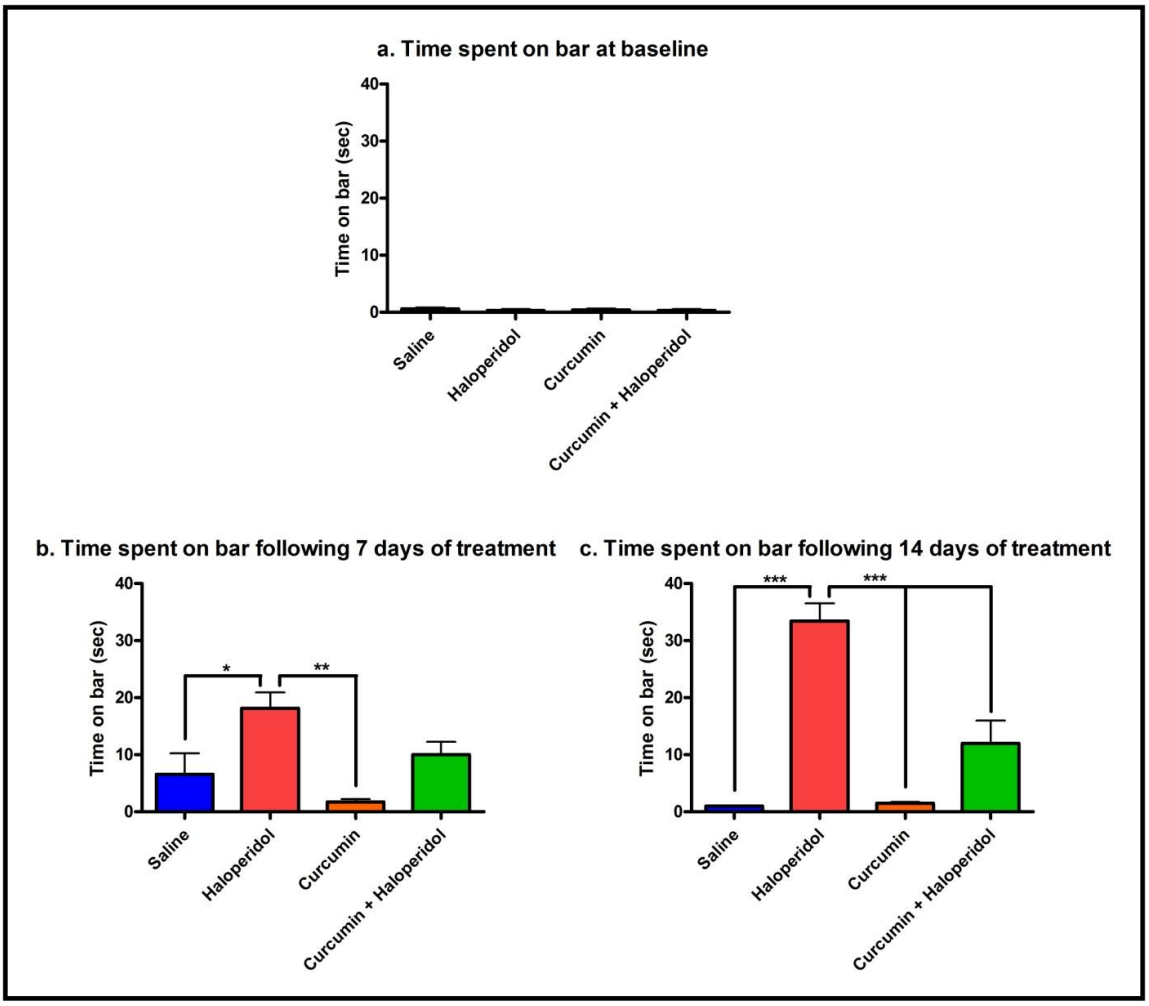


Figure 19. The effect of curcumin and haloperidol administration on catalepsy. Average time spent on the bar, as an assessment of catalepsy in rats at **a.** baseline, **b.** day 7 and **c.** day 14. Haloperidol induces a significantly increased cataleptic response in rats following seven and fourteen days of treatment. Concurrent curcumin treatment prevents haloperidol induced catalepsy following fourteen days of treatment. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

6.1.3. (iii) The effect of curcumin on anti-psychotic drug induced locomotion

Chronic haloperidol treatment, for fourteen days or twenty eight days, significantly decreases total locomotor activity in rats in a 180 minute trial (**Figure 20**). Concurrent curcumin treatment does not prevent haloperidol induced hypo-locomotion. Analysis without habituation (**Figure 21**) was performed to determine whether the differences observed were biased due to the novel environment of the locomotor chambers. Results closely resemble those observed with total locomotor activity by days 27/28 which suggest that curcumin does not interrupt haloperidol induced hypo-locomotion.

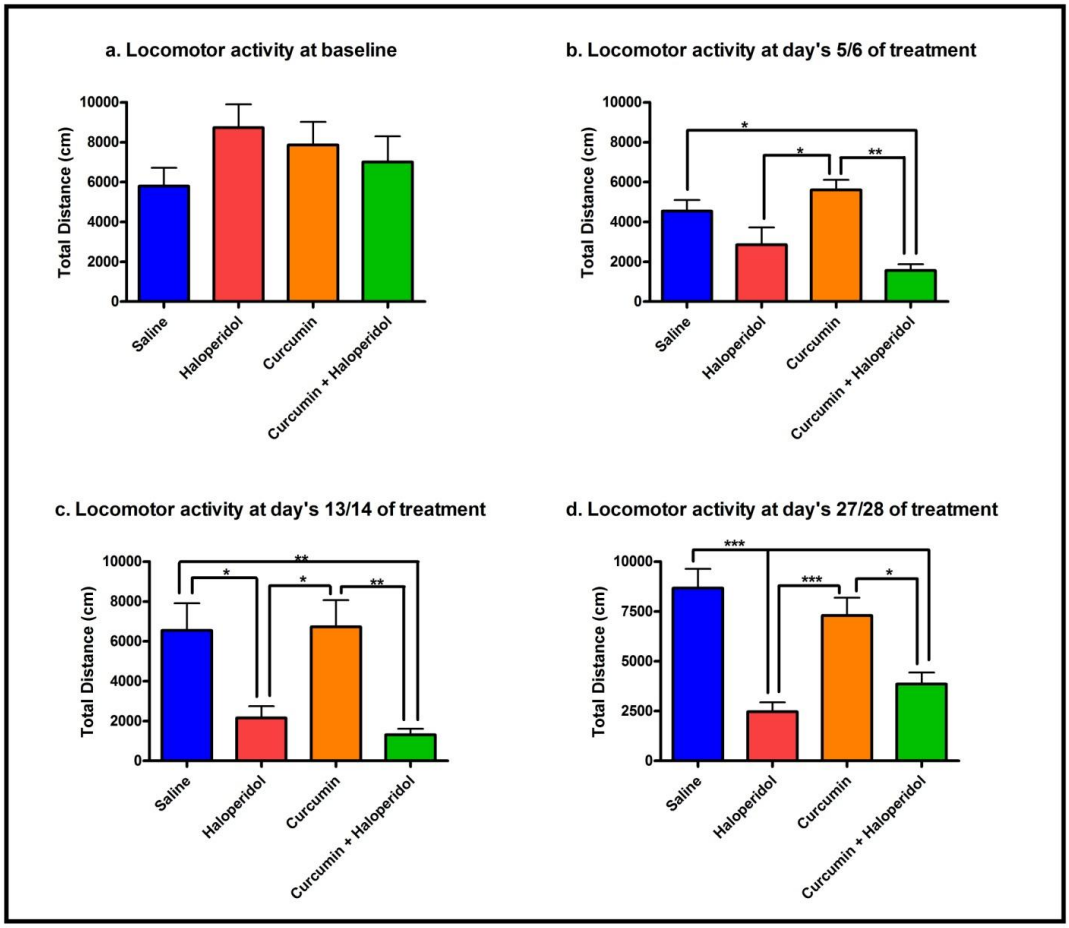


Figure 20. The effect of curcumin and haloperidol administration on locomotor activity. Locomotor activity at **a.** baseline, **b.** days 5/6, **c.** days 13/14 and **d.** days 27/28 following daily intra-peritoneal injections of haloperidol and orally adminstered curcumin in jell-o®. Curcumin does not prevent haloperidol induced hypo-locomotion following fourteen nor twenty eight days of the drug regimen. (*p<0.05, **p<0.01, ***p<0.001)

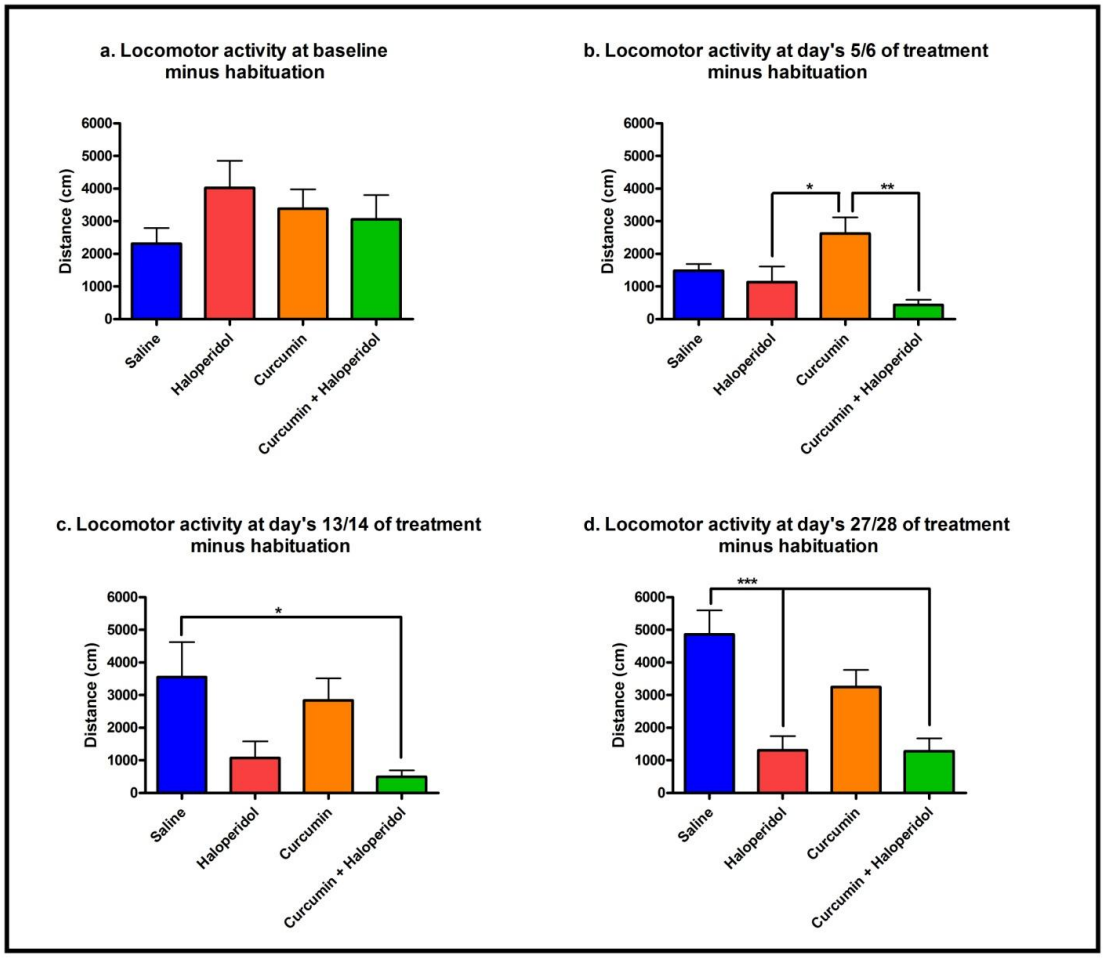


Figure 21. The effect of curcumin and haloperidol administration on locomotor activity, without habituation. Locomotor activity minus habituation at **a.** baseline, **b.** days 5/6, **c.** days 13/14 and **d.** days 27/28 following daily intra-peritoneal injections of haloperidol and orally adminstered curcumin in jell-o®. Curcumin does not prevent haloperidol induced hypo-locomotion following fourteen nor twenty eight days of the drug regimen. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

OBJECTIVE 2

Biochemical assessments on Rat Tissues

6.2.1. Tissue preparation

Sample and tissues preparations were all standard protocols and were effectively carried out with no un-anticipated challenges.

6.2.2. Ultra performance liquid chromatography assessments of curcumin levels in the brain and CSF

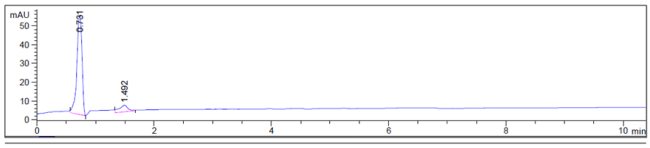
6.2.2. (i) Conformation that curcumin is present in the striatum following 14 days of oral administration

Our PBS controls display a peak at 0.7minutes and this peak is observed in a couple of our other plots (**Figure 22 (i)**). This suggests that the solvent extraction solutions and/or the mobile phase might produce some background peaks detectable at 420nm. 40μg of the internal standard quercertin was observed to produce a peak at 0.9minutes. Finally, curcumin samples produced two peaks: a major peak at 1.5minutes and a minor peak at 1.3minutes after injection.

The identity of the peak of interest for our curcumin was initially confirmed by comparing between the injection of 10μg and 100μg curcumin samples preparations (**Figure 22**). However, two peaks were observed (1.5minutes and 1.3minutes) to be increased in the 100μg curcumin preparation relative to the 10μg curcumin preparation (**Figure 22 (ii)**). The identities of these peaks were distinguished by mass spectrometry in as described in ***sections 5.2.3*** and ***6.2.3.***

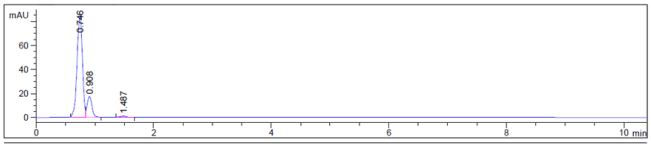
A peak was observed at 1.5 minutes in our curcumin treated brain samples following solvent extraction that was much greater than that observed in control treated rat striata (**Figure 23**). The levels of curcumin that was assessed by these investigations suggest that curcumin is appearing in the rat striatum at 81μg curcumin per g striatal tissues a ten-fold increase over the residual levels observed in control tissues. The levels of curcumin being observed in our control treated rat striata were less than that observed residually in PBS controls and Quercetin controls as observed in **Figure 22 (i)-(iii)**.

PBS Control: NO CURCUMIN OR QUERCETIN



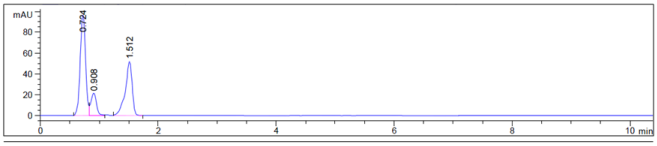
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.731	316.82764	53.48658	89.6161736
2	1.492	36.71082	3.51444	10.3838264

40µg QUERCETIN



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.746	535.03168	85.50737	83.409786
2	0.908	99.06736	17.48253	15.4442954
3	1.487	7.35049	1.00016	1.14591868

10µg CURCUMIN PLUS 40 µg QUERCETIN



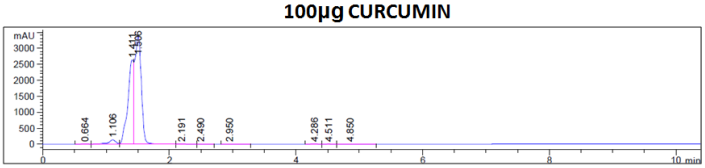
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.724	608.67834	97.06528	51.797263
2	0.908	143.74901	21.48969	12.2327423
3	1.512	422.68945	52.04526	35.9699946

10µg CURCUMIN: 40µg QUERCETIN = 422.68945 / 143.74901 = 2.94

1µg CURCUMIN: 40µg QUERCETIN = (422.68945/10) / (143.74901) = 0.2940

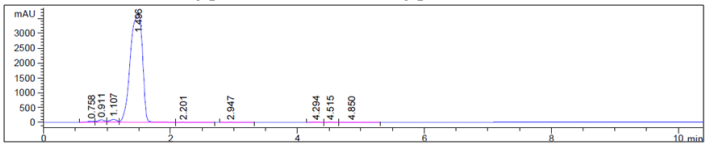
Figure 22 (i). Control UPLC Plots for the assessment of curcumin. PBS Control, 40ug Quercetin Internal Standard, and 10µg Curcumin with 40µg Quercetin. Based on the area under the peaks, it was determined that the ratio of 1µg curcumin to 40µg quercetin is 0.29. A residual peak at 1.5minutes was observed in both the PBS and quercetin controls.

100µg CURCUMIN



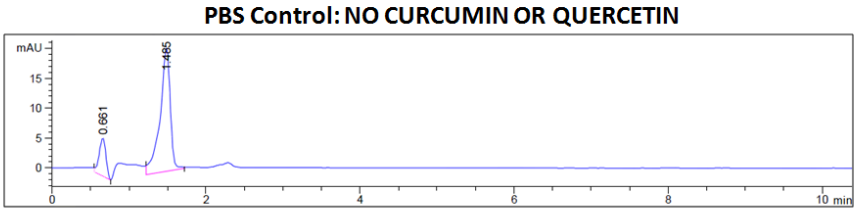
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.664	44.23529	7.04233	0.0528
2	1.106	1107.2418	132.08459	1.3219
3	1.411	1.66008e4	2634.09058	19.8187
4	1.506	2.40680e4	3410.0285	28.7334
5	2.191	49.43971	3.13994	0.0590
6	2.490	19.32021	1.75868	0.0231
7	2.950	21.71782	1.88983	0.0259
8	4.286	32.17336	4.47049	0.0384
9	4.511	20.20429	2.73650	0.0241
10	4.850	18.30630	1.25088	0.0219

100µg CURCUMIN PLUS 40 µg QUERCETIN



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.758	290.93533	37.67221	0.2856
2	0.911	608.91437	83.05109	0.5978
3	1.107	773.45862	98.44067	0.7593
4	1.496	4.85175e4	3660.60474	47.6282
5	2.201	57.28392	2.61905	0.0562
6	2.947	32.13688	2.53105	0.0315
7	4.294	47.64226	5.56598	0.0468
8	4.515	27.96889	2.99651	0.0275
9	4.850	28.45124	1.63762	0.0279

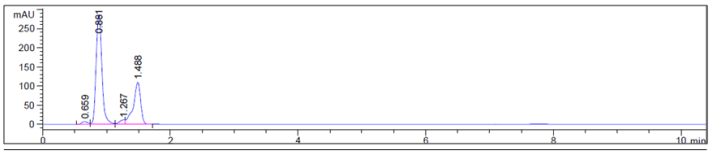
Figure 22 (ii). Control UPLC Plots for the assessment of curcumin. At 100µg curcumin the area produced by the peak at 1.5minutes increased 100 fold from that observed with 10µg curcumin.



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.661	38.72984	6.34626	16.9234138
2	1.485	190.12375	20.69456	83.0765862

Figure 22 (iii). Control UPLC Plots for the assessment of curcumin. PBS Control re-run at the end of UPLC assessments illustrating the largest residual peak for curcumin observed in our investigations.

RAT 7: CURCUMIN ADMINSTRATION



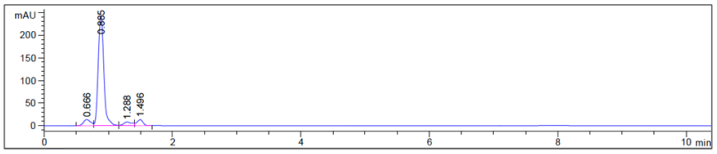
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.659	46.17154	7.14153	1.64310615
2	0.881	1825.64709	289.16873	64.9692855
3	1.267	67.05499	11.43680	2.38628529
4	1.488	871.14203	110.25773	31.0013231

Xμg CURCUMIN: 40μg QUERCETIN = 871.14203 / 1825.64709 = 0.4772

Since,
1μg CURCUMIN: 40μg QUERCETIN = 0.2940
Amount of curcumin = 0.4772 / 0.294 = 1.623μg curcumin
Mass of striatal tissue obtained from rat 7 = 0.0199g

Therefore,
Curcumin getting to the striata = 1.623 / 0.0199 = 81.56μg curcumin per g striatal tissue

RAT 8: CONTROL ADMINSTRATION



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	0.666	105.46698	13.54832	6.01928694
2	0.885	1482.43506	245.01787	84.6065943
3	1.288	76.72692	7.94169	4.37901367
4	1.496	87.52177	13.34212	4.99510507

Xμg CURCUMIN: 40μg QUERCETIN = 87.52177 / 1482.43506 = 0.0590

Since,
1μg CURCUMIN: 40μg QUERCETIN = 0.2940
Amount of curcumin = 0.0590 / 0.294 = 0.201μg curcumin
Mass of striatal tissue obtained from rat 7 = 0.02340g

Therefore,
Curcumin getting to the striata = 0.201 / 0.02340 = 8.581μg curcumin per g striatal tissue

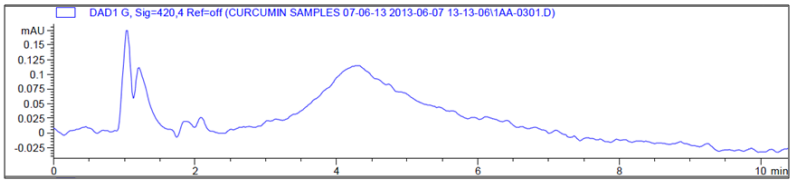
Figure 23. The levels of curcumin in rat strital tissues following 2 weeks of oral administration on curcumin or control treatments. Calculations were performed based on the curcumin to quercetin ratios in our samples relative to that observed ratio in control treatments as outlined in **Figure 22.**

6.2.2. (ii) Conformation that curcumin is present in the CSF of rats following 14 days of oral administration

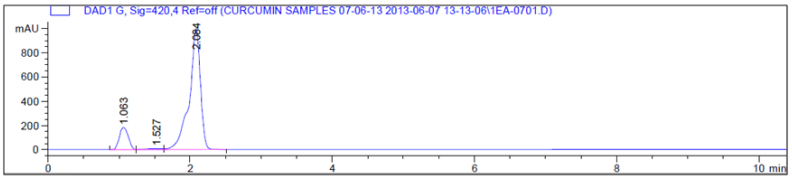
The mobile phase in this modified approach did not display any significant peaks after a thorough pre-wash of the column. The solvent A was identified to produce a peak at 1.063 minutes. The peak for curcumin was identified to be eluted at 2.1minutes in a 10µg control sample of curcumin powder and this time was confirmed in a sample with 100-fold increased curcumin concentration (**Figure 24**). The internal standard was identified to be eluted at 1.35minutes. Based on these observations the ratio of curcumin to the internal standard quercetin was determined to be 0.89 (**Figure 25**). This was confirmed in a replicated sample of this standard. This ratio was utilized to deduce the concentrations of curcumin in the CSF of our rats.

The level of curcumin in the CSF of rat # 9, 11 and 15 from cohort 4 were calculated to be 1.66ng/µL, 0.79ng/µL and 0.24ng/µL CSF. No peak was observed at 2.1minutes in rats 10 and 12 which received control treatments (**Figures 26 - 28**).

MOBILE PHASE

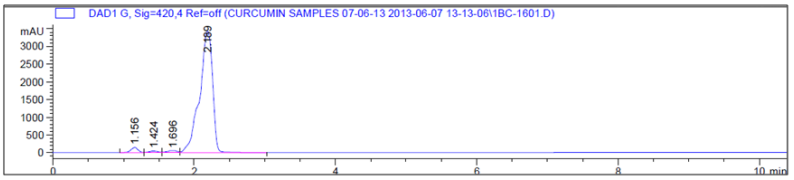


10µl CURCUMIN NO QUERCETIN



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.063	1615.82910	184.14966	12.7464
2	1.527	162.61711	9.73618	1.2828
3	2.084	1.08983e4	998.40857	85.9708

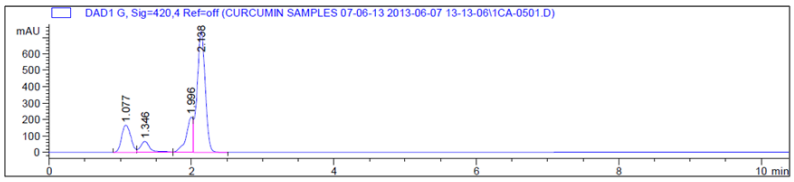
100µg CURCUMIN PLUS 40µg QUERCETIN



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.156	1101.77075	148.23436	2.3952
2	1.424	453.60602	48.50357	0.9861
3	1.696	621.43414	60.76799	1.3509
4	2.189	4.38231e4	3430.80811	95.2678

Figure 24. Control UPLC Plots for the assessment of curcumin for CSF samples. Injection of mobile phase produced no recognizable peaks. In the presence of quercetin a peak is apparent that is not apparent in solutions with no quercetin at 1.3-1.4minutes. When the concentration of curcumin is increased the peak at 2.1minutes is amplified. These control assessments allow the deduction that the peak at 1.3-1.4minutes represents our internal standard and the peak at 2.1minutes represents curcumin.

10µg CURCUMIN PLUS 40µg QUERCETIN

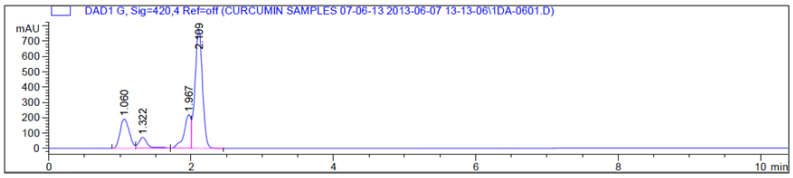


Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.077	1492.35669	166.50079	15.9354
2	1.346	651.86536	67.59598	6.9606
3	1.996	1435.79163	214.54237	15.3314
4	2.138	5785.05322	740.19092	61.7727

10µg CURCUMIN: 40µg QUERCETIN = 5785.05322 / 651.86536 = 8.87

1µg CURCUMIN: 40µg QUERCETIN = (5785.05322/ 10) / (651.86536) = 0.89

10µg CURCUMIN PLUS 40 µg QUERCETIN



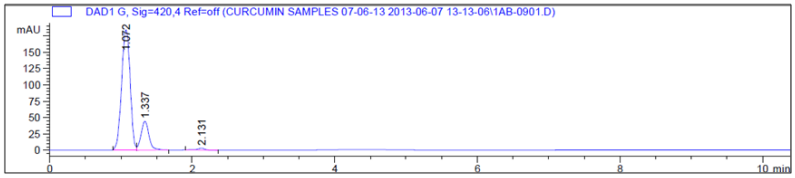
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.060	1726.19055	191.21309	18.1346
2	1.322	637.45514	71.05778	6.6968
3	1.967	1485.09387	218.50621	15.6017
4	2.109	5670.04297	781.56152	59.5669

10µg CURCUMIN: 40µg QUERCETIN = 5670.04297 / 637.45514 = 8.89

1µg CURCUMIN: 40µg QUERCETIN = (5670.04297/ 10) / (637.45514) = 0.89

Figure 25. Replicates of the curcumin and quercetin standard preparations. These samples confirm that the curcumin:quercetin ratio of 0.89 for this protocol was fairly consistent in spite of the extensive processing which was necessary.

Rat 9 CSF: CURCUMIN ADMINISTRATION



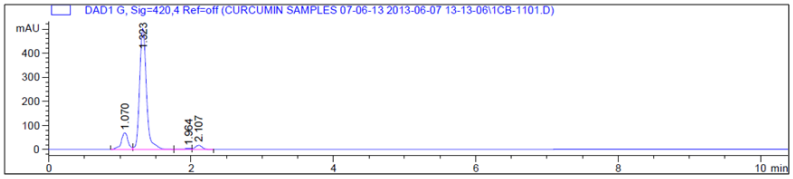
Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.072	1555.53064	184.81137	80.8157
2	1.337	343.92172	44.24898	17.8680
3	2.131	25.33499	2.77833	1.3162

Xμg CURCUMIN: 40μg QUERCETIN = 25.33499 / 343.92172 = 0.074

Since,
1μg CURCUMIN: 40μg QUERCETIN = 0.89
Amount of curcumin = 0.074/0.89 = 0.083μg curcumin = 83ng curcumin
Volume of CSF obtained from rat 9 = 50μL

Therefore,
Curcumin getting to the striata = 83/50 = 1.660ng curcumin per μL CSF

Rat 11 CSF: CURCUMIN ADMINISTRATION



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.070	464.56223	69.53849	11.5996
2	1.323	3383.24219	505.15021	84.4755
3	1.964	38.85144	4.96626	0.9701
4	2.107	118.34295	17.53691	2.9549

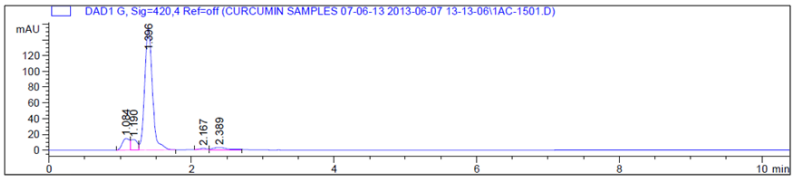
Xμg CURCUMIN: 40μg QUERCETIN = 118.34295/3383.24219 = 0.035

Since,
1μg CURCUMIN: 40μg QUERCETIN = 0.89
Amount of curcumin = 0.035/0.89 = 0.039μg curcumin = 39ng curcumin
Volume of CSF obtained from rat 11 = 50μL

Therefore,
Curcumin getting to the striata = 39/50 = 0.788ng curcumin per μL CSF

Figure 26. The levels of curcumin in rat CSF following 2 weeks of oral administration on curcumin.

Rat 15 CSF: CURCUMIN ADMINISTRATION



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.084	107.15061	14.69663	7.6544
2	1.190	82.14924	13.27153	5.8684
3	1.396	1159.17590	154.35516	82.8066
4	2.167	12.14596	1.60694	0.8677
5	2.389	39.23693	2.82694	2.8029

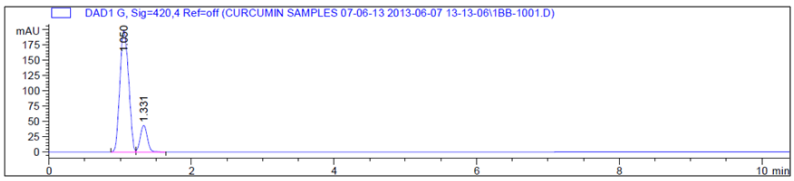
Xµg CURCUMIN: 40µg QUERCETIN = 12.14596/1159.17590 = 0.010

Since,
1µg CURCUMIN: 40µg QUERCETIN = 0.89
Amount of curcumin = 0.010/0.89 = 0.012µg curcumin = 12ng curcumin
Volume of CSF obtained from rat 15 = 50µL

Therefore,
Curcumin getting to the striata = 12/50 = 0.236ng curcumin per µL CSF

Figure 26 (ii) The levels of curcumin in rat CSF following 2 weeks of oral administration on curcumin. Curcumin was detected at 1.66ng/µl, 0.788ng/µl and 0.236ng/µl in rats administered curcumin at 200mg/kg orally.

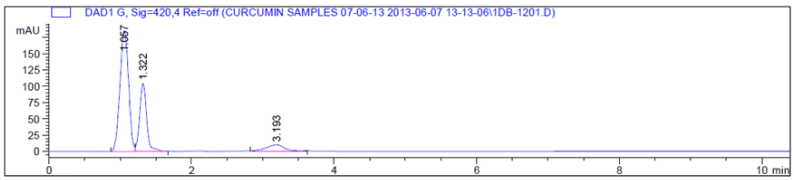
Rat 10 CSF: CONTROL ADMINISTRATION



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.050	1717.61194	198.23436	84.8326
2	1.331	307.09457	43.98883	15.1674

No peak for curcumin at 2.1minutes

Rat 12 CSF: CONTROL ADMINISTRATION



Peak #	Retention Time (min)	Area (mAU/sec)	Height (mAU)	Area %
1	1.057	1573.47644	186.13680	63.8764
2	1.322	715.99054	104.85348	29.0661
3	3.193	173.84900	9.80401	7.0575

No peak for curcumin at 2.1minutes

Figure 27. The levels of curcumin in rat CSF following 2 weeks of control administration. No peaks were observed at 2.1minutes.

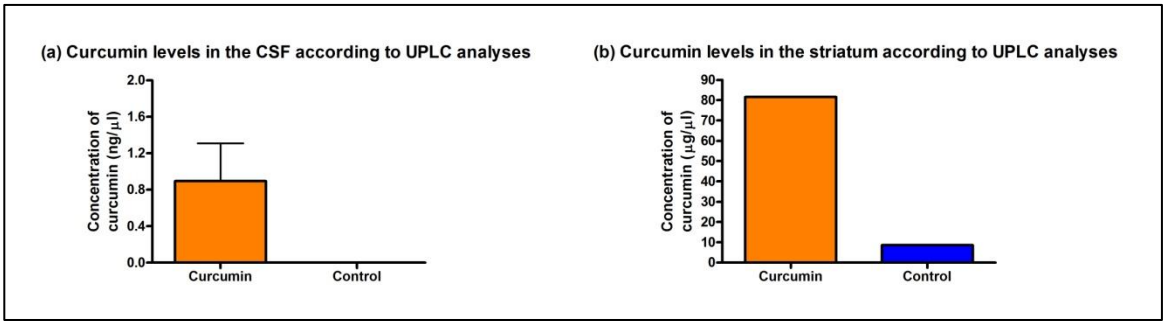


Figure 28. Graph depicting relative curcumin expression in the (a) CSF and (b) striatum of rats following two weeks of oral curcumin or control administration. P values could not be calculated for these comparisons due to control group curcumin levels being zero in the CSF, and low sample sizes for striatal samples.

6.2.3. Mass Spectrometry confirmation of the identity of the peaks identified for curcumin via UPLC assessments

Since two peaks show up for curcumin in both standards with curcumin and the brain tissues from animals administered curcumin, it was necessary to confirm which peak represented curcumin and the identity of the second peak. LC/MS was used for this purpose. One minor peak showed up on both the positive channel (positive ions that are detected) and negative channel. The major peak that appeared was confirmed to be curcumin detected at its approximate molecular weight, a mass of 361g/mol. The minor peak showed up 339g/mol, suggesting that it might be a curcumin molecule minus two methyl groups (**Figure 29-30**). For the purpose of this investigation only the major peak was utilized for calculations of the level of curcumin in the brain.

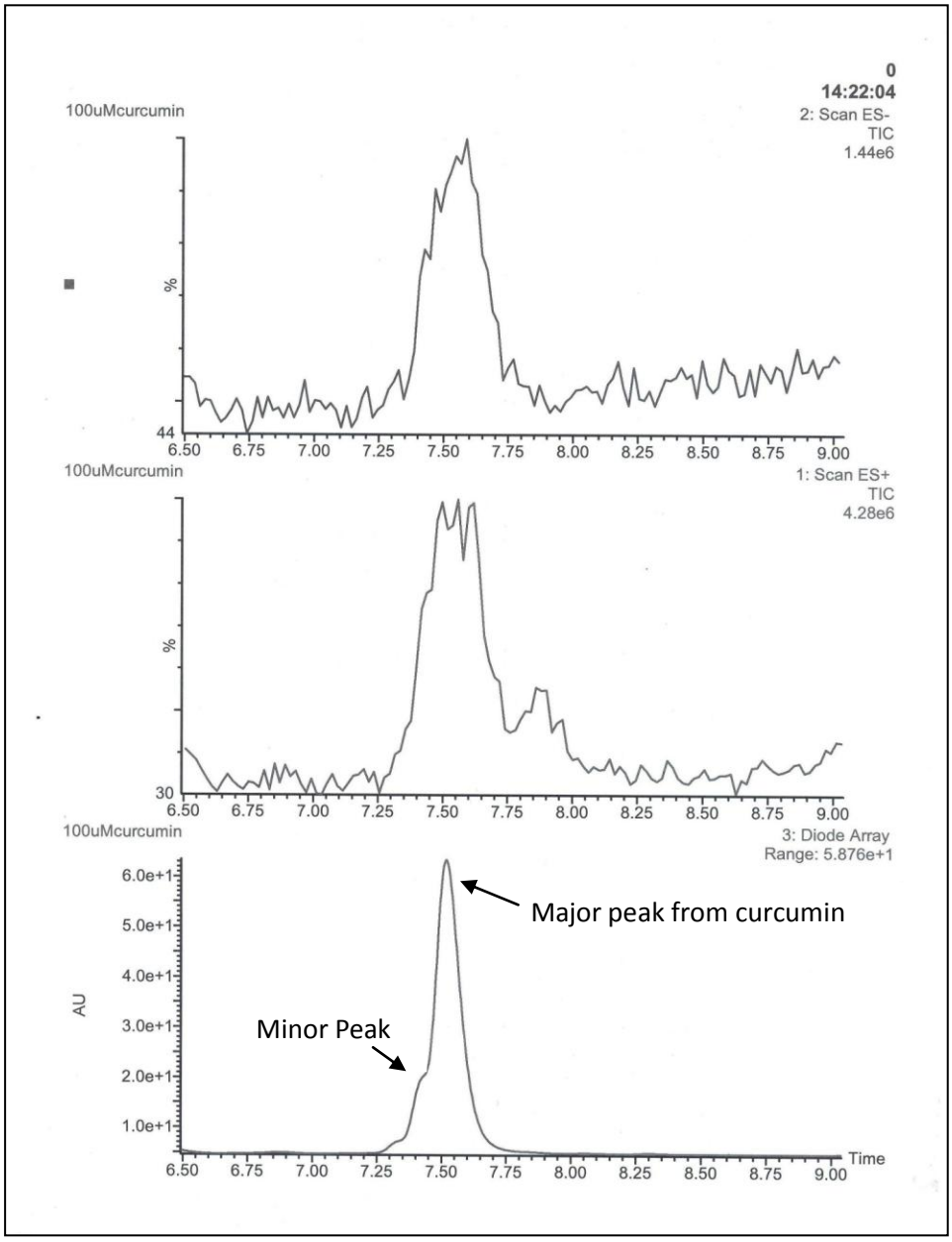


Figure 29. Curcumin detection via liquid chromatography/mass spectrometry, detecting both positive and negative ions and liquid chromatography plot. Plots are reflective of the previously identified trend for curcumin samples when assessed via UPLC wherein both a major and minor peak are observed.

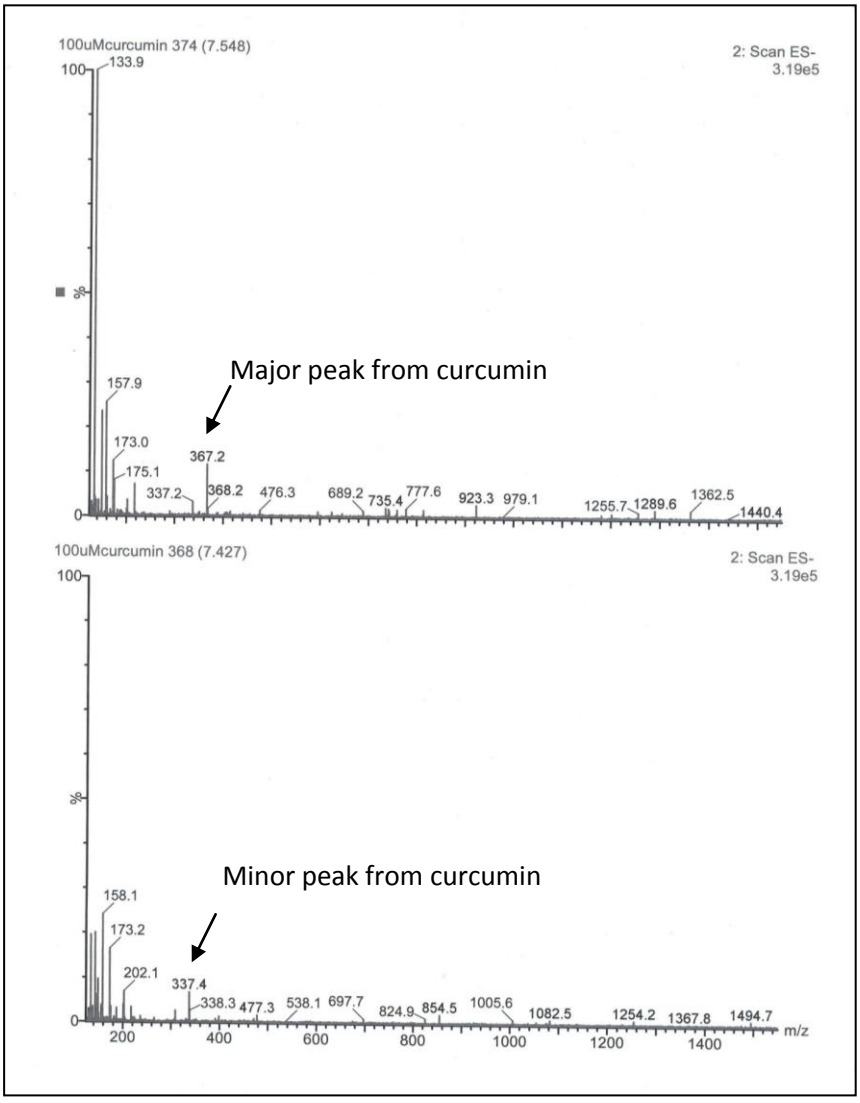


Figure 30. The detection of curcumin via mass spectrometry, plot of negative ions. Based on the molecular weight of curcumin being 361g/mol it is likely that the major peak is the peak of interest and that both represent curcuminoids with the minor peak being a modification of the major peak.

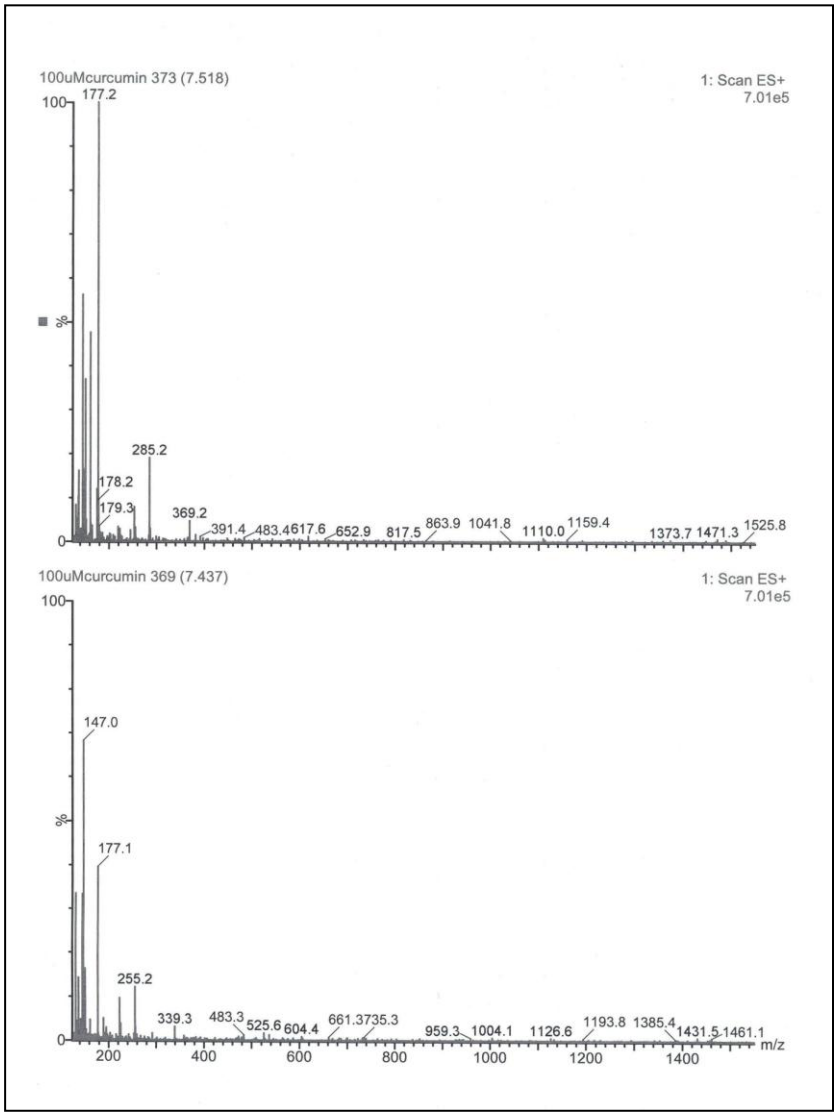


Figure 31. The detection of curcumin via mass spectrometry, plot of positive ions. Based on the molecular weight of curcumin being 361g/mol it is likely that the major peak is the peak of interest and that both represent curcuminoids with the minor peak being a modification of the major peak.

6.2.4. The effect of curcumin on protein expression as assessed by proteomic microarray

The 20 proteins with the highest up-regulation of %CFC are listed in **Table 4**. The 20 proteins with the highest down-regulation of %CFC are listed in**Table 5**. The four fold increase observed in B-cell lymphoma-2 like protein 1, BclXL, was of particular relevance to our study since it is an anti-apoptotic molecule that can interrupt the AIF pathway of cell death. As such, it might provide a mechanistic direction for assessment of the anti-apoptotic potential of curcumin in our rat model of haloperidol induced behavioural and biochemical abnormalities.

Table 4. Kinexus result: Top 20 up-regulated proteins after 35 days of curcumin treatment, relative to controls. *(Filtered according to Kinexus' instruction)*
(Sookram et al., 2011)

Target Protein Name	Full Target Protein Name	Treated Average/Control Average (Fold Change)	Log2(Treated Average/Control Average)	% CFC
PCNA	Proliferating cell nuclear antigen	6.55	2.71	555
PERP	p53-induced protein PIGPC1	6.06	2.60	506
Bcl-xL	Bcl2-like protein 1	4.56	2.19	356
Paxillin 1	Paxillin 1	2.94	1.56	194
PP2B/Aa	Protein-serine phosphatase 2B - catalytic subunit - alpha isoform	2.90	1.53	190
Raf1	Raf1 proto-oncogene-encoded protein-serine kinase	2.86	1.52	186
PI3K	Phosphatidylinositol 3-kinase regulatory subunit alpha	2.84	1.50	184
CASP1a	Pro-caspase 1 (Interleukin-1 beta convertase) alpha isoform	2.76	1.47	176
PP2A/Bg2	Protein-serine phosphatase 2A - B regulatory subunit - gamma isoform	2.69	1.43	169
Kit	Kit/Steel factor receptor-tyrosine kinase	2.62	1.39	162
Arrestin b1	Arrestin beta 1	2.51	1.33	151
4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1 (PHAS1)	2.44	1.29	144
DNAPK	DNA-activated protein-serine kinase	2.40	1.26	140
Erk4	Extracellular regulated protein-serine kinase 4	2.35	1.23	135
Erk1	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	2.32	1.21	132
DFF35	DNA fragmentation factor alpha (ICAD) 35-kDa subunit	2.24	1.16	124
Caveolin 2	Caveolin 2	2.20	1.14	120
ATF2	Activating transcription factor 2 (CRE-BP1)	2.18	1.13	118
Ubiquitin	Ubiquitin	2.13	1.09	113
PKCd	Protein-serine kinase C delta	2.12	1.08	112

Table 5. Kinexus result: Top 20 down-regulated proteins after 35 days of curcumin treatment, relative to controls. *(Filtered according to Kinexus' instruction)*

Target Protein Name	Full Target Protein Name	Treated Average/Control Average (Fold Change)	Log2(Treated Average/Control Average)	% CFC
PKCg	Protein-serine kinase C gamma	0.13	-2.98	-87
Ret	Ret receptor-tyrosine kinase	0.16	-2.61	-84
S6Ka (p70/p85 S6Ka)	p70/p85 ribosomal protein-serine S6 kinase alpha	0.20	-2.29	-80
PKCg	Protein-serine kinase C gamma	0.21	-2.23	-79
GAP-43	Growth associated protein 43 (Neuromodulin)	0.22	-2.17	-78
Bad	Bcl2-antagonist of cell death protein	0.24	-2.05	-76
Chk2	Checkpoint protein-serine kinase 2	0.25	-1.99	-75
FAK	Focal adhesion protein-tyrosine kinase	0.26	-1.93	-74
PKCh	Protein-serine kinase C eta	0.28	-1.84	-72
Cyclin B1	Cyclin B1	0.28	-1.83	-72
IRAK2	Interleukin 1 receptor-associated kinase 2	0.28	-1.82	-72
IKKa	Inhibitor of NF-kappa-B protein-serine kinase alpha (CHUK)	0.32	-1.66	-68
IKKa	Inhibitor of NF-kappa-B protein-serine kinase alpha (CHUK)	0.33	-1.60	-67
EGFR	Epidermal growth factor receptor-tyrosine kinase	0.34	-1.57	-66
PKCg	Protein-serine kinase C gamma	0.34	-1.57	-66
Hsp90a	Heat shock 90 kDa protein alpha	0.34	-1.56	-66
PKBa (Akt1)	Protein-serine kinase B alpha	0.34	-1.56	-66
PKBb (Akt2)	Protein-serine kinase B beta	0.36	-1.48	-64
Hsp90	Heat shock 90 kDa protein alpha/beta	0.36	-1.46	-64
ErbB2 (HER2)	ErbB2 (Neu) receptor-tyrosine kinase	0.37	-1.44	-63

6.2.5. The effect of curcumin on protein expression as assessed by Immunoblotting

Immunoblotting was used to characterize changes in apoptosis relevant molecules in the striatal tissues of rats from cohorts 1, 2 and 4 (**Figures 12, 13** and **15**).

6.2.5. (i) The effect of curcumin and haloperidol on the expression of AIF

AIF translocation was previously determined to be intregral to the pathophysiology of anti-psychotic drug induced neurodegenration. To address the question of whether curcumin exerted it therapeutic effect by interrupting AIF regulation the mRNA and protein expression of this molecule was quantified. PCR and immunoblotting analyses do not indicate any change in the expression of AIF. Furthermore, concurrent curcumin and haloperidol treatment does not appear to effect AIF protein levels (**Figure 32**).

6.2.5. (ii) The effect of curcumin and haloperidol on the expression of Bcl2

The Bcl2 protein was selected for investigation because it is an anti-apoptotic molecule that can interrupt AIF translocation. Bcl2 expression was significantly increased by curcumin treatment, however in the presence of concurrent haloperidol and curcumin the levels of Bcl2 are comparable to controls (**Figure 33**).

6.2.5. (iii) The effect of curcumin and haloperidol on the expression of BclXL

The expression of the anti-apoptotic molecule BclXL was analyzed by immunoblotting to confirm the proteomic result and to assess the effect of concurrent

curcumin and Haloperidol on BclXL expression. Our study revealed no significant differences when analyzed by ANOVA followed by Tukey in the BclXL expression following curcumin treatment alone. However, there is a significant increase in BclXL when curcumin and haloperidol are administed concurrently (**Figure 34**).

6.2.5. (iv) The effect of curcumin and haloperidol on the expression of Bax

The pro-apoptotic molecule Bax exists as an monomer of molecular weight: 21kDa, but can dimerize and oligomerize. As such oligomers of Bax (typically 96kDa and 240kDa) are frequently observed via Immunoblotting and the oligomer at molecular weight 96kDa was analyzed herein in addition to the monomer analyses. Curcumin administration decreased the expression of the Bax monomers (**Figure 34**).

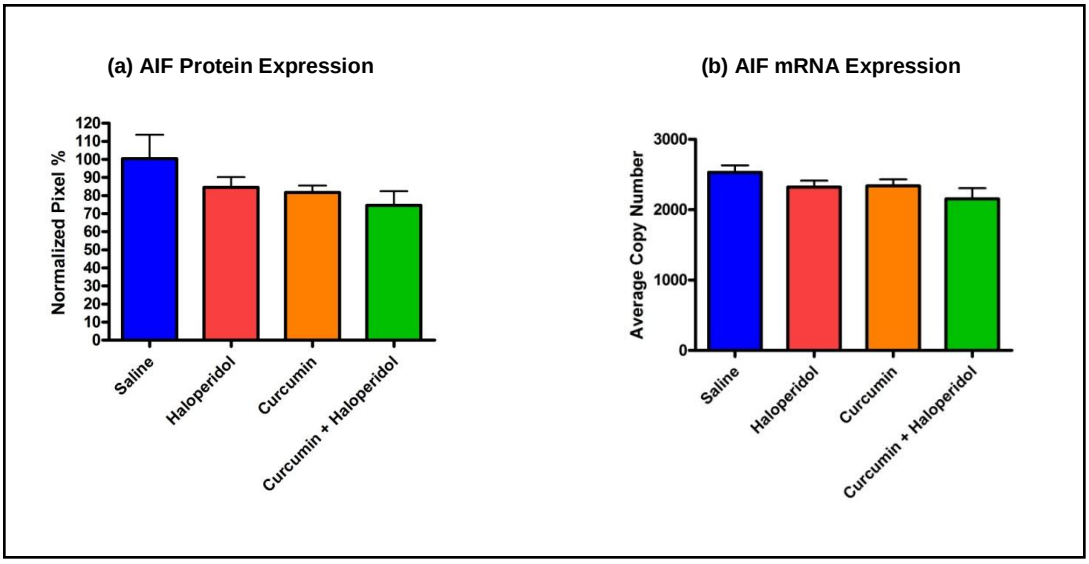


Figure 32. Immunoblot and PCR assessments of (a) AIF protein and (b) mRNA expression in the striatum of rats administered curcumin, haloperidol, concurrent curcumin and haloperidol and the respective control. Neither AIF protein or mRNA expression are affected by curcumin and haloperidol administration.

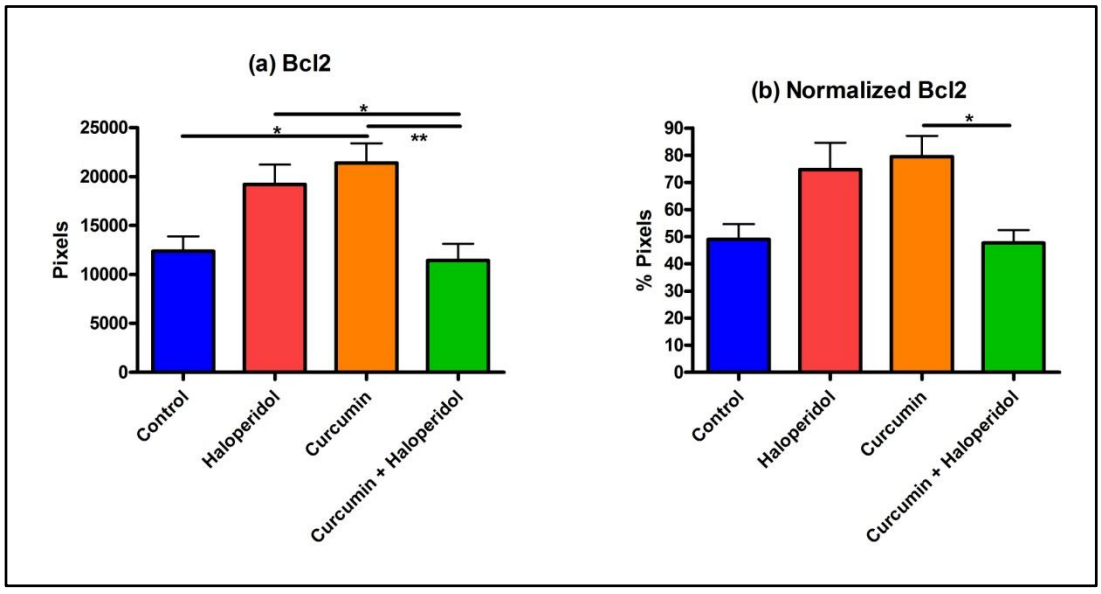


Figure 33. Immunoblot assessments of Bcl2 protein expression in the striatum of rats administred curcumin, haloperidol, concurrent curcumin and haloperidol and the respective control. Curcumin significantly increased Bcl2 expression. However, in the presence of haloperidol, curcumin does not alter the overall Bcl2 expression in the striatum of rats (*$p<0.05$, **$p<0.01$)

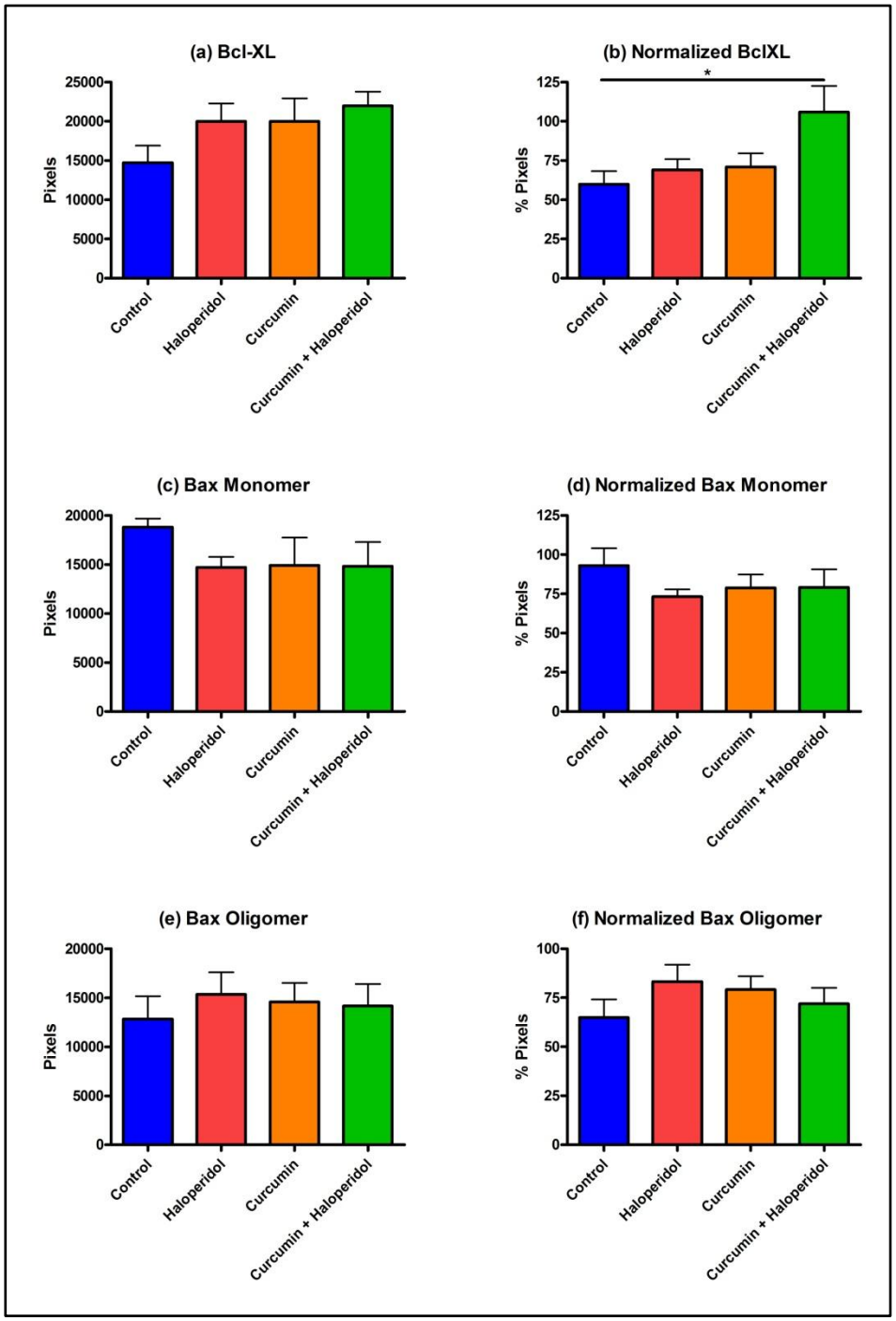


Figure 34. Immunoblot assessment of the expression of Bcl-XL and Bax protein expression in the striatum of rats administered curcumin, haloperidol, concurrent curcumin and haloperidol and the respective control. Concurrent curcumin and haloperidol administration significantly increased the expression of BclXL (*$p<0.05$).

6.2.6. The effect of curcumin on markers for neuronal and glial cells as assessed by real time reverse transcription PCR

The expression of neurofilament (NF) and glial fibrillary acidic protein (GFAP) have previously been shown to be a measure of cell viability in brain tissues (Cabras et al., 2010). However the mRNA expression of NF and GFAP in our investigations remained unchanged in striatal tissues from rats administered curcumin, haloperidol, concurrent curcumin and haloperidol and control treatments for two weeks (**Figure 35**).

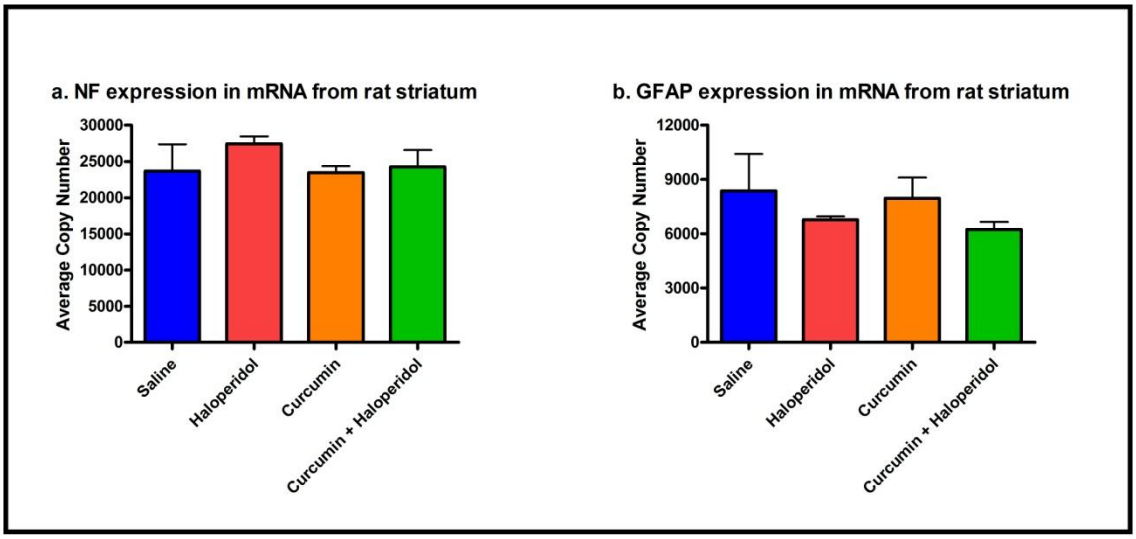


Figure 35. PCR assessment of the mRNA expression of neurofilament (NF) and glial fibrillary acidic protein (GFAP) in rat striatal tissues. NF is a marker of neuronal cell populations and GFAP is a glial cell marker. Curcumin and Haloperdiol administration did not alter GFAP nor NF expression in the striata of rats.

Biochemical assessments in Cells

6.2.7. The establishment of SHSY-5Y and HEK cell lines

SHSY-5Y and HEK cell lines were cultured without un-anticipated challenges. These cultures were well established in our laboratory.

6.2.8. The effect of curcumin on the expression of BclXL protein in SHSY-5Y cells

Increasing concentrations of curcumin treatment decreased the expression of the anti-apoptotic molecule BclXL in SHSY-5Y cells relative to DMSO control (**Figure 36**).

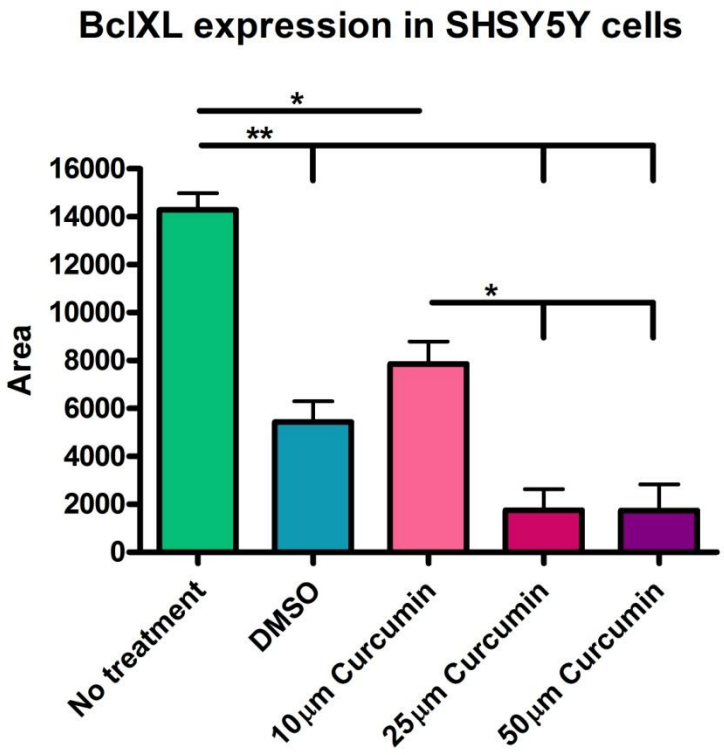


Figure 36. Immunoblot assessment of BclXL protein expression following overnight curcumin treatment in SHSY-5Y cells. 10µM curcumin increased BclXL expression. However, 25-50 µM curcumin decreased BclXL expression. (*$p<0.05$, **$p<0.01$)

6.2.9. The effect of curcumin on cell viability in SHSY-5Y cells

Cell viability was 50% higher than untreated control cells with curcumin administration. Haloperidol decreased cell viability from control treatments by approximately 20% with overnight treatments. The drug vehicles did not decrease cell viability. On the contrary, DMSO (the drug vehicle for curcumin) increased cell viability by almost 80% over control levels when administered alone. Concurrent DMSO and acetic acid treatments did not however on their own cause any effect on cell viability in SHSY-5Y cells beyond normal control levels.

Increasing concentrations of curcumin from 5μM to 10μM displayed a trend toward decreased cell survival with no significant differences in survival between haloperidol induced cell death and the cell death observed in the presence of concurrent curcumin administration. However, by 15μM cell death is observed to be even greater in the presence of curcumin than that observed by haloperidol treatment alone (**Figure 37**).

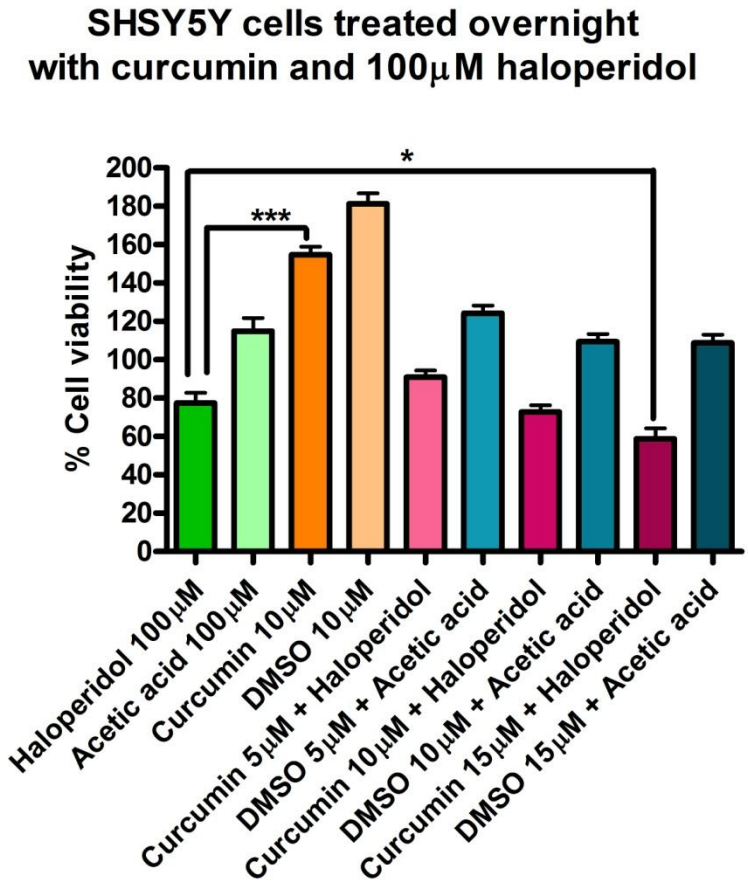


Figure 37. MTT assay to characterize the apoptotic and anti-apoptotic nature of curcumin when administered concurrently with 100μM haloperidol. The Curcumin concentrations utilized include: 5μM, 10μM and 15μM. Curcumin vehicle: DMSO, Haloperidol vehicle: Acetic acid. Increasing curcumin concentrations exacerbated haloperidol induced cell death. (* $p<0.05$, *** $p<0.001$)

6.2.10. The establishment of primary striatal cell cultures

Primary striatal cells of C57BL/6J mice were successfully cultured (**Figure 38**). These were not previously established in our lab. Culturing techniques were optimized for MTT investigations and successful culturing was illustrated herein labeled with neurofilament to identify neuronal cells grown in culture.

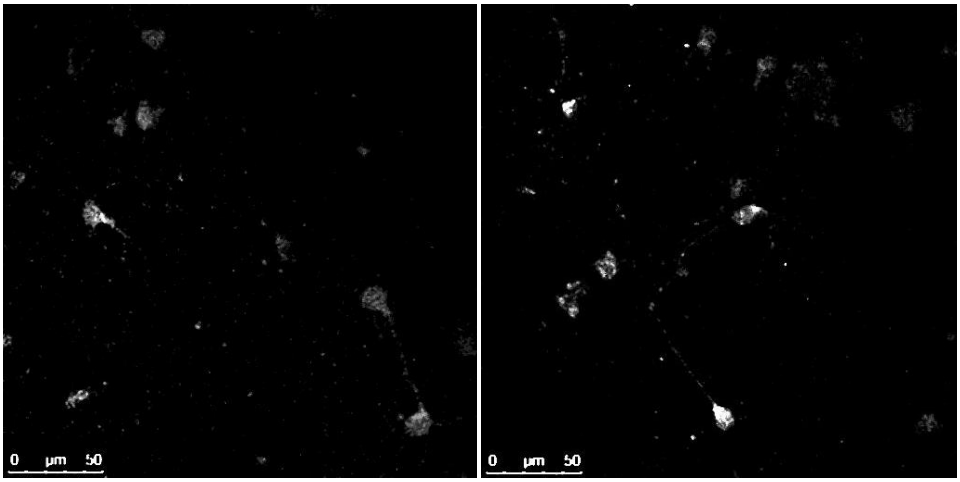


Figure 38. Neurofilament expression in C57BL/6J primary striatal cell cultures illustrating the establishment of these cultures for our investigations

6.2.11. The effect of curcumin on anti-psychotic drug induced neurodegeneration

Cell viability was illustrated herein as both direct absorbance that resulted from viabile cells in the MTT assessment as well as calculated viability relative to untreated control cells. Haloperidol administration to 10μM with one hour incubation significantly reduced cell viability by 20%. Concurrent curcumin treatment prevented this cell loss, increasing cell viability to normal untreated levels (**Figure 39**).

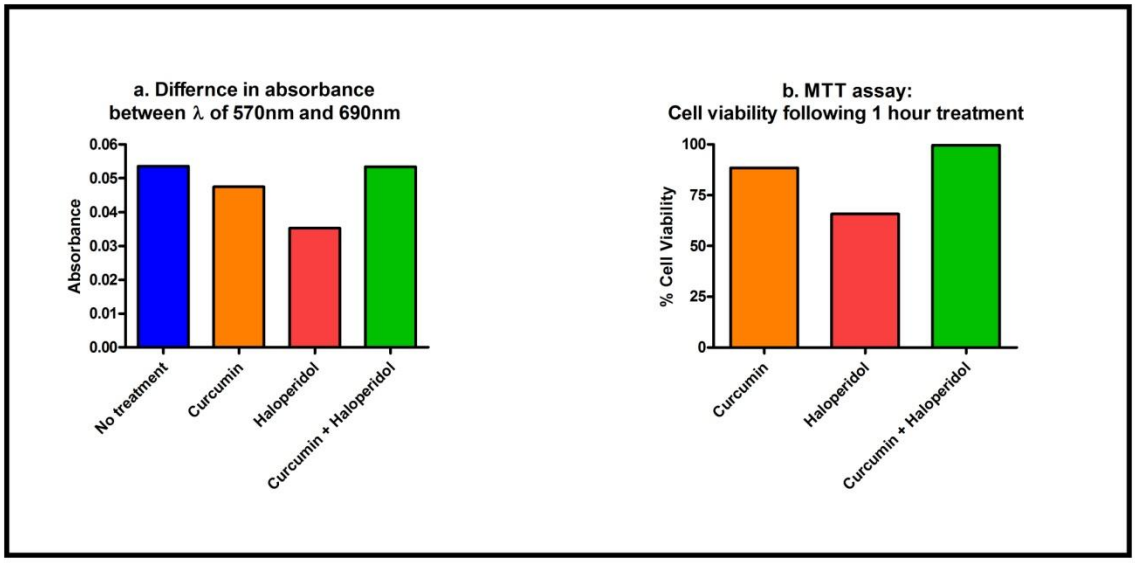


Figure 39. MTT assessment of cell viability in C57BL/6J primary mouse striatal cultures. **a.** Difference in the absorbance wavelengths (λ) of the primary cell cultures following curcumin, haloperidol and curcumin + haloperidol treatment. **b.** MTT assessment of % change in cell viability relative to untreated control cultures. Curcumin prevented the haloperidol induced reductions in primary striatal cell cultures.

OBJECTIVE 3

<u>Biochemical assessments in Rat tissues</u>

6.3.1. The effect of curcumin on protein expression of the Dopamine D2 Receptor

In correlation with the current literature, haloperidol treatment in rats increases dopamine D2 receptor protein levels. Curcumin treatment has herein been demonstrated to also increase dopamine D2 receptor expression. Concurrent curcumin and haloperidol treatment also causes an increase in dopamine D2 receptor expression, however this effect does not appear to be additive (**Figure 40**).

6.3.2. The effect of curcumin on mRNA expression of the Dopamine D2 Receptor

The changes in mRNA expression mirror those observed via Immunoblot assessments. Haloperidol and curcumin both increase mRNA expression of the dopamine D2 receptor. Concurrent curcumin and haloperidol administration also increased the expression of the dopamine D2 receptor, however this effect was not seen to be additive (**Figure 41**).

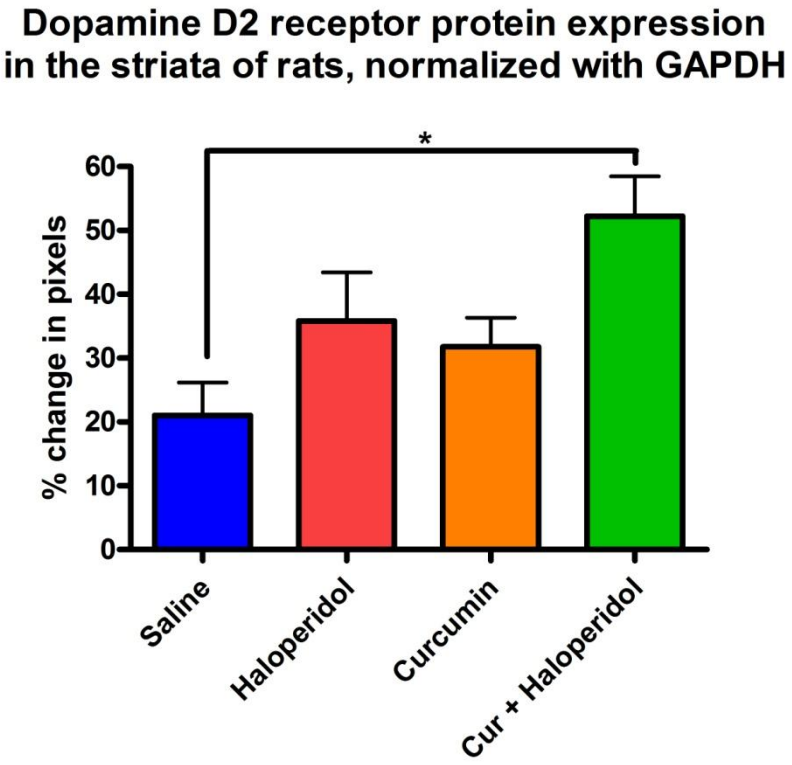


Figure 40. D2 receptor protein expression in the striata of rats administered haloperidol, curcumin, concurrent curcumin and haloperidol and respective controls for two weeks. Concurrent Curcumin and Haloperidol administration increases the expression of the dopamine D2 receptor (*$p<0.05$).

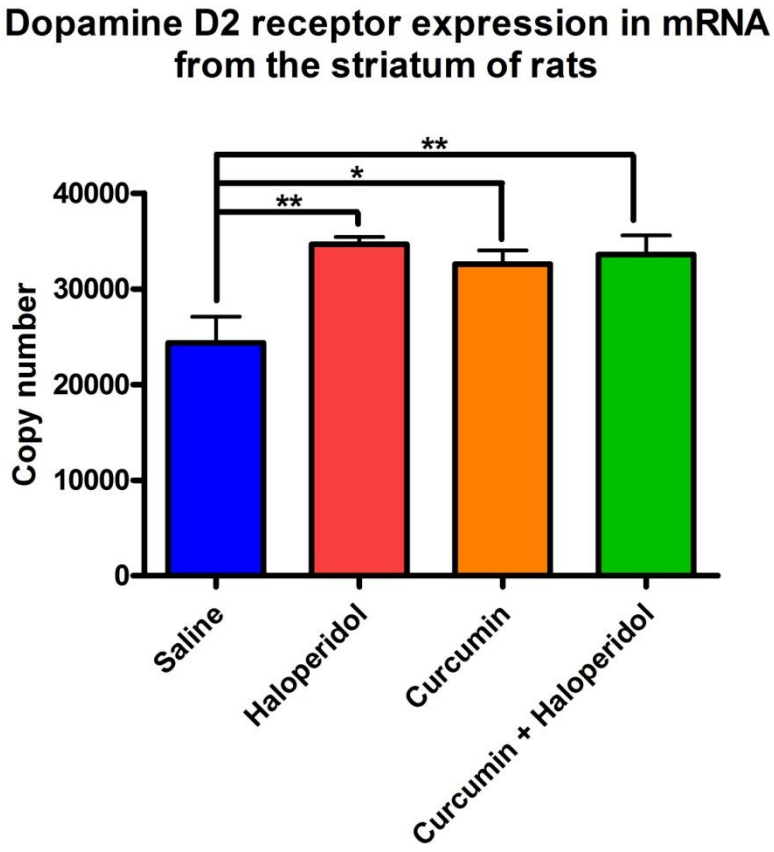


Figure 41. Dopamine D2 Receptor mRNA expression in the striata of rats treated with curcumin. Curcumin and Haloperidol administration and their concurrent administration increase the expression of the dopamine D2 receptor at the level of mRNA (*$p<0.05$, **$p<0.01$).

Biochemical assessments in Cells

6.3.3. Establishment of cell cultures

Cell cultures were well established in our lab and there were no un-anticipated challenges.

6.3.4. The effect of curcumin on dopamine D2 receptor expression in SHYSY5Y and HEK cell lines

Dopamine D2 receptor expression is decreased following curcumin treatment in HEK cells, however in the SHSY-5Y cell lines curcumin treatment increases dopamine D2 receptor expression. Thus, suggesting that the effect of curcumin on the dopamine D2 receptor is cell line specific. Furthermore, when n=5 replicates of treatments were performed in SHSY-5Y cells, the changes observed were confirmed to be significant (**Figure 42-43**).

5.3.4. Receptor Binding

Curcumin treatment significantly increases D2 receptor binding, indicative of increased binding sites and thus increased D2 receptor levels in SHSY-5Y cells (**Figure 44**).

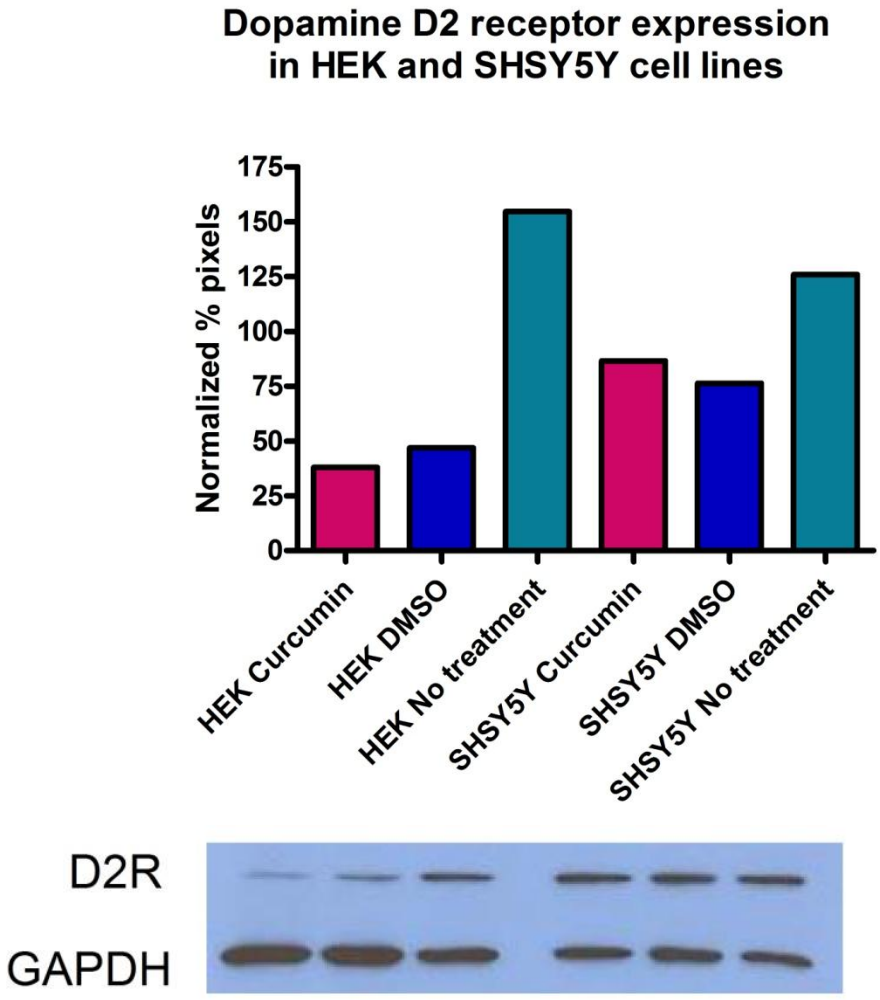


Figure 42. Dopamine D2 Receptor protein expression in HEK and SHSY-5Y cell lines. The expression of dopamine D2 receptors is increased by curcumin incubation in SHSY-5Y cells but decreased in HEK cells.

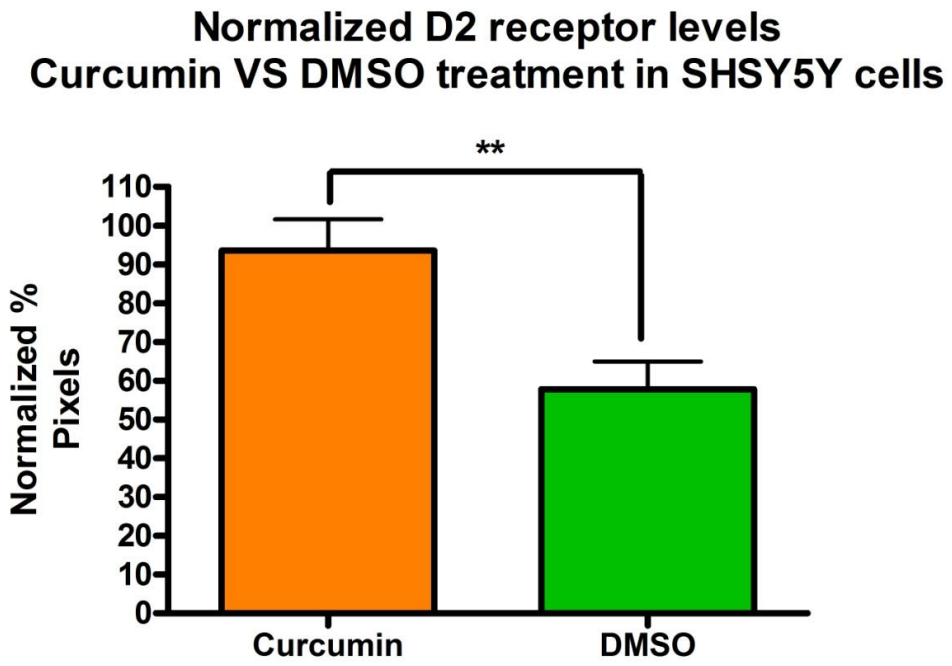


Figure 43. Dopamine D2 Receptor protein expression in SHSY-5Y cell line. Curcumin significantly increases the expression of the D2 receptor in SHSY-5Y cells (**$p<0.01$).

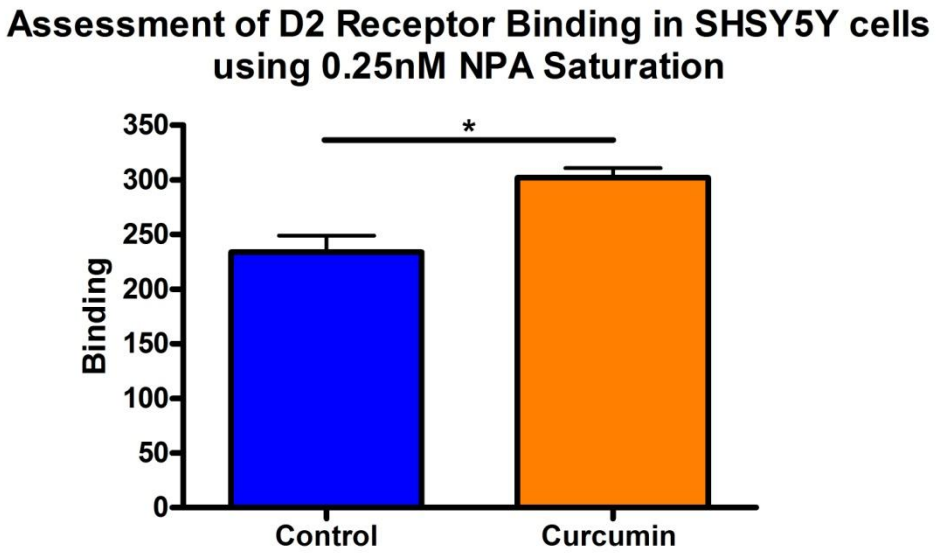


Figure 44. Dopamine D2 Receptor displacement in SHSY-5Y cells treated overnight with 10μM curcumin. Binding = Maximum binding – Non-specific Counts. Curcumin treatment significantly increases the D2 receptor binding sites in SHSY-5Y cells reflecting the increase in D2 receptor population (*$p<0.05$).

7. DISCUSSION

The results described in this thesis provide strong support for the effectiveness of curcumin in the prevention of haloperidol-induced vacuous chewing movements as a model of tardive dyskinesia. We have for the first time demonstrated the effectiveness of curcumin in preventing catalepsy but not locomotor activity. Further, we have identified novel features of this ancient molecule such as its capacity to up-regulate dopamine receptors and we have provided further support for its anti-apoptotic capacity.

OBJECTIVE 1

7.1. Curcumin prevents Haloperidol induced vacuous chewing movements

Curcumin prevents the development of haloperidol-induced vacuous chewing movements. This finding has been published by our laboratory and is in agreement with Bishnoi *et al* who also considered this molecule as a potential therapeutic for tardive dyskinesia (Bishnoi et al., 2008; Sookram et al., 2011). The intra-peritoneal injection of haloperidol, as a model of tardive dyskinesia, did not however produce persistent vacuous chewing movements beyond day 21 under our testing paradigm. Animals that received haloperidol for more than 21 days were visibly distinguishable from all other treatment groups. These animals displayed severe vacuous chewing movements when left undisturbed in their home cages. Testing conditions however masked this effect. This is in correlation with the human condition wherein individuals who experience tardive dyskinesia often do not display symptoms during attentive tasks nor in clinical settings (Faurbye et al., 1964). Furthermore, it is possible that the other haloperidol-induced extra-

pyramidal symptoms become more prominent with extensive drug administration and as such these symptoms additionally mask the dyskinetic phenotype.

Concurrent curcumin administration prevented the development of haloperidol induced vacuous chewing movements up to day 14. Since curcumin has been widely demonstrated to be physiologically safe in humans this finding provides strong support for the potential of curcumin to be useful in the treatment of tardive dyskinesia as an adjuvant to typical anti-psychotic drug treatment and in particular for co-administration with haloperidol. In an attempt to address the potential of curcumin to attenuate other extra-pyramidal side effects catalepsy was also considered in our investigations.

7.2. Haloperidol decanoate produces stable and pronounced vacuous chewing movements in rats

Intra-muscular haloperidol decanoate administration produced more persistent vacuous chewing movements that were not inhibited under our testing conditions. This phenotype required a prolonged period of drug administration for the development of symptomology but once established there was minimal fluctuation in the prevalence of this phenotype. Thus, the haloperidol decanoate induction of vacuous chewing movements may provide a more stable model for tardive dyskinesia in future investigations. However, the major limitation of this approach is the length of time necessary for the establishment of this model. The available literature on this model of tardive dyskinesia propose 2 months to be a minimum with chronic administration

ranging from 2 months to 24 months (Andreassen et al., 2000). As such, the economic feasibility and logistics of adding the daily administration of curcumin into the experimental set up, pose the major challenge in utilizing this approach to model tardive dyskinesia and the assessment of prospective therapeutic options.

7. 3. Curcumin prevents Haloperidol induced catalepsy

The effect of curcumin on haloperidol-induced catalepsy was considered for the first time in our investigation. Catalepsy is a characteristic consequence of anti-psychotic drug administration to rats. Most commonly the drug-induced cataleptic effect in rats has been characterized as a reflection of the potential of these drugs to produce extra-pyramidal side effects in humans including Parkinsonianism, akathesia and tardive dyskinesia (Hoffman & Donovan, 1995). However, some manuscripts have considered it more specifically a model of Parkinsonian-like bradykinesia (Naidu & Kulkarni, 2004). The predictive capacity of the catalepsy test in identifying extra-pyramidal side effects rendered this behavioural test a relevant assessment in our investigations.

Our work confirms that chronic curcumin co-treatment prevents the haloperidol-induced cataleptic effect. These results are in correlation with other groups, primarily in India, who have provided evidence for the effectiveness of anti-oxidants in the prevention of catalepsy. The NR-ANX-C polyherbal formulation which contains the bio-active components of Withania somnifera, Ocimum sanctum, Camellia sinensis, triphala and shilajit was demonstrated to prevent catalepsy in albino mice who were administered haloperidol at 1mg/kg (Nair et al., 2007). The same group in India demonstrated the

effectiveness of *Withania somnifera* extract alone in the prevention of catalepsy (Nair et al., 2008). These behavioural observations extend the potential effectiveness of anti-oxidants, like curcumin, to the prevention of extra-pyramidal side effects overall. While the underlying mechanisms of induction of the cataleptic effects are not yet clearly delineated nor differentiated from the underlying pathophysiology of dyskinesia, it is curcumin's anti-oxidant capacity that continues to be the most accepted mechanistic hypothesis (Nair et al., 2008).

7. 4. Curcumin does not affect Haloperidol induced hypolocomotion

The dopamine D2 receptor has been widely acknowledged to be involved in the modulation of locomotor activity. It has been demonstrated that agonism of the D2 receptor induces hyper-locotion, while antagonism induces hypo-locomotion (Storey et al., 1995). Since anti-psychotic drugs exert their therapeutic effect via the antagonism of the D2 receptor they concurrently induce hypo-locomotor state in animals. Thus, the question of curcumin impact on locomotion was an important one in terms of identifying and interpreting the mechanism of action and inter-action between curcumin and haloperidol. Our results indicate that chronic haloperidol treatment causes significantly reduced locomotor activity and that curcumin does not alter this effect. This may suggest that curcumin is not affecting the dopamine D2 receptor activity directly. This then raises the question of whether curcumin is interrupting any of the anti-psychotic drug's therapeutic effects. Interestingly, this association might provide some clues as to a means of un-coupling the psychological therapeutic effect of anti-psychotic drugs from their

extra-pyramidal symptoms. While these studies are still preliminary, curcumin's capacity to prevent vacuous chewing movements and catalepsy but not locomotor changes following haloperidol treatment is inarguably and important contribution to the future interpretations of the pathophysiology and therapy of tardive dyskinesia.

OBJECTIVE 2

7.5. Oral administration of curcumin at 200mg/kg in a cherry jell-o® vehicle is an effective dose that allows curcumin to get to the brain

The most significant challenge in the clinical use of curcumin has been its low bio-availability. Curcumin demonstrates low aqueous solubility and is readily metabolized by the liver and intestine (Anand et al., 2007; Wahlang et al., 2011; Ireson et al., 2002). Consequently, curcumin administration in humans at 12g per day has produced blood serum levels of just 51ng/ml serum (Anand et al., 2007; Sharma et al., 2004). In rats, curcumin administration at 1-2g/kg produced blood serum levels of 0.5-1.35µg/ml (Shoba et al., 1998; Maiti et al., 2007; Anand et al., 2007). A group in the USA assessed the levels of curcumin getting to the brain following oral administration of approximately 50mg/kg per day added to the diet of rats via a general integration into their food source. The route of administration, which was selected in this study, would only provide an approximate dose and therein exhibited some limitations to the potential interpretations of this work. Nevertheless, this group suggested that oral administration would produce overall brain curcumin levels of 2-3ng/g tissue (Bansal et al., 2012). Since this investigation and others like it have utilized a different approach to drug administration

than our work it could not as such be directly translated to our study. In order to address the question of whether curcumin was causing some biochemical change that would account for the behavioural changes that we observed in objective 1, it was necessary to assess whether curcumin was getting to the brain at all when administered at 200mg/kg in cherry jell-o®.

(i) Justinfication for the selection of cherry jell-o® as a vehicle of choice

Cherry jell-o® was selected as the most optimal drug vehicle for curcumin administration in our investigations primarily to circumvent the challenges of curcumin's low solubility. The supplier's details on physical properties of this formulation of curcumin was in agreement with the available literature stating that curcumin was insoluble in water, but dissolves in DMSO at 11mg/ml and ethanol at 1mg/ml (Tonnesen et al., 2002). However these concentrations were too low for us to administer an effective dose within the approved volumes for intra-peritoneal injections into rats. Additionally, surgical approaches were considered, however, organic solvents were not compatible with the use of the available mini-pump technology. Oral administration of curcumin was widely characterized in literature and it was demonstrated that in spite of potentially losses during absorption, once administered at sufficiently high dosages (350-2000mg/kg) curcumin could be detected in the blood plasma or serum of rats (Marczylo et al., 2007; Shoba et al., 1998; Yang et al., 2007; Maiti et al., 2007). Furthermore, previous investigations had suggested that at concentrations as low as 25-50mg/kg curcumin could produce a detectable behavioural change, indicative of an effective dose (Bishnoi et al.,

2008). In light of these studies curcumin was administered at 200mg/kg since it would permit an effective dose within a feasible level for daily oral intake, without the need for gavage, in these animals. Furthermore, the use of cherry jell-o® would allow for the suspension of a calculated amount of curcumin within a solid for which rats had a natural propensity.

(ii) Curcumin levels in the brain

Curcumin administered at 200mg/kg in cherry jell-o® produced striatal curcumin concentrations of 81μg/g striatal tissue a ten-fold increase over control levels. The identity of the curcumin peak was confirmed by liquid chromatography/mass spectrometry. However, the main limitation of this assessment was its low sample size and that residual curcumin was observed in control and mobile phase injections. Thus, the protocol for the assessment of curcumin levels underwent minor modifications and curcumin levels were assessed in CSF tissues (as outlined in ***section 5.2.2.***). The average curcumin concentration identified by this protocol in rat CSF was 0.895ng/μL with no peak observed in control tissues.

It is anticipated that while curcumin has been recognized to cross the blood brain barrier that only a portion of that observed in the blood would get to the brain. In spite of the high oral dose of curcumin administered to our animals only a very small percentage of the curcumin administered appears to be getting to the brain. However, our results in the striatal tissues indicate a significantly higher than anticipated level of curcumin in the

striatum. In lieu of the fact that these results were obtained in small samples sizes, these foundational investigations must be replicated. This difference in detected levels of curcumin in brain tissues versus CSF may reflect a number of complicating factors. Firstly, it is plausible that processing might better accommodate the extraction and detection of curcumin in one type of tissue versus the other. These protocols were primarily collected and modified from UPLC protocols designed for brain tissues specifically (Cheng et al., 2010, Marczylo et al., 2009). Alternatively, curcumin has been demonstrated to be taken up by specific cell types more than others e.g. cancer cells, this high level of accumulation of striatal curcumin may reflect the accumulation of curcumin in the brain region wherein there is a build-up of reactive oxygen species (Hanif et al., 1997). Finally, it may simply reflect experimental error, since the sample sizes are very low. Consequently, while these investigations cannot provide a final answer to date to address the question of why curcumin is appearing at a higher concentration in the striatal tissues than the CSF, these experiments provide strong support that curcumin is getting to the brain. As such, the presence of curcumin may account for the biochemical underpinnings of its therapeutic effect in the prevention of anti-psychotic drug induced extra-pyramidal side effects.

7.6. Curcumin exerts it's therapeutic effect in the prevention of extra-pyramidal side effects via the up-regulation of anti-apoptotic molecules and down-regulation of pro-apoptotic molecules

Curcumin has been widely characterized as both a direct and indirect anti-oxidant. It functions by the direct scavenging of reactive oxygen species and by promoting the activity of native antioxidant enzymes. Bishnoi et al (2008) were amongst the first to suggest that curcumin was functioning in an animal model of tardive dyskinesia by the up-regulation of the anti-oxidant enzymes superoxide dismutase and catalase (Bishnoi et al., 2008). Our investigations extend their preliminary work to implicate the anti-apoptotic molecule BclXL.

Previous work from our lab distinguished AIF translocation and consequent apoptosis as the primary mechanism of haloperidol-induced cell death (Skoblenick et al., 2006). Our work extends this investigation suggesting that it is not the over-expression of AIF but the translocation of AIF that accounts for haloperidol-induced neurodegeneration. Furthermore, BclXL, which is an inhibitor of AIF release and translocation, was up-regulated by curcumin treatment (according to the kinexus result) and by concurrent curcumin and haloperidol treatment in Immunoblotting assessments in striatal tissues. Our investigations also indicate potential curcumin-induced increases in the anti-apoptotic molecule Bcl2. Bcl2 functions by inhibiting the formation of mitochondrial permeability pores and thus AIF release. However, concurrent curcumin and haloperidol treatment did not produce a change in the Bcl2 beyond that of controls. Still, these results provide support for an anti-apoptotic role of curcumin in the prevention of anti-psychotic drug-induced cell death. Importantly however, up-regulation alone does not necessarily

correlate to activity of these molecules. As such assessments of cell survival were considered in our investigation.

7.7. Curcumin does not affect GFAP or NF expression

PCR assessments of GFAP and NF have been previously utilized to investigate cell viability in brain tissues (Cabras et al., 2010). Our attempts to use this tool in our assessment of the extent of cell loss did not reveal any significant changes in the expression of these molecules in the different treatment groups. These results suggest that the cell losses following two weeks of haloperidol administration were not quantifiable by this approach. Striatal cell loss has been a well established phenotype of anti-psychotic drug-induced tardive dyskinesia as such it is unlikely that these results contradict the oxidative stress hypothesis, rather it is more plausible that the lack of differences observed herein reflect cell losses that are below the detection limit of this technique.

7.8. Increasing concentrations of curcumin render SHSY-5Y cells more susceptible to cell death

SHSY-5Y cells are a neuroblastoma cancer cell line. When curcumin was administered at 10μM overnight to these cells there was a tendency towards increased BclXL expression relative to DMSO controls which quickly drops off at 25-50μM (**Figure 36**). Based on the observation that the dose of curcumin could potentially promote or inhibit the anti-apoptotic molecule BclXL, cell death was assessed within the

likely neuroprotective range of 5-15μM curcumin (**Figure 37**). Contrary to the anticipated neuroprotective function however, concurrent curcumin and haloperidol treatment exacerbated haloperidol-induced cell death. This un-anticipated result may reflect curcumin's anti-carcenogenic character (Chen et al., 2005). While a dopamine D2 receptor transfected neuroblastoma cell line is useful in modelling many dopaminergic functions and as such has been a relevant tool to address numerous effects of anti-psychotic drugs in our laboratory, it did not provide an effective model for our work with curcumin. As such a more accurate model for the investigation of curcumin's effect was sought in primary striatal cell cultures.

7.9. Curcumin is neuroprotective in primary striatal cell cultures

The oxidative stress hypothesis of tardive dyskinesia proposed heightened reactive oxygen species and cell loss as the primary mechanism of induction of this disorder. In an attempt to address the question of the underlying mechanism for curcumin's therapeutic effect curcumin's potential role as a neuroprotective agent was investigated. Our results support a neuroprotective function, for curcumin, in primary striatal cell cultures. Since this association was not observed in SHSY-5Y cells curcumin's neuroprotective capacity is cell-type specific. The primary striatal cell cultures are more representative of the effects *in vivo* that we were attempting to quantify and as such are herein considered a more accurate representation of *in vivo* occurances. This result complements the oxidative stress hypothesis implicating curcumin as a neuroprotective agent that is capable of preventing haloperidol-induced cell loss.

OBJECTIVE 3

7.10. Curcumin increases dopamine D2 receptor levels in rat striatal tissues and D2 receptor transfected SHSY-5Y cell lines but not in D2 receptor transfected HEK cells

The dopamine supersensitivity hypothesis of tardive dyskinsia proposes that the increased expression of the dopamine D2 receptor in the striatum that accompanies anti-psychotic drug administration accounts for the development of dyskinetic symptoms. The association between anti-psychotic drug administration and dopamine D2 receptor has been well established, however it does not easily distinguish between the individuals who develop tardive dyskinesia and those who do not. Nor does it account for the chronic nature of tardive dyskinesia. Our results confirm the previously established relationship between the haloperidol treatment and increased dopamine D2 receptors. However, the biochemical effect of curcumin is counter-intuitive. Our investigation has demonstrated for the first time that curcumin administration increases dopamine D2 receptor levels in striatal tissues. The animals that were utilized for these Immunoblots were from cohorts 2 and 4 (**Figures 13** and **15**). These were the same animals wherein the behavioural therapeutic effect of curumin was established. As such if dopamine supersensitivity was the primary means of induction of vacuous chewing movements the therapeutic effect should be observed in these animals also.

However, there are multiple predicaments that might account for this result. Firstly, it is possible that dopamine supersensitivity is not primarily responsible for the behavioural changes observed in these animals and that the primary means of induction is oxidative stress. Alternatively, curcumin may alter D2 receptor levels in the striatum but this does

not exclude the possibility that curcumin is having an effect elsewhere in the basal ganglia circuitry. If curcumin alters other receptors that are implicated in the direct or indirect circuit this may still account for curcumin's therapeutic effect via the interruption of a dopamine supersensitivity mechanism.

Interestingly, our investigations did not show any direct interaction with curcumin on the D2 receptor dopamine agonist site (data not shown but can be produced upon request). Thus, while curcumin regulates dopamine D2 receptor expression it does not interrupt binding. Furthermore, curcumin increased D2 receptors in SHSY-5Y cells but preliminary work has suggested that curcumin decreases D2 receptors in HEK cells. Thus, the dopaminergic regulatory effect of curcumin is cell type specific. Collectively, the dopaminergic effects of curcumin provide clues to its capacity in the brain, however, whether these effects are directly implicated or irrelevant and independent of its therapeutic potential in the prevention of tardive dyskinesia remains to be determined.

FUTURE DIRECTIONS

Our work provides strong support for a role of curcumin in the prevention of an animal model of tardive dyskinesia, however many questions still remain. While curcumin exerts great therapeutic effect in the prevention of vacuous chewing movements and catalepsy it does not affect locomotor activity. As such the question of why this phenomenon occurs is an important one. Furthermore, while curcumin is preventing the side effects of tardive dyskinesia, it is yet unknown whether curcumin will disrupt the effectiveness of anti-psychotic drug therapy. Thus, a relevant future experiment should explore the effect of curcumin in an animal model of schizophrenia to determine whether curcumin interrupts the therapeutic effects of anti-psychotic drug administration.

Curcumin administration in our investigations was confirmed to get to the brain in very small sample populations and as such this portion of our investigations must be replicated. Additionally, our neuroprotective investigations utilized only an MTT assay to quantify and characterize the overall neurodegenerative effect of haloperidol and the protective effect of curcumin. This test was selected since it was a fast economical approach that we optimized in other cell lines. However, due to limitations in the use of primary cell cultures such as low cells counts from each mouse at E18 and a low survival rate, we were unable to replicate this work. Furthermore, this measure does not distinguish the anti-apoptotic capacity of curcumin. Future investigations using a TUNNEL assay, annexin V or propidium iodide are alternative approaches to specifically differentiate apoptosis in our cell cultures.

Finally, in light of the limitations of investigating curcumin's effect on the D2 receptor alone, the diversity of targets of curcumin is being investigated using a receptor screen. A sample of the curcumin powder administered to our rats was sent to the NIH PDSP for assessment of potential targets, including other receptors upon which curcumin might have an effect. As was outlined in the methods section, we have collected tissues from other brain and body regions from the animals during sacrifice and still retained some protein and mRNA from the striatal tissues prepared for Immunoblot and PCR assessments. These tissues will be used to confirm receptor changes identified by PDSP and further investigate other receptors of interested to the basal ganglia circuitry.

8. CONCLUSIONS

These investivations provide strong support for the therapeutic effect of curcumin in the prevention of haloperidol-induced vacuous chewing movement's and catalepsy but not hypo-locomotion. Using ultra performance liquid chromatography we confirmed that curcumin is in the CSF and striata of rats following oral administered curcumin at 200mg/kg for two weeks. Curcumin up-regulates of the anti-apoptotic molecule Bcl-XL in the striata of rats when administered concurrently with haloperidol. Additionally, we provided preliminary data suggesting that curcumin is neuroprotective in primary striatal cell cultures. Finally we demonstrated that curcumin increases dopamine D2 receptor levels in the striatum of rats and in SHSY-5Y cell lines.

The development of anti-psychotic drugs in the 1950's marked a transition in our understanding of mental health and psychoses. It was the beginning of a journey towards a biochemical understanding of the brain and its psychological challenges. Over half a decade later, our investigations complement those fundamental discoveries. We propose a novel means of circumventing the shortcomings of those innovative drugs with the unified goal of developing a safe therapeutic approach for the treatment of psychoses.

9. NOVEL CONTRIBUTIONS TO THE FIELD

- Support for a therapeutic role of curcumin in the prevention on an animal model of tardive dyskinesia

- First to demonstrate that curcumin mitigates the cataleptic effect which is predictive of a mitigated extra-pyramidal side effect profile in humans

- First to argue that curcumin does not alter haloperidol-induced hypo-locomotion

- First to show that curcumin administered orally at 200 mg/kg curcumin in jello is an effective therapeutic dose that would allow for curcumin to get to the brain and prevent the haloperidol induced-behavioural abnormalities

- First to provide data confirming that curcumin can increase dopamine D2 receptors in rat striatal tissues and SHSY-5Y cells

10. PUBLICATIONS ARISING OUT OF THIS AND OTHER WORK

Peer Reviewed Journal Articles:

M Tan, B Dyck, J Gabriele, R Daya, N Thomas, C Sookram, D Basu, M Ferro, and R Mishra. Synapsin II Gene Expression in the Dorsolateral Prefrontal Cortex of Schizophrenic and Bipolar Disorder Brain Specimens. *The Pharmacogenomics Journal.* 2013 Mar. doi: 10.1038/tpj.2013.6. [Epub ahead of print]

B Dyck, M Tan, R Daya, N Thomas, D Basu, C Sookram, and R Mishra. Behavioural effects of non-viral mediated RNA interference of synapsin II in the medial pre-frontal cortex of the rat. *Schizophrenia Research.* 2012 May;137(1-3):32-8.

C Sookram, N Argintaru, R Mishra. The implications of oxidative stress in tardive dyskinesia. *Current Topics in Pharmacology.* 2011;15 (1):43-56.

C Sookram, M Tan, R Daya, S Heffernan, R Mishra. Curcumin prevents haloperidol induced development of abnormal oro-facial movements: Possible implications of BclXL in its mechanism of action. *Synapse.* 2011 Aug;65(8):788-94.

B Dyck, K Guest, C Sookram, D Basu, R Johnson, and R Mishra. PAOPA, an allosteric modulator of the dopamine D_2 receptor, prevents NMDA receptor antagonist (MK-801)-induced deficits in social interaction in the rat: Implications for the treatment of negative symptoms in schizophrenia. *Schizophrenia Research.* 2011 Jan;125(1):88-92

R Daya[±], M Tan[±], C Sookram, K Skoblenick and R Mishra. Free Radicals Trapping Agent Alpha-Phenyl-N-tert-Butylnitrone attenuates Haloperidol-Induced Behavioural Supersensitivity: Implications for the role of Apoptosis Inducing Factor. *Brain Research.* 2011 Sep 15;1412:28-36.

Book chapters:

C Sookram, M Tan, R Daya, J Mah, R Mishra. Curcumin Implication in the treatment of CNS disorders. BOOK CHAPTER. *Nova Science Publishers, Inc. NY,* November 2011.

Abstracts and Poster presentations:

C Sookram. R Mishra. Investigating curcumins therapeutic role in the dopamine supersensitivity versus the oxidative stress hypotheses of tardive dyskinesia. *Society for Neuroscience*. 2012. New Orleans

C Sookram. R Mishra. Investigating the role of the antioxidant curcumin in the prevention of vacuous chewing movements. *Faculty of Health Sciences Research Pleanary.* 2012. McMaster University, Hamilton Ontario

C Sookram. R Mishra. Investigating the mechanisms underlying the therapeutic use of curcumin in the prevention of tardive dyskinesia. *Psychiatry Research Day.* 2012. McMaster University, Hamilton Ontario

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C Sookram. The Dopamine supersensitivity hypothesis versus Oxidative stress hypothesis of Tardive Dyskinesia: contradicting theories or complementary hypotheses.

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