# INVESTIGATING THE ROLE OF INDOLEAMINE 2,3 DIOXYGENASE IN HUNTINGTON DISEASE

by

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### Abstract

The striatum is predominantly affected in Huntington disease (HD). To address this selective degeneration, we previously studied the gene expression profile in mouse brain and compared the striatum to other brain regions to identify novel striatal-enriched genes. One identified gene was Indoleamine 2,3 dioxygenase (Ido1), the first and the rate-limiting enzyme of the kynurenine pathway (KP), which was differentially expressed in the striatum of YAC128 mouse model of HD. KP leads to the production of both neuroprotective and neurotoxic metabolites, the imbalance of which has been implicated in several neurodegenerative disorders. This PhD thesis initially focuses on the age-dependent changes of the KP in YAC128 mice with a main focus on Ido1 expression and activity. I was able to demonstrate a chronic induction of Ido1 expression and activity in the striatum of YAC128 mice, which correlated with different substrate or product levels during the course of the disease. Using a liquid chromatography mass spectrometry method, I was also able to identify changes in the downstream metabolites, which seemed to follow a biphasic pattern where neurotoxic metabolites were reduced in presymptomatic mice and increased in symptomatic mice. We propose that the striatal-specific induction of Ido1 and downstream KP alterations suggest involvement in HD pathogenesis, and should be taken into account in future therapeutic developments for HD. To follow up, this thesis project also assesses the sensitivity of brain to NMDA-mediated excitotoxicity in the absence of Ido1 expression under in vivo and ex vivo settings. I was able to demonstrate decreased sensitivity to NMDA receptor-mediated neurotoxicity in the brain of Ido1 constitutive null mice compared to that of WT. These data suggest that lack of Ido1 expression in vivo provides protection against NMDAreceptor-mediated excitotoxic stress, a well-described mechanism in HD pathogenesis.

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## Preface

Some material of chapter 1 is currently *in press* with Elsevier Publishing and will be published as a book chapter named 'Murine models of HD' in the second edition of the textbook 'Movement disorders: Genetics and models'.

Chapter 2 is based on the work conducted in Dr. BR Leavitt's laboratory in collaboration with Dr. E. Tomlinson Guns (PhD) and H. Adomat at the Vancouver Prostate Centre and Drs. David Budac (PhD) and Thomas Möller (PhD) at the Lundbeck Institute in New Jersey, USA. A version of this chapter is now *in press* in the *Jounral of Neurochemistry*. Mazarei G. Budac D., Adomat H., Lu G., Guns E., Möller T., Leavitt BR. (2013). Under BRL (MD) supervision, I designed and conducted the majority of the experiments for this work except the Mass Spec experiments, which were performed and analyzed by collaborators at the Prostate Centre and Lundbeck. Ge Lu was responsible for dissections of all brain samples. Austin Hill and Jasmine Yang helped with colony maintenance. I was responsible for writing the entire manuscript.

Chapter 3 is based on the work conducted in Dr. BR Leavitt's laboratory. Under BRL supervision, I was responsible for the majority of experimental designs as well as conducting all the testing. Austin Hill and Jasmine Yang helped with the colony maintenance. Pamela Wagner assisted in primary cell culture experiments. Ge Lu did the intrastriatal brain injections. Hyeri Lee helped with quantitative analysis of the injected brains. The first three figures and the only table in this chapter have been submitted for publication as a brief communication manuscript.

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## **List of Abbreviations**

HD Huntington disease Htt Huntingtin Ido1 Indoleamine 2,3 dioxygenase Tdo2 Tryptophan 2,3 dioxygenase Ido2 Indoleamine 2,3 dioxygenase 2 KP Kynurenine pathway Kyn Kynurenine LC/MS/MS liquid chromatography tandem mass spectrometry Tryp Tryptophan QA Quinolinic acid KA Kynurenic acid **3-HAA** 3-hydroxyanthranilic acid **3-HK** 3-hydroxykynurenine 3-HAO 3-hydroxyanthranilic acid oxidase MSN medium spiny neurons mHtt mutant Huntingtin CNS Central Nervous Systems Kyn Kynureninne Trp Tryptophan BBB Blood Brain Barrier NMDA N-methyl-D-aspartate

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To my parents

#### **Chapter 1: INTRODUCTION**

#### 1.1 Huntington disease

Huntington disease is an autosomal dominant neurodegenerative disorder characterized by motor, cognitive, and neuropsychiatric symptoms. The prevalence of HD is estimated to be approximately 1 in 10,000 individuals of European descent, which is reduced by 10-fold in populations of Asian and African ancestry. HD manifests commonly as a late-onset disorder (age > 35), but juvenile forms of the disease also exist. The physician George Huntington is generally acknowledged to be the first to fully describe HD in an article entitled "On Chorea" which he published in The Medical and Surgical Reporter of Philadelphia in 1872. "There seems to exist a hidden power, something that is playing tricks and keeping the poor victim in a continual jigger as long as he remains awake" said Huntington of the choreic movements he observed in his patients. Furthermore, he accurately described the hereditary nature of the chorea and explained the association of chorea with psychiatric disease (Huntington, 2003). More than a century later, in 1983, the genetic defect was mapped to chromosome 4p16.3 and 10 years later in 1993, the HD Collaborative Research Group discovered the cause for HD to be a CAG repeat expansion mutation in the huntingtin gene (*HTT*) initially called *IT15* for interesting transcript 15 (Anon, 1993). This mutation results in the production of a huntingtin protein with an abnormally long polyglutamine (polyQ) tract near the N terminus (Anon, 1993).

#### 1.1.1 Clinical features of Huntington disease

Classic signs and symptoms of HD can be divided into three general categories: motor dysfunction, cognitive impairment, and neuropsychiatric disturbances. Chorea, derived from the Greek word for a kind of dance, is the classic feature of motor dysfunction in HD. Involuntary choreic movements of face, limbs, or trunk, are often seen in early phases of the disease and generally become more pronounced with disease progression. Chorea can often be reduced or disappear in advanced HD being replaced by a more rigid and bradykinetic phenotype. Another type of involuntary movement in HD patients is dystonia, a slower twisting and repetitive movement across a joint. The movement disorder in HD also consists of abnormal voluntary motor function such as bradykinesia (slowness in the execution of the movement), rigidity, dysphagia, dysarthria, gait problems, and abnormal eye movements (Harper, 2005). Symptoms of chorea, hypotonia, and hyper-reflexia at early stages of the disease gradually become replaced by rigidity, bradykinesia, and general functional impairment as HD progresses (Harper, 2005). Juvenile HD occurs in about 5-10% of patients and it is characterized by onset under the age of 20. Patients with Juvenile HD often develop rapid manifestation of symptoms such as spasticity, bradykinesia, dystonia, and seizures and interestingly, may never develop chorea. Changes in personality, failure in school, irritability, violence, and deterioration of intellect are common features in Juvenile HD (Nance and Myers, 2001).

Cognitive impairments in HD begin with executive dysfunction manifest by subtle slowing of intellectual processes, personality changes, dysinhibition, and reduced mental flexibility which can manifest prior to the onset of motor symptoms. However, many studies have failed to show strong and consistent cognitive defects in presymptomatic HD (Paulsen et al., 2001). Cognitive deficits generally become worse as the disease progresses through motor onset and can be considered a form of "subcortical dementia" characterized by learning and memory disturbances (memory recall more than memory storage) (Butters et al., 1985; Schmidtke et al., 2002), difficulty with complex intellectual tasks such as strategy generation and problem solving (Ho et al., 2003).

Neuropsychiatric dysfunction has been reported in pre-manifest or presymptomatic HD (Duff et al., 2007). Recently, extensive neuropsychological evaluations, have characterized some significant measurable differences between pre-manifest gene-mutation carriers and non-HD gene mutation control individuals, but studies suggest that neuropsychiatric changes, like cognitive changes may be subtle and highly variable (Stout et al., 2012). Consistent with original observations by George Huntington who described "a tendency to insanity and suicide" in HD patients (Huntington, 2003), signs and symptoms of depression, apathy, feeling of suicide, and anxiety are common in symptomatic HD patients (Anderson and Marder, 2001). Additionally patients with HD have been reported to exhibit psychosis, paranoia, and signs and symptoms of obsessive-compulsive disorder. Specific psychiatric features in HD often tend to be similar among patients from the same family suggesting an involvement of other genetic modifiers or environmental factors in manifestation of these symptoms.

Finally, weight loss due to altered metabolic state (Sanberg et al., 1981; Pratley et al., 2000), sleep and circadian rhythm disorders (Morton et al., 2005), and testicular degeneration (Van Raamsdonk et al., 2007) have also been reported in HD.

### 1.1.2 Genetics of Huntington disease

HD is caused by a CAG repeat expansion in the first exon of the *HTT* gene, which is 210 kb long and located on the short arm of chromosome 4. Due to alternative polyadenylation, two different-sized transcripts are formed; the larger transcript is dominant in the brain while the smaller transcript predominant elsewhere (Lin et al., 1993). Both transcripts are predicted to encode a 350 kDa protein product which is expressed ubiquitously throughout the body with higher expression in brain and testis (Sharp et al., 1995). The mutant *HTT* gene encodes for a

protein with an abnormally long polyQ tract near its N-terminus. An expanded CAG repeat size of greater than 35 is considered pathological, with repeat sizes between 36-39 having reduced penetrance and/or very late age of onset (Semaka et al., 2010; 2012). The size of the CAG repeat expansion is inversely correlated with the age of onset in HD. The expansion is unstable and CAG length can dynamically change during somatic development resulting in somatic mosaicism (Goldberg et al., 1993; 1995) as well as during intergenerational parent to child transmission (Semaka et al., 2012). Paternal transmission of large expanded alleles more often results in expansion rather than contraction of the repeat tract in offspring (Kremer et al., 1995; 1995). This leads to a phenomenon called anticipation, which is defined as decreasing age of onset, or increasing severity of disease in successive generations (Mahadevan et al., 1992; Trottier et al., 1994). CAG repeat sizes of greater than 60 are associated with juvenile HD cases and very rarely repeat sizes of 80-100 have been observed.

#### 1.1.3 Neurophathology in HD

An important pathological hallmark of HD is the selective neuronal degeneration in the caudate and putamen (together known as the striatum) which can be characterized by striatal atrophy and selective neuronal loss (Reiner et al., 1988; Halliday et al., 1998; Huntington, 2003; Rosas et al., 2003; Harper, 2005). It has also been shown that the severity of striatal pathology is correlated with the degree of motor and cognitive impairments (Bamford et al., 1989; Rosenblatt et al., 2003) suggesting a central role for striatal degeneration in HD symptomatology. Interestingly, this selective neuronal degeneration occurs despite the fact that mutant HTT (mHTT) protein is expressed widely throughout the brain and body of HD patients.

Magnetic resonance imaging (MRI) reveals that atrophy in the striatum of the HD patients begins many years prior to motor diagnosis in HD (Aylward et al., 2004). In early-mid stages of the disease, striatal volume loss has been measured as 53% compared with controls using MRI or post-mortem volume replacement (Halliday et al., 1998; Rosas et al., 2001). Volume loss in other brain regions such as globus pallidus and cortex is not as severe and has been estimated as 41% and 23% respectively (Halliday et al., 1998; Rosas et al., 2001).

At the cellular level, neuronal loss occurs predominantly in the striatum early on during the course of HD. This phenomenon has been reported as 90% loss of the total number of cells in the striatum (Macdonald and Halliday, 2002) and it selectively affects the main population of striatal neurons, the GABAergic medium spiny neurons (MSNs) (Vonsattel et al., 1985). Within GABAergic MSNs, the loss of enkephalinergic neurons occurs early on and is followed by substance P neurons in later stages of the disease (Reiner et al., 1988; Albin et al., 1992). This is supported by further studies that showed decreases in mRNA for preproenkephalin in striatal MSNs in early stages of HD, and only in later stages of illness there were reductions in substance P mRNA levels (Richfield et al., 1995a; 1995b). The other major population of striatal neurons, the aspiny cholinergic interneurons, remains relatively intact through the course of the disease (Ferrante et al., 1985; Albin et al., 1992; Cicchetti and Parent, 1996). Interestingly, some studies suggest that striatal pathology in HD would manifest as neuronal dysfunction before the welldocumented neuronal death in this region (Hedreen and Folstein, 1995). This indicates that early symptoms seen in the early stages of the disease in the absence of obvious neuropathology (e.g. early manifestation of chorea) may result from neuronal dysfunction rather than neuronal loss (Rosenblatt et al., 2003; Levine et al., 2004). Additionally, there has been indication of

inflammation including astrocytosis, microgliosis, and complement activation in the striatum of HD patients (Singhrao et al., 1999).

Striatal neurons however, are not the only brain cells affected in HD. At later stages of the disease, neuronal loss manifests in the pyramidal projection neurons in layers V and VI of cerebral cortex (Hedreen et al., 1991) and the CA1 region of hippocampus (Spargo et al., 1993). Interestingly, expressing mHTT only in pyramidal cortical neurons of mice did not create neurologic phenotypes similar to models where mHTT is expressed ubiquitously leading to the idea that inter-communication between brain regions is crucial in HD pathogenesis (Gu et al., 2005). As HD progresses, neuronal degeneration generalize and include other brain regions such as the globus pallidus, hypothalamus, thalamus, and substantia nigra (Vonsattel et al., 1985). This generalized pattern of degeneration in later stages of the disease resembles patterns seen in the juvenile HD brain (Nance and Myers, 2001).

Formation of nuclear and cytoplasmic mHTT aggregates is another feature of HD brain which was first noticed in a mouse model of HD (Davies et al., 1997) and was later found in the striatum and cortex of post-mortem HD brains (DiFiglia et al., 1997) Interestingly, in cases of juvenile HD, aggregates are more wide-spread and are present at earlier stages than in adult onset HD (DiFiglia et al., 1997). Uncertainty exists on whether these aggregates are involved in the pathogenesis of HD; it is not known whether they play a causal role in the HD pathology or whether they are simply the results of defense mechanism of neurons against cell death.

Curiously, the selective degeneration of neurons occurs despite the widespread expression of mHTT throughout the brain and body of HD patients. The mutation is well known to cause a gain of toxic function in the protein; however, there is speculation and recent evidence suggesting the

involvement of loss of wild-type (WT) HTT function in HD (Nasir et al., 1995; Cattaneo et al., 2001; Van Raamsdonk et al., 2005b).

#### **1.2** The use of mice to study disease

*Mus Musculus* (mice) have been considered as useful model organisms to understand and study human disease. As vertebrates and mammals, mice develop diseases that naturally affect immune, endocrine, nervous, skeletal, digestive, and cardiovascular systems. Aside from its close resemblance to humans in many complex physiologic systems, what makes the mouse an even more attractive model organism is its ease in reproduction, relatively low cost, and short gestation period (Gama Sosa et al., 2012; 2012). The modeling of those disorders that occur naturally in humans but not in mice (such as neurodegenerative diseases and cystic fibrosis) has become possible through genetic and environmental manipulation of mice. For the past decade, genetic engineering has focused on improving techniques to develop mutant embryonic stem (ES) cells, knock-out, knock-in and conditional strategies, N-ethyl-N-nitrosurea (ENU) mutagenesis, gene trapping, and multi-parental recombinant inbred lines to be used in mapping and studies involving quantitative trait loci (QTL).

The ability to manipulate the mouse genome using these techniques is a powerful tool for the discovery of relationships between genotype and phenotype, unveiling disease mechanisms, and developing therapeutics. Currently, there are over 1000 human diseases modeled with one or more mouse models (Gama Sosa et al., 2012).

The first complete draft of the mouse genome was sequenced and published by the Mouse Genome Sequencing Consortium (MGSC) in December 2002 issue of Nature, a year after the publication of the complete human genome sequence. The mouse genome sequence included the assembly from ~7X coverage of the "Black 6" or C57BL/6 strain mouse genome. By 2011, the sequence of 17 strains of mice was obtained using next-generation sequencing. The mouse genome sequence has revealed over 17,000 mouse genes with identifiable human orthologs (Mouse genome Informatics, 2011), which in theory, all have the potential to be manipulated and studied.

#### 1.3 Mouse models of Huntington disease

Since there are no naturally occurring animal models of HD, a great deal of effort has been put into engineering various animal models in an attempt to recapitulate this disease. An ideal animal model of HD mimics the main features of the human disease with respect to etiology, symptomatology, and pathophysiology. Additionally, in order to be useful in pre-clinical therapeutic trials, an animal model must exhibit quantifiable and biologically relevant differences from WT animals. Mus musculus has been the species of choice for generating models of HD since it reproduces rapidly, produces large litters, has a reasonably short lifespan, and takes up little space. Moreover, using inbred strains allows a better understanding of the specific manipulation by ruling out genetic variations. Four general categories of HD mouse models have been generated thus far: i) chemical models generated by the administration of specific neurotoxins, ii) fragment transgenic models generated by the insertion of an N-terminal fragment of human HTT gene containing the expanded CAG repeat into mouse genome iii) full-length transgenic models generated by the insertion of the human full-length mutated HTT gene into the mouse genome, and iv) knock-in transgenic models generated by the selective introduction of CAG expansion into the endogenous mouse *Htt* gene. Although species differences complicate the exact phenotype comparisons that can be made between mice and humans, genetic HD mice

overall recapitulate the cognitive failure, motor dysfunction, and striatal neurodegeneration seen in human HD patients.

#### 1.3.1 Chemical models of HD

Glutamate is the main excitatory neurotransmitter in the brain and it also provides the major excitatory input of the cortical projections into the striatum, the brain region predominantly affected in HD (Nasir et al., 1995; Cattaneo et al., 2001; Van Raamsdonk et al., 2005b). In the late 1970s, prior to the discovery of the HTT gene, several chemical mouse models of HD were developed in which direct injection of glutamatergic agonists (excitotoxins) into the striatum of rodents caused selective damage to MSNs (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976; Coyle, 1979). The first compound tested and shown to cause striatal lesions was a structural analog of glutamate called kainic acid (Coyle and Schwarcz, 1976). Upon administration, kainic acid, originally isolated from a type of seaweed, binds to kainate receptors in the striatum and leads to striatal degeneration. Reminiscent of pathology in the brain of patients, kainic acid mainly affected the susceptible population of striatal neurons, MSNs, while interneurons remained relatively intact. Similarly, quinolinic acid (QA), an N-methyl-D-aspartate (NMDA) receptor agonist replicates HD-like striatal degeneration by binding to extrasynaptic receptors and producing axon-sparing lesions in the striatum (Schwarcz et al., 1983; Beal et al., 1986). The intra-striatal administration of these compounds caused an acute but selective striatal degeneration and some HD-like motor phenotypes. In addition to excitotoxic models of HD in rodents, toxins causing mitochondrial dysfunction have also proved useful in modeling the disease. 3-nitroproprionic acid (3-NP) was a mycotoxin found to cause striatal-specific lesions in kids who had ingested moldy sugarcane in China (Ludolph et al., 1991; Beal, 1998). 3-NP is a

succinate dehydrogenase inhibitor and when administered systemically to rodents it causes mitochondrial dysfunction and striatal lesions in the brain (Beal et al., 1986; Beal, 1998). Conveniently, chemical models of non-human primates have also been developed using both excitotoxic compounds as well as 3-NP.

Overall, even after the discovery of the *HTT* gene and subsequent development of *HTT* gene transgenic models, the use of these chemical models elucidated important pathophysiological mechanisms such as mitochondrial dysfunction and NMDA receptor mediated excitoxicity, potential pathogenic pathways which are still being investigated in HD today.

## 1.3.2 Genetic mouse models of HD: start of a new era

Although chemical models of HD may reveal underlying pathological mechanisms and recapitulate some features of HD including selective striatal degeneration, these models are forms of acute neurotoxicity and are not suitable for studying many aspects of the disease. The progressive nature of HD requires a time-course where gradual worsening of phenotype can be observed and measured. Additionally, it has been demonstrated that progression of symptoms in HD happens as a result of gradual but severe dysfunction of MSNs rather than sudden synchronous neuronal degeneration (Levine et al., 2004). The discovery of mutations in the *HTT* gene in 1993 precipitated the development of mouse models based on the genetic defect observed in patients and paved the way for our current understanding of disease mechanism, disease progression and has enabled the development of novel therapeutic strategies. Various mouse models of HD have been developed over the last 15 years using different genetic strategies and will be reviewed in this chapter. Many therapeutic approaches based on work in mouse models of

HD are currently being assessed at different phases of human clinical trials, with the hope of developing effective treatments for HD.

As routinely pursued following the discovery of a novel gene, *Htt* knockout mice were generated as the first step towards elucidating the function of the gene with the hope of understanding the disease.

## 1.3.2.1 Knock out models

*Htt* knockout mice were developed in order to investigate the role of this gene during animal development and particularly in brain (Nasir et al., 1995; Riethmacher et al., 1995; Zeitlin et al., 1995). It was found that Htt deficiency leads to mouse embryonic lethality between days 8.5 and 10.5 of gestation (Nasir et al., 1995; Zeitlin et al., 1995). Additionally, mice with <50% *Htt* expression displayed characteristic aberrant brain development, indicating a critical function for *Htt* in neurogenesis (White et al., 1997). To examine the effect of loss of Htt post-developmentally, conditional knockouts were generated using the Cre/*LoxP* system to inactivate mouse *Htt* in brain (Dragatsis et al., 2000). Interestingly, these mice displayed a partial HD-like phenotype with increased apoptosis in the brain and testes resulting in progressive neurodegenerative phenotype as well as sterility (Dragatsis et al., 2000). Despite the fact that knockouts are not considered as mouse models of HD, they have been serving as valuable tools in understanding *HTT* and its role during development and disease. Additionally, when designing therapeutics targeting HTT, one should take into account the importance of WT HTT and the consequences of any potential loss of function.

#### 1.3.2.2 Fragment models of HD

The first genetic mouse models of HD generated (Mangiarini et al., 1996) were fragment transgenic models created by the random insertion of an N-terminal fragment of the human *HTT* gene containing the expanded CAG repeat into the mouse genome using pronuclear injection. This small fragment of mHTT is expressed in addition to the expression of the normal mouse endogenous *Htt* gene. These fragment models were initially created in an attempt to study the CAG repeat stability of the *HTT* gene in mice while avoiding the technical challenges of working with the large *HTT* gene (~180 kb). These mouse models exhibit highly progressive and severe motor symptoms developed in mice as young as only a few weeks old. The best characterized of these models are the R6/2, R6/1, and N171-82Q models.

The R6/2 mice contain human *HTT* exon 1 carrying a (CAG)<sub>150</sub> expansion and ~1kb of the human *HTT* promoter region (Mangiarini et al., 1996). Motor symptoms manifest as early as 5-6 weeks as deficits in swim tests, beam walking, and rotarod performance and are observed as motor choreiform and involuntary stereotypic movements, tremor, and epileptic seizures (Mangiarini et al., 1996). Neuropathological features include a decrease in brain weight, volume and neuron counts, and intranulear inclusions (NIIs) formation which are all non-selective (Mangiarini et al., 1996; Davies et al., 1997; Stack et al., 2005). Interestingly, neuropathology in the form of NIIs appears before development of motor deficits suggesting an underlying involvement of these species in the neurological dysfunction in R6/2 mice (Davies et al., 1997). NIIs and dystrophic neurites were first described in the striatum and cortex of HD patients as structures to contain mHTT protein fragments as well as ubiquitin (DiFiglia et al., 1997; Morton et al., 2000). Although NIIs are present in HD, their role in pathogenesis is still under debate.

Some other reported symptoms in R6/2 mice include weight loss (Mangiarini et al., 1996), electrophysiological abnormalities (Stack et al., 2005), and diabetes (Hurlbert et al., 1999). In the R6/2 mice, the accelerated exacerbation of the motor (Carter et al., 1999) and cognitive phenotypes (inability to switch from one set of learned responses to a new and lack of inhibitory control) (Lione et al., 1999) progresses up to ~15 weeks which marks the end of the lifespan in these animals (Mangiarini et al., 1996).

From the founder of R6/2 mice, another R6 line was also established (Mangiarini et al., 1996). R6/1 carries similar transgene with a slightly shorter CAG repeat expansion. R6/1 develops rotarod performance deficiency at around 18 weeks and a more slowly progressive phenotype compared with the R6/2 line. R6/1 mice has been shown to develop aberrant cortical synaptic plasticity (Cummings et al., 2006). These mice die around 32-40 weeks of age (Mangiarini et al., 1996).

The N171-82Q model was generated with a slightly longer N-terminal *HTT* gene fragment than the R6 line expressed under a prion promoter (Schilling et al., 1999). Transgenic mice expressing a cDNA construct encoding an N-terminal fragment (171 amino acids) with 82, 44, or 18 glutamine repeats were created. The N171-82Q model has been studied extensively and has been shown to develop robust and early motor phenotypes such as loss of coordination, tremors, gait abnormalities, and hypoactivity starting at 3 months. Progressive weight reduction and motor dysfunction eventually leads to premature death at ~ 5 months of age in these mice (Schilling et al., 1999). An important pathological feature in these mice is the formation of nuclear inclusions and neurite aggregates in both nucleus and cytoplasm of neurons in different parts of the brain (Schilling et al., 1999). Later on, it was shown that nuclear targeting of the N171-82Q fragment using a nuclear localization signal produced HD-like phenotypes in WT mice similar to what is seen in N171-82Q mouse model (Schilling et al., 2004).

#### 1.3.2.3 Full-length models of HD

Full-length models have been generated using two different strategies: The use of the yeast artificial chromosomes (YACs) containing the entire region of the full length human *HTT* gene with 18, 46, 72 and 128 polyglutamine repeats as well as all regulatory elements (~25 kb of upstream regulatory sequence), and the use of a bacterial artificial chromosome (BAC) expressing full-length human *mHTT* with 97 glutamine repeats under the control of endogenous *HTT* regulatory machinery.

### **1.3.2.3.1** YAC full-length mouse models

YAC72 mice were among the first full-length transgenic HD models generated and developed progressive motor and electrophysiological abnormalities that precede nuclear translocation and aggregation of Htt (Hodgson et al., 1999). YAC72 mice develop late-onset behavioural abnormalities at around 7 months, electrophysiological deficits between 10 and 12 months, a selective loss of striatal neurons by 12 months, and striatal Htt aggregates by 18 months. Interestingly, the occurrence of the selective neurodegeneration was shown to be independent of aggregate formation and nuclear translocation of mHtt (Hodgson et al., 1999). In general, the motor and neurological phenotypes in YAC72 mice were subtle and late appearing.

The YAC128 model, the best-characterized full-length mouse model of HD, was developed with larger CAG expansions in an effort to decrease the age of onset in animals and thus to create a more suitable mouse model for the use in pre-clinical trials. The YAC128 model was developed by random insertion of a YAC containing the entire human *mHTT* gene with 120 CAG repeats and ~25 kb of upstream endogenous promoter elements into the mouse genome (Slow et al., 2003). This model shows a spectrum of measurable behavioral, cognitive, motor, and neuropathological changes amenable to its use in therapeutic testing. Behavioral changes in this mouse model occur first at 2 months of age evident as hyperactivity and progression to hypoactivity by 12 months. Motor deficit is evident on the rotarod beginning at 6 months of age and progressively worsening with age (Slow et al., 2003; Van Raamsdonk et al., 2005c). Neurodegeneration of specific brain regions occurs in these mice with striatal atrophy beginning at 9 months of age and cortical atrophy arising by 12 months (Van Raamsdonk et al., 2005a). Although nuclear translocation of mHTT is observed as early as 2 months in the brain of YAC128 mice, intranuclear inclusions in MSNs do not form up until 18 months (Slow et al., 2003; Van Raamsdonk et al., 2005a). YAC128 mice develop testicular degeneration and parallel studies showed testicular abnormalities to also be a feature of human disease (Van Raamsdonk et al., 2007).

## **1.3.2.3.2** BAC full-length mouse models

BACHD mice were generated using a BAC-expressing human mHTT with 97 repeat expansions under the control of endogenous HTT regulatory elements (Gray et al., 2008). The flanking *lox*P sites located on either sides of the first exon (the one containing the trinucleotide expansion) allow deletion of the first exon of *mHTT* using the enzyme Cre recombinase. Accordingly, BACHD is considered a conditional HD mouse model in which the expression of full-length mHTT can be switched off in specific cell types expressing Cre recombinase (Gray et al., 2008). The phenotype of this model is similar to the YAC128 model and exhibits progressive motor deficits starting as early as 2 months progressively worsen by 6 and 12 months mainly revealed by rotarod analyses. Striatal and cortical volume loss, accumulation of mHTT aggregates, and selective striatal neuropathology all occur beyond 12 months of age in the BACHD mice (Gray et al., 2008). The BACHD model also suggests that early and diffuse nuclear accumulation of aggregated mHTT is not a requirement for selective and progressive pathology observed in these mice (Gray et al., 2008).

In general, although the phenotypes in HD full-length models are less severe compared to fragment models, these mice display robust and consistent progressive phenotypes including motor dysfunction and selective neuronal loss as a result of placing the CAG expansion within the context of the full-length human *HTT* gene.

## 1.3.2.4 Knock-in models of HD

In an attempt to generate HD mouse models without the use of human transgenes and to overcome potential problems resulting from over-expression of human transgenes and potential unknown genetic interactions, several strains of so called "knock-in" mice have been created. Generation of these models relies on the fact that murine Htt and human HTT are 86% identical at DNA level and 91% similar at protein level. These mice were generated by introducing long human CAG repeat expansions into the mouse endogenous *Htt* gene. Most of these have been generated in both homozygous and heterozygous forms. The knock-in construct is generally composed of chimeric human/mouse exon 1 containing expanded CAG repeats between 50 and 140 units. The CHL2 (Htt<sup>(CAG)150</sup>) and HttQ200 has been generated by inserting respectively 150 and 200 CAG repeats into the mouse endogenous *Htt*.

### 1.3.2.4.1 CAG repeat < 80

HttQ50 (White et al., 1997), Htt6/Q72, Htt4/Q80 (Shelbourne et al., 1999), and CHL1 (Htt<sup>(CAG)80</sup>) have been generated. However, none of these models have been reported to exhibit any neuropathological abnormalities such as reduced brain weight, neuronal loss, or nuclear inclusions. Minor isolation-induced aggression has been detected in Htt6/Q72 and CHL1 mice (Lin et al., 1993; Shelbourne et al., 1999) but in general, no significant HD phenotype or behavioural abnormalities have been observed in these mice.

## 1.3.2.4.2 CAG repeat >80

With the hope of producing knock-in models which manifest earlier and more robust motor and pathological phenotypes during mouse's short life span, mice with longer CAG repeats were generated. HttQ92 and HttQ111 (Wheeler et al., 2000) were among the first models generated with this goal in mind. Interestingly, the two models did not display any symptoms of motor and neuropathology until about 2 years. The only neuropathological early features included diffuse striatal nuclear staining between 3-5 months and striatal nuclear inclusions and microaggregates in mice older than 1 year. Subsequently, the HttQ140 mouse model (Menalled et al., 2002) was generated and shown to display relatively early onset nuclear inclusions and neurophil aggregates at about 4-6 months. Additionally this model developed subtle motor symptoms such as abnormal rearing and increased locomotor activity at around 1 month, which was followed by hypoactivity at 4 months and gait abnormalities at 1 year.

The CHL2 (Htt<sup>(CAG)150</sup>) mouse model was generated using a different method of construction by insertion of 150 CAG repeats without the rest of human exon 1 into the mouse endogenous *Htt* gene (Lin et al., 2001; Woodman et al., 2007). Despite the large repeat size and double

dosage of the gene in homozygous mice, the median age at onset of motor symptoms is at 6 months. For heterozygotes, the median age of motor onset is ~15 months. A proportion of homozygous CHL2 mice show some rotarod deficits at around 10 months. Additionally, the selective late-onset neuropathology is reported as formation of nuclear inclusions and astrogliosis by 10-18 months and significant loss of striatal neurons at 2 years of age. This phenotype is highly delayed compared with other transgenic models.

Among the knock-in mice developed to date, HttQ200 (Heng et al., 2010b) contains the largest CAG repeat and has the most severe phenotype even at the heterozygous state . This model was derived from the Htt<sup>(CAG)150</sup> mouse model and exhibits a more severe and accelerated phenotype compared with the parent line. This includes formation of intranuclear inclusions by 5 months, progressive imbalance and decreased motor coordination by 15 months and gross motor impairment by 20 months of age. Cortical and striatal astogliosis is also an important feature of 20-month-old mice. Very interestingly, an early abnormal autophagosomal/lysosomal response at 10 months has been shown as a hallmark of neuropathology in this model. Autophagy has been suggested to have a crucial role in the clearance and degradation of aggregates containing mHTT and ubiquitin in HD (Kegel et al., 2000; Ravikumar et al., 2002; 2004; Pandey et al., 2007a; 2007b; Martinez-Vicente et al., 2010; Heng et al., 2010a)

In general, most of the knock-in models that have been developed to date fail to display the equivalent early, robust, and consistent motor and neuropathological phenotypes seen in transgenic mice and are generally felt to reflect a pre-symptomatic state of the disease. This relative lack of strong phenotype occurs despite the fact that many of these models contain very large stretches of CAG and have been used in a homozygous state. This may indicate that

chromosomal context is highly important or that the expansion of polyglutamine in the context of human *HTT* sequence may influence the development of HD-like phenotypes in mice.

#### 1.3.2.5 Conditional models of HD

HD conditional mouse models were generated with two general objectives in mind: 1) To demonstrate proof of concept that progression of the phenotype in mice is caused by continuous expression of mHTT protein and stopping the expression at different stages of the disease can prevent pathological manifestations or/and halt disease progression and 2) To investigate the contribution of mHTT expression to HD pathology in discrete brain cell types.

## **1.3.2.5.1** Tet-HD94: a temporal approach

The Tet/HD94 conditional mouse model was generated containing chimeric murine/human *HTT* exon 1 fragment with 94 CAG repeats, expressed under a tetracycline-regulated promoter (Yamamoto et al., 2000; Díaz-Hernández et al., 2005). This system allows the expression of a transgene to be turned off with oral administration of tetracycline analogs (Furth et al., 1994). The use of this mouse model has provided evidence that reversing motor dysfunction and halting the progression of neuronal loss is possible through switching off *mHTT* at either early (Yamamoto et al., 2000) or late time points (Díaz-Hernández et al., 2005) in disease progression. Interestingly, it was shown that despite the irreversibility of inclusion formation at later stages of disease, animals fully recovered from motor deficits suggesting that turning off the expression of mutant protein can be considered as a potential intervention strategy even at late disease stage (Díaz-Hernández et al., 2005).

## 1.3.2.5.2 Cre/lox system: a cell-type specific approach

Selective striatal degeneration is a hallmark of pathology in HD despite the ubiquitous expression of mHTT throughout the body. However, the idea of cell non-autonomy, meaning the involvement of neighboring cells in pathology, has always been of interest in the field of HD. A glial-specific mouse model of HD was generated by expressing an N-terminal fragment of mHTT under the control of the human glial fibrillary acidic protein (GFAP) promoter that has been widely used to express a variety of genes in astrocytes (Bradford et al., 2009; 2010; Faideau et al., 2010). Interestingly, it was found that the selective expression in astrocytes causes agedependent neurological symptoms in mice such as clasping, rotarod deficits, and premature death (Bradford et al., 2009). The severity of symptoms induced by astrocytic expression of mHTT seemed to be CAG length dependent (Bradford et al., 2010).

To investigate the contribution of mHTT expression to HD pathology in corticostriatal pathway, conditional mice were generated using the Cre/*lox* system (Sauer, 1987) where they show expression in a particular cell population (Gu et al., 2005). For example, Emx1-Cre mice were crossed to *lox*P mice to create offspring which expressed mHTT only in pyramidal cortical neurons and glia and not in cortical interneurons and striatal MSNs. Interestingly, neuropathological analysis of these mice revealed that aggregate formation is a cell-autonomous phenomenon which occurs merely in cell types expressing mHTT but motor dysfunction and cortical neuropathology are only observed if mHTT is expressed in multiple cell types including cortical interneuron's and striatal MSNs (Gu et al., 2005). This was shown by crossing the Nestin-Cre mice to *lox*P mice, which resulted in the generation of offspring expressing mHTT in all neurons and glia.

#### 1.3.3 Humanized model of HD

Most HD transgenic models contain a fragment or a full-length construct of human *HTT* gene in addition to the mouse endogenous *Htt* gene. In an effort to move toward a more accurate recapitulation of HD genetics in mice, a group has recently generated a model, which includes two equally expressed doses of human full-length *HTT* genes with heterozygosity of HD mutation and no mouse endogenous *Htt* gene (Southwell et al., 2012). The so-called Hu97/18 mice have been generated by cross breeding the BACHD and YAC18 on the *Htt-/-* background mice. These mice display many symptoms associated with HD including a motor learning and climbing deficits at 2 months and which worsened by age, behavioral and cognitive changes such as anxiety and depressive-like symptoms at 3 month. At 12 months, these mice develop neuropathological changes such as reduced striatal and cortical volume (Southwell et al., 2012). The humanized model will serve as an invaluable tool for evaluating the functional differences between mouse and human *HTT* genes as well as for developing therapeutics specifically targeting human *HTT*.

## 1.4 Comparison of mouse models of HD

Although all of the described mouse models have been designed and subsequently generated to recapitulate the features of HD as closely as possible, there are many differences that separates these models in terms of their applications in studying physiological, cellular, and biochemical mechanisms of HD as well as their usage in therapeutic development. Fragment mouse models of HD are attractive models since they display robust and rapid motor phenotypes as well as reduced survival, both of which are considered as well-established endpoints for use in therapeutics trials. Despite these advantages, the fragment models fail to recapitulate some important features of the

disease. Neuropathological changes including reduction in brain size and inclusion formation occur in a non-selective manner throughout the brain. This is contrary to the selective striatal degeneration, which occurs in the brain of HD patients and the full-length transgenic mouse models. Additionally, due to the acute and early manifestation of many 'end-stage' symptoms of HD including severe phenotype and short lifespan, fragment models may 'skip' many stages of pathology such as post-translational modification and processing of the mHTT protein.

The full-length models exhibit slower progression of the motor phenotypes and a relatively normal lifespan compared with the fragment models. The use of these models however, makes it possible to study and understand earlier behavioural and pathologic abnormalities. Very importantly, these mice develop, in a progressive manner, selective degeneration of striatal neurons similar to what occurs in the brain of HD patients. This makes these mice suitable models for therapeutics. The full-length models have also been successfully used in understanding the modifications of the human full-length HTT in the presence of specific Nterminal mutations in HTT. These changes include post-translational modifications, proteinprotein interactions, and particular HTT fragments formations that are not necessarily present in the N-terminal fragment of the gene (Wellington et al., 2002; Warby et al., 2005; Graham et al., 2006; Huang et al., 2010; Metzler et al., 2010; Carroll et al., 2011; Gafni et al., 2012; Gil-Mohapel, 2012). Knock-in models of HD exhibit modest and inconsistent behavioural phenotypes, and neuropathological changes are essentially absent or very late and subtle. Moreover, HD is a human genetic disease and does not occur spontaneously in mice. Therefore, when considering using these mice in pre-clinical therapeutic studies, one must keep in mind that the slight genomic/protein differences between mouse and human could potentially account for difference in phenotypes and accordingly the outcome measures. The knock-in models however,
have provided valuable evidence of the behavioural and neuropathological differences surrounding homozygosity and heterozygosity of the *mHTT* gene.

Figure 1.1 demonstrates a comparison between the various genetic constructs used in the generation of different transgenic mouse models of HD. The construct schematics are depicted in relation to the human HD allele in a patient.



**Figure 1.1 Schematic representation of gene constructs used for the generation of various HD mouse models.** The figure demonstrates engineered gene constructs used in generating fragment (R6/1, R6/2, N171-82Q), full-length (BACHD, YAC128), and knock-in models of HD.

The last construct includes the tetracycline-regulated promoter system (Tet) as a tool for generating conditional knockout mice. The construct schematics are depicted in relation to the human HD allele in a patient (at the top).

#### 1.5 Recommendations on use in therapeutics and preclinical trials

#### 1.5.1 Sample size

Ideally, a trial should be designed in a way that the number of animals used in each particular treatment would provide sufficient information about the efficacy of the particular therapeutic approach being used. Therefore, the so-called 'power analysis' will be performed according to inter-animal variability for specific mouse models to determine how many animals will be needed to provide a high power (80%) in order to detect a significant improvement (p < 0.05). For example, in the study by Slow et al. it was demonstrated that eight YAC128 animals are needed to show striatal atrophy in this mouse model (Slow et al., 2003); therefore one can imply that striatal atrophy is a reasonable endpoint in this particular mouse model to be used in therapeutics with the goal of showing a significant benefit.

#### 1.5.2 Using standardized protocols among laboratories for the purpose of replication

It is highly recommended that laboratories all around the world put their effort into developing thorough and careful standard operating protocols and reach a consensus on a set of different aspects such as the outcome measures, testing conditions and equipment, statistical analyses, genetic background of the mouse model to be studied, and environmental enrichment for the animals. Only after paying careful attention to the above aspects, can the results of a pre-clinical

trial be confidently compared among different investigators. As an example of a standard operating protocol, one can refer to standardized rotarod testing which has been used widely to assess motor coordination in various HD mouse models (Hockly et al., 2003; Slow et al., 2003; Gray et al., 2008)

#### 1.5.3 The importance of publishing negative results

In general, publication is more likely in a pre-clinical study when the treatment effect is significantly large; conversely, publication is less likely when there is no significant effect of a particular treatment strategy. This leads to 'publication bias' that tends to exaggerate the true magnitude of a treatment effect and lead to an over-interpretation of the results of pre-clinical trials. This phenomenon can be prevented by reporting all results from pre-clinical trials, including negative results, the data which fail to support the main hypothesis (i.e. a benefit in therapeutics). Ruling out the effectiveness of a particular therapeutic approach in a system can be positive knowledge and can also save time and money, both of which are crucial factors when undertaking to develop a cure for a disease such as HD.

#### 1.5.4 Use of multiple models

The ultimate goal for pre-clinical studies with HD mice is to develop therapeutic approaches that can ultimately be tested in human clinical trials. For this reason, ideally, an approach should be assessed in more than one mouse model before entering human trials. We recommend that for pre-clinical testing of a specific compound or targeting strategy at a minimum, both a full-length model and a fragment mouse model should be used. In this case, validating a specific therapeutic approach in one or both mouse models can help with our understanding of its mechanisms and factors that need to be taken into account before taking the therapy to the next step.

#### 1.6 Conclusion

Since the discovery of the gene in 1993, thousands of studies have been pursued with the goal of unveiling the cellular and molecular pathways involved in HD. The resulting discoveries have paved the way for the development and testing of new therapeutics. The various mouse models of HD that have been developed have played a crucial role in increasing our understanding of HD pathogenic mechanisms and the potential effects of different therapeutics. Many of these approaches are currently being assessed at different phases of human clinical trials with the hope of developing effective treatments for HD.

#### **1.7** HD mouse model in current study

The YAC128 mouse model of HD has been selected as the model of choice throughout this project. As explained before, this model contains the entire human HD gene with 128 CAG repeats under the control of the endogenous human *HTT* promoter (Slow et al., 2003). First of all, this allows the study of the disease within the context of human full-length protein. Secondly, the YAC128 mouse model recapitulates robustly many features of HD including progressive motor and behavioural deficits (Slow et al., 2003; Van Raamsdonk et al., 2005c). Behavioural changes in this mouse model occur first at 3 months of age evident as hyperactivity and progression to hypoactivity by 12 months. Motor deficit is evident on the rotarod beginning at 6 months of age and progressively worsening with age (Slow et al., 2003; Van Raamsdonk et al., 2005c). Unlike the fragment models of HD, YAC128 mice develop phenotypes in a progressive and gradual

manner and are thus known to represent an early stage of HD featuring pre-symptomatic/early symptomatic phenotypes. Another advantage of the YAC128 mouse model is the selective neuronal loss, which manifests as the shrinkage of 18% of striatal volume and loss of 15% of medium spiny neurons by 12 months. This feature mimics the selective striatal degeneration in the brain of human HD patients and thus provides effective outcome measures for conducting therapeutic trials.

#### 1.8 Transcriptional dysregulation in HD

The hallmark neuropathologic feature of HD is early neuronal loss in the caudate and putamen (striatum). Interestingly, this selective degeneration in the striatum occurs despite the fact that the mHTT protein is expressed ubiquitously throughout the brain and body of HD patients. Consequently, this finding has led to many studies investigating the unique physiology of the striatum compared with other brain regions. Increasing evidence suggests that transcriptional dysregulation may be an important pathogenic mechanism in HD (Cha, 2000; Okazawa, 2003; Sugars and Rubinsztein, 2003). There have been several proposed mechanisms describing how mHTT protein disrupts transcriptional processes in neuronal cells. First, it has been suggested that mHTT interacts with ubiquitous transcription factors such as specificity protein 1 (Sp1) (Dunah et al., 2000; Li et al., 2002), the nuclear receptor co-repressor (N-CoR) (Boutell et al., 1999), CREB-binding protein (CBP) (Nucifora et al., 2001), TATA-box binding protein (TBP) (van Roon-Mom et al., 2002), TAFII130 (Shimohata et al., 2000), Sin3A (Boutell et al., 1999), and p53 (Steffan et al., 2001) via soluble or insoluble complexes. The mHTT has also been shown to disrupt the members of the core transcriptional machinery such as the pre-initiation complex (Zhai et al., 2005), as well as to interfere with acetylation and deacetylation states of

histones (Steffan et al., 2001) resulting in repression of general transcription (Steffan et al., 2001; Freiman and Tjian, 2002; Zhai et al., 2005). Finally, intranuclear inclusions are highly suspected to alter general gene expression by decreasing association of transcription factors to DNA binding sites (Chen-Plotkin et al., 2006). These proposed mechanisms are not mutually exclusive and may have synergistic roles in disrupting transcription.

#### 1.8.1 Global transcriptional changes

Many studies have been pursued to examine gene expression changes in HD. Comparative transcriptomics have been performed in fragment models as well as full-length HTT models of HD (Luthi-Carter et al., 2000; Chan et al., 2002; Desplats et al., 2006; Becanovic et al., 2010). Recently, microarray analyses were carried out on brain samples from human subjects with HD (Hodges et al., 2006; Kuhn et al., 2007). As a result, a large number of genes were found to be altered in mice (1.2%) and human postmortem brains (21%)(Thomas, 2006). Although these studies have been performed in striatum, many of the genes with altered levels of expression show widespread patterns of expression throughout the brain. These changes can potentially correspond to pathology in other brain regions, clinical heterogeneity in HD, and/or different regulatory mechanisms in the striatum compared to other brain regions. Results from these studies have also demonstrated that mHTT protein directly or indirectly can reduce the expression of a distinct set of genes involved in signaling pathways known to be critical to striatal neuron function (Luthi-Carter et al., 2000; Hodges et al., 2006).

#### 1.8.2 Striatal-enriched systems

Although insightful, results from global transcriptional analyses do not establish how only specific neuronal populations are affected in HD. The relative abundance of genes in different brain regions can provide clues to this selective vulnerability. Good examples are 'striatalenriched' genes, which are associated with several biological processes previously implicated in HD. These processes encompasses a wide variety of functional groups such as transcription factors, and genes involved in calcium homeostasis and G-protein signaling (Desplats et al., 2006; Thomas, 2006; Desplats et al., 2008). The exceptionally high percentage of striatalenriched genes whose expression levels are altered in HD compared to other genes expressed in the striatum strongly suggests that striatal-enriched genes are functionally relevant to HD pathogenesis (Thomas, 2006). Interestingly, a more recent study revealed that in vivo cellautonomous transcriptional abnormalities could be displayed in mice with mHTT expression exclusive to MSNs and in the absence of expression in cortical neurons (Thomas et al., 2011). In 2010, as part of my MSc thesis project, we identified 34 novel striatal-enriched transcripts through an unbiased high-throughput gene expression study using a Serial Analysis of Gene Expression (SAGE) database, and analyzed the expression of each transcript in the striatum of YAC128 mice (Mazarei et al., 2010). A striatal-enriched candidate gene, Indoleamine 2,3 dioxygenase (Ido1), was shown to be upregulated in the striatum of YAC128 animals. As a result, this PhD thesis focuses on understanding the importance of *Ido1* expression in the normal brain and investigating its potential role in HD.

#### 1.9 Indoleamine 2,3 dioxygenase

IDO1 is the first and the rate-limiting enzyme of the kynurenine pathway which was first discovered in 1960s (Higuchi and Hayaishi, 1967). Kynurenine pathway is the principle route of tryptophan catabolism and has been implicated in infection, immunoregulation, autoimmunity (Ravishankar et al., 2012), tumouregenesis (Munn and Mellor, 2007), antioxidant activity (Thomas and Stocker, 1999), and neuropathology (Guillemin et al., 2005a; Bonda et al., 2010). IDO1 is a heme-containing enzyme that catalyzes the conversion of the essential amino acid Ltryptophan (Trp) to N-formylkynurenine and then L-kynurenine (Kyn) through the oxidative cleavage of the indole ring of Trp (Higuchi and Hayaishi, 1967). IDO1 is highly expressed in mammalian organs such as lung, epididymis, small intestine and placenta (Yuasa et al., 2009). In most other organs the expression is low and induced by upregulation of cytokines such as interferon gamma (IFNy) (Yuasa et al., 2009). During infection, IDO1 upregulation depletes Trp which in turn inhibits the proliferation of intracellular parasites (Pfefferkorn, 1984; Nettelnbreker et al., 1998). During pregnancy, IDO1 induction prevents allogeneic fetal rejection by reducing the availability of Trp to maternal T cells and thus enhancing activation induced T cell death (Munn et al., 1998). IDO1 has also been implicated in immune tolerance by maintaining the T cell homoeostasis of self-antigen tolerance during inflammation (Sakurai et al., 2002; Kwidzinski et al., 2005; Platten et al., 2005; Ravishankar et al., 2012). Similarly, by increasing IDO1, cancer tumours create a tolerogenic microenvironment in which they can suppress the host T cells and enhance local Treg-mediated immunosuppression through Trp depletion (Munn and Mellor, 2007). IDO1 therefore has the potential to be used as a therapeutic target under multiple clinical settings such as cancer, chronic infections, autoimmunity, allergy, and even transplantation (Munn and Mellor, 2012). In an in silico analysis performed using Gemma

(http://www.chibi.ubc.ca/Gemma/home.html), we found mouse *Ido1* to be co-expressed with many striatal-enriched genes that were found in previous studies (Mazarei et al., 2010). Gemma is an open-source database and software system for the meta-analysis of gene expression data and contains data from hundreds of public microarray data sets, referencing hundreds of published papers. Table 1.1 demonstrates the list of genes retrieved by Gemma to be co-expressed with *Ido1*.

Gene	Gene name	Striatal-enriched
Symbol		Mazarei <i>et al.</i>
Rgs9	Regulator of G protein signaling 9	
Cd4	Cd4 antigen	
Pde10a	Phosphodiesterase 10A	
Ppp1r1b	Protein phosphatase 1 regulatory subunit 1B	
Drd2	Dopamine receptor D2	
Cyld	Cylindromatosis (turban tumor syndrome)	$\checkmark$
Sh3rf2	SH3 domain containing ring finger 2	
Gpr88	G-protein coupled receptor 88	
Psmb10	Proteosome subunit, beta type 10	
Rasgrp2	RAS, guanyl releasing protein 2	
Rarb	Retinoic acid receptor, beta	
Rasd2	RASD family, member 2	
Irgm2	Immunity-related GTPase family M member 2	
Gm12185	Predicted gene 12185	
Dock9	Dedicator of cytokinesis 9	

Table 1. 1 Striatal-enriched genes found by Gemma to be co-expressed with Ido1

#### 1.10 Role of Indoleamine 2,3 dioxygenase and kynurenine pathway in brain

Within the past decade, there has been more focus on the characterization of the kynurenine pathway within the brain and in different brain cell types. Furthermore, there is currently accumulating evidence on the perturbation of the pathway and an imbalance in the synthesis of its metabolites at many neurologic disease states. Kynurenine pathway diverges into two branches that can lead to production of either neuroprotective or neurotoxic metabolites. In one branch, kynurenine produced as a result of Trp degradation is further catabolized to neurotoxic metabolites such as 3-hydroxykunurenine (3-HK) and guinolinic acid (OA). Although 3-HK is converted to QA though a few steps in the pathway, the toxic effect of 3-HK is independent of NMDA receptor mediated toxicity and solely depends on its ability to generate free radicals (Szalardy et al., 2012). QA is an N-methyl-D-aspartate (NMDA) receptor agonist and thereby has the potential of mediating NMDA mediated neuronal damage and dysfunction, a mechanism well described in HD pathogenesis (Heyes et al., 1992). In the other branch, kynurenine is converted to the neuroprotective metabolite kynurenic acid (KA), which is an endogenous glutamate antagonist. In addition to KA inhibitory action on NMDA receptors, it also acts on kainate- and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-sensitive ionotropic glutamate receptors (Vécsei et al., 2012). Another antiglutamatergic function of KA is through the inhibition of the  $\alpha$ 7 nicotinic acetylcholine receptors (nAChRs) known to mediate the enhancement of glutamate release (Marchi et al., 2002). Finally, KA acts as a potent free radical scavenger (Lugo-Huitrón et al., 2011). Degradation of Trp through this pathway also results in production of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) in mammalian tissues (Yuasa et al., 2009). Figure 1.2 depicts all metabolites and enzymes of the kynurenine pathway (vertical) and parts of the serotonin pathway (horizontal).



**Figure 1.2** Tryoptophan entering the CNS becomes the substrate for kynurenine pathway (depicted vertically) and serotonin pathway (depicted horizontally)

Studies have used IFN $\gamma$ , as the most potent inducer of IDO1 (Werner-Felmayer et al., 1989), to stimulate the activation of the pathway in neurons, astrocytes and microglia in culture (Guillemin et al., 2005b) to successfully show that the complete pathway is present in microglia

and macrophages while neurons and astrocytes seem to express only parts of the pathway by expressing the required enzymes partially (Guillemin et al., 2005b). Investigations involving the role of IDO1 in the brain have mostly focused on immune tolerance associated with brain tumours (Munn and Mellor, 2007; Adams et al., 2012) as well as infiltrating macrophages in the case of autoimmune disease such as multiple sclerosis (MS) (Sakurai et al., 2002; Kwidzinski et al., 2005; Kwidzinski and Bechmann, 2007).

Development of depression and depressive-like phenotypes as a result of IDO1-induced Trp degradation and thus reduced serotonin levels has been of recent interest (Dantzer et al., 2008; Moreau et al., 2008; O'Connor et al., 2009b; Fu et al., 2010; Laugeray et al., 2010; Leonard and Maes, 2012). A new study has been successful in showing that IDO1 contributes to the comorbidity of pain and depression (Kim et al., 2012).

More recently, there has also been increasing evidence for the involvement of kynurenine pathway in general, and IDO1 specifically, in neuroinflammatory/neurodegenerative diseases such as amyotropic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) stroke, cerebral malaria, HIV-induced dementia, and schizophrenia (Wonodi and Schwarcz, 2010; Sathyasaikumar et al., 2011) (Sardar et al., 1995; Huengsberg et al., 1998; Baran et al., 2000; Widner et al., 2002; Clark et al., 2005; Guillemin et al., 2005a; Walker et al., 2006; Darlington et al., 2007; Yamada et al., 2009; Bonda et al., 2010; Chen et al., 2010; Fu et al., 2011; Szabó et al., 2011).

#### 1.11 Other catalysts for the first step of tryptophan degradation (Tdo2 and Ido2)

While IDO1 is expressed widely in most extrahepatic tissues, a second enzyme named tryptophan 2,3 dioxygenase (TDO2) has been considered the main enzyme converting Trp to kyn

in the liver. Recently however, there has been increasing evidence for the role of TDO2 in the brain (Kanai et al., 2009; Laugeray et al., 2010). TDO2 and IDO1 are different in their regulation and are induced/inhibited through different physiological mechanisms. For example, the family of interferons ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), platelet activating factor, T-antigen 4, HIV1 proteins (Nef and Tat), Amyloid beta peptide 1-4, Trp, and Trp analogues are well-known inducers of IDO1 while TDO2 is mainly induced by Trp, Trp analogues, and glucocorticoids (Chen and Guillemin, 2009). On the other hand, IL-4, nitric oxide, and COX-2 inhibitors are known to inhibit IDO1 expression whereas indoleamines, nicotinamide analogues, and several antidepressant drugs are effective inhibitors of TDO2 (Chen and Guillemin, 2009). Interestingly, inhibition of TDO2-mediated Trp degradation has been shown to delay aging and aging-associated protein homeostasis in *C. elegans* (van der Goot et al., 2012).

*IDO2* or *IDO*-like gene was first discovered in 2007 to be a paralog of *IDO1*. Both genes have very similar genomic structures and are located adjacent to each other on chromosome 8 in both mouse and human genomes. These genes are likely to be the result of gene duplication and seem to have similar enzymatic activity (ie. conversion of Trp to kyn) but are different in their expression patterns (Ball et al., 2007) and their selectivity for some inhibitors (Ball et al., 2009). Evolutionarily, the *IDO* ancestor gene was found to be more similar to *IDO2* than *IDO1* and while *IDO1* is found in mammals and yeast, *IDO2* is present only in mammals (Yuasa et al., 2009).

The kinetic parameter Michaelis–Menten constant ( $K_m$ ) which is a reverse measure of enzymatic affinity for substrate, was shown to be different for each of the three enzymes.  $K_m$ value is ~20 µM for human IDO1 and ~ 100 µM for human TDO2 (Yuasa et al., 2009). The  $K_m$ value of IDO2 is estimated to be 500-1000 fold larger than that of IDO1 (Yuasa et al., 2009).

These findings demonstrate that among the three enzymes, IDO1 has the highest affinity for substrate L-Trp.

#### 1.12 Indolemeamine 2,3 dioxygenase and kynurenine pathway in HD

As mentioned before, IDO1 activity takes place at the first and the rate-limiting step of the kynurenine pathway favouring the production of neurotoxic metabolite QA and thus linking the susceptibility to NMDA-mediated neurotoxicity in HD to kynurenine pathway activity. An increase in the plasma L-kyn to L-Trp ratio as a measure of IDO1 activity has also been observed in HD (Stoy et al., 2005a). Interestingly, despite the absence of IDO1 expression in flies, TDO inhibition has been shown to be neuroprotective in the drosophila model of HD (Campesan et al., 2011) indicating an important role for the first step of the kynurenine pathway. Among the downstream metabolites of the pathway, there are implications for a reduction in KA and an increase in QA and 3-hydroxykynurenine (3-HK) production in the brain of HD patients as well as mouse models of HD (Beal et al., 1990; Guidetti et al., 2006; Sathyasaikumar et al., 2010). Mice deficient for kynurenine aminotransferase ii (KAT2), an enzyme of the pathway that converts L-L-kyn into KA, are more susceptible to QA-induced toxicity (Rossi et al., 2008). Similarly, an increased neostriatal QA/KA ratio, caused by a rise in QA levels has been observed in early grade HD brain at the first stages of the disease (Guidetti et al., 2004). Importantly, the result of feeding neurotoxic metabolites such as KA and 3-HK to flies expressing mHTT demonstrated a *causal* involvement of kynurenine pathway metabolites in mHTT dependent neuron loss (Campesan et al., 2011).

Very recently, the enzyme kynurenine 3-hydroxylase (KMO) has attracted a lot of attention as a potential therapeutic target for HD. This enzyme is known to be expressed primarily in

microglia where it converts L-kyn to 3-HK (Foster et al., 1986; Giorgini et al., 2008). A chronic oral administration of a KMO inhibitor was shown to extend lifespan, prevent synaptic loss and decrease microglial activation in the brain of R6/2 model of HD (Zwilling et al., 2011). The latter study is a valuable example of how manipulating specific enzymes in the kynurenine pathway can divert the pathway to one branch or another and modify the net result of the pathway in terms of neurotoxicity.

#### 1.13 Hypothesis

In the first part of this PhD thesis, I have performed a thorough analysis of the kynurenine pathway in the brain and periphery of the YAC128 mouse model of HD at different stages of disease progression. Focusing initially on the first step of the pathway, I discovered changes in transcription and activity of Ido1 in the striatum of YAC128 mice, which were further demonstrated to be chronically present at multiple time points of the animal's life. Accordingly, we tested the hypothesis that a change in the first step of the kynurenine pathway in the brain of YAC128 mice is followed by an imbalance of downstream neurotoxic and neuroprotective metabolites of the pathway. For this purpose, I collaborated with a group that were expert in liquid chromatography mass spectrometry (LCMS) technique, to measure all the downstream metabolites of the pathway in the striatum and cerebellum of 3 and 12 months. An imbalance of neurotoxicity versus neuroprotection was observed at both time points, which were different in presymptomatic versus symptomatic animals; this is reminiscent of a biphasic state of kynurenine pathway during disease progression which will be discussed further in much detail. Based on this study, I propose that the early and region-specific induction of Ido1 should be taken into account when assessing therapeutic targets for HD in this pathway. Additionally, systemic versus central

changes of the pathway reveal important differences which are worth considering in any future work.

The chapter 3 of this PhD thesis was built upon the first segment. Based on our previous findings, we hypothesized that the chronic induction of *Ido1* expression and activity in the striatum of YAC128 mice leads to increased neurotoxicity in this mouse model; accordingly, chronic absence of *Ido1* expression can render less sensitivity to neurotoxicity in mice. To test this hypothesis, we sought to assess the sensitivity of brain (particularly the striatum) to excitotoxic insult in constitutive *Ido1* null mice (*Ido<sup>-/-</sup>*). Upon characterization of enzymatic expression and activity in these mice, we tested the sensitivity of *Ido<sup>-/-</sup>* brain to QA-induced toxicity and demonstrated that the striatum of *Ido<sup>-/-</sup>* mice is less sensitive to this type of excitotoxic insult. To learn more about the sensitivity of *Ido<sup>-/-</sup>* striatum to NMDA receptor mediated excitotoxic stress, we also performed thorough analyses of the response to NMDA in *Ido<sup>-/-</sup>* isolated MSNs as well as neuronal-glial co-cultures.

### Chapter 2: IDENTIFICATION OF AGE-DEPENDENT CHANGES OF THE KYNURENINE PATHWAY IN THE YAC128 MOUSE MODEL OF HD

#### 2.1 Introduction

Previously, in a high-throughput expression study of mouse brain using serial analysis of gene expression (SAGE), we identified Indoleamine 2,3 dioxygenase (*Ido1*) as a striatal-enriched transcript (Mazarei et al., 2010). Additionally, we showed that the relative *Ido1* transcription level was upregulated in the YAC128 mouse model of HD (Mazarei et al., 2010).

This led to the investigation of *Ido1* expression and IDO1 activity in multiple time points during development in YAC128 mice. We first studied the spatial and temporal expression patterns of *Ido1* and assessed its expression level and activity in the striatum of YAC128 mice at different developmental time points. In order to increase our understanding of enzymatic expression in the first step, we analyzed the expression of other catalysts of Trp to kyn breakdown, *Ido2* and *Tdo2*, and compared them to *Ido1* expression. To test the hypothesis that changes in the first step of the KP in the brain of YAC128 mice will be followed by an imbalance of downstream neurotoxic versus neuroprotective metabolites, the majority of downstream KP metabolites were also measured.

Overall, this chapter demonstrates the age-dependent changes in the KP, with an initial focus on Trp to kyn conversion, in the brain of presymptomatic (3 months) and symptomatic (12 months) YAC128 mice and discusses the potential implication of these findings. Based on this study, we propose that the early and region-specific induction of *Ido1* should be taken into account when assessing therapeutic targets for HD in this pathway.

#### 2.2 Methods and materials

#### 2.2.1 Mice

Animals of both genotypes (WT and YAC128) and mixed sex were used in this study at different time points. This time frame ranged from embryonic day 17 up to 12 months of age. All mice used in this study were on the FVB/N strain background. Mice were group-housed with a normal light–dark cycle and given free access to food and water. Mice were euthanized in accordance with our institutional animal care guidelines and all experiments were approved by the University of British Columbia's Committee for Animal Care. No perfusion was performed on animals and dissected brain regions for RNA extraction and liquid chromatography mass spectrometry LC/MS/MS were immediately snap-frozen at -80 °C. The chart below is the summary of mouse gender distribution for each figure/experiment in our study. Sex was generally assigned to animals when they were weaned (~ 21 days post birth).

 Table 2.1
 Gender distribution for figure/experiment in chapter 2

	WT		YAC128	
Figure/experiment (chapter 2)	Male	Female	Male	Female
1.2 (a, b, c)	2	1		
1.2 (d)	equal distribution of male and female per time point			
2.1	2	3		
2.3	3	2	3	3
2.4 (1 month)	2	6		8
2.4 (3 months)		5		5
2.4 (12 months)	2	2	2	2
mass spec (3-month-old mice)	8	4	8	4
mass spec (12-month-old mice)	6	2	4	4

#### 2.2.2 Isolation of primary cells

#### Neuron cultures

Striatum and cortex were isolated from the brain of WT FVB/N background strain neonatal mouse pups and were kept in Hybernate A (-Ca) (BrainBits). The brain pieces for each brain region were trypsinized in 0.05% trypsin 0.25% EDTA (Hyclone) including 65.5U/ml of

Deoxyribonuclease I (Invitrogen) for 15 minutes at 37 °C. Trypsinization was stopped by the addition FBS (PAA) solution. The tissue pieces were triturated in the solution using a 5 ml pipette and the homogenate was passed through a 40-micron cell strainer. The homogenate was pelleted at 1,000 rpm and the pellet was resuspended and washed in Hank's Balanced Salt Solution (HBSS) (Hyclone) twice. Finally, the pellet was resuspended in neurobasal A medium (Gibco) containing B27 supplement (Gibco), 0.5 mM L-glutamine (Hyclone) and 1% penicillin/streptomycin (Hyclone). Neurons were counted in a hemacytometer (VWR) and seeded in tissue culture plates (BD Falcon) and kept in complete neurobasal A for 9 days at 37°C at 5% CO<sub>2</sub>. The media was changed every 2-4 days. At day *in vitro* (DIV) 9, neurons were harvested for RNA isolation.

Cerebellum was isolated from postnatal day 5 WT mouse pups and cerebellar neurons were isolated according to the protocol described by Swanson et al. (Swanson et al., 2005). *Astrocyte and Microglia cultures* 

Brain was isolated from post-natal day 1 WT mouse pups and placed in HBSS on ice. Meninges were removed carefully and the striatum was dissected out and placed into the growth medium [DMEM, 10% FBS (PAA), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen)] and homogenized using a 5 ml pipette. Striatal cells were pelleted at 1,000 rpm, resuspended in the growth medium and transferred into a T75 flask that was cultured at 37°C at 5% CO<sub>2</sub>. The growth medium was replaced with fresh media after 24 h and then every 7 days. At DIV 18–21, loosely attached microglia were harvested for RNA isolation. Tightly attached astrocytes were scraped off the flask and harvested for RNA isolation.

#### 2.2.3 Quantitative real-time PCR

For the *in vivo* analysis of spatial expression patterns of *Ido1*, *Ido2*, and *Tdo2* mRNA expression, striatum, cortex, cerebellum and hippocampus were dissected from the brains of 9-day-old, and 3 and 12-month-old WT mice (n = 3-5) and were immediately snap-frozen at  $-80^{\circ}$ C. Similarly, for transgenic vs. WT comparison experiments, striata from YAC128 mice and age-matched controls (n = 5 -10 per genotype and age group) from 6 different time points (E17, postnatal day 7 and 15, and 1, 3, and 12-month-old) were dissected immediately snap-frozen at  $-80^{\circ}$ C. Homogenization of each individual tissue in lysis buffer (Ambion) was performed using a Fastprep Homogenizer (ThermoScientific). RNA was extracted from each homogenized brain region using PureLink® RNA Mini Kit (Ambion).

For the *ex vivo* analysis of *Ido1*, *Ido2*, and *Tdo2* mRNA expression in different cell types, cells were harvested and RNA was extracted immediately. For neurons, lysis buffer (Ambion) was added to plate wells and neurons were scraped off the plate using cell scrapers. To obtain n of 1, we pooled neurons from three wells of 12-well plates. For astrocytes and microglia, cells were harvested from different flasks. Subsequently, cells were homogenized in lysis buffer (Ambion) and by passing them through 21 gauge needles multiple times.

RNA was then extracted from each cell type using an RNA extraction kit (Ambion). Genomic DNA was eliminated from all samples by using the PureLink® DNase set (Ambion). cDNA was then obtained by the reverse transcription (RT) of 500 ng of total RNA from freshly dissected brain regions or cell types using the SuperScript® VILO<sup>TM</sup> cDNA Synthesis Kit (Invitrogen). Primers for *Ido2* (forward 5'- CAAAGTCAGAGCATGACGCTG-3', reverse 5'- CGCTGCTCACGGTAACTCTTTA-3'), *Rplp0* (forward 5'-TGGGCATCACCACGAAAAT-3', reverse 5'-ATCAGCTGCACATCACTCAGAATT-3'), *Hprt1* (forward 5'-

CGTCGTGATTAGCGATGATGA-3', reverse 5'-TCCAAATCCTCGGCATAATGA-3', *Rn18s* (forward 5'- AGAAACGGCTACCACATCCAA-3', reverse

5'GGGTCGGGAGTGGGTAATTT-3', *Actb* (forward 5'-CCAGCCTTCCTTGGGTAT-3', reverse 5'-TGTGTTGGCATAGAGGTCTTTACG-3'), *Gapdh* (forward 5'-

AACTTTGGCATTGTGGAAGG-3', reverse 5'-ACACATTGGGGGGTAGGAACA-3')

and were designed using the Primer Express software version 3.0 (Applied Biosystems).

Additionally, the following primers were used from previous studies: Ido1 (forward 5'-

AGGATCCTTGAAGACCACCA-3', and reverse 5'-CCAATAGAGAGAGAGGAAG-3')

(Okamoto et al., 2007): Tdo2: forward 5'-ATGAGTGGGTGCCCGTTTG-3' and reverse 5'-

GGCTCTGTTTACACCAGTTTGAG-3' (Ohira et al., 2010). Amplicons spanned introns where possible and were between 110 and 400 bp for efficient amplification. Primer efficiencies were determined using dilution series of adult striatum or cerebellum cDNA. Only primer pairs with an efficiency greater than 0.9 were used in subsequent analyses.

The ABI 7500 real-time PCR system (Applied Biosystems) and the Fast SYBR® Green Master mix (Applied Biosystems) were used for all qPCR experiments.

All reactions were carried out in duplicate using 1  $\mu$ l of cDNA in each reaction. Duplicates with inconsistent amplification were removed from the analysis.

Absolute quantity of the targets in each sample was calculated based on the standard curve method. Standard curves were created using 10-fold serial dilutions of either mouse striatum, or cerebellum cDNA. The relative amount of mRNA in each well was calculated as the ratio between the target mRNA and a normalization factor (NF) described below.

#### 2.2.4 Calculation of normalization factor for qPCR data

We analyzed multiple reference genes for normalization of the qPCR data based on the normalization strategy suggested by Vandesompele et al. (Vandesompele et al., 2002). Similar to the previously published work by our group (Becanovic et al., 2010), 18S ribosomal RNA (Rn18s), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), actin-beta (Actb), ribosomal protein, large, P0 (Rplp0), and hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) were measured in our samples. We applied the GeNorm software analysis for the calculation of the most accurate normalization factor (NF) for our data measurements in samples from different age groups. The normalization factor was calculated based on the geometric averaging of multiple reference genes. Calculations were based on the average expression stability and pairwise variation analysis using the GeNorm-software (http://medgen.ugent.be/~jvdesomp/genorm/).

#### 2.2.5 Kynurenine pathway metabolite LC/MS/MS

#### Mouse Tissue

Striatum and cerebellum were collected from 3-month-old (n = 12 WT, 12 YAC128) and 12month-old (n= 10 WT, 6 YAC128) mice and snap-frozen at  $-80^{\circ}$ C. Plasma samples from 12month-old WT and YAC128 mice were separated by the centrifugation of blood in microvettes for haematological testing (SARSTEDT) at 3000 rpm for 15 minutes and were kept at -80°C immediately.

*Brain Tissue Preparation*: Large mouse brain samples (>40mg) were homogenized (1 minute) using an Omni-Prep Multi-Sample Homogenizer equipped with disposable blades, after addition of a 3x mass of an aqueous solution containing 0.2% acetic acid and internal standards into a 2mL eppendorf tube. Smaller tissue samples (<40mg) were homogenized after the same dilution

but using a Covaris Sonication apparatus (4 minutes). All resultant samples were filtered using a 3kDa Amicon Ultra filter from Millipore which was centrifuged at 13500 g for 60 minutes at 4°C followed by either direct transfer to CMA tubes ( $300\mu$ L) for injection onto the LC/MS/MS or a further 10x dilution with 0.2% acetic acid solution prior to injection. Additional dilutions were performed to allow measurement of the highly concentrated analytes such as TRP and NTA. Amicon Ultra filters retain roughly 50µL of solution and therefore when smaller homogenate volumes were available a Vivaspin 2 filter with a 2kDa mol. wt. cutoff was utilized as they retain less than 20µL of solution. Isolation efficiencies with respect to KP metabolites in brain tissue samples were determined by comparing the levels of recovered material from each filter. Similar concentrations of all metabolites were observed.

*Plasma Sample Preparation*: Mouse plasma sample  $(50\mu L)$  preparation involved dilution by 5 fold using a 0.2% acetic acid aqueous solution containing deuterated internal standards followed by filtration as described above. Direct injections of the resultant solution as well as a 10x diluted (diluted with 0.2% aqueous solution) version were then performed.

*Materials*: Formic acid, acetic acid and KYN pathway (KP) standards (KYN, TRP, KYNA, QA, 3-HK, anthranilic acid (AA), 3-HAA, xanthurenic acid (XT), nicotinamide (NTA), picolinic acid (PA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5HIAA) were all purchased from Sigma Aldrich. Internal standards <sup>2</sup>H<sub>5</sub>-TRP, <sup>2</sup>H<sub>5</sub>-KYNA, <sup>2</sup>H<sub>4</sub>-NTA, <sup>2</sup>H<sub>4</sub>-PA and <sup>2</sup>H<sub>4</sub>-5HT were purchased from CDN Isotopes and prepared in a 0.2% acetic acid solution at a concentration of 100ng/mL for addition to tissue or plasma samples. High purity acetonitrile, (B&J) was purchased from Fisher Scientific and water was purified in house using a MilliQ system (Millipore) equipped with a biofilter.

LC/MS/MS Method: A Waters Acquity HPLC system equipped with an YMC ODS AQ 2x100mm, 3um particle column provided separation of the KP analytes prior to detection by a Waters Quattro Premier XE triple quadrupole mass spectrometer operating in the MS/MS mode. Column and pre-column tubing were maintained at 40°C while eluting KP metabolites with a mobile phase consisting of an aqueous component (A: 0.5% formic acid in milliQ water) and an organic component (B: 1% formic acid in acetonitrile). Gradient elution included a 2-minute hold at 100% A followed by a shallow gradient of 0-30% B over 4.4min. Later eluting materials were then brought off the column using a stronger gradient of 30-70% B over 2 minutes with a total run time of 12 minutes. The HPLC was equipped with a  $5\mu$ L loop which was filled using a full loop injection with a 3x overfill factor for a total sample consumption of 15µL. Operating conditions of the triple quadrupole mass spectrometer were as follows: capillary voltage 1.5kV, cone voltage 40V, source temperature 150°C, desolvation temperature 500°C, cone gas flow 50L/hr, desolvation gas flow 1000L/hr. For each analyte (presented in order of elution) the following transitions were monitored using the collision energy (CE) indicated and any deviation from the above operating conditions are noted: 1) NTA 122.75-79.7, CE 18, cone 28V, 2) D-NTA 126.8-83.7, CE 20, 3) PA 123.75-77.7, CE 17, 4) D-PA 127.8-81.75, CE 15, 5) QA 167.85-77.7, CE 20, 6) 3HK 225.12-109.8, CE 18, 7) 5HT 176.9-114.8, 131.8, CE 22, 8)D-5HT 181.0-118.0, 136.0, CE 24 and 22 respectively, 9) 5HTP 220.91-161.8, CE 18, cone 15, 10) KYN 209.05-93.7, CE 15, 11) 3HAA 153.85-79.75, 107.75, CE 25 and 20 respectively, 12) TRP 205.1-145.8, CE 18, 13) D-TRP 210.2-150.1, CE 20, 14) XT 206.05-131.8, CE 27, cone 28, 15) KYNA 190.0-143.85, CE 18, cone 28, 16) D-KYNA 195.1-149.0, CE 20, 17) 5HIAA 191.75-145.75, CE 15, 18) AA 137.82-64.75, 91.7, CE 25 and 20 respectively, 19) CBA 301.2-236.95, 265.0, CE 30 and 24 respectively.

#### 2.2.6 Statistics

Expression data were analyzed using two-tailed student's *t* test, one-way ANOVA, or 2-way ANOVA followed by post hoc comparisons using the Tukey or Bonferroni tests where appropriate. Two-tailed student's t-test was used for the comparisons of metabolite levels in WT and YAC128 mice. (GraphPad Prism version 5.0).

#### 2.3 Results

#### 2.3.1 Spatial and temporal patterns of *Ido1* expression in mouse brain

Previously, we showed that *Ido1* is a striatally-enriched transcript in the adult mouse brain using SAGE analysis with quantitative real-time PCR (qRT-PCR) validation. To assess *Ido1* expression patterns during brain development, relative abundance of *Ido1* mRNA was assessed in four different brain regions at three time points of 9 days postnatal (dpn), 3 months, and 12 months by qRT-PCR. Comparison of the expression levels in striatum, cortex, hippocampus, and cerebellum revealed that *Ido1* mRNA was significantly elevated in the striatum at all time points tested (Fig.2.1 a, b, c). At all three time points, *Ido1* expression was higher in cortex compared to hippocampus and cerebellum and was not detectable in the cerebellum suggesting similar spatial expression patterns for *Ido1* within the brain at multiple time points. Similarly, the relative abundance of *Ido1* transcript was compared at multiple time points in the striatum. The striatum was isolated from embryonic day 17 (E17), post-natal day 7 and 15, 1 month, 3 months, and 12 months of age. Analysis of all the time points demonstrated that *Ido1* expression was significantly lower at E17 and significantly higher dpn15, when it reached its maximal expression during the 12-month period analyzed. *Ido* expression was reduced again at 1 month where it

remained relatively constant till 12 months of age (Fig. 2.1d). *Ido1* expression was assessed in primary cultures of different brain cell types known to express KP enzymes (Guillemin et al., 2005b). *Ido1* mRNA levels were higher in isolated primary MSNs, compared to primary cortical and cerebellar neurons isolated from the brain of postnatal mice (Fig. 2.2a). The expression of *Ido1* transcript in isolated MSNs was similar to that of isolated astrocytes from the striatum but was barely detectable in isolated microglia (Fig. 2.2b). This *ex vivo* data suggests that astrocytes and neurons may contribute to the most of the striatal *Ido1* expression in brain.



**Figure 2.1 Temporal and spatial patterns of** *Ido1* **expression.** qRT-PCR was used to measure the relative abundance of *Ido1* mRNA expression in brain at 3 different time points during mouse development. A comparison between the expression levels in the striatum, cortex, hippocampus,

and cerebellum revealed *Ido1* relative mRNA expression to be significantly higher in the striatum at 9 days postnatal (a), 3 months (b), and 12 months (n = 3) (c). (d) Analysis of *Ido1* relative abundance in the striatum at 6 time points during mouse development in a pairwise comparison. *Ido1* expression is significantly lower at E17 and significantly higher at postnatal day 15 (P15) compared with any other time points. (n = 5 -10 per age group). NS = not significant. Data are the mean  $\pm$  SEM. (\*\*\*\*p<0.0001, \*\*\*p<0.001, \*p<0.05, one-way ANOVA followed by a Tukey multiple comparisons post test).



**Figure 2.2 Expression of** *Ido1* **in different brain cell types**. qRT-PCR was used to measure the relative abundance of *Ido1* mRNA expression in different types of neurons including striatal, cortical, and cerebellar neurons isolated from postnatal mice (a). *Ido1* mRNA relative abundance is significantly higher in striatal neurons. Neurons, astrocytes, and microglia were isolated from the striatum of postnatal animals and *Ido1* mRNA relative abundance was measured under basal conditions. Microglia express significantly less *Ido1* at baseline (b) Data are the mean  $\pm$  SEM. (\**p*<0.05, one-way ANOVA followed by a Tukey multiple comparisons post test).

## 2.3.2 Different expression patterns of *Ido1*, *Ido2* and *Tdo2* in adult mouse brain: considerations for functional analysis

IDO1, IDO2, and TDO2 are the currently known enzymes catalyzing the first step of the KP; Trp to kyn conversion. Although TDO2 was originally described as a primarily hepatic enzyme, there has been increasing evidence for an involvement in the brain and brain pathophysiology (Kanai et al., 2009; van der Goot et al., 2012). Little is currently known about the role of the recently discovered enzyme IDO2 in brain (Ball et al., 2007; Yuasa et al., 2009). We investigated the expression patterns of *Ido1*, *Ido2*, and *Tdo2* in two brain regions using qRT-PCR (Fig. 2.3); the striatum, a brain region predominantly affected in HD and in YAC128 mouse model and the cerebellum, a brain region relatively spared in HD. Ido2 has a similar expression pattern as Ido1, being enriched in the striatum with no expression detected in the cerebellum (Fig. 2.3a, b). Tdo2 expression is exclusive to cerebellum (Fig. 2.3c) and was not seen in striatum. Revisiting the SAGE data from cerebellum and striatum confirmed the presence of *Ido1* mRNA only in the striatum and Tdo2 mRNA exclusively in cerebellum. Additionally, the absolute abundance of striatal Ido1 SAGE tag was twice as large as that of cerebellar Tdo2 indicating higher transcription of striatal *Ido1* compared with cerebellar *Tdo2*. The *in silico* SAGE tag retrieved for Ido2 was not detected in either striatum or cerebellum. The findings of these experiments raise new considerations for the performance of functional analyses investigating kyn/Trp ratios in brain as a measure of activity for these region-specific enzymes.



Figure 2.3 *Ido1, Ido2,* and *Tdo2* mRNA display different expression patterns in the striatum and cerebellum of 3-month-old WT mice. Using qRT-PCR, the abundance for each enzyme's transcript was assessed in striatum and cerebellum. *Ido2* display striatal-enriched expression pattern (c) similar to that of *Ido1* (a). *Tdo2* on the other hand is barely detected in the striatum and is enriched in the cerebellum (c) Data are the mean  $\pm$  SEM. (\*\*\*\**p*<0.0001, \*\*\**p*<0.001, two-tailed student *t* test).

# 2.3.3 *Ido1* is upregulated in the striatum of YAC128 mouse model of HD at multiple time points during development

We had previously shown that *Ido1* mRNA is upregulated in the striatum of YAC128 adult mice compared to wild-type mouse striatum (Mazarei et al., 2010). The striatal expression of *Ido1 in* YAC128 mice was assessed at the six different time points previously used for temporal expression analysis and *Ido1* mRNA relative abundance compared between wild-type and YAC128 mice. *Ido1* mRNA was significantly upregulated in YAC128 compared to wild-type striatum at all time points assessed (Fig. 2.4).



Figure 2.4 *Ido1* mRNA relative expression is upregulated in the striatum of YAC128 mice at multiple time points. Relative quantification of *Ido1* mRNA was analyzed at 6 time points of E17, P7, P15, 1 month, 3 months, and 12 months as a pairwise comparison between the striatum of WT and YAC128 at each age group. The striatum was isolated from WT (open bar) and YAC128 (solid bar) mice of different ages (n = 5 -10 per genotype and age group). *Ido1* mRNA abundance in YAC128 striatum has been reported as the percent WT control, the expression of which has been shown in Fig. 2.1d. Data are the mean  $\pm$  SEM. (\*\*\*\*p<0.0001, \*\*\*p<0.001, \*p<0.05, one-way ANOVA followed by a Bonferroni multiple comparisons post test).

#### 2.3.4 Expression analysis of *Ido2* and *Tdo2* in the YAC128 mice

Based on transcriptional analysis (Fig. 2.5), *Ido1* and *Ido2* are striatally-enriched and *Tdo2* is enriched in the cerebellum. The expression of each of the three enzymes was assessed using qRT-PCR in YAC128 and wild-type mice for both striatum and cerebellum at 3 months of age. The

analysis of striatal *Ido2* and cerebellar *Tdo2* revealed no significant change and *Ido1* was the only enzyme in the first step of KP, which was differentially expressed in the brain of the YAC128 mouse model of HD (Fig. 2.5 a, b, c). This expression change was specific to the striatum, no increased expression and was found in the cerebellum where the abundance of *Ido1* was negligible in wild-type mice (Fig. 2.5a).



Figure 2.5 The expression of *Ido2* and *Tdo2* is not changed in the brain of YAC128 mice. Transcripts from *Ido1* (a), *Ido2* (b), and *Tdo2* (c) were quantified in the striatum and cerebellum of 3-month-old WT and YAC128 mice using qRT-PCR (n = 4-5 per genotype and tissue). *Ido1* mRNA was the only enzyme with a differential expression in the striatum of YAC128 compared

with that of WT mice (a). No change of expression was observed for any of the enzymes in cerebellum. Data are the mean  $\pm$  SEM. (genotype\*\*\*\*p<0.0001, two-way ANOVA followed by a Bonferroni multiple comparisons post test).

#### 2.3.5 Tryptophan-to-kynurenine ratio is increased in the striatum of YAC128 mice

Having analyzed *Ido1* mRNA expression spatially and temporally, the kyn-to-Trp (kyn/Trp) ratio in YAC128 and wild-type mice was measured using liquid chromatography tandem mass spectrometry (LC/MS/MS), as an estimate of Ido1 and Ido2 enzymatic activity in the striatum and Tdo2 activity in the cerebellum (Fig. 2.6). Plasma kyn/tyrp was also assessed in these mice, as the state of the KP in the periphery can affect the KP in the CNS and several KP metabolites can traverse the blood brain barrier (BBB) (Vécsei et al., 2012). The kyn/Trp ratio had a trend to increased levels in the YAC128 mice at 3 months (p = 0.06) (Fig. 2.6 a) and was significantly increased (p = 0.001) at 12 months (Fig. 2.6 b). No significant change in kyn/Trp was observed in cerebellum at either time points (Fig. 2.6 c, d). There was also a trend toward an increase in kyn/Trp ratio in plasma for YAC128 compared to control at twelve months of age (Fig. 2.6 e). The increases in YAC128 striatal kyn/Trp ratio result from a significant depletion of tryptophan at 3 months and a significant accumulation of kyn at 12 month (Table1). This could indicate that the chronic induction of Ido1 mRNA expression over time in the striatum of YAC128 mice can result in an accumulation of kyn at 12 months which is subsequently used as a substrate for downstream reactions in the pathway.


Figure 2.6 Kyn/Trp ratio is elevated in the striatum of the YAC128 mice. To determine the relationship between relative mRNA expression and enzymatic activity, striatum and cerebellum concentrations of kyn and Trp were measured using LC/MS/MS at 3 and 12 months. (n = 7-12 per genotype and tissue). The ratio of kyn/Trp was determined as a measure of Ido1 and Ido2 enzymatic activity in the striatum (a, b) and Tdo2 activity in the cerebellum (c, d). To compare the central vs. systemic changes of kyn/Trp ratio, kyn and

Trp concentrations were measured in plasma (e). Kyn/Trp ratio in the striatum shows a strong trend towards elevation at 3 months (a), and a significant increase at 12 months (b). Kyn/Trp in plasma shows a trend toward upregulation as well (e) No significant changes were observed in the cerebellum at any of the time points tested (c, d). Data are the mean  $\pm$  SEM. (\*\*\*\**p*<0.001, \**p*<0.05, two-tailed student *t* test).

#### Table 2.2 KP metabolites in brain and plasma

KP metabolites in the striatum and cerebellum at 3 and 12 months and in plasma at 12 months in wild-type and YAC128 mice (Mean  $\pm$  SEM) KP metabolites were measured using LC/MS/MS and the levels are presented as fmoles of metabolite per mg of brain tissue or ml of plasma (\*\*\*\*p<0.0001, \*\*\*p<0.001, \*\*p<0.01, \*p<0.05)

	3 months											
		Striatum				Cerebellum						
Metabolite (fmol/mg)	WT	YAC128	p value	Significance	WT	YAC128	p value	Significance				
Tryptophan	9.25e4 ± 801	5.72e4 ± 3.22e3	0.0003	***	3.60e4 ± 2.18e3	3.79e4 ± 1.93e3	0.5	NS				
Kynurenine	231 ± 24.2	286 ± 69.2	0.4	NS	335 ± 79.7	304 ± 33.1	0.7	NS				
Kynurenic acid	26.8 ± 5.13	22.0 ± 8.00	0.6	NS	7.78 ± 0.715	7.72 ± 0.601	0.9	NS				
Anthranilic acid	22.7 ± 2.93	19.5 ± 3.02	0.4	NS	31.3 ± 2.9	26.1 ± 1.65	0.1	NS				
Xanthurenic acid	12.7 ± 0.58	6.32 ± 0.597	<0.0001	****	3.68 ± 0.546	5.70 ± 1.28	0.09	Trend				
3-hydroxykynurenine	180 ± 17.5	124 ± 11.8	0.01	*	501 ± 62.9	496 ± 47.9	0.9	NS				
3-hydroxyanthranilic acid	6.54 ± 0.9	3.82 ±0.574	0.02	*	31.1 ± 6.07	33.9 ± 2.95	0.67	NS				
Picolinic acid	438 ± 57.0	288 ± 13.7	0.014	*	705 ± 23.9	663 ± 36.9	0.3	NS				
Quinolinic acid	386 ± 178	76.8 ± 30.2	0.09	Trend	49.2 ± 6.4	29.2 ± 1.9	0.005	**				
Nicotinamide	5.45e5 ± 3.48e4	4.54e4 ± 1.03e4	0.1	NS	4.70e4 ± 2.55e3	4.54e4 ± 1.03e4	0.1	NS				
Serotonin	1.46e3 ± 132	2.47e3 ± 82.7	< 0.0001	****	1.73e4 ± 1.81e3	1.53e4 ± 641	0.3	NS				
5-hydroxyindoleacetic acid	4.73e3 ± 489	3.42e3 ± 343	0.03	*	820 ± 46.0	870.0 ± 33.7	0.37	NS				
<b>5</b>	12 months											
	Striatum				Cerebellum				Plasma (fmol/ml)			
Metabolite (fmol/mg)	WT	YAC128	p value	Significance	WT	YAC128	p value	Significance	WT	YAC128	p value	Significance
Tryptophan	4.65e4± 6.95e3	5.02e4 ± 5.06e3	0.7	NS	3.45e4 ± 2.87e3	4.14e4 ± 3.26e3	0.1	NS	2.39e4 ± 1.7e3	2.02e4 ± 1.91e3	0.22	N/S
Kynurenine	194 ± 67.5	347 ± 29.2	0.03	*	535 ± 163	893 ± 184	0.15	NS	949 ± 168	1451 ± 190	0.07	Trend
Kynurenic acid	14.2 ± 1.9	16.5 ± 3.63	0.55	NS	8.66 ± 1.01	8.05 ± 0.683	0.65	NS	204 ± 96.7	50.9 ± 5.39	0.2	N/S
Anthranilic acid	14.3 ± 2.11	13.4 ± 1.92	0.7	NS	9.71 ± 3.02	7.65 ± 1.36	0.6	NS	44.2 ± 19.3	24.0 ± 2.07	0.4	N/S
Xanthurenic acid	6.89 ± 0.9	4.22 ± 1.70	0.27	NS	7.24 ± 0.984	7.12 ± 0.614	0.9	NS	147 ± 104	23.3 ± 2.84	0.4	N/S
3-hydroxykynurenine	80.7 ± 7.25	155 ± 9.22	<0.0001	****	350 ± 100	573 ± 81.0	0.1	NS	88.0 ± 13.2	81.0 ±23.1	0.7	N/S
3-hydroxyanthranilic acid	30.4 ± 7.71	6.46± 1.11	0.03	*	5.69 ± 0.634	6.89 ± 5.69	0.2	NS	14.6 ± 2.02	8.65 ± 0.31	0.05	Trend
Picolinic acid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Quinolinic acid	126 ± 12.6	178 ± 35.8	0.12	NS	44.2 ± 6.55	80.6 ± 8.55	0.002	**	435 ± 109	856 ± 208	0.05	Trend
Nicotinamide	3.7e5 ± 1.03e4	4.01e5 ± 3.61e3	0.04	*	4.25e5 ± 5.01e3	4.05e5 ± 1.26e4	0.08	Trend	3.82e3 ± 372	4.36e3 ± 506	0.4	N/S
Serotonin	1.86e3 ± 223	2.67e3 ± 103	0.018	*	740 ± 77.3	717 ± 59.8	0.8	NS	368 ± 259	62.7 ± 12.1	0.4	N/S
5-hydroxyindoleacetic acid	2.25e3 ±186	2.21e3 ± 217	0.9	NS	688 ± 38.1	679 ± 50.3	0.9	NS	203 ± 61.7	180 ± 24.8	0.8	N/S
5-HTP	124 ± 46.6	147 ± 20.2	0.73	NS	11.1 ± 0.637	14.1 ± 0.926	0.01	*	3.98 ± 4.95	4.08 ± 3.98	0.84	N/S

#### 2.3.6 Kynurenine pathway metabolites in the striatum of 3-month-old YAC128 mice

LC/MS analysis of neuroprotective and neurotoxic metabolites downstream of kyn was performed on the striatum and cerebellum from YAC128 and wild-type mice at 3 months-of-age. No change was observed in the levels of the neuroprotective metabolite kynurenic acid (KA), but a general reduction in the downstream metabolites of the pathway at 3 months, the majority of which are neurotoxic, was observed (Table 2.2). This included reduction in xanthurenic acid (XA), 3-HK, 3- hydroxyanthranilic acid (3-HAA), and quinolinic acid (QA) in YAC128 mice. The majority of changes in these metabolites were present only in the striatum. As shown in Table 2.2, the only metabolite reduced in YAC128 for both striatum and cerebellum at 3 months was QA.

#### 2.3.7 Kynurenine pathway metabolites in the striatum of 12-month-old YAC128 mice

Kynurenine pathway metabolites were assessed using LC/MS in the striatum and cerebellum of 12 month-old wild-type and YAC128 mice. Peripherally generated kyn can pass through the BBB to influence the CNS KP (Vécsei et al., 2012), therefore KP changes in the plasma of these mice were also measured to tease apart the changes that occur systemically versus centrally. A shift in the levels of neurotoxic metabolite 3-HK, from a significant reduction at 3 months (Fig 2.7 a, b) to a significant increase in the striatum of YAC128 mice at 12 months was measured (Fig. 2.7 c, d). 3-HK is a known free radical generator which leads to oxidative stress resulting in neuronal damage (Eastman and Guilarte, 1989; Nakagami et al., 1996; Okuda et al., 1998) whose levels are reported to be elevated in HD brain (Reynolds and Pearson, 1989; Guidetti et al., 2000). Elevated 3-HK change was not measured in the cerebellum and plasma of these mice, suggesting 3-HK-mediated toxicity is specific to the striatum. The increase in striatal 3-HK at 12

months was coincident with a significant elevation of its substrate kyn at this time point (Fig. 2.7

c).



Figure 2.7 Age-dependent and tissue-specific changes of kynurenine and 3-HK in YAC128 mice

To determine the neurotoxic changes following changes in kyn, the concentrations of 3-HK were measured at 3 and 12 months. Following no changes in kyn at 3 months, 3-HK was significantly reduced in the striatum (a). No changes were detected in cerebellum (b). At 12 months, following a significant elevation in kyn levels, 3-HK was significantly increased in the striatum (c). No changes in 3-HK were observed at 12 moths in the cerebellum (d) or plasma (e). The levels are presented as fmoles of metabolite per mg of brain tissue or ml of plasma (n = 7-12 per genotype and tissue), Data are the mean  $\pm$  SEM. (\*\*\*\*p<0.0001, \*\*\*p<0.001, \*p<0.05, two-tailed student *t* test)

### 2.3.8 **3-hydroxyanthranilic acid (3-HAA) levels are decreased in the striatum of YAC128 mice.**

3-HAA is converted to QA through the action of 3-hydroxyanthranilic acid (3-HAO) in brain. Therefore, 3-HAA levels are important determinant for production of endogenous QA in brain. LC/MS analysis of 3-HAA levels in the striatum and cerebellum of 3 and 12-month-old YAC128 and wild-type mice (and the plasma of 12-month-old mice) revealed that 3-HAA is significantly reduced in the striatum of YAC128 mice at both presymptomatic and symptomatic stages of the disease progression (Table 2.2). Reduced 3-HAA levels were also found in plasma from YAC128 mice compared to controls, but were not found in the cerebellum (Table 2.2).

#### 2.3.9 Quinolinic acid levels in the brain and plasma of YAC128 mice

QA is an NMDA receptor agonist produced endogenously through KP and increased levels have been implicated in NMDA receptor-mediated excitotoxic stress in HD. This was initially inferred when intrastriatal injection of QA replicated HD-like striatal degeneration by binding to extrasynaptic receptors and producing axon-sparing lesions in the striatum (Schwarcz et al., 1983; Beal et al., 1986). Using LC/MS/MS, QA was decreased in the brain of 3-month-old YAC128 mice compared to wild-type mice. Interestingly, this change was more pronounced in the cerebellum (p = 0.005) compared with the striatum (p = 0.08) (Fig. 2.8 a, b). KP metabolites were also measured in the striatum, cerebellum, and plasma at 12 months, and elevations were significant in the cerebellum (p = 0.002) (Fig. 2.8 d). There were also a trend toward an increase QA level in the plasma (p = 0.05) at 12 months but changes in the striatum were not significant (p = 0.1) (Fig. 2.8 a, c). These data suggest that unlike increases in 3-HK, increased QA at 12 months is not specific to the striatum, where *Ido1* induction is most obvious. Similarly, QA changes may not be specific to the brain, as there is a strong trend toward elevation in the periphery in symptomatic YAC128 mice (Fig. 2.8 e).



**Fig. 2.8 QA changes are similar across different tissues tested.** QA was measured at 3 months in striatum and cerebellum (a, b) and 12 months in striatum, cerebellum and plasma (c,d,e). There was a reduction in both striatum and cerebellum at 3 months that manifested as a trend towards a decrease in striatum (a) and a significant decrease in cerebellum (b). At 12 months however, the change in QA was shifted toward an elevation displayed as a trend in striatum (c), a significant increase in cerebellum (d) and a strong trend toward an increase in plasma. The levels are presented as fmoles of metabolite per mg of brain tissue or ml of plasma (n = 7-12 per genotype and tissue), Data are the mean  $\pm$  SEM. (\*\*p < 0.01 \*p < 0.05, two-tailed student *t* test).

#### 2.3.10 Serotonin levels are increased in the striatum of YAC128 mice

Within the brain, tryptophan can enter the serotonin pathway in addition to the KP and can act as the precursor for serotonin production. We investigated whether altered levels of Trp at 3 and 12 months in YAC128 striatum can be attributed to changes in serotonin. As shown in table 2.2, serotonin levels are significantly elevated in the striatum of YAC128 mice compared to wild-type mice at both time points, and do not correlate with measured Trp levels. Accordingly this elevation may be due to changes in the decarboxylation step converting 5-HTP to serotonin. Interestingly, elevation of serotonin was only observed in the striatum and is absent in the cerebellum and plasma of YAC128 mice (Table 2.2).

Figure 2.9 depicts a summary of the KP with all the significant metabolite changes measured in the striatum of YAC128 mice at 3 and 12 months.



**Figure 2.9** An illustration of KP including the summary of all the significant changes observed in the striatum at 3 and 12 months

#### 2.4 Summaries and conclusions

The work of this chapter investigated age-dependent and brain-region specific alterations of KP metabolites in the YAC128 mouse model of HD, with a primary focus on the expression and function of the first enzyme in the pathway, Ido1. Most studies investigating KP metabolites in HD have focused on the pathway metabolites downstream of kyn. For example, it was demonstrated that the inhibition of Kynurenine 3-monooxygenase (KMO), the enzyme that converts kyn to 3-HK has the potential to shunt KP metabolism toward enhanced KYNA production with reduced neuronal vulnerability in the R6/2 mouse model of HD (Zwilling et al., 2011). Evidence emphasizing the relevance of *Ido1* in various forms of neurodegeneration has been previously reported. The induction of Idol occurs in Alzheimer's disease brain (Guillemin et al., 2005a) and monocytic cells primed with amyloid ß peptide 1-42 (Yamada et al., 2009). In HD, increased kyn/Trp ratio in the plasma of end-stage patients compared to controls has been reported (Stoy et al., 2005b), consistent with increased IDO1 activity. Genetic inhibition of enzyme Tdo2 was neuroprotective in a *Drosophila* model of HD (Green et al., 2012). These studies all indicate a potential role for the enzyme(s) converting Trp to kyn (Ido1, Ido2, and Tdo2 in mammalian cells) in HD pathology. Accordingly, we measured the temporal and spatial expression of these enzymes and KP metabolite levels in the YAC128 mouse model of HD. In chapter 2, we show for the first time that *Ido1* and *Ido2* mRNAs are striatally-enriched, and that *Tdo2* displays a cerebellar-enriched pattern in mouse brain. Many studies in the past have used kyn/Trp ratio in the whole brain (or forebrain) as a *de facto* surrogate for Ido1 enzymatic

activity and thus, have neglected the region-specific expression of *Ido2* and *Tdo2* in different parts of brain. These findings raise important considerations regarding regional specificity of the three enzymes in the brain, and have implications for these enzymes as potential therapeutic targets in HD. This is particularly important since chemical inhibitors for each of these enzymes are available (Chen and Guillemin, 2009).

Based on our data, within the striatum and under basal conditions, neurons and astrocytes seem to be the major contributors to *Ido1* expression in mouse brain. The kinetic parameter Michaelis–Menten constant ( $K_m$ ) which is a reverse measure of enzymatic affinity for substrate, was previously shown to be different for each of the three enzymes (Yuasa et al., 2009).  $K_m$  values suggest that among the three enzymes, IDO1 has the highest affinity for substrate Trp (Yuasa et al., 2009), supporting a role for IDO1 as the primary enzyme for this step of the KP in the striatum. While many studies have investigated the activation of IDO1 following cytokine stimulation (e.g. interferon gamma) (Pfefferkorn et al., 1986; Werner-Felmayer et al., 1989; Alberati-Giani et al., 1996; Connor et al., 2008; Yamada et al., 2009; O'Connor et al., 2009a), our data indicate that in the absence of exogenous inflammatory stimulation, *Ido1* expression is barely detectable in microglia. This is relevant to HD pathology, keeping in mind that the expression of both *Ido1* and *Kmo* increases markedly in microglia upon immune activation, a phenotype previously described in HD (Guillemin et al., 2003a; 2005b; 2007; Björkqvist et al., 2008).

Consistent with the expression changes of *Ido1* mRNA in the striatum of YAC128 mice, the kyn/Trp ratio was increased only in the striatum and not cerebellum at both presymptomatic and symptomatic stages of disease progression in the YAC128 mice. Induction of *Ido1* in the brain of YAC128 mice is not only specific to the most susceptible brain region in HD and YAC128 mice,

but also occurs before the onset of overt motor symptoms. It is unclear whether depletion of striatal Trp at 3 months is the cause or the result of *Ido1* induction and this is an area of on-going investigation. It is known that *Ido1* transcription is down-regulated by Trp and its analogue (Okamoto et al., 2007). Hypothetically, reduced Trp in the striatum of YAC128 mice as a result of a defect in the transport of this essential amino acid across the BBB would lead to increased *Ido1* transcription. Alternatively, consistent upregulation in *Ido1* transcription within the first three months of life in these animals could cause Trp depletion. The trend toward elevation of kyn and thus increased kyn/Trp ratio in plasma may indicate a cross talk between the periphery and striatum at 12 months occurring through the transfer of Trp and kyn across BBB.

An interesting finding in this study was the reduction in several KP metabolites downstream of kyn in the striatum of presymptomatic YAC128 mice. At 3 months, YAC128 mice are considered presymptomatic since no major motor/behavioural phenotypes have developed (Slow et al., 2003). At 12 months however, these mice display major motor phenotypes and selective neuronal loss (Slow et al., 2003). A recently-described phenotype in the YAC128 mice at 12 months is the inflammatory phenotype which manifests as a result of striatal microglial activation (Franciosi et al., 2012). This phenotype is absent at 3 months in these mice. The YAC128 mouse model also has a biphasic sensitivity to NMDA excitotoxicity (Graham et al., 2009). This biphasic response to excitotoxic stress manifests as an initial sensitivity to intrastriatal QA in presymptomatic mice progressing to resistance, as the disease progresses, in symptomatic animals. The specific line of YAC128 mice used in this study is line 55, which carries the transgene expressing a full-length *HTT* as a homozygote (+/+). This line also demonstrates increased sensitivity to excitotoxicity at ages younger than 12 months of age. This phase is as short as only 1 month in a heterzygous line of YAC128 mice, line 53 (+/-) (Graham et al., 2009)

where some metabolites such as 3-HK and QA are significantly increased at 12 months (Guidetti et al., 2006). In YAC128 line 55 mice, changes in the KP occurring at 3 months included a significant reduction in 3-HK, XA, 3HAA, picolinic acid (PA), and a strong trend toward reduction in QA. While many of these metabolites have important physiological roles in brain, this general reduction of neurotoxic KP metabolites occurs in young YAC128 mice that are intrinsically sensitive to excitotoxic stress.

At 12 months-of-age, YAC128 mice display an obvious motor and neuropathological HD phenotype, develop inflammation in the striatum, and become resistant to exogenous excitotoxic stress (Slow et al., 2003; Van Raamsdonk et al., 2005a; Franciosi et al., 2012). The KP changes at 12 months include *Ido1* induction shown both at mRNA level and increased kyn/Trp ratio (as a result of a significant elevation in kyn levels). The significant increase in striatal 3-HK at 12 months may be a consequence of an inflammatory environment secondary to increased microglial activity in the striatum at this time point (Franciosi et al., 2012). Unlike resting microglia, activated microglia produce elevated levels of IDO1 and KMO (Guillemin et al., 2007). These activated cells are capable of taking up the striatal kyn produced by other cell types such as astrocytes and neurons at 12 months and catabolizing it into neurotoxic metabolites such as 3-HK and QA. This state of ongoing intrinsic NMDA excitotoxic stress may prime MSNs of the YAC128 mice and make them more resistant to exogenous toxic NMDA receptor stimulation. It is estimated that  $\sim 40\%$  of the total brain kyn is synthesized in the brain, and the rest comes from plasma (Gál and Sherman, 1980). Tryptophan and 3-HK are other KP metabolites that can cross BBB and enter the brain (Vécsei et al., 2012). In this study, we analyzed KP in the plasma of symptomatic mice and concluded that the changes observed in YAC128 striatum are either absent in plasma (e.g. 3-HK) or display similar trends in plasma but do not reach a statistical

significance (e.g. elevations in kyn/Trp ratio and kyn concentrations). Consistent with YAC128 line 53 and other mouse models of HD (Guidetti et al., 2006), the levels of 3-HK and QA were increased at 12 months in the YAC128 line 55 mice. While the elevation in 3-HK seems to be present only in the striatum, the changes in QA seem to be also present in cerebellum and plasma. Minor increases in plasma kyn and kyn/Trp could potentially contribute to the increase in the pool of kyn in the striatum where it can get converted to 3-HK in a region-specific manner. 3-HAA was consistently lower in the striatum of YAC128 mice compared with that of wild-type mice at both 3 and 12 months. Significant reduction in 3-HAA was also observed in the plasma of YAC128 mice, reminiscent of lower 3-HAA levels in the plasma of HD patients (Stoy et al., 2005b). Increased QA despite low 3-HAA levels may be secondary to increased 3-HAO enzymatic activity, a change that has been previously described in HD brain (Schwarcz et al., 1988). Interestingly, no changes in the levels of the neuroprotective metabolite KA were found in the brain or plasma of YAC128 mice, suggesting that even in the striatum, chronic *Ido1* induction *in vivo* does not lead to increased KA concentrations.

Finally, we were able to demonstrate that changes in striatal serotonin levels are likely to be independent of tryptophan levels. This is consistent with previous findings that IDO1 activation shifts Trp metabolism from the serotonin pathway toward KP (Vécsei et al., 2012). Serotonin does not pass through the BBB (Vécsei et al., 2012), and the elevated levels in YAC128 mice measured in our study are likely generated within the striatum. *Ido1* induction and decreased IDO1-mediated serotonin levels have been implicated in human depression studies (Kohl and Sperner-Unterweger, 2007) and depressive-like behaviour in mice (Connor et al., 2008; O'Connor et al., 2009b; Kim et al., 2012). YAC128 mice have been shown to develop a depressive-like behaviour, similar to the symptoms of depression in HD patients (Pouladi et al., 2009). Unlike

many depression models in which reduced serotonin levels are observed, serotonin was significantly increased in the striatum of YAC128 mice.

In conclusion, in this part of the thesis, we identified age-dependent biphasic alterations in metabolites of the Kynurenine pathway in the YAC128 mouse model with the majority of changes occurring in the striatum. These changes followed an induction of the first step of the pathway (IDO1) in the striatum of both presymptomatic and symptomatic YAC128 mice, and displayed a shift from reduced to increased neurotoxic metabolites compared with wild-type mice. Altered kyn/Trp ratio was present in the striatum and absent in the cerebellum providing clues regarding the KP state in vulnerable brain regions in HD. This study demonstrates a potential imbalance between neuroprotective and neurotoxic KP metabolites in the YAC128 mouse model, providing insight into the involvement of the KP in HD.

## CHAPTER 3: THE ABSENCE OF INDOLEAMINE 2,3 DIOXYGENASE EXPRESSION PROTECTS FROM NMDA RECEPTOR-MEDIATED EXCITOTOXIC STRESS IN MOUSE BRAIN

#### 3.1 Introduction

*N*-methyl-*D*-aspartate (NMDA) receptor-mediated excitotoxicity is a well-established mechanism in Huntington disease. NMDA receptors are ionotropic glutamate receptors involved in synaptic plasticity in brain. Under normal conditions, the binding of glutamate to these receptors results in a voltage-dependent activation, which subsequently leads to the removal of magnesium blocks from these ion channels. This results in a flow of sodium and calcium into the neurons and potassium out of the neurons. Within the cortico-striatal pathway, NMDA receptors in medium spiny neurons of the striatum receive input from the glutamatergic neurons of the cortex. Previous studies have robustly demonstrated that the over-activation of these receptors plays an important role in the cellular processes involved in neuronal death in HD (Levine et al., 1999; Zeron et al., 2004). Enhanced excitotoxicity as a result of increased NMDA receptormediated currents have also been demonstrated in several HD models early in the pathogenesis (Levine et al., 1999; Cepeda et al., 2001; Zeron et al., 2004; Graham et al., 2006). It was also found that while stimulation of synaptic NMDA receptors renders protection from neurotoxicity in neurons, the excitation of the extrasynaptic NMDA receptor is a key pathogenic mechanism in neurodegeneration (Okamoto et al., 2009). Intrastriatal injection of NMDA receptor agonists such as quinolinic acid (QA) into the mouse brain has been shown to lead to selective degeneration of medium spiny neurons in the striatum reminiscent of striatal degeneration in HD (Schwarcz et al., 1983). Injections of exogenous QA have been used for decades for the generation of chemical models of HD (Schwarcz et al., 1983; Beal et al., 1986). Administration of QA has been shown to reproduce patterns of cell loss similar to that observed in HD. Intrastrial injection of QA also recapitulates the progressive course of degeneration of MSNs in the striatum while selectively sparing the aspiny neurons in this region (Schwarcz et al., 1983; Beal et al., 1986). Additionally, the levels of endogenous QA, a neurotoxic metabolite of the kynurenine pathway, is elevated in the brain and plasma of HD patients and mouse models (Guidetti et al., 2004; 2006). This was also confirmed by our study and has been described in chapter 2 of this thesis.

As described in chapter 1, Indoleamine 2,3 dioxygenase (IDO1) is the first and the ratelimiting enzyme of the kynurenine pathway. It is a heme-containing enzyme that catalyzes the conversion of the essential amino acid L-tryptophan (Trp) to N-formylkynurenine and then Lkynurenine (Kyn) through the oxidative cleavage of the indole ring of Trp (Higuchi and Hayaishi, 1967) . IDO1 has been implicated in neuropathology of diseases such as amyotropic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) stroke, cerebral malaria, HIV-induced dementia, and schizophrenia (Levine et al., 1999; Cepeda et al., 2001; Zeron et al., 2004; Graham et al., 2006; Wonodi and Schwarcz, 2010; Sathyasaikumar et al., 2011) (Sardar et al., 1995; Huengsberg et al., 1998; Baran et al., 2000; Widner et al., 2002; Clark et al., 2005; Guillemin et al., 2005a; Walker et al., 2006; Darlington et al., 2007; Okamoto et al., 2009; Yamada et al., 2009; Bonda et al., 2010; Chen et al., 2010; Fu et al., 2011; Szabó et al., 2011).

We previously showed that *Ido1* levels are chronically elevated in the striatum of YAC128 mouse model of HD. This is consistent with the finding that *Ido1* is induced in the plasma of HD patients (Stoy et al., 2005b). The chronic induction of *Ido1* in the striatum of YAC128 mice throughout development was followed by increased production of neurotoxic metabolites such as 3 hydroxykynurenine (3-HK) and QA in symptomatic animals. We therefore hypothesized that the chronic induction of *Ido1* expression and activity in the striatum of YAC128 mice leads to

increased neurotoxicity in this mouse model; based on this hypothesis, we would predict that absence of *Ido1* expression would result in decreased sensitivity to neurotoxicity in mice. To test this prediction, we sought to assess the sensitivity of brain (particularly the striatum) to excitotoxic insult in the absence of *Ido1* expression in the brain of constitutive *Ido1* null mice (*Ido<sup>-/-</sup>*). These mice were developed by replacing *Ido1* exons 3-5 (encode critical portions of the enzyme catalytic site) with the beta-galactosidase and neomycin resistance genes as well as a translational stop codon which was inserted into exon 2 (Mellor and Munn, 1999).

The work described in this chapter will encompass the characterization of *Ido*<sup>-/-</sup> striatum in terms of enzymatic expression and activity in the first step of the pathway. The other KP metabolites as well as a few serotonin pathway metabolites were also measured in the striatum of *Ido*<sup>-/-</sup> mice. Finally, the striatal sensitivity to NMDA-receptor-mediated excitotoxicity was assessed in these mice.

#### 3.2 Methods

#### 3.2.1 Mice

Animals of both genotypes (WT and Ido<sup>-/-</sup>) used in this study were 3-month-old adult mice. All mice used in this study were on the C57BL/6 strain background. Mice were group-housed with a normal light–dark cycle and given free access to food and water. Mice were euthanized in accordance with our institutional animal care guidelines and all experiments were approved by the University of British Columbia's Committee for Animal Care. No perfusion was performed on animals and dissected brain regions for RNA extraction and LC/MS/MS were immediately snap-frozen at -80 °C. The chart below is the summary of mouse gender distribution for each figure/experiment in our study.

 Table 3.1
 Gender distribution for figure/experiment in chapter 3

	W	Т	Ido-/-		
Figure/experiment (chapter 3)	Male	Female	Male	Female	
3.1	2	2	2	2	
3.3	2	3	2	3	
mass spec	2	3	2	2	

#### 3.2.2 Intra-striatal injection of quinolinic acid

Quinolinic acid (QA; Sigma) was dissolved into 0.1M PBS. Three-month-old mice of mixed sex were anesthetized by inhalation of 4% isoflurane with 1% oxygen and positioned in a stereotaxic frame. Mice received unilateral intrastriatal injections of 6 nmol QA dissolved in 0.5ml PBS (7-11 mice/genotype). Coordinates of the injection site are as follows: +0.8mm anterior to Bregma, 1.8mm mediolateral, and 3.5mm dorsoventral to the neocortex.

#### 3.2.3 LC/MS/MS

See methods in chapter 2.

#### 3.2.4 Quantitative analysis

#### Fluorojade B staining for lesion analysis

Fluorojade B (FJB; Millipore, Temecula, CA) staining was performed in serial 25um coronal sections to evaluate cell death after QA injection. For QA, every 8<sup>th</sup> section through the entire striatum (Bregma +1.10mm to Bregma -0.94mm, total of 15 sections) was mounted on a glass slide and processed for FJB staining. Staining was performed according to the manufacturer's suggested protocol. All slides were processed in parallel for each set of injections.

#### Lesion quantification and neuronal cell counts

FJB-positive lesions in QA-injected sections were quantified using Stereoinvestigator (Microbrightfield). All stereology was performed blinded to genotype and treatment. In a set of adjacent sections, the number of NeuN-positive neurons in the striatum was estimated using the Optical Fractionator method (400 x 400mm grid, 25 x 25mm probe). Because FJB staining in adjacent sections showed lesions only in the anterior part of the striatum, counting was performed on every 6<sup>th</sup> section in a total of 5 sections from Bregma 0.98mm to Bregma -0.10mm.

#### 3.2.5 **Primary cell culture**

#### Isolation of primary microglia and astrocytes

Primary microglia and astrocytes were cultured from IDOKO and WT C57BL/6N p0-p3 pups. Brains were dissected in ice cold HBSS (Hyclone), and the cortex and striatum were isolated. The tissue was then triturated through a 5mL pipette 3-5 times, and again 3-5 times with a 200uL tip added on the end in a 15mL falcon tube. The tubes were spun at 200g for 5 minutes. The supernatant was removed and the pellets were resuspended in 5mL warm DMEM (Hyclone) containing 10% FBS (PAA), 1% L-glutamine (Hyclone) and 1% penicillin/streptomycin solution (Hyclone) and transferred to a T150 flask containing 15mL of complete DMEM. Media was changed the day following culture and once a week thereafter.

At 17-21 days in culture, the culture flasks were tapped about 20 times to detach the microglia. The culture media was collected in 50mL falcon tubes and spun at 200g for 10 minutes. The conditioned media (supernatant) was reserved for plating of microglia and astrocytes. Microglial pellets were resuspended in conditioned media. The number of cells was counted, and 1mL of cells was plated in each well at a density of 124 000 cells/mL in Primeria 24-well plates (Falcon).

After removal of culture media containing microglia, flasks were washed twice with 10mL of serum-free DMEM and 12mL of trypsin mixture (1 part 0.25% trypsin (Hyclone) to 3 parts serum-free DMEM) was added to remove the remaining astrocyte layer. The flasks were then incubated for 30-45 minutes at 37°C. The cell suspension was collected in a 50mL falcon tube and spun at 200g for 10 minutes. The supernatant was removed completely and the astrocyte pellet was resuspended in same conditioned media saved during microglia isolation. The astrocytes were further resuspended by triturating 3 times through a 5mL pipette and another 3 times with a 200uL tip added to the end. Finally, the suspension was passed through a sterile 70µm filter. Cells were counted using a hemocytometer, and 1mL of cells was plated at a density of 64 000 cells/mL in Primeria 24-well plates (Falcon).

#### Primary culture of medium spiny neurons

Embryos were collected from E15.5 C57BL/6N mice, and brains were removed and dissected in ice-cold HBSS. The striatum was isolated and kept in ice-cold Hibernate-E (without Ca<sup>2+</sup>, BrainBits) for the duration of the dissections. The striata from approximately 10 pups were pooled into one 15mL falcon tube. The tissue was then washed twice with HBSS and trypsinized

for 15 minutes at 37°C in 3mL of a 0.05% trypsin (Hyclone) solution in HBSS containing 80-90U/mL of DNaseI (Life Technologies). The trypsinization reaction was stopped by the addition of 3mL of 20% FBS in HBSS (Hyclone). The tissue was triturated 5 times through a 5mL pipette, and another 5 times through the same pipette with a 200uL tip added. The cell suspension was passed through a 70µm filter and spun at 200g for 5 minutes in a 15 mL falcon tube. The supernatant was removed and pellet was resuspended in 5 mL of HBSS and spun again for 5 minutes. After removing the supernatant, the cells were resuspended in warm neuronal growth media: Neurobasal A (Life Technologies) containing 2% B-27 supplement (Life Technologies), 1% penicillin/streptomycin (Hyclone) and 0.5mM L-glutamine (Hyclone). Cells were counted and 1mL of cells were plated at a density of 175 000 cells/well in a 24-well plate with a 12mm glass coverslip (James International Sales) coated for 4 hours with 50µg/mL poly-D-lysine (Sigma). Cells were maintained at 37°C in humidified air containing 5% CO<sub>2</sub> with half media changes every 3-4 days.

#### Co-culture of primary MSNs with glia and NMDA stimulation

Mice were paired for microglial/astrocyte culture 2.5 weeks ahead of mice paired for embryonic neuronal culture. At 17-21 days *in vitro* (DIV), microglia and astrocytes were plated (see above). The following day, conditioned media was removed and replaced with 500uL of fresh neuronal growth media. Using sterile forceps, one coverslip with MSNs at DIV8 was transferred to each well containing glia, astrocytes, or no cells (control). Neurons were facing up. Six hours after transfer, half of all wells were stimulated by the addition of 1mM NMDA and incubated for 24 hours at 37°C.

#### 3.2.6 LDH cytotoxicity assay

Cytotoxicity detection kit Lactose dehydrogenase - LDH (Roche) was used as a measure of cell cytotoxicity. Cells release the enzyme lactose dehydrogenase (LDH) into the media when their membrane integrity is compromised. LDH activity was measured in the cell-free neuronal supernatant according to the manufacturer's instructions. As the high control, 20% TritonX was added to two wells of neurons one hour before the start of the assay and the amount of LDH activity was considered as max cell death after one hour. The data is presented as percent max cell death.

#### 3.2.7 MTT reduction test

For determination of cytotoxicity in co-culture and isolated primary neurons, 0.5mg/ml MTT (Sigma) was added to the co-culture and isolated primary neuron media four hours prior to the end of the 24 hr-stimulation period and were maintained at 37°C in humidified air containing 5% CO<sub>2</sub>. 24 hrs post stimulation (4 hours after MTT addition), all coverslips bearing neurons were placed in a new dish, and the reaction was stopped by the addition of 500 µl of DMSO. The amount of reduced MTT (formazan) solubilized in DMSO was then determined spectrophotometrically using absorbance at 570 nm (PolarStar Omega Multimode Microplate Reader). Results are expressed as OD reads at 570 nm wavelengths.

#### RESULTS

Before assessing the sensitivity of the mouse brain in the absence of *Ido1* expression, we performed some characterization on enzymatic expression and activity in the brain of *Ido<sup>-/-</sup>* mice. First, we sought to measure the expression of other enzymes facilitating Trp to kyn conversion to analyze whether there are any compensatory expression changes of these enzymes in the absence of Ido1 expression. Secondly, we investigated the effect of loss of Ido1 on KP and serotonin pathway metabolites. For this reason, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure these metabolites in the wild-type and *Ido<sup>-/-</sup>* mice.

# 3.2.8 Expression analysis of *Ido1*, *Ido2* and *Tdo2* in the striatum and cerebellum of *Ido<sup>-/-</sup>* mice

The reaction of Trp to *N*-formylkynurenine conversion is considered the first and the ratelimiting step of the kynurenine pathway and is catalyzed by one or more of the three enzymes Ido1, Indoleamine 2,3 dioxygenase (Ido2), and Tryptophan 2,3 dioxygenase (Tdo2) throughout the body. First, we confirmed the presence of *Ido1* mRNA only in the striatum and not in the cerebellum and subsequently showed that its expression is completely abolished in *Ido<sup>-/-</sup>* striatum (Fig. 3.1a). *Ido2* is considered the paralog of *Ido1* and is located adjacent to *Ido1* on both human and mouse chromosome 8. Apart from Ido2 structural and functional resemblance to Ido1, not much is known about the role of this recently discovered enzyme in brain (Higuchi and Hayaishi, 1967; Ball et al., 2007; Yuasa et al., 2009). Based on our findings described before, *Ido2* was only found in the striatum and not in the cerebellum of wild-type animals (Fig. 3.1b). To assess whether the lack of *Ido1* expression and activity are compensated by *Ido2*, we measured *Ido2* mRNA expression in the striatum and cerebellum of *Ido<sup>-/-</sup>* mice. We found that not only is *Ido2*  expression not upregulated, but it appears that its expression is also abolished in the striatum and cerebellum of  $Ido^{-/-}$  mice (Fig. 3.1b). These data suggest for the first time that *Ido2* is not expressed in the brain of *Ido1* knockout mice and that *Ido1* and *Ido2* have very similar expression patterns in the brain of wild-type and *Ido<sup>-/-</sup>* mice. We propose that these knockouts are likely to be *Ido1* and *Ido2* deficient at least in the striatum and this must be considered when utilizing these mice in research. Tdo2 was originally described as a primarily hepatic enzyme but there has been increasing evidence for an involvement in the brain and brain pathophysiology (Kanai et al., 2009; Ravishankar et al., 2012; van der Goot et al., 2012). Similar to *Ido2*, the expression pattern of *Tdo2* mRNA was investigated in the striatum and cerebellum of wild-type and *Ido<sup>-/-</sup>* mice. As expected, *Tdo2* was predominantly expressed in the cerebellum where its expression did not differ in wild-type and *Ido<sup>-/-</sup>* mice (Fig. 3.1c). Overall, these data demonstrate that the lack of *Ido1* expression in the brain of *Ido<sup>-/-</sup>* animals was not compensated by alterations in the mRNA expression of other enzymes of tryptophan-degradation.



Fig 3.1 The expression of *Ido2* mRNA is abolished in *Ido<sup>-/-</sup>* striatum. *Tdo2* is not changed in the striatum or cerebellum of the *Ido<sup>-/-</sup>* mice. Transcripts from *Ido1* (a), *Ido2* (b), and *Tdo2* (c) were quantified in the striatum and cerebellum of 3-month-old WT and *Ido<sup>-/-</sup>* mice using qRT-PCR (n = 4-5 per genotype and tissue). *Ido1* mRNA was absent in the striatum of *Ido<sup>-/-</sup>* mice and 84

in the cerebellum of both genotypes (a). *Ido2* mRNA was also absent in the striatum of *Ido<sup>-/-</sup>* mice and in the cerebellum of both genotypes (b). *Tdo2* mRNA was equally expressed in the cerebellum of WT and *Ido<sup>-/-</sup>* mice. Data are the mean  $\pm$  SEM. (genotype\*\*\*\**p*<0.0001, two-way ANOVA followed by a Bonferroni multiple comparisons post test).

#### 3.2.9 The effect of Ido deficiency on the levels of the KP downstream metabolites

Having analyzed *Ido1, Ido2*, and *Tdo2* mRNA expression in *Ido<sup>-/-</sup>* brain tissues, the kyn-to-Trp (kyn/Trp) ratio in the striatum of *Ido<sup>-/-</sup>* and WT mice was measured using liquid chromatography tandem mass spectrometry (LC/MS/MS). The other KP metabolites were assessed using LC/MS/MS in the striatum of *Ido<sup>-/-</sup>* mice and were compared to that of WT mice. Table 3.2 summarizes the levels of the majority of KP and a few of serotonin pathway metabolites in the striatum of *Ido<sup>-/-</sup>* and WT mice. Kyn/Trp was significantly lower in the striatum of *Ido<sup>-/-</sup>* mice but was not completely depleted (Fig. 3.2 a). Among the downstream neuroactive metabolites, the free radical generator 3-HK and XA were significantly reduced in the striatum of Ido<sup>-/-</sup> mice (Fig.3.2 c, d). QA and KA levels were not changed significantly (Fig.3.2 b, e) in the striatum of these mice.

### Table 3.2 KP and SP metabolites in the striatum of WT and *Ido<sup>-/-</sup>* mice

Metabolite (fmol/mg)	WT	Ido-/-	p value	Significance	
Tryptophan	3.85e4 ± 9.9e3	3.4e4 ± 4.70e3	0.7	NS	
Kynurenine	88.7 ± 15.0	$59.1 \pm 5.86$	0.09	Trend	
Kynurenic acid	$14.8 \pm 1.61$	$50.5 \pm 50.7$	0.4	NS	
Anthranilic acid	$6.05 \pm 0.348$	$5.33 \pm 0.515$	0.2	NS	
Xanthurenic acid	$8.12 \pm 0.834$	2.39±1.54	0.016	*	
3-hydroxykynurenine	$154 \pm 9.85$	97.3±20.4	0.038	*	
3-hydroxyanthranilic acid	6.74±0.94	$5.53 \pm 0.818$	0.3	NS	
Picolinic acid	$89.2 \pm 9.58$	86.4±7.30	0.8	NS	
Quinolinic acid	79 ±10.9	62.5±8.66	0.22	NS	
Nicotinamide	$4.22e5 \pm 1.45e4$	4.80e5 ± 2.87e4	0.11	NS	
5-hyroxytryptophan	94.3 ± 14.7	$68.2 \pm 41.6$	0.51	NS	
Serotonin	2.1e3 ± 115	2.32e3 ± 72.5	0.18	NS	
5-hydroxyindoleacetic acid	$1.6e3 \pm 156$	$1.65e3 \pm 211$	0.9	NS	



**Figure 3.2 Examples of the KP metabolites in the striatum of** *Ido*<sup>-/-</sup> **mice.** Kyn/Trp ratio is significantly decreased in the striatum of  $Ido^{-/-}$  mice (a). 3-HK and XT are the only downstream metabolites of the pathway that are significantly reduced (see table 3.2) (c,d). No significant change was observed in KA and QA levels (b, e). The levels are presented as fmoles of metabolite per mg of brain tissue (*n* =3-4 per genotype). Data are the mean ± SEM. (\**p*<0.05, two-tailed student *t* test)

# 3.2.10 The striatum of *Ido<sup>-/-</sup>* mice is less sensitive to NMDA receptor-mediated excitotoxic stress induced by QA.

In order to understand the effect of *Ido1* induction in the brain of YAC128 mice, we asked whether lack of *Ido1* expression and activity would affect the susceptibility of neurons to NMDA receptor-mediated excitotoxic stress. To determine this we assessed the vulnerability to intrstriatal injections of OA in  $Ido^{-/-}$  mice compared to their WT counterparts. As a first approach. we used Fluoro-Jade B (FJB) to stain QA-induced degenerating neurons in the striatum of WT and  $Ido^{-/-}$  mice. This revealed a trend toward smaller OA-induced lesion volume in the striatum of  $Ido^{-/-}$  mice. which approached statistical significance (Fig. 14 a,b, p = 0.07). As a secondary approach, we used neuronal nuclear antigen (NeuN) to stain and count live neurons in the striatum of mice used in the FJB experiment. Consistent with the trend observed in the FJB experiment, we could show a significantly greater number of striatal neurons in *Ido<sup>-/-</sup>* mice (Fig. 14 c.d. p = 0.003) compared to WT mice. In order to rule out the potential baseline genotypic difference as an explanation for the observed neuronal cell count difference in the two mouse strains, we also counted neurons in the brain of  $Ido^{-/-}$  and WT animals at baseline (i.e. with no injections). Our data indicates that there is no baseline difference in the number of striatal neurons in *Ido<sup>-/-</sup>* and WT mice (data not shown). These data suggest that the absence of *Ido1* and Ido2 expression in vivo decreases neuronal sensitivity to NMDA receptor-mediated excitotoxicity induced by QA in the striatum.



Figure 3.3.  $Ido^{-t-}$  striatum is less sensitive to NMDA receptor-mediated excitotoxic stress induced by QA injection. Two endpoint measures were used to assess the susceptibility of  $Ido^{-t-}$ striatum to intrastriatal injection of QA in 3-month-old WT and  $Ido^{-t-}$  mice. (a) Quantification of lesion volume demonstrates a trend toward enhanced lesion volume in WT compared with  $Ido^{-t-}$ striata (p=0.07). Figure (b) is a representative image of the lesion area in each genotype shown by Fluorojade B staining. Counting the NeuN-positive neurons revealed a significant decrease in the number of live cells in WT striatum compared with  $Ido^{-t-}$  (p=0.003) (c). Figure (d) is a representative image depicting the lack of NeuN-staining in the lesion area. Lesion volume and mean number of NeuN-positive cell is Mean ± SEM. (\*\*p<0.01, two-tailed student *t* test).

### 3.2.11 Isolated *Ido<sup>-/-</sup>* primary striatal neurons are more sensitive to NMDA receptormediated excitotoxicity.

To determine whether reduced sensitivity of  $Ido^{-/-}$  striatum to NMDA receptor-mediated excitotoxic stress observed *in vivo* could be replicated *ex vivo*, we assessed the sensitivity of  $Ido^{-/-}$  isolated striatal neurons at baseline and upon stimulation with NMDA. Using the LDH cytototoxicity assay and MTT viability assay, we found that  $Ido^{-/-}$  primary striatal neurons isolated from E16.5 embryos were more sensitive to NMDA compared to WT neurons at both baseline and upon 500 µM NMDA excitotoxic stress. Primary embryonic striatal neurons from  $Ido^{-/-}$  brains were much more sensitive to NMDA-induced toxicity caused by 500 µM NMDA compared to the brain of WT mice (Fig. 14). These data suggest that unlike the *in vivo* findings that displayed less sensitivity to NMDA receptor-mediated excitotoxicity in the striatum of  $Ido^{-/-}$ mice, isolated neurons from these animals seem to be more sensitive at baseline and upon neurotoxic insult.



Figure 3.4. Primary striatal neurons isolated from  $Ido^{-/-}$  embryos are more sensitive to NMDA-induced toxicity. Striatal neurons from WT and  $Ido^{-/-}$  E16.5 embryos were treated with 500  $\mu$ M of NMDA for 24 hours. The figure on the left exhibits the amount of lactose

dehydrogenase (LDH) secreted into the media by dying cells, which is presented as the percent maximum cell death (measured as LDH secreted into the media an hour post 20% TritonX treatment). The figure on the right shows the results of the MTT assay as a measure of activity of living cells via mitochondrial dehydrogenases. The results represent two independent experiments. Data are the mean  $\pm$  SEM. (\*\*p<0.01, \*p<0.05, two-way ANOVA followed by a Bonferroni multiple comparisons post test).

# 3.2.12 No increased sensitivity to NMDA was observed in WT neurons co-cultured with glia; A slight hint for the action of microglial genotypic differences

To address the discrepancy that exists between the *in vivo* and *ex vivo* data, we hypothesized that  $Ido^{-/-}$  glia, including microglia and/or astrocytes, are responsible for the decreased sensitivity of  $Ido^{-/-}$  neurons to NMDA receptor-mediated excitotoxicity in the brain of  $Ido^{-/-}$  animals. This hypothesis is built upon previous findings that activated microglia produce QA through induction of Ido1 and kynurenine 3-hydroxylase (KMO) (Heyes et al., 1996; Thomas and Stocker, 1999; Guillemin et al., 2005b; Munn and Mellor, 2007). Accordingly,  $Ido^{-/-}$  microglia may be intrinsically less toxic than WT microglia. Astrocytes have also been shown to protect neurons from NMDA receptor-mediated toxicity through QA uptake (Vécsei et al., 2012). It is possible that  $Ido^{-/-}$  astrocytes *in vivo* would have the ability to protect neurons, more efficiently than their WT counterparts resulting in less sensitivity of  $Ido^{-/-}$  striatum to neurotoxic stress. To test these hypotheses, we asked the question of whether sensitivity of neurons to NMDA is modified as a result of co-culturing with glia and whether there is a genotypic difference in the glial effect on neurons.

We conducted co-culture experiments to assess the effect of *Ido<sup>-/-</sup>* glia on neuronal viability. For this reason, WT neurons were co-cultured with astrocytes and microglia isolated from the forebrain of WT and  $Ido^{-/-}$  neonatal mice (as described in methods). These co-cultures were treated with NMDA for 24 hours and the sensitivity of these neurons was assessed at basal levels and upon NMDA-receptor mediated excitotoxic using the MTT assay. The data show that unlike the scenario of isolated WT neurons, there is no increased sensitivity to NMDA stimulation in WT neurons co-cultured with either microglia or astrocytes (Figs. 3.5a and 3.6a). Analysis of figure 3.5b demonstrates that viability of neurons has been affected by the microglia genotype. The statistical difference (using two-way ANOVA) between the effects of microglia from the two genotypes may have resulted from the differences at baseline. There is no difference between the effects of treatment in the two groups. Figure 3.6 b demonstrates that there is no effect of astrocyte genotype or treatment on the viability of neurons. These data suggests that co-culturing neurons with glia from WT or Ido-/- brains does not result in increased sensitivity to NMDA unlike the increased sensitivity observed in Ido-deficient neurons. There is some evidence that *Ido<sup>-/-</sup>* microglia may have a protective effect on neurons, as we observed that neurons co-cultured with  $Ido^{-/-}$  microglia are more viable at baseline (Fig. 3.5 b).



Figure 3.5 Impact of microglia on neuronal viability in co-culture at baseline and upon NMDA stimulation. Isolated striatal neurons at DIV8 were exposed to microglia or media only (WT neurons) (a) for six hours. After six hours, the co-cultures and the neurons only were stimulated with 1000  $\mu$ M NMDA for 24 hours. Neuron coverslips were removed and MTT was done as a measure of cell viability (measured at 570 nm). Figure (a) shows that unlike neurons alone, there is no increased sensitivity to NMDA stimulation in WT neurons co-cultured with microglia from either genotype. Figure (b) demonstrates that difference in the viability of neurons co-cultured with WT vs  $Ido^{-/-}$  microglia at baseline, but there is no NMDA treatment effect or interaction. Data are the mean  $\pm$  SEM. (\*p<0.05, two-way ANOVA followed by a Bonferroni multiple comparisons post test).




**Figure 3.6 Impact of astrocytes on neuronal viability in co-culture at baseline and upon NMDA stimulation.** Isolated striatal neurons at DIV8 were exposed to astrocytes or media only (WT neurons) (a) for six hours. After six hours, the co-cultures and the neurons only were stimulated with 1000 μM NMDA for 24 hours. Neuron coverslips were removed and MTT was done as a measure of cell viability (measured at 570 nm). Figure (a) shows that unlike neurons alone, there is no increased sensitivity in WT neurons co-cultured with astrocytes from either genotype. Figure (b) demonstrates no difference in the viability of neurons exposed to WT vs

*Ido*<sup>-/-</sup> astrocytes. Data are the mean  $\pm$  SEM. (\*p<0.05, two-way ANOVA followed by a Bonferroni multiple comparisons post test).

### 3.3 Discussion

The work in this chapter was developed as a follow-up to the second chapter where we discovered a chronic induction of expression and activity of *Ido1* in the striatum of YAC128 mice. To follow up on this finding, we hypothesized that *Ido1* induction is involved in HD pathogenesis and therefore, *Ido1* deficiency may decrease neuronal sensitivity to toxicity in the brain. We particularly focused on NMDA receptor-mediated excitotoxicity, a well-established mechanism in the pathogenesis of HD (Levine et al., 1999; Zeron et al., 2004). Surprisingly, we observed that both *Ido1* and *Ido2* transcripts were not expressed in the striatum of *Ido<sup>-/-</sup>* mice. This can be explained by the high degree of sequence similarity between *Ido1* and *Ido2* genes (Yuasa et al., 2009), which potentially led to targeting of both genes in the knockouts. In addition, the mRNA level of *Tdo2*, the enzyme normally expressed at very low levels in the striatum, did not change in either striatum or cerebellum of *Ido<sup>-/-</sup>* mice.

Since *Ido1* and *Ido2* are the dominant enzymes catalyzing the conversion of Trp to kyn in the striatum, we sought to analyze the effect of the absence of expression of these two genes on downstream metabolites in the striatum of *Ido<sup>-/-</sup>* mice. As expected, analysis of kyn/Trp revealed a significant decrease of the ratio in the striatum of YAC128 mice. This ratio however, was not completely depleted indicating the presence of alternative mechanisms facilitating the reaction in the striatum. Additionally, one must keep in mind that ~60% of brain kyn is supplied from the peripheral system (Gál and Sherman, 1980). A portion of this kyn is produced by Tdo2 catalysis in the periphery and can enter the striatum through the BBB. This peripheral kyn prevents the

kyn/Trp ratio in the striatum from reaching zero. Additionally, despite the absence of *Ido1* expression, there was no accumulation of Trp in the striatum of *Ido<sup>-/-</sup>* mice indicative of alternative mechanism(s) involved in Trp homeostasis in the striatum.

Intrastriatal injection of the NMDA receptor agonist, QA, was used in this study to induce excitotoxicity in the brain of *Ido*<sup>-/-</sup> mice. We showed that these mice are less sensitive to QA compared to their WT counterparts. Having measured the downstream metabolites of the KP, no change of KA and endogenous QA were observed in the striatum of Ido -/- mice, suggesting that synthesis of these metabolites in the striatum is likely to be independent of *Ido1* and *Ido2* expression. 3-HK however was significantly reduced in the striatum of *Ido<sup>-/-</sup>* mice. 3-HK is an endogenous oxidative stress generator which is known to increase in the brain of HD patients and mouse models of HD (Guidetti et al., 2006; Vécsei et al., 2012). 3-HK causes neuronal cell death with apoptotic features and region selectivity to the striatum (Sapko et al., 2006). Whether reduction in 3-HK level facilitates neuroprotection in the striatum of these mice needs to be further investigated.

Unlike the reduced sensitivity to QA observed *in vivo*, isolated striatal neurons from *Ido*<sup>-/-</sup> embryos were more sensitive to cell death than WT neurons both at baseline and upon NMDA stimulation. To address this discrepancy, we sought to generate micro-environments which partially mimicked the *in vivo* settings. For this reason, we performed co-culture experiments where we assessed the neuroprotective capacity of *Ido*<sup>-/-</sup> microglia and astrocytes. Co-culturing neurons with either microglia or astrocytes did not increase neuronal sensitivity to NMDA stimulation. This suggests that both types of glia tend to protect neurons from NMDA toxicity. Although we have found some evidence for a neuroprotective effect of *Ido*<sup>-/-</sup> microglia (at least at baseline) on MSNs, in this co-culture system there was no drastic genotypic effect of glia on

neuronal death induced by NMDA stimulation. Further work is needed to unveil the role of *Ido*<sup>-/-</sup> in different brain cell types. Potential future work will be discussed in more detail in chapter 4 of this thesis.

## **Chapter 4: DISCUSSION**

This PhD project has confirmed the importance of tryptophan catabolism in the brain and in the context of HD pathogenesis through an age-dependent and tissue-specific study in a fulllength mouse model of HD. The investigation of KP in this mouse model has also involved a detailed analysis of the metabolites and the enzymatic activity in the first step of the pathway. This has revealed that the conversion of Trp to kyn by Ido1 is dysregulated in this mouse model in a chronic and tissue-specific manner and is followed by an imbalance in the downstream metabolites in both presymptomatic and symptomatic mice.

The delineation of expression patterns and changes of *Ido1*, *Ido2* and *Tdo2* can be considered the major novelty of this PhD thesis and provides new considerations for studying the first step of the KP in brain. In addition, this work suggests for the first time that induction of *Ido1* among the three enzymes is a central player in dysregulation of the KP in the YAC128 mouse model.

# 4.1 Striatal-enrichement of *Ido1* in mouse brain makes it an attractive candidate gene to study in HD.

In a previous project, we identified *Indoleamine 2,3 dioxygenase (Ido1)* in a high-throughout gene expression study using a SAGE database as a striatal-enriched transcript (Mazarei et al., 2010). That study identified ~ 40 novel transcripts that were highly enriched in the striatum compared to other brain regions at multiple time points during development, which were not previously described as striatal-enriched. Many high-throughput expression studies are not followed up by detailed analyses of discovered candidates. Taking a different approach, we analyzed the expression of these candidate genes in the striatum of the YAC128 mouse model of HD. *Ido1* was one of the candidate genes which showed a differential pattern of expression in the

striatum of YAC128 mice compared to that of WT mice. This finding led to the current PhD thesis project, which has investigated the role of *Indoleamine 2,3 dioxygenase (Ido1)* in HD.

Ido1 catalyzes the conversion of Trp into kyn as the first and rate-limiting enzyme of the KP. This reaction takes place throughout the body and brain and in multiple cell types as a required step in the degradation of the essential amino acid Trp. Trp enters the CNS through the blood brain barrier (BBB) and subsequently enters the KP. In the CNS under no stimulations, *Ido1* is expressed at low levels but is induced during many pathological processes in the brain, which can result in rather complex downstream effects.

During this investigation, we characterized the temporal and spatial expression patterns of *Ido1* in the normal mouse brain. Quantification of *Ido1* mRNA in striatum, cortex, hippocampus, and cerebellum at three time points of 9 and 15 days post-natal as well as 3 months revealed a striatalenriched pattern of expression. *Ido1* mRNA was also quantified through a relatively large time frame in the animal's lifespan starting from embryonic time points up until adulthood (a year). In different cell types of striatum at baseline, *Ido1* was equally expressed in neurons and astrocytes while the expression was significantly lower in microglia. When activated however, monocytic cells including microglia and infiltrating macrophages have been shown to produce high levels of Ido1 similar to that of astrocytes and neurons and are known to be the most potent QA producers during brain inflammatory diseases (Guillemin et al., 2003a; 2005b). Our findings at baseline along with these data suggest a change in the state of *Ido1* activity and thus KP in resting versus activated microglia in the brain.

# 4.2 *Ido1* induction in the striatum of YAC128 mice provides clues regarding the regionspecificity of the state of KP in HD.

The consequence of *Ido1* induction in brain has been implicated in many neurologic conditions ranging from neuroinflammatory to neurodegenerative diseases. Intriguingly, induction of *Ido1* in the CNS can be both beneficial and harmful (Kwidzinski and Bechmann, 2007; Vécsei et al., 2012). For example, in multiple sclerosis (MS), Ido1-induced Trp depletion acts as an endogenous immunosuppressive response to counteract the autoimmune activation mediated by autoreactive T cells (Vécsei et al., 2012). This induction however, leads to the elevation of the potentially neurotoxic downstream metabolites of KP such as 3-HK and OA (by induction of NMDA receptor-mediated neurotoxicity and generation of free radicals, respectively), which can contribute to the pathology of MS through their neurotoxic properties (Vécsei et al., 2012). In Alzheimer disease (AD), amyloid beta 1-42 (AB 1-42) peptide has been shown to induce *Ido1* expression with consequent elevation in QA in primary macrophages and microglia (Guillemin et al., 2003a; 2003b; 2003c). Moreover, Ido1 immunoreactivity increases in AD hippocampus in association with senile plaques (Guillemin et al., 2005a) and its activity is elevated in the patients' serum correlating with cognitive behaviour in these patients (Widner et al., 2002). Accordingly, activation of *Ido1* in the CNS has been referred to as a 'double-edge sword' (Kwidzinski and Bechmann, 2007; Vécsei et al., 2012).

The work in this thesis shows for the first time that *Ido1* mRNA levels are significantly upregulated in the striatum of an HD mouse model and this is a chronic induction. Increased Ido mRNA in the striatum of YAC128 mice is first detectable at an asymptomatic embryonic time point tested and perseveres into symptomatic stages of pathogenesis (a year). Consistent with the chronic increase in mRNA expression of *Ido1* in the striatum, kyn/Trp ratio as a measure of Ido

activity was also shown to be elevated in the striatum of both presymptomatic and symptomatic mice. In addition, *Ido1* upregulation and increased activity was specific to the striatum and was absent in the cerebellum, providing clues regarding the state of Ido1 activity in vulnerable brain regions in HD.

According to our data in the YAC128 mouse model of HD and previous data in other neurodegenerative disorders, it is possible that *Ido1* induction can initially occur as a response against neurotoxicity caused by the presence of mHTT in the striatum but its chronic activation through time will eventually lead to accumulation of KP downstream neurotoxic metabolites that can further cause damage to neurons. Figure 4.1 depicts an overview of our findings and proposes potential mechanisms downstream and upstream of Ido1 induction that may be involved in HD pathogenesis.





# A model describing potential mechanism(s) involved in the selective vulnerability of the striatum in HD

The normal striatum consists of medium spiny neurons (MSNs) that are intrinsically sensitive to excitotoxic stress. Additionally, the quinolinic acid (QA) level is higher in the striatum at baseline compared to other brain regions such as cerebellum. In the striatum of YAC128 mice and in the presence of mutant huntingtin (mHTT) expression, Ido1 expression and activity is elevated only in the striatum and not in the cerebellum. At 12 months, Ido1 induction is followed by the accumulation of kynurenine (kyn) in the striatum. This does not result in increased

kynurenic acid (KA) but instead, is followed by an elevation of 3-HK specifically in the striatum at 12 months. The elevation of 3-HK could consequently result in more sensitivity of MSNs through induction of oxidative stress and conversion into QA synthesis. Our data also provides clues regarding induction of Trp to kyn conversion and increased kyn levels in the plasma of YAC128 mice, which may cross the blood brain barrier (BBB) and add to the pool of striatal kyn.

# 4.3 A biphasic pattern of change is observed in the state of striatal KP correlating with the stage of disease progression in YAC128 mice.

As described in the previous chapter 2, we discovered that the increased *Ido1* expression and activity in the striatum of YAC128 mice was both chronic and striatal-specific. In investigating these changes, our study took a unique approach. First, unlike most previous studies where whole brain or forebrain is usually studied to assess kyn/Trp levels as a measure of Ido1 activity, we used micro-dissected brain regions such as striatum and cerebellum and measured kyn/Trp ratio in each region independently. Striatum and cerebellum were selected as the brain regions of interest in this study since the former is predominantly affected in HD (and in YAC128 mice) and the latter remains relatively intact during the course of the disease. A similar study measured the recovery of kyn from injection of radiolabeled Trp in the striatum of the R6/2 fragment mouse model of HD, but did not observe any significant change in the rate of recovery (Sathyasaikumar et al., 2010).

As mentioned in previous chapters, kyn, Trp, and 3-HK are the metabolites of KP that can pass through the BBB and enter the brain from the periphery. We therefore investigated the potential cross talk between the periphery and CNS in symptomatic YAC128 mice, which are thought to mimic the early stages of human disease. A trend towards elevation of plasma kyn/Trp

was found in these animals, which was found not to be statistically significant. A study in 1995 reported an increase in kyn/Trp in the plasma of HD patients at very advanced stages of the disease (Stoy et al., 2005b) but this was only tested at a single time point. Monitoring the changes of this ratio longitudinally in the plasma of HD patients as well as presymptomatic mutation carriers may open doors towards the development of new biomarkers for HD patients.

In this study, in addition to the measurements of kyn/Trp ratio for comparison between the genotypes, we also analyzed the levels of metabolites making up the ratio (kyn and Trp). This was a useful analysis as it revealed a biphasic change in the first step of the pathway despite the constant induction of Ido1 activity. We concluded that higher kyn/Trp ratio is due to significantly lower Trp levels in presymptomatic mice and higher kyn levels in the striatum of symptomatic animals. This can create a scenario at the symptomatic stage where more kyn, as a result of chronic *Ido1* induction, will be available to get shunted into the pathway as the precursor for the production of downstream metabolites. While tryptophan and its analogues have been shown to inhibit *Ido1* transcription (Okamoto et al., 2007), it is not clear in this study whether induction of *Ido1* in presymptomatic mice is the cause or the effect of lower Trp levels at this time point. The question of whether lower Trp is a potentially inherent defect in the striatum of presymptomatic YAC128 mice and HD patients also needs to be addressed. This will be assessed in future studies involving long-term tryptophan supplementation and depletion under *in vivo* and *ex vivo* settings.

# 4.4 The imbalance in the state of KP in the striatum of presymptomatic and symptomatic mice suggests involvement of tryptophan catabolism in HD pathogenesis

Many changes of downstream KP metabolites have been implicated in HD. In fact, metabolites downstream of kyn have been studied extensively compared to those upstream of kyn. There are implications for the reduction of KA in the brain (Beal et al., 1992) and CSF (Beal et al., 1990) of end-stage HD patients. KA is a neuroprotective metabolite which acts by the inhibition of glutamate receptor mediated excitotoxicity. Furthermore, the enzymatic activity of KAT, the enzyme responsible for converting kyn into KA has also shown to be reduced in HD (Beal et al., 1990). Interestingly, our data revealed no observable change in KA levels in either brain or plasma of YAC128 mice. This was despite the significantly higher levels of its substrate kyn, in the striatum. This can be explained by either lower KAT enzymatic activity or a preference for kyn to get shunted into the neurotoxic route. While there are no changes of the neuroprotective metabolite KA in these mice, neurotoxic metabolites such as 3-HK and QA were elevated. This is consistent with previous studies showing an increase in these metabolites in the striatum at early stages of the disease in the brain of HD patients as well as HD mouse models (Beal et al., 1990; Guidetti et al., 2006; Sathyasaikumar et al., 2010). While 3-HK changes were highly tissue-specific, the changes in QA were more widespread.

At the pre-symptomatic stage (3 months), we noticed that many downstream metabolites including 3-HK, XA, 3-HIAA, and PA were reduced in the striatum. Similarly, QA was significantly reduced in the cerebellum. I hypothesize that such a global reduction in neurotoxic metabolites at this time point is a protective response against NMDA neurotoxicity in the YAC128 mice used in our study. As described earlier in this thesis, this strain is homozygous for the *HTT* transgene (+/+) and displays increased sensitivity to NMDA receptor-mediated excitotoxic stress at this timepoint. This general reduction in neurotoxic metabolites of KP was not detected in another line of YAC128 mice whose brains were resistant to neurotoxic insult. This suggests that there may be a link between instrinsic sensitivity to neurotoxicity and the state of the KP in the brain of YAC128 mice.

At 12 months of age, we found a significant increase in striatal 3-HK, a well-known free radical generator. This followed the significant elevation in kyn levels at this time point. While promising therapeutic trials for the inhibition of KMO (the enzyme converting kyn to 3-HK) in HD mouse models are currently underway (Thevandavakkam et al., 2010), I hypothesize that reduction of kyn through inhibition of Ido1 using the currently available Ido1 inhibitors may also be an effective therapeutic approach.

3-HAA is another intermediate metabolite in KP, which is converted to QA through the catalysis of 3-HAO. 3-HAA seems to play a complex role in the brain. It can act as a neurotoxin whose intracerebral injection can damage neurons by reducing choline acetyltransferase in brain (Chen and Guillemin, 2009). 3-HAA also acts as a free radical generator in the presence of copper but can also have antioxidant properties (Chen and Guillemin, 2009). In my study, 3-HAA was significantly reduced at both presymptomatic and symptomatic time points in the striatum of YAC128 mice and thus did not display a biphasic pattern. Similar reduction had been previously described in the plasma of end stage HD patients (Stoy et al., 2005b). The striatal-specific reduction in 3-HAA levels, in this mouse model can potentially be caused by the reduction in kynureninase enzymatic activity and/or an elevation in 3-HAO activity both of which have been previously reported in HD patients and in the R6/2 fragment model (Schwarcz et al., 1988; Sathyasaikumar et al., 2011; Vécsei et al., 2012). Whether increased 3-HAO activity in the striatum of YAC128 mice, despite the low 3-HAA levels, accounts for the increased baseline level of QA in these mice should be a subject for future studies.

The detailed analysis of KP metabolites in this part of the thesis has shed light on the agedependent and brain region-specific alterations of the pathway in the YAC128 mouse model of HD.

### 4.5 *Ido1* knockout mouse: a valuable tool to study *Ido1* in brain

During this project, *Ido<sup>-/-</sup>* mice were used as a tool to study the role of Ido1 in brain. These mice breed normally and are on the C57BL/6J background (Baban et al., 2004) (http://jaxmice.jax.org/strain/005867.html). I showed in this study that both *Ido1* and *Ido2* are not expressed in the striatum of *Ido<sup>-/-</sup>* mice. As expected, kyn/Trp is significantly lower in the striatum of adult *Ido<sup>-/-</sup>* mice but is not completely depleted. Among the downstream metabolites in *Ido<sup>-/-</sup>* striatum, 3-HK and XA were significantly reduced. 3-HK generates hydrogen peroxide and other reactive oxygen species and has been shown to result in neuronal cell death with brain region selectivity that has been implicated in the pathology of neurodegenerative disorders including HD (Okuda et al., 1998).

While 3-HK has no known function on NMDA receptors, its ability to exacerbate QA-induced lesions in striatum has always been a question. Intrastriatal co-application of 3-HK, at a concentration that did not produce neuronal damage on its own, increased the efficacy of intrastriatal QA injections (Schwarcz et al., 2010). Consistent with these findings, we showed that the striatum of *Ido*<sup>-/-</sup> mice are less sensitive to NMDA-mediated excitotoxicity induced by QA. This may be explained by the reduced 3-HK production in the striatum of these mice. Much to my surprise however, I noticed that isolated MSNs from *Ido*<sup>-/-</sup> embryos displayed increased sensitivity both at baseline and as a response to NMDA receptor-mediated excitotoxic stress.

The discrepancy between the *in vivo* and *ex vivo* data may be the result of the involvement of other brain cell types *in vivo*. Accordingly, I hypothesized that *Ido*<sup>-/-</sup> microglia, due to their inability to have a fully functional KP, produce a less toxic microenvironment and therefore cause less sensitivity of *Ido*<sup>-/-</sup> neurons as a response to NMDAR-mediated excitotoxicity. This hypothesis roots from the fact that activated microglia are known to produce high levels of 3-HK

and QA through the induction of Ido1 (Heyes et al., 1996). Accordingly, the absence of Ido1 expression in microglia may render the brain less sensitive to excitotoxicity by producing less of these neurotoxic species (Guillemin et al., 2005b). We have shown that co-culturing of neurons with microglia from WT or *Ido<sup>-/-</sup>* neonatal brains does not increase the sensitivity of neurons to NMDA. We found some differences between the two genotypes of glia specifically at baseline, in their effectiveness on neuronal viability. Our conclusion from this ex vivo co-culture setting is that microglia and astrocytes help to protect neurons from excitotoxic stress and this may be different depending on the glial genotype. While interpreting such experiments however, one should keep in mind that an *in vivo* system includes highly complex and dynamic environments, which are very difficult to mimic in *ex vivo* settings. Future culture experiments would involve the assessment of WT and  $Ido^{-/-}$  microglial and astrocytic effect on WT as well as  $Ido^{-/-}$  neurons. Different properties of QA and NMDA in their ability to potentiate excitotoxicity have been previously described (Guidetti and Schwarcz, 2003). Future studies will utilize both NMDA receptor agonist compounds for in vivo and ex vivo experiments to rule out any potential differential effects.

#### 4.6 Ido1, Ido2 and Tdo2

Bearing in mind the involvement of Ido2 and Tdo2 in facilitating Trp to kyn conversion, we sought to assess the expression patterns of the transcripts corresponding to these enzymes in the brain of YAC128 mice and *Ido<sup>-/-</sup>* adult mice. Ido1 and Ido2 proteins are encoded by genes adjacent to each other on human and mouse chromosome 8 and are 43% similar in amino acid sequence. These two genes are paralogs that have resulted from the duplication of an ancestral *Ido* gene. Interestingly, while *Ido1* exists only in yeast and mammalian cells, *Ido2* is also found

in lower vertebrates such as zebrafish. Organisms such as chicken have only one *Ido* gene, similar to *Ido2*. These results indicate that the ancestral protein was more similar to Ido2. Tdo2 on the other hand is structurally unrelated to Ido1 and Ido2 but has similar functions (Yuasa et al., 2009). While Tdo2 function has mainly been postulated in the liver, its newly described function as the regulator of brain Trp degradation and serotonin pathway as well as adult neurogenesis has led to the finding that Tdo2 modulates anxiety-related behavior in mice (Kanai et al., 2009). This suggests a role of this enzyme in higher brain functions.

In chapter 3 of this thesis, we showed that *Ido2* expression pattern is similar to that of *Ido1* in that it is highly enriched in the striatum compared with the cerebellum. Similar to *Ido1*, and unlike our expectations, *Ido2* mRNA was not expressed in the striatum of *Ido<sup>-/-</sup>* mice. This suggests that the knockout construct designed for the generation of *Ido<sup>-/-</sup>* mice also targeted *Ido2*. We therefore propose that these animals are null for both *Ido1* and *Ido2*. *Ido2* expression however, did not change significantly in the striatum of YAC128 mice. *Tdo2* expression was not detectable in the striatum and was only present in the mouse cerebellum. Levels of cerebellar *Tdo2* did not change in either of the YAC128 or *Ido<sup>-/-</sup>* mice.

#### 4.7 Potential pitfalls

## 4.7.1 Lack of proper Ido1 antibodies for studies in brain

During this thesis project, a series of different monoclonal and polyclonal antibodies were tested in different brain regions of WT and *Ido*<sup>-/-</sup> mice. This included a variety of different commercially available antibodies as well as an antibody donated by another laboratory. In our Western blots, all of these antibodies detected strong bands at the correct size for epididymus, a tissue known to produce high levels of Ido1. In tissues of mouse brain, a faint band was detected

at the right size and the signal seemed to have the same level of intensity in all brain regions tested (striatum, cortex, cerebellum, and hippocampus). When we tested *Ido<sup>-/-</sup>* brain tissue samples as a negative control, the majority of these antibodies also detected a band at the correct size. Similarly, immunohistochemistry (HIS) and immunocytochemistry experiments (using both immuno-fluorescence and 3,3'-Diaminobenzidine (DAB) systems) led to a cytoplasmic staining in neurons and potentially microglia which was similar between WT and *Ido<sup>-/-</sup>* tissues and cells. While several studies have used these antibodies as tools in their analysis of Ido1 expression, we did not consider them reliable and thus decided not to utilize them in our *Ido1* expression studies. (Kim et al., 2012; Yamada et al., 2009; Guillemin et al., 2005a; Guillemin et al., 2005b).

# 4.7.2 The genetic composition of $Ido^{-/-}$ mice

Several findings still leave the question of whether or not  $Ido^{-/-}$  mice are complete knockout mice. For example, we have found that kyn/trp ratio is not fully depleted in the striatum of  $Ido^{-/-}$  mice. While this finding may indicate that other enzymes provide the striatum with a pool of kyn, which results in a non-zero ratio, it does not rule out the possibility of a functional Ido isoform which has resulted from an unpredicted recombination event while generating this mouse model. Secondly, we found that monocolonal Ido1 antibodies detect a signal in IHS as well as a similar sized band in Western blots) in the brain of  $Ido^{-/-}$  mice. While this may be due to the detection of non-specific species by this antibody, it does not preclude that the antibody is detecting a protein resulting from faulty targeting of Ido1 locus. Additionally, the fact that both Ido1 and Ido2 mRNA expressions were absent in these mice indicate that the targeting strategy may have resulted in some unknown rearrangements in the genomic region encompassing Ido1 and Ido2, which is not necessarily based on the original genetic design of this mouse. Finally, the original work by Baban et al., (2004) describing the generation of  $Ido^{-/-}$  mice fails to address some

important issues such as the incorporation of the stop codon into the genome and the expression of the  $\beta$ -galactosidase gene within the construct. All the above points indicate that the genetic/genomic composition of this mouse model needs to be further investigated. This study was pursued based on the assumption that the *Ido*<sup>-/-</sup> mice are indeed *Ido* depeleted.

# 4.7.3 Autoimmune response in $Ido^{-/-}$ mice

While it appears that the absence of *Ido1* in brain is beneficial with regards to brain response to neurotoxicity, one should consider the potential systemic as well as CNS side effects as a result of absence of this enzyme. A recent study showed that Ido1 regulates immune tolerance to apoptotic cells and chronic administration of these cells into *Ido<sup>-/-</sup>* mice induced a lupus-like disorder (Ravishankar et al., 2012). This was demonstrated by serum autoreactivity to double-stranded DNA associated with renal pathology and increased mortality (Ravishankar et al., 2012).

Similar to what was reported by the Mellor laboratory where these mice were generated (Baban et al., 2004), *Ido*<sup>-/-</sup> mice in our colony have lived with no obvious defects in breeding and exhibited no spontaneous autoimmune disorders for up to one year now. While there may have been no triggers to induce autoimmunity in mice in our colony, the information regarding their phenotype and survival is purely observational. More testing is needed to assess their general health including any signs of autoimmune response and pregnancy defects. If these mice turn out to be susceptible to autoimmunity during their lifespan, an interesting experiment will be to assess the presence of an autoimmune response in a conditional knockout mouse that is Ido-deficient specifically in brain.

### 4.7.4 Functional redundancy in higher organisms including human

The functional homology of enzymes involved in Trp to kyn conversion step of the KP in mammalian cells is an important issue to be considered when using the enzymatic step of this reaction as a therapeutic target. The studies done in *Drosophila* and *C. elegans* take advantage of the fact that Tdo2 is the sole enzyme facilitating the first step of the pathway in these organisms. As a result, inhibition of Tdo2 can completely inhibit the conversion into kyn. This was shown to result in an almost complete depletion of kyn and an accumulation of Trp in *C. elegans*. In mammalian cells however, there are a few issues to deal with: First, Ido1, Ido2, and Tdo2 all facilitate the first step of the pathway. Interestingly, we found the expression of both *Ido1* and *Ido2* absent in the striatum of  $Ido^{-/-}$  mice. Despite the fact that we did not report a compensatory expression of Tdo2 in the striatum of  $Ido^{-/-}$  mice, no complete depletion of kyn and accumulation of Trp were observed. This suggests that there are potential unknown mechanisms involved in brain Trp homeostasis that could be pursued as important future studies investigating the role of Trp breakdown in brain.

# 4.8 Future directions: Further investigation of the role of Ido1 in HD and agingassociated neurodegeneration

While this PhD project has built the stepping-stones towards understanding the involvement of tryptophan catabolism in the brain and in the context of HD, it has raised important questions that need to be further addressed. Some of the potential future studies are discussed below:

## 4.8.1 Further analysis of the role of Ido1 in the context of mHTT

The work in this thesis is one of the very first studies describing a potential role for Ido1 and the first step of the KP in HD. As previously mentioned, investigating tryptophan metabolism and oxidative stress in patients with Huntington disease, Stoy *et al.* (2005) examined kyn/Trp ratios in the plasma of end-stage HD patients and showed that it was significantly elevated (Stoy et al., 2005b) but they never investigated this further. In a recent *ex vivo* study by Van der goot et al. (2012), it was shown that the inhibition of Tdo2 suppresses mHTT-mediated toxicity in *C*. *elegans* neurons and this is independent of downstream metabolites in the KP (van der Goot et al., 2012). We showed in our study that the striatum of the  $Ido^{-/-}$  mice on a C57BL/6 background is less vulnerable to NMDA receptor-mediated excitotoxicity induced by QA. In addition, we showed that these mice have significantly lower levels of 3-HA. Whether less toxicity in the striatum of  $Ido^{-/-}$  is due to Ido deficiency alone or whether it is also dependent on decreased downstream 3-HK must be further investigated.

Based on this data, we decided to generate a cross between *Ido<sup>-/-</sup>* and YAC128 mice. The offspring of this cross will be YAC128 mice that are Ido-deficient throughout development. We hypothesize that this cross has the potential to ameliorate the pathogenic HD phenotype in the YAC128 mice. As a long-term future plan, these mice will be assessed in longitudinal studies where behaviour, neuropathology, and sensitivity to neurotoxic stress will be assessed.

Another field of study that would be compelling to pursue is to investigate the role of the first step of KP in longevity. Ageing and neurodegenerative diseases are thought to follow similar metabolic and molecular processes that result from toxicity of aggregation prone proteins (van der Goot et al., 2012). The recent study by Van der goot also showed that *Tdo2* inhibition not only leads to decreased mHTT- associated toxicity in worm neurons, but it also extends lifespan in these *C. elegans* (van der Goot et al., 2012). Similarly, a future study will include the monitoring of the survival and analysis of lifespan in *Ido-<sup>/-</sup>* mice and YAC128x *Ido-<sup>/-</sup>* mice. This experiment will test the hypothesis suggesting that the absence of Ido not only decreases the mHTT-associated toxicity in the striatum of YAC128 mice, but also increases their lifespan.

### 4.8.2 Assessing the effect of mHTT expression on Ido1 transcription

As mentioned previously, *Ido1* is induced through different mechanisms. IFN $\gamma$  is known to be a potent inducer of *Ido1*. Additionally, IFN $\alpha$ , IFN $\beta$ , TNF $\alpha$ , platelet activating factor, T-antigen 4, HIV1 proteins (Nef and Tat), A $\beta$  1-42, Trp and Trp analogues have been shown to induce *Ido1*. In contrary, Interleukin-4 (IL-4), Nitric oxide, and cytochrome oxydase 2 (COX-2) act as inhibitors of *Ido1* (Yuasa et al., 2009). The *Ido1* promoter region is composed of INF $\gamma$  activation site (GAS), IFN-stimulated response element-1 (ISRE-1), and ISRE-2 regions which are bound by transcription factors such as Stat1 or IFN regulatory factor-1 (IRF-1) (Sotero-Esteva et al., 2000). In our study, using a meso-scale platform for the cytokine measurements, no increase in the levels of INF $\gamma$  was detected in the in the striatum of YAC128 with significantly higher *Ido1* levels indicating that INF $\gamma$  may not be the cause of Ido1 induction in these animals (data not shown).

*Ido1* mRNA is upregulated in the striatum of YAC128 mice even at very early time points during development. Whether or not *Ido1* transcription in the striatum of these mice is regulated directly by the mHTT protein or indirectly through any of the above mechanisms is yet to be understood. Studies could be designed to assess the effect of mHTT vs WT HTT expression, in a dose-dependent manner, on Ido1 transcription. Assessement of *Ido1* transcription levels in response to mHTT versus WT HTT expression modification would be a necessary step in this pursuit. Additionally, the investigation of the *Ido1* promoter and the analysis of direct interactions of mHTT and WT HTT with this promoter would reveal whether mHTT functions directly to regulate Ido1 expression.

## 4.8.3 Understanding the role of Ido1 in different brain cell types

While *Ido<sup>-/-</sup>* mice can serve as a valuable tool to study the role of Ido in brain, these mice fail to address the role of Ido in different brain cell types. We showed in our work that there is cell-type specificity of *Ido1* expression in brain. While neurons and astrocytes were found to express *Ido1* mRNA at baseline, transcription in microglia was not detectable. Immune-induced Ido1 activation however is a known feature of activated microglia. The activation of Ido1 in different cell types leads to the activation of the downstream metabolites depending on how the pathway enzymes are expressed in each cell type. For example, it has been shown that the net result of KP induction in astrocytes and primary neurons is neuroprotective while the activated microglia are known to shunt the pathway to the more neurotoxic branch. Accordingly, a reasonable future direction is to use the Cre/lox system to generate cell-type specific conditional Ido1 knockout mice to investigate the role of *Ido1* induction and the resulting KP activation in different brain cell types. The use of the Cre/lox system would involve engineering mice in which Ido1 is floxed between the two loxP sites. These mice will be subsequently crossed to mice that express Cre under the control of the promoters for expression of cell-type specific proteins/markers such as Nestin, GFAP, etc that has been widely used to express genes in different types of neurons and glia (Bradford et al., 2009; 2010; Faideau et al., 2010). The offspring resulting from these crosses will be used to further investigate the effect of Ido1 absence of expression in different brain cell types.

## 4.8.4 *Ido1 as a therapeutic target*

The targeting the KP in HD has drawn a great deal of attention in the past few years. This involves strategies that shift the pathway toward generation of KA and away from production of

3-HK and QA. In other words, the therapeutic strategy is considered successful when the imbalance in KP resulted from increased neurotoxicity over neuroprotection is reversed. So far, KMO inhibition has been validated as a therapeutic strategy for HD. It has been shown that the peripheral inhibition of this enzyme by a novel orally bioavailable prodrug (JM6) decreased synaptic loss in brain and increased the lifespan of the R6/2 mouse model (Thevandavakkam et al., 2010; Zwilling et al., 2011).

While the first step of the KP is located upstream of both neuroprotective and neurotoxic branches, there is increasing evidence that the inhibition of this step may render neuroprotection (van der Goot et al., 2012). A study in Drosophila models of HD also showed that the reduction in the activity of the first step through genetic inhibition of Tdo2 (flies do not express Ido1 or Ido2) protected flies from mHTT-mediated neurotoxicity (Campesan et al., 2011). While in the mammalian brain Ido1, Ido2, and Tdo2 seem to catalyze the first step of the pathway, our study shows a dysregulation in Ido1 activity in the striatum. Ido1 inhibitors such as 1-methyl- DLtryptophan and methyl-thiohydantoin- tryptophan used in cancer research have been shown to potentiate the efficacy of chemotherapy in a mouse model of breast cancer (Chen and Guillemin, 2009). Interestingly, a study has shown that Ido2 is the preferred biochemical target of the D-1methyl-tryptophan (Metz et al., 2007). While inhibitors are also available for Tdo2 (Chen and Guillemin, 2009), an ideal future therapeutic strategy would encompass the selective inhibition of Ido1 and Ido2 in the striatum (and possibly in the plasma) of the YAC128 mouse model of HD. The potential mode of administration of various Ido1 inhibitors required to get the compounds into the brain remains a confounding element in the development of effective therapies for HD based on these compounds.

Over-expression of *Ido1* has been shown to lead to tumour cell proliferation by contributing to tumour-induced tolerance (Mellor and Munn, 1999). Interestingly, inhibitors of both Ido1 and Tdo2 have been *systemically* administered for cancer treatment in mice and have been shown to improve response to chemotherapy (Muller et al., 2005; Pilotte et al., 2012). On the other hand, while intra-hippocampal microinjection of Ido inhibitor 1-MT, showed an amelioration of Ido1-induced comorbidity of pain and depression in mice, the systemic 1-MT treatment alone did not alter depressive-like behaviors (Kim et al., 2012). While these data imply that the CNS versus systemic modes of Ido1 inhibition may result in different outcomes, the effects of intrastriatal versus intraperitoneal administration of inhibitors in the context HD mouse models will remain to be examined.

## 4.9 Conclusions

This PhD dissertation has investigated the potential role of Ido1 in Huntington disease. This study initially focuses on the enzymatic expression and activity of the first step of the kynurenine pathway in the normal mouse brain as well as in the brain of YAC128 mouse model of HD and for the first time reports a chronic and striatal-specific induction of the enzyme Ido1 in the striatum of a widely used HD mouse model. Under *in vivo* and *ex vivo* experimental settings, this work subsequently explores the potential involvement of *Ido1* expression in NMDA-receptor mediated excitotoxicity, a well-established mechanism in HD, and shows that the absence of *Ido1* expression reduces the sensitivity of striatal neurons to neurotoxic stress.

Further analysis of the levels of downstream metabolites in the kynurenine and serotonin pathways sheds light on the age-dependent and brain region-specific alterations of these metabolites in the YAC128 mouse model. Through investigating these metabolites, this work not

only confirms several previously described modifications in HD by describing them for the first time in the YAC128 mouse model, but also reveals novel age-dependent and tissue-specific changes of both kynurenine and serotonin pathways in the brain of these mice.

Based on this work, I propose that the early and region-specific induction of Ido1 plays a central role in the observed imbalance of the kynurenine pathway in HD and should be taken into account when assessing therapeutic targets in this pathway. In addition, the findings of this study open new doors toward understanding novel mechanisms involving tryptophan degradation and suggest potential future therapies for HD.

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