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Reduced vesicular storage of catecholamines in the pathogenesis of Parkinson's Disease

By

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Abstract

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Parkinson's disease (PD) is a neurodegenerative disorder which affects millions of people worldwide. Although the cause of PD is unknown, it is thought to be due to a combination of genetic and environmental factors; many therapies aim to restore dopamine (DA) within the brain. Animal models, both chemical and transgenic, have been used to uncover potential mechanisms of PD, but none have successfully recapitulated the symptoms of PD on an appropriate timescale. Genetic perturbation of the vesicular monoamine transporter (VMAT2) yielded the VMAT2-deficient mice, which display many of the motor and non-motor symptoms associated with PD. With a 95% reduction in VMAT2 expression, VMAT2-deficient animals have decreased novelty-induced locomotor activity and a shortened stride length at older ages. VMAT2-deficient animals also displayed progressive deficits in olfactory discrimination without changes in other sensory systems, which appear to be possibly correlated with previously seen age-dependent reduction in dopamine transporter expression. Moreover, VMAT2-deficient mice have a shorter latency to behavioral signs of sleep, delayed gastric emptying, anxiety-like behaviors at younger ages, and a progressive depressive-like phenotype. Taken together, these data suggest that reduced storage of monoamines may contribute to the development of many features of Parkinson's disease. Progressive neurodegeneration in the substantia nigra (SNpc), locus coeruleus (LC), and dorsal raphe (DR) has been observed, accompanied by α-synuclein accumulation. Moreover, primary cultures from the SNpc, LC, and DR of postnatal mice exposed to physiological concentrations of monoamines were found to undergo increased oxidative stress via formation of H_2O_2 and other oxidative species; whereas, cultures from wildtype mice were unaffected. These studies demonstrate that reduced vesicular storage may play a role in the pathogenesis of parkinsonian symptoms and neurodegeneration. Perturbing the catecholaminergic cytosolic environment may ultimately lead to the development of several behavioral phenotypes and neuronal death. Using the VMAT2-deficient mice as a new model of PD, could potentially lead to new therapeutic strategies beyond DA replacement therapy.

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This dissertation is dedicated to my parents and my brother:

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease and affects approximately 4-6 million people worldwide. Initially characterized by James Parkinson in 1817, PD is a progressive disorder, most notably affecting the motor processes of the brain. PD is distinguished by the cardinal symptoms of resting tremor, rigidity, bradykinesia, and postural instability (Parkinson, 1817; Olanow and Tatton, 1999; Fahn and Sulzer, 2004). The incidence of PD is positively correlated with age; there is a greater than 40-fold increase in prevalence between the ages of 55 and 85 (Fahn and Sulzer, 2004). Approximately 5-10% of PD patients have a familial form of Parkinsonism with either an autosomal dominant or autosomal recessive pattern of inheritance. These familial forms are characterized by an age of onset before 40 years and a slow, progressive course (Saito et al., 2000). The tetrad of motor symptoms is relatively mild in these cases with differing patterns of pathology compared to sporadic PD cases (Saito et al., 2000). Unfortunately, mutations in many genes associated with familial PD have not been identified in patients with sporadic forms of the disease (Spillantini et al., 1997). Although these familial mutations have been suggested as causes or risk factors for the development of PD, the low penetrance of some mutations and the low disease concordance in relatives of affected patients suggests that there are interactions between multiple factors (Sulzer, 2007). Epidemiological studies indicate that exposure to environmental toxins may play a role in the pathogenesis of PD. Factors such as rural living, pesticide use, well-water consumption and certain occupations, including mining and welding, are associated with an increased risk for PD development (Hernan et al., 2002). Due to the multifactorial nature of PD, it has thus far proven difficult to pinpoint an exact cause of the disease.

Dopamine and PD

The monoamine dopamine (DA) has been associated with PD since the early 1950s, soon after the neurotransmitter's discovery. DA is synthesized by the rate-limiting enzyme tyrosine hydroxylase (TH) from tyrosine into L-3,4-dihydroxylphenylalanine (L-DOPA), which is then decarboxylated by aromatic acid decarboxylase (AADC) yielding DA. DA has been implicated in numerous processes in the brain and is associated with many behaviors including reward, drug addiction, schizophrenia, and locomotion (Beninger, 1983; Di Chiara et al., 1992; Gray et al., 1995). Pathogenically, PD is mainly typified by the depletion of DA neurons in the substantia nigra pars compacta (SNpc) and development of proteinaceous cytoplasmic inclusions known as Lewy bodies (Olanow and Tatton, 1999; Fahn and Sulzer, 2004). Moreover, striatal DA denervation leads to the appearance of the motor phenotype; at the onset of symptoms, DA in the putamen is depleted by approximately 80% and 50 % of SNpc dopaminergic neurons have already been lost (Dauer and Przedborski, 2003). However, dopaminergic neurons of the ventral tegmental area (VTA), which is adjacent to the SNpc, are relatively less affected in PD (Uhl et al., 1985).

Dopaminergic cells are believed to die by programmed cell death rather than necrosis in PD, but this is a highly disputed point (Jellinger, 1990; Hirsch et al., 1999; Olanow, 2007). Overall, studies have focused on three types of cellular dysfunction that may be important in the pathogenesis of PD: oxidative stress, mitochondrial respiration defects, and abnormal protein aggregation, most notably of α-synuclein (Dauer and Przedborski, 2003). However, there are problems surrounding each of these hypotheses. For example, it is unknown whether oxidative damage that occurs in PD is a primary event or if it occurs secondary to an alternate etiology, drugs, or postmortem events (Olanow and Tatton, 1999). Moreover, an increase in glial cells in the SNpc and a loss of neuromelanin have also been found to accompany neuronal loss, potentially implicating inflammatory pathways (Fahn and Sulzer, 2004; Farrer, 2006). Even with

competing theories as to why nigral dopaminergic neurons die, the mainstay of parkinsonian therapy since its discovery in the late 1960s, has been L-DOPA.

PD treatment relies on neurotransmitter replacement therapy using L-DOPA or DA agonists, which compensate for the loss of midbrain dopaminergic neurons (Farrer, 2006). The early symptoms, such as tremor and bradykinesia, are usually correctable by these treatments, but these drugs fail to halt disease progression and patients suffer from an eventual decline (Fahn and Sulzer, 2004; Olanow et al., 2004). As the disease worsens, bradykinesia no longer fully responds to L-DOPA and symptoms develop that do not respond to L-DOPA, such as flexed posture, freezing, and loss of reflexes (Fahn and Sulzer, 2004). The ultimate inefficacy of L-DOPA and the DA agonists is presumably due to the involvement of other neurotransmitter systems in the pathogenesis of PD.

Pathogenic changes in PD are extensive and include the degeneration of the norepinephrine (NE) neurons of the locus coeruleus (LC), acetylcholine (Ach) neurons of the nucleus basalis of Meynert and peripeduncular nucleus, serotonin (5-HT) neurons of the raphe nuclei, nerve cells in the olfactory system, the dorsal motor nucleus of the vagus, and the peripheral autonomic nervous system (Jellinger, 1990; Fahn and Sulzer, 2004; Jenner and Olanow, 2006). Furthermore, Lewy body pathology can also be found in the LC, nucleus basalis of Meynert, hypothalamus, cerebral cortex, and in components of the peripheral nervous system (Olanow and Tatton, 1999; Braak et al., 2002; Fahn and Sulzer, 2004). As the acknowledgement of pathology associated with PD extends beyond the dopaminergic system, symptoms beyond the cardinal motor phenotype so too are recognized. Most PD patients experience a prodromal phase in addition to the motor phenotype typically diagnosed by clinicians. These symptoms often play a large role in the quality of life and disease etiology, potentially re-directing PD research to examine a more heterogeneous disorder.

Non-motor symptoms in PD

After the advent of L-DOPA and similar dopamine augmenting therapies, little advancement has been made in achieving a lasting therapy or cure. This is due to the fact that beyond the cardinal motor phenotype, disease progression is associated, in part, with the development of non-dopaminergic features that are not adequately controlled with existing medications (Olanow et al., 2008). The onset of classic parkinsonism is frequently preceded by a prodromal phase lasting from anywhere to decades to a few years; this prodromal phase is characterized by the development of non-motor symptoms including hyposmia, sleep disturbances, gastrointestinal dysfunction, anxiety, depression, and autonomic disturbances (Gonera et al., 1997; Ziemssen and Reichmann, 2007). Over the years, these non-motor features, which are largely perceived to be non-dopaminergic, have proven to be the major sources of disability and the primary cause of nursing home placement in patients (Shulman et al., 2001; Olanow, 2007; Simuni and Sethi, 2008). Consistently, PD has been shown to involve much more than depletion of the nigrostriatal system, a multisystem disorder, both pathologically and clinically (Langston, 2006; Ziemssen and Reichmann, 2007). Areas implicated in the preclinical stages 1 and 2 of PD are also thought to be key areas that mediate non-motor symptoms such as olfaction, sleep homeostasis, and other autonomic characteristics (Braak et al., 2002; Chaudhuri et al., 2006). Even in James Parkinson's initial description of the disease, he noted many abnormalities beyond motor impairments, including notations of disturbed sleep, constipation, and depression (Parkinson, 1817). These non-motor symptoms contribute significantly to the clinical picture of PD, impairing quality of life and shortening life expectancy; only 12% of patients have reported the absence of non-motor symptoms, and the majority have more than one non-motor symptom (Shulman et al., 2001; Chaudhuri et al., 2006).

Even with the acknowledgement of the prodromal phase, non-motor symptoms are often poorly recognized and inadequately treated. Dopaminergic treatment is relatively unhelpful for most of the non-motor symptoms, unless they happen to be related to motor dysfunction

(Chaudhuri et al., 2006). While non-motor symptoms are not synonymous with a distinctly non-dopaminergic cause, the recognition that non-motor symptoms occur in the premotor phase is slowly shifting the understanding of PD, and expanding the possibility for an earlier diagnosis (Chaudhuri and Schapira, 2009; Tolosa et al., 2009). To this end, the addition of a non-motor screening questionnaire and the new unified Parkinson's disease rating scale (UPDRS) should aid clinicians in earlier diagnosis and treatment of PD (Zesiewicz et al., 2006). During the prodromal phase, the neuropathological processes have been shown to continually progress even without concomitant motor manifestations (Gonera et al., 1997; Tissingh et al., 2001). Moreover, exactly which neural structures are involved and whether synuclein deposition, cell loss, or neuronal dysfunction, unrelated to α-synuclein accumulation, is responsible for the premotor symptoms is still unclear (Tolosa et al., 2009).

Norepinephrine and PD

In addition to DA, norepinephrine (NE) has long been believed to play a role in PD pathogenesis. After the L-DOPA revolution, the importance of NE was downplayed. But since research and clinical trials have demonstrated that DA restoration is not enough to overcome the Parkinson's symptomatology, NE has reemerged as a driving force of research. Centered in the locus coeruleus (LC), NE is synthesized from DA by dopamine beta hydroxylase (DβH) within vesicles. NE also contains a catechol ring, and therefore has a similar ability to auto-oxidize in the cytosol unless taken up by the vesicular monoamine transporter (VMAT2). DA and NE share a very intimate relationship. NE is critical for the firing of SNpc neurons and striatal DA release; moreover, there are moderate levels of α and β adrenergic receptor binding sites present in the SNpc and striatum (Dolphin et al., 1979). Activity in NE-containing neurons exerts an indirect modulatory influence on DA systems, regulating their function (Antelman and Caggiula, 1977). Because of this, NE is thought to influence the pathogenesis of PD.

Clinically, LC has consistently been shown to degenerate in PD patients (Chan-Palay and Asan, 1989; Forno, 1996). Severe decreases (~78%) in LC neuronal number, accompanied by

reductions in DβH and monoamine oxidase (MAO) activities have been seen (Mann, 1983; Zarow et al., 2003). Furthermore, DA loss in PD has been negatively correlated with healthy NE levels (Tong et al., 2006). Suggesting that NE may exert a trophic influence, consequently increasing the sensitivity of nigrostriatal DA neurons to neurotoxic insults (Gesi et al., 2000). However, even though LC degeneration occurs and can be more severe than nigral degeneration, the temporal relationship of degeneration between the two catecholaminergic centers has yet to be elucidated (Rye and DeLong, 2003). It has also been theorized that degeneration of the LC neurons masks the debilitating effects of progressive nigrostriatal DA death and delays the onset of clinical signs until considerable DA depletion has occurred (Antelman and Caggiula, 1977).

The importance of LC degeneration has been corroborated experimentally using various animal models of PD. Decreased NE produced by pretreatment with the noradrenergic toxin, DSP-4, or direct lesioning of the LC has been shown to not only reduce basal release of DA, but also to exacerbate MPTP toxicity (Lategan et al., 1990; Mavridis et al., 1991; Lategan et al., 1992; Marien et al., 1993; Rommelfanger et al., 2007). Conversely, genetically or pharmacologically upregulating NE has proven to be effective in reducing parkinsonian symptoms in reserpinized and MPTP-treated animals (Colpaert, 1987; Rommelfanger et al., 2004). These data show that although it is unlikely that lack of NE is the primary factor responsible for neuronal death, it may have the ability to compensate for DA deficiency (Antelman and Caggiula, 1977; Tong et al., 2006).

Clinically, PD is not diagnosed until the cardinal motor symptoms appear. However, motor impairments do not develop until ~80% of DA terminals have been lost and 40-50% of SN neurons have died (Olanow and Tatton, 1999; Jenner and Olanow, 2006). The presymptomatic phase of PD, which includes olfaction and neuropsychiatric symptoms may be related to extra-dopaminergic degeneration, such as the LC (Chaudhuri et al., 2006). Additionally, the primary therapies available for PD, including L-DOPA, and surgical techniques only provide temporary relief of motor symptoms; the patient's quality of life often continues to deteriorate due to non-

dopaminergic clinical manifestations (Lang and Obeso, 2004; Ahlskog, 2007). The death of LC neurons may also complicate current therapeutic interventions as it may modulate the eventual behavioral pathology associated with long-term L-DOPA treatment (Rommelfanger and Weinshenker, 2007). NE has the potential to wear many hats in PD progression, including inhibition of DA-activated neuroinflammation, and protection against neurotoxicity to nigrostriatal DA neurons (Kilbourn et al., 1998; Heneka et al., 2003; Heneka et al., 2006; Rommelfanger and Weinshenker, 2007). Rather than the traditionally lauded DA, NE may dictate both the onset and the progression of damage to the nigrostriatal tract in PD (Gesi et al., 2000).

Serotonergic involvement in PD

Serotonin (5-HT) is biologically derived from tryptophan, instead of tyrosine like the catecholamines, and consequently has an indole ring as opposed to a catechol ring. As an indolamine, 5-HT auto-oxidizes in the cytosol unless it is sequestered into vesicles via VMAT2 (Sinhababu et al., 1985; Guillot and Miller, 2009). This third major monoamine is centered in the raphe nuclei, and is involved in basic physiological functions including: sleep, arousal, feeding, mood, and emotion (Fox et al., 2009). Traditionally noted in PD due to its relationship with DA neurotransmission, 5-HT has come into prominence because it has been shown to be involved with intracellular changes leading to protein aggregation and formation of Lewy neurites. Moreover, Lewy bodies may originate in the caudal raphe nucleus, among other non-dopaminergic nuclei, before changes occur in the SNpc (Del Tredici et al., 2002). This is important because serotonergic neurons stemming from the dorsal and median raphe innervate midbrain dopaminergic areas to impose an inhibitory regulatory tone on DA function (Jenner et al., 1983). Recently, 5-HT has taken on an even greater role in PD; serotonergic dysfunction appears to play a role in a number of parkinsonian symptoms, including: mood, psychosis, motor function, and L-DOPA-induced dyskinesias (Fox et al., 2009). Serotonergic receptors located in

the peripheral nervous system may also play a role in PD pathogenesis in the contributing to gastric dysmotility (Fox et al., 2009).

Postmortem analysis has shown substantially reduced levels of 5-HT in the raphe nuclei cortex and in the basal ganglia of PD patients (Hornykiewicz, 1966; Scatton et al., 1983; Halliday et al., 1990). Researchers have also noted a decline in serotonergic markers including 5-HIAA, serotonin transporter (SERT), and tryptophan hydroxylase (TPH) in the striatum (Kish et al., 2008). Interestingly, the parts of the raphe initially affected are the lower raphe nuclei, which project to the spinal cord (Braak et al., 2003). The rostral group (dorsal raphe (DR)), which projects to forebrain structures affecting mood, is not affected until Braak stage 3, in concert with Lewy body pathology in the SNpc and the appearance of motor dysfunction (Braak et al., 2002). Despite its later pathology, there is evidence that depression in PD is dependent upon 5-HT dysfunction, seen through reduced 5-HT metabolites in the cerebrospinal fluid and tryptophan depletion in depressed PD patients (Mayeux et al., 1986; McCance-Katz et al., 1992). However, no relationship has been demonstrated between the degree of reduction in 5-HT and the overall severity of parkinsonian deficit (Jenner et al., 1983).

Teasing out the role of 5-HT in the pathogenesis of PD has been a daunting task thus far, and alternative hypotheses exist in addition to the degeneration of serotonergic neurons. It has long been known that the DA and 5-HT systems are extensively interconnected; the SNpc, striatum, and nucleus accumbens all contain significant amounts of 5-HT and TPH, the content of which is reduced by lesions of the raphe (Hajdu and Hassler, 1973; Marsden and Guldberg, 1973). The degeneration of nigrostriatal DA neurons may or may not lead to the reactive hyperinnervation of 5-HT, potentially masking the true extent of 5-HT loss in PD (Karstaedt et al., 1994). These results have been confirmed in animal studies; after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or 6-OHDA (6-hydroxydopamine) lesioning, a hyperinnervation of serotonergic fibers was shown to occur (Scholtissen et al., 2006). It has also been theorized that reduction of 5-HT in PD patients is a compensatory mechanism for reduced DA levels in the

striatum (Scholtissen et al., 2006). With progressive dopaminergic neuronal death, DA production has been shown to take place in striatal serotonergic nerve terminal (Tanaka et al., 1999). Synthesis of DA in 5-HT neurons is accompanied by the possibility that remaining 5-HT neurons innervating the striatum might release DA as a false neurotransmitter (Ng et al., 1970; Tanaka et al., 1999). This supposition has the potential to be harmful and exacerbate PD dysfunction as DA might be released from 5-HT neurons in a non-physiological manner, leading to swings of DA causing dyskinesias (Kish et al., 2008). Taken together, these data suggest that decreased 5-HT content and altered 5-HT metabolism in patients may reflect functional rather than structural changes in the serotonergic centers of the brain (Chase, 1972). If these nuances in serotonergic function can be better elucidated, perhaps serotonin's contribution to PD can be more fully understood.

Hypotheses of PD

Oxidative stress has come to the forefront of research on PD because of the oxidative capacity of DA and its involvement in neuronal death. The brain is especially susceptible to oxidative damage because neurons are highly aerobic cells, consuming large amounts of energy and oxygen, producing free radicals and reactive oxygen species (ROS). Their capacity to withstand oxidative stress is limited because of the following features: (1) high content of easily oxidizable substrates such as polyunsaturated fatty acids and catecholamines, (2) mitochondrial production of superoxide and downstream ROS, which can be dramatically enhanced under conditions of high energy demands or dysfunction of the electron transport chain (ETC), and (3) low antioxidant capacity, reflected by relatively low levels of glutathione (GSH) and GSH peroxidase (Jenner, 1998; Chinopoulos and Adam-Vizi, 2006; Maher, 2006). Dopaminergic neurons are particularly susceptible to oxidative damage because they intrinsically have lower levels of GSH compared to other brain regions and because DA can auto-oxidize in the cytosol. For example, many studies have found that GSH levels are specifically decreased in the SNpc of PD patients, and a positive correlation exists between the severity of the disease and the extent of

GSH loss (Jenner, 1998; Olanow and Tatton, 1999; Zigmond et al., 2002; Dauer and Przedborski, 2003). GSH levels are not reduced in other non-dopaminergic brain areas in PD patients or in patients with other neurodegenerative disease that also affect dopaminergic neurons (Sian et al., 1994). The decrease in GSH precedes other PD-associated changes in the SNpc, such as decreases in mitochondrial complex I activity and DA levels. GSH and the thioredoxins (Trx1 and Trx2) both function in the reduction of peroxides, which are produced through catecholamine metabolism, through the action of multiple GSH peroxidases and peroxiredoxins (Das and White, 2002; Maher, 2006). GSH has the ability to reduce peroxides, scavenge free radicals, and be conjugated with electrophilic compounds (Meister and Anderson, 1983; Dickinson and Forman, 2002). Trx, on the other hand, has a dithiol motif that makes it useful in reversing oxidative changes to proteins, including reduction of protein disulfides and cysteinyl sulfenic acids. Trx also acts to maintain the activity of other antioxidant enzymes, such as the peroxiredoxins (Patenaude et al., 2005; Maher, 2006). There are two isoforms of Trx: Trx1, found in the cytosol and the nucleus, and Trx2, found in the mitochondria. Both types of Trx are expressed copiously in regions of high metabolic activity and ROS production, such as the brain, and a strong correlation has been found between Trx1 upregulation and neuronal survival after neuronal insult (Maher, 2006). Due to the involvement of GSH and Trx in the progression of PD, evidence points to characterizing PD as an oxidative stress disorder, rather than simply a disease of the dopaminergic system.

Many chemical models of PD take advantage the nigrostriatal system's heightened susceptibility to cell death by employing oxidative damage or inducing mitochondrial dysfunction as their primary mechanism of action. For example, MPTP and rotenone both inhibit mitochondrial complex I to induce a PD-like pathology (Schapira et al., 1990; Jenner, 2001). However, no chemical model has been able to successfully reproduce all the behavioral symptoms of the disease. In mice, the inability to fully recapitulate PD is most likely due to researchers' focus only the redox state of dopaminergic neurons, and not the parallel destruction

of dopaminergic and other neurotransmitter systems. When a dopaminergic neurotoxin is given in concert with a noradrenergic toxin, such as DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride), nigrostriatal damage is exacerbated, amplifying parkinsonian behavioral deficits (Mavridis et al., 1991; Dauer and Przedborski, 2003; Marien et al., 2004). Moreover, mice lacking NE have been shown to have parkinsonian motor deficits, despite their normal striatal dopamine content, suggesting that both catecholamines play a significant role in the pathogenesis of PD (Rommelfanger et al., 2007; Rommelfanger and Weinshenker, 2007).

When the neuron cannot manage high levels of cytosolic catecholamines, the cell's antioxidant defenses are presumably activated. These defenses include intracellular GSH and vitamin E on the cell membrane (Cadet, 1988). Alternatively, extracellular DA and NE have been proposed to act as antioxidants *in vitro* via inhibition of lipid peroxidation and chelation of iron ions, and could potentially offset the deleterious effects of intracellular catecholamines (Liu and Mori, 1993). However, it is unknown whether extracellular catecholamines are effective as antioxidants *in vivo*. Catecholaminergic neuron's primary defense against exogenous and endogenous toxicants is the vesicular monoamine transporter (VMAT2). Normally, catecholamines are highly regulated within the neuron. Their respective reuptake transporters, DA transporter (DAT) or NE transporter (NET), regulate selective uptake of DA and NE from the synapse, modulating the magnitude and duration of neuronal signaling. VMAT2 pumps cytosolic DA and NE into vesicles for regulated exocytotic release, inhibiting their auto-oxidation potential and metabolism in the cytosol. In order to study the role of VMAT2 in PD, many groups generated VMAT2 KO mice (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). Complete ablation of VMAT2 is lethal by postnatal day 14. However, Mooslehner et al generated VMAT2 hypomorph mice that only express ~5% of the VMAT2 protein (Mooslehner et al., 2001). Reduced expression of VMAT2 diminishes the neuron's ability to package DA and dopaminergic neurotoxicants; as a result, DA accumulates in the cytosol with the ability to induce oxidative stress without the use of other exogenous toxicants.

While the oxidative stress hypothesis of PD has the potential to explain the slow progressive nature of the disease, along with its selective degeneration of monoaminergic neurons, other hypotheses have been proposed involving the contribution of α-synuclein. Alpha-synuclein is a 140 amino acid, natively unfolded soluble protein that is normally localized on presynaptic nerve terminals in close proximity to synaptic vesicles (Jakes et al., 1994; Goedert, 2001; Ischiropoulos, 2003). Alpha-synuclein, which is the major component of Lewy bodies (LB), has been thought to interact with vesicular membranes and VMAT2 (Spillantini et al., 1997; Abeliovich and Flint Beal, 2006). Although there have been many theories about the contribution of α-synuclein to the development of PD, the function of the protein remains largely unknown.

One hypothesis incorporates the oxidative nature of catecholamines with the ability of α-synuclein to fibrillize into protein aggregates. DA and other catecholamines have been proposed to inhibit α-synuclein fibrillization through oxidative modification of critical residues in α-synuclein inducing a conformational change that prevents full fibril formation (Conway et al., 2001; Norris et al., 2005). This activity was found to be reversed or inhibited by antioxidants; however, under inhibitory conditions, protofibril concentration increases eventually seeding the fibrils present in LBs (Goldberg and Lansbury, 2000; Conway et al., 2001; Lotharius and Brundin, 2002). Assuming that protofibrils are pathogenic, decreasing VMAT2 in neurons can have lethal implications, causing the cytosolic autooxidation of catecholamines to increase, amplifying protofibril concentration. Based upon the oxidative stress and α-synuclein hypotheses, the vesicular storage of catecholamines has the potential to greatly influence PD pathogenesis, placing VMAT2 at the center of investigation.

Vesicular monoamine storage

Evidence for the monoamine theory of PD surfaced as early as the 1950s but was not fully appreciated until recently. Reserpine, an inhibitor of vesicular monoamine transport, was first introduced as a potent anti-hypertensive drug (Freis, 1954). Reserpine acts by depleting cells

of their monoamine stores; however, it is not selective for the periphery and affects the central nervous system as well (Freis, 1954; Peter et al., 1994). Patients who took reserpine chronically began to display lethargy similar to that seen in depression, giving rise to the monoamine hypothesis of affective disorders (Freis, 1954). In order to study lethargy associated with reserpine, animal models were used. Apparently, unbeknownst to researchers, the symptoms observed in mice dosed with reserpine reproduced many of the hallmarks of PD. Using the expanded definition of PD, reserpinized mice displayed a decrease in locomotor activity, L-DOPA responsive stride length, a depressive-like phenotype, and cognitive decline (Schneider, 1954; Fernagut et al., 2002; Silva et al., 2002; Skalisz et al., 2002). Acute depletion of monoamine stores was found to reproduce a similar symptom profile as mice dosed with MPTP, the gold standard of PD models. Moreover, reserpinization of mice faithfully reproduces more features of the disease than the PARK loci mutants and chemical models of PD. Despite this, the site of action for reserpine was not discovered until the early 1990s (Erickson et al., 1992; Liu et al., 1992).

VMAT2 is a 12 transmembrane domain H^+ ATPase antiporter that sequesters monoamines in secretory vesicles for regulated exocytotic release. VMAT2 has a similar selectivity for all monoamines and is present throughout the central nervous system and in the periphery in mast cells and platelets. Reserpine irreversibly inhibits VMAT2, to the extent that new protein must be synthesized to halt reserpine's effects. Researchers began to investigate the possibility of making animals models with altered levels of VMAT2 expression, beginning with VMAT2 knockout mice. These mice were originally made to study the importance of VMAT2 in maintenance of presynaptic function (Fon et al., 1997; Wang et al., 1997). However, these mice had limited utility since they did not survive long after birth and displayed reduced locomotion and feeding activity (Fon et al., 1997; Wang et al., 1997). Using cultured neurons from these mice, researchers observed that expression of VMAT2 dictates the size of the vesicle, the amount of neurotransmitter stored within the vesicle, and general brain monoamine content (Fon et al.,

1997; Wang et al., 1997; Pothos et al., 2000). Given that VMAT2 expression directs a significant part of neurotransmitter activity within the presynaptic cell, it also has the potential to regulate the lifespan of the neuron. Using the aforementioned oxidative stress hypothesis of PD, the indirect antioxidant capabilities of VMAT2 are vast.

Dopamine, norepinephrine, and, to a lesser extent, serotonin all have the ability to spontaneously oxidize in the cytosol and create free radicals during metabolism (Graham, 1978). These free radicals can then oxidize lipids or protein, thereby damaging the cell. VMAT2 acts as the first line of defense, protecting the cell from monoamines that have the ability to act as endogenous toxins that directly increase oxidative stress (Liu and Edwards, 1997). However, no human has complete ablation of VMAT2, so the direct utility of VMAT2 knockouts or reserpine as a model of PD approaches irrelevance. Moreover, it is impossible to measure VMAT2 levels in current PD patients because of the cell death that has already occurred. Genetic studies have demonstrated that there are variations in VMAT2 expression within the human population shown by small nucleotide polymorphisms in VMAT2 in humans, and a gain of function haplotype that has shown to be protective in women against PD (Burman et al., 2004; Glatt et al., 2006). Even though the VMAT2 KO mice do not survive, it is possible to have very low levels of VMAT2. As mentioned above, mice that only express ~5% of the VMAT2 protein were made (Mooslehner et al., 2001). Reduced expression of VMAT2 diminishes the neuron's ability to package monoamines and dopaminergic neurotoxicants; as a result, DA accumulates in the cytosol with the ability to induce oxidative stress independent of any oxidative stress caused by exogenous toxicants. These multisystem dysfunctions more closely mimic the pathogenesis of PD, suggesting a greater involvement of the monoamine systems than previously thought.

Introduction to specific aims

Parkinson's disease (PD) is a neurodegenerative disease that affects more than one million people in the United States and is characterized by a marked decrease in mobility and the onset of resting tremors. Many of the symptoms of PD are caused by the death of nigrostriatal

dopamine neurons in the brain, and usually are not diagnosed until over 80% of striatal DA is lost. Recent studies have also implicated neurodegeneration in the locus coeruleus in the progression of PD. Current treatments for PD include MAO inhibitors, DA and NE agonists, and the DA precursor, L-DOPA. Due to this widespread catecholaminergic deficiency in the brain, it is very difficult to identify the factors that contribute to the prevalence of this disease.

Many chemical models of PD utilize the inhibition of mitochondrial function or the induction of an oxidative environment to cause neuronal cell death. It has been suggested that these models typically utilize catecholamines' unique ability to auto-oxidize and induce oxidative stress. Moreover, it is theorized that treatments such as L-DOPA can enhance oxidative stress by increasing endogenous oxidation of DA into ROS. Because of this, the role of VMAT2 in the progression of PD is becoming increasingly important in etiologic and therapeutic PD research. Normally, VMAT2 protects the cell from damage induced by cytosolic DA and exogenous toxicants by sequestration into vesicles. Reduced expression of VMAT2 diminishes the neuron's ability to package catecholamines and catecholaminergic neurotoxicants. As a result, catecholamines accumulate in the cytosol with the ability to induce oxidative stress independent of any oxidative stress caused by exogenous toxicants. Increased expression of VMAT2 may be able to prevent oxidative damage and subsequent death of the cell. It was hypothesized that VMAT2 can contribute to the development of parkinsonian pathology and symptoms by causing neuronal dysfunction as a consequence of reduced vesicular storage.

Previously it has been shown that mice with genetic reduction of the vesicular monoamine transporter (VMAT2-deficient) display progressive loss of striatal dopamine, L-DOPA responsive motor deficits, synuclein aggregation, and nigral dopamine cell loss (Caudle et al., 2007). This work aims to further probe the development of parkinsonian pathology and behavioral phenotypes resulting from reduced vesicular monoamine storage in these mice.

CHAPTER 2

BEHAVIORAL PHENOTYPING OF MOUSE MODELS OF PD

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Abstract

Parkinson's disease (PD) is a common neurodegenerative movement disorder afflicting millions of people in the United States. The advent of transgenic technologies has contributed to the development of several new mouse models, many of which recapitulate some aspects of the disease; however, no animal model has been demonstrated to faithfully reproduce the full constellation of symptoms seen in human PD. This may be due in part to the narrow focus on the dopamine-mediated motor deficits. As current research continues to unmask PD as a multi-system disorder, animal models should similarly evolve to include the non-motor features of the disease. This requires that typically cited behavioral test batteries be expanded. The major non-motor symptoms observed in PD patients include hyposmia, sleep disturbances, gastrointestinal dysfunction, autonomic dysfunction, anxiety, depression, and cognitive decline. Mouse behavioral tests exist for all of these symptoms and while some models have begun to be reassessed for the prevalence of this broader behavioral phenotype, the majority has not. Moreover, all behavioral paradigms should be tested for their responsiveness to L-DOPA so these data can be compared to patient response and help elucidate which symptoms are likely not dopamine-mediated. Here, we suggest an extensive, yet feasible, battery of behavioral tests for mouse models of PD aimed to better assess both non-motor and motor deficits associated with the disease.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and is widely thought to primarily affect the dopamine (DA) neurons of the substantia nigra pars compacta (SNpc). PD is considered to be due to the combination of genetic and environmental factors (Olanow and Tatton, 1999; Jenner and Olanow, 2006; Sulzer, 2007). Most animal models of the disease have stemmed from this concept, and have employed a myriad of genetic manipulations and/or endogenous/exogenous toxic insults to recapitulate the symptomatology and/or neuropathology of PD (Meredith and Kang, 2006). Thus far, the standard of animal model behavioral assessment has been the presence of a parkinsonian motor phenotype (Sedelis et al., 2001; Brooks and Dunnett, 2009). Various behavioral tests have been routinely used to qualify PD mouse models including, locomotor activity, rotarod, forepaw stride length, grid test, and pole test (Schallert et al., 2000; Sedelis et al., 2001; Tillerson et al., 2002; Colebrooke et al., 2006; Rommelfanger et al., 2007; Brooks and Dunnett, 2009) (see Table 1). While these techniques have proven useful for verifying a parkinsonian motor phenotype and gaining insight to possible causes of motor dysfunction, a disconnect still remains between the wealth of mouse models for PD and the slow progress towards restorative therapeutics. This disconnect may be due to the evolving definition of PD as a multisystem syndrome (Langston, 2006; Jenner, 2008; Olanow et al., 2008).

Clinically, PD is not diagnosed until the onset of motor deficits (Lang and Obeso, 2004; Langston, 2006); this has likely contributed to the dominance of motor-based animal models. However, there are many non-motor symptoms associated with PD that can appear years, sometimes decades, before the onset of the motor phenotype (Shulman et al., 2001; Chaudhuri et al., 2006; Ziemssen and Reichmann, 2007). These symptoms include hyposmia, sleep abnormalities, gastrointestinal disturbances, anxiety, depression, autonomic dysfunction, and impaired cognition (Shulman et al., 2001; Chaudhuri et al., 2006; Ziemssen and Reichmann,

2007). Some of these symptoms respond to dopaminergic therapies; others do not and contribute to diminished quality of life for PD patients (Comella, 2003; Ponsen et al., 2004; Chaudhuri et al., 2006). This highlights the importance of shifting attention to non-motor symptomatology in mouse models of the disease. The non-motor phenotype is more difficult to treat as the underlying pathophysiology remains unclear. This may be a reflection of the widespread neuronal loss that occurs in neurotransmitter systems other than the nigrostriatal pathway (Lang and Obeso, 2004; Jenner, 2008). Though, at present, the effects of nondopaminergic drugs in mouse models do not effectively translate into clinical efficacy (Jellinger, 1991; Ziemssen and Reichmann, 2007; Olanow et al., 2008). Expanding the required behavioral phenotype in mouse models could lead to progress towards more effective therapeutics. Here, we propose a battery of behavioral tests designed to assess a larger array of behavioral symptoms associated with PD.

General Health

Although Parkinson's disease is a debilitating disorder with symptoms spanning a wide spectrum of organ systems, there are many aspects that remain healthy in PD patients. When characterizing a mouse model, it is important to confirm that any aberrant phenotypes are not due to generally poor health. For example, after acute administration of MPTP or rotenone, animals become quite ill. After the animals have had the chance to recover from the acute toxicity, PD-like behaviors can then be evaluated. Moreover, PD is a relatively selective disorder in that it primarily targets regions innervated by monoamines (Braak et al., 2003; Langston, 2006); even within this category some symptomatology remains rare. While characteristic phenotypic behaviors help define the disease, lack of deficits in other systems is also important in showing relative selectivity.

Tactile, gustatory function and trigeminal nerve response are used as indices of gross sensory function, independent of olfactory deficits. Responsiveness to tactile stimulation is commonly assessed by latency to remove a small adhesive dot from the animal's forehead (Schallert et al., 2000; Tillerson et al., 2006; Taylor et al., 2009). In a two minute trial, an

adhesive dot is placed between the ears on the top of the head in their home cage and monitored for latency to removal. Despite dopamine abnormalities, dopamine transporter knockout (DAT -/-), D_2 receptor knockout (D_2 -/-), and vesicular monoamine transporter 2 (VMAT2) deficient mice, all of which have some level of altered dopamine homeostasis, were found to respond normally to tactile stimulation relative to their wildtype littermates (Tillerson et al., 2006; Taylor et al., 2009).

Gustatory function can be tested using a taste aversion paradigm. Quinine is frequently used due to its unpalatable bitterness (Tillerson et al., 2006; Taylor et al., 2009). In this test, a cotton swab is used to expose the mouse to either an aliquot of quinine or water; the latency to groom or drag the jaw along the ground is then measured during a one minute test session (Grill and Norgren, 1978; Schallert and Whishaw, 1978; Tillerson et al., 2006). Again, despite abnormalities in dopamine and other monoamines, DAT -/-, D_2 -/-, and VMAT2-deficient mice displayed similar taste aversion to quinine as their wildtype littermates (Tillerson et al., 2006; Taylor et al., 2009). Finally, trigeminal nerve function is assessed by exposing the mice to ammonia, which is a known irritant, or water. While the trigeminal nerve innervates the olfactory epithelium and nasal mucosa, it is not responsible for olfactory responses. Rather it is responsible for non-odor sensations such as mild irritation and burning (Tillerson et al., 2006). In a two minute session, the animal's response to ammonia or water is measured by time spent sniffing (nose <1 cm away from stimulus) each scent. Normally, mice show preferential exploration of water due to the mild irritation induced by ammonia (Tillerson et al., 2006; Taylor et al., 2009). Collectively, these three simple tests can indicate deficits in general sensory function, independent of any perceived olfactory deficits.

Finally, even though vision has not been found to be significantly affect in PD, normal vision is a prerequisite for many behavioral tests, especially for visuospatial learning tasks. For more general tests of visual acuity, the visual cliff test can be employed. Visual cliff measures the ability of a mouse to see the drop-off at the edge of a horizontal surface (Crawley, 1999). The

test consists of a box with a ledge covered with patterned contact paper to emphasize the drop-off; a piece of clear Plexiglas covers the ledge so there is only a perceived drop-off. If a mouse is blind, it will not see the appearance of the edge and explore the Plexiglas immediately (Crawley, 1999). Visual cliff serves to assess gross visual function and give insight as to if more complex visual tests should be completed. Gross visual function can be quantified by electroretinogram (ERG). The ERG can detect abnormalities in retinal function and in the electrical responses of photoreceptor cells (Sugawara et al., 1998; Cameron et al., 2008). Studies in younger VMAT2-deficient mice have demonstrated zero deficiencies in retinal function as compared to wildtype littermates (Taylor et al., 2009).

Motor phenotype of PD

Parkinson's disease is not usually diagnosed until over 80% of the striatal dopamine innervation has been lost (Jellinger, 1991; Fahn and Sulzer, 2004; Chaudhuri et al., 2006). When the neurons in the SNpc die, the levels of dopamine in the striatum decrease resulting in a loss of voluntary motor control. The cardinal symptoms of PD include tremor, rigidity, postural instability, and bradykinesia, which have contributed to the basis of most behavioral testing in mouse models of PD. The most prominent model of PD was discovered because of its ability to recapitulate many of the features associated with the severe motor phenotype of PD (Langston, 1987). One of the most basic ways to assess the presence of a motor phenotype in a mouse model of PD is to use locomotor activity chambers. Mice are typically placed in transparent locomotor chambers, and activity is measured by consecutive photobeam breaks or ambulations in a certain time period. As shown in Table 1, most mouse models of PD reflect a basic motor phenotype as evidenced by decreased locomotor activity in open field behavioral chambers. When challenged with L-DOPA, the LRRK2, Pitx3-aphakia, MitoPark, and VMAT2-deficient mice all displayed increases in locomotor activity (Hwang et al., 2005; Caudle et al., 2007; Ekstrand et al., 2007; Li et al., 2009). The shuffling gait observed in PD patients can be considered analogous to forepaw stride length, which is also easily evaluated in mouse models of PD. In this test, mice are trained

to walk down a narrow corridor and their forepaws are inked to analyze any deficits in stride length (Tillerson et al., 2002). The α-synuclein transgenic mice, DJ-1 mice, and mice dosed with MPTP or reserpine all demonstrate deficits in forepaw stride length; however, only MPTP-dosed and reserpinized mice respond positively to acute doses of L-DOPA (Fernagut et al., 2002; Tillerson et al., 2002; Gispert et al., 2003; Chandran et al., 2008; Plaas et al., 2008). Other features of the parkinsonian motor phenotype such as coordination, rigidity, and tremor can also be tested behaviorally and are outlined in Table 1.

Modeling PD in animals has, until recently, focused on behaviors that involve striatal function and that should improve with dopamine replacement therapy (Dauer and Przedborski, 2003); and previously, criteria for a functional PD animal model only demanded a motor phenotype. While all parkinsonian behaviors should be tested for their response to L-DOPA, it is paramount that the primary motor deficits observed in mouse models respond positively to this primary PD therapeutic. However, research suggests that PD is more than a dopamine-based motor disorder, thus exploring non-motor symptoms and non-dopamine mediated behaviors is warranted. By using a more comprehensive battery of behavioral tests, new pharmacological treatments can be developed that treat more than the dopaminergic deficit (see Table 3).

Olfactory deficits in PD

Since the preclinical phase of PD begins long before the degeneration of the substantia nigra, assessment of non-motor symptoms can further the understanding of the true PD disease process. Olfactory disturbances are one for the first non-motor symptoms observed in PD; hyposmia can be multifaceted and are not restricted to one modality. PD patients have demonstrated impairments in odor detection, differentiation, and identification (Ward et al., 1983; Doty et al., 1992; Tissingh et al., 1998). Moreover, this non-motor symptom is not responsive to dopaminergic therapies (Kranick and Duda, 2008), and occurs with a similar frequency to resting tremor (Doty et al., 1992). Behavioral testing of olfaction can facilitate an earlier detection of

PD, since impaired olfaction has been positively correlated with an increased risk of developing the disease (Ross et al., 2008).

To measure general olfactory function, the buried pellet test can be used. This test relies on the mouse locating a hidden object, usually a food pellet, by odor (Crawley, 1999; Nathan et al., 2004; Fleming et al., 2008). The amount of time a food-restricted animal takes to find and uncover a food pellet or reward is measured. Alternately, the latency to locate buried food versus the latency to locate food placed on the surface can also be measured (Fleming et al., 2008). In a study with mice overexpressing human wildtype α-synuclein (Thy1-aSyn), Thy1-aSyn mice displayed a longer latency to find a buried pellet than wildtype littermates (Fleming et al., 2008) (see Table 2). However, Thy1-aSyn mice have a similar latency to wildtype mice when forced to locate a food pellet on the surface (Fleming et al., 2008). Studies using MPTP-treated mice and ApoE knockout mice yielded similar results (Nathan et al., 2004; Schintu et al., 2009).

The novel scent test and block test can be used to measure olfactory acuity and discrimination. The novel scent test is a simple way to quantify time spent sniffing/exploring a novel odor. This test can be made more relevant to human PD by using scents tested in the University of Pennsylvania Smell Identification Test, which is commonly used to diagnose hyposmia/anosmia in humans (Doty et al., 1984). Commonly used olfactory cues are attractive to mice, but vary over a range of scent classes. The mouse is presented small aliquots of either a novel scent (lemon, peppermint, or vanilla) or water simultaneously (Taylor et al., 2009). Time spent sniffing each odor is recorded for a three minute session. When given the choice between a novel odor and water, both wildtype and VMAT2-deficient mice show a preferential exploration of the novel scent at 2 months of age; however, VMAT2-deficient mice lose this ability by 18 months of age (Taylor et al., 2009) (see Table 2). The block test evaluates the ability of mice to discrimination between social odors, specifically self and non-self (Tillerson et al., 2006; Taylor et al., 2009). The animal is presented with a wooden block scented with its own bedding and a block scented with another mouse's bedding (of the same sex). The time spent in contact with

each block is recorded for a two minute trial (Tillerson et al., 2006; Taylor et al., 2009). In experiments with DAT knockout and D_2 knockout animals, DAT and D_2 -/- mice do not display a preferential exploration of the block scented with a foreign animal's bedding, which was not due to decreased exploratory activity (Tillerson et al., 2006). This is in contrast to wildtype littermates that always display a preferential exploration for the block scented with a foreign animal's bedding (Tillerson et al., 2006). Similarly, VMAT2 wildtype mice show preferential exploration of the block scented with the foreign animal's bedding at all ages; whereas, VMAT2-deficient mice exhibit preferential exploration until 4 months of age, but not by 6 and 12 months of age (Taylor et al., 2009) (see Table 2).

To examine more subtle olfactory deficits, habituation/dishabituation paradigms can be employed. Briefly, a small plastic cartridge is packed with cotton scented with a novel odor and placed in the home cage of the subject for one minute over four trials. In the fifth dishabituation trial the subject is presented with a cotton ball scented with a different novel odor. The time spent in olfactory investigation for each trial is recorded, and olfactory investigation is defined as direct nasal contact with the cartridge (Bielsky et al., 2004; Fleming et al., 2008). DAT and D_2 -/- mice were both able to habituate to the novel odors of paprika or cinnamon, but neither mouse demonstrated increased investigation to novel odor versus the habituated odor, unlike their wildtype littermates (Tillerson et al., 2006). Thy1-aSyn mice were able to habituate and then discriminate between habituated and novel odors at younger ages (Fleming et al., 2008) (see Table 2).

Sleep abnormalities in PD

Nocturnal sleep disturbances affect 60-98% of patients suffering from Parkinson's disease, including night-time awakenings, sleep fragmentation, and REM sleep disorder (Comella, 2007). Because PD patients often do not get a full night's sleep, they also suffer from excessive daytime sleepiness (EDS). EDS has also been found to increase the risk for development of PD and is correlated with advanced stages and longer duration of PD (Ondo et

al., 2001; Abbott et al., 2005). Sleep dysfunction is an important non-motor symptom associated with PD and has been correlated with other non-motor symptoms such as anxiety, depression, and GI disturbances (Borek et al., 2006; Dhawan et al., 2006). These problems occur more frequently as the PD disease state advances; early untreated patients often report nocturia, night-time cramps, dystonia, and tremor (Chaudhuri et al., 2006; Dhawan et al., 2006; Zesiewicz et al., 2006). However, some night-time problems can be reduced by optimizing PD medications, since motor symptoms can contribute to disturbed sleep and EDS (Zesiewicz et al., 2006).

Measuring latency to behavioral signs of sleep is the simplest test used to evaluate sleep abnormalities in mice. This test assesses the time it takes for mice to attain behavioral signs of sleep during their circadian nadir. During sleep, mice exhibit a distinctive posture and breathing pattern that allows the observer to determine onset (Mitchell et al., 2008). On test day, mice are removed from their home cages, placed individually in behavioral chambers, and allowed to acclimate for 4 hours during their light cycle. During circadian nadir, mice are handled, injected with saline to ensure they are awake, placed back into the behavioral chamber and monitored for latency to achieve behavioral signs of sleep (Mitchell et al., 2008; Taylor et al., 2009). Sleep is defined as two minutes of uninterrupted sleep behavior, and 75% of the next 10 minutes spent asleep; this behavioral scoring paradigm has been shown to reliably correlate with onset of sleep using electroencephalography (EEG) measurements (Hunsley and Palmiter, 2004; Mitchell et al., 2008). Although this test has not been completed in many mouse models of PD, sleep latency has been conducted in a mouse model of catecholamine deficiency. Dopamine β-hydroxylase knockout (*DβH* −/−) mice, that lack norepinephrine after birth, have a shorter sleep latency than *DβH* +/− mice, which have wildtype noradrenergic levels (Hunsley and Palmiter, 2003; Mitchell et al., 2008). Treating DβH -/- mice with the wakefulness promoting drug, modafinil, dose-dependently increases sleep latency in both *DβH* +/− and *DβH* −/− mice, but the *DβH* −/− mice were hypersensitive to the wake-promoting effects of modafinil (Mitchell et al., 2008).

If a mouse model displays an altered latency to behavioral signs of sleep, more sophisticated tests involving polysomnography and electromyography (EMG) can be employed. Polysomnography can help determine the underlying characteristics of sleep abnormalities, and which stages of the sleep-wake cycle may be affected. These studies are most similar to methods used to detect aberrant sleep phenotypes in humans (Monaca et al., 2003; Laloux et al., 2008b). EMG electrodes are inserted into the neck muscles and the mouse is recorded for 24-48 hour periods. Polysomnographic recordings are then scored visually in five second epochs as wakefulness, slow wave sleep (SWS), or paradoxical sleep (PS) according to standard criteria (Valatx and Bugat, 1974; Tobler et al., 1997). Studies in mice that were exposed to MPTP 20 days prior reveal increased PS during the dark phase and increased number of PS bouts during a 24 hour period compared to mice dosed with vehicle (Laloux et al., 2008a; Laloux et al., 2008b). When MPTP mice were treated with an acute dose of L-DOPA, PS latency and amounts of wakefulness were found to increase (Laloux et al., 2008a). However, 40 days after MPTP exposure, no differences between treated and control mice were observed in the number of PS episodes during the dark cycle or in a 24 hour period (Laloux et al., 2008b) (see Table 2).

An alternate test to polysomnography is using a 2-dimensional state map with EMG to identify behavioral sleep states. This test is used to differentiate rapid eye movement (REM) sleep specifically from wakefulness, and is confirmed using behavioral observations (Dzirasa et al., 2006). Novelty exposed DAT -/- mice were observed to enter a novel awake state resembling REM sleep (Dzirasa et al., 2006). Interestingly, when DAT -/- mice are acutely depleted of dopamine, they enter a different novel awake state, which resembles SWS, but with suppression of SWS and REM sleep (Dzirasa et al., 2006). Treatment with D_2, but not D_1, agonists recovers only REM sleep (Dzirasa et al., 2006). Despite these sophisticated sleep behavioral paradigms, the underlying causes of sleep disorders in PD remain unknown and could be due to disease progression, dopaminergic or noradrenergic pathology, current medications, or a combination of factors.

Gastrointestinal dysfunction in PD

Gastrointestinal (GI) dysfunction affects more than 70% of PD patients and has been attributed to a variety of factors including lack of activity, inadequate hydration, and autonomic/enteric neuronal dysfunction (Edwards et al., 1993; Abbott et al., 2001; Pfeiffer, 2003). Pathologically, Lewy bodies have been found in the myenteric and submucosal plexuses of the enteric nervous system (Wakabayashi et al., 1988; Braak et al., 2006; Anderson et al., 2007). Moreover, there is evidence of decreased dopamine neurons in the enteric nervous system of PD patients, and for the involvement of the dorsal motor nucleus of the vagus (DMV) due to α-synuclein pathology independent of nigral degeneration (Singaram et al., 1995; Braak et al., 2003; Tian et al., 2008). Some manifestations of GI dysfunction in PD are early satiety and nausea from delayed gastric emptying, bloating from poor small bowel coordination, and constipation (Anderson et al., 2007). However, GI dysfunction is not exclusive to late stage PD patients, and can also manifest in the early stages of the disease before motor involvement (Bassotti et al., 2000). Delayed gastric emptying can also interfere with drug action by disrupting drug absorption in the intestinal tract (Pfeiffer, 2003). An association between the frequency of bowel movements and risk for developing PD has been found in patients who are constipated, defined as having fewer than three bowel movements in a week; these patients have 2-7 times higher risk of developing PD later in life (Abbott et al., 2001; Pfeiffer, 2003).

Screening for GI dysfunction in mouse models of PD can be done using solid gastric emptying to evaluate stomach motility and stool collection to examine colon motility. Solid gastric emptying is tested after the mouse undergoes a 12-hour fast. Mice are then allowed free access to food for a defined period and, the amount of food consumed is calculated. After food removal (15-120 minutes), animals are killed, the stomach contents are weighed (wet and dried), and the percentage of food remaining in the stomach is measured (Whited et al., 2006; Anderson et al., 2007; Taylor et al., 2009). When compared to wildtype animals, VMAT2-deficient mice were found to have delayed gastric emptying overall, with a greater apparent effect at 12 months

of age (Taylor et al., 2009) (see Table 2). MPTP has been shown to deplete dopamine neurons in the enteric nervous system; however, solid gastric emptying remained unaffected (Anderson et al., 2007) (see Table 2).

To screen for constipation in mouse models, the mouse is monitored for one hour and stool is collected. Each mouse is placed in a separate clean cage and observed throughout the 60 minute collection period. Fecal pellets are collected immediately after expulsion and placed in sealed (to avoid evaporation) 1.5 mL tubes. Tubes are weighed to obtain the wet weight of the stool; these are then dried overnight at 65°C and reweighed to obtain the dry weight (Li et al., 2006; Anderson et al., 2007; Taylor et al., 2009). Even though PD patients have been found to be constipated, several mouse models of PD have demonstrated increased stool frequency compared to wildtype or saline treated animals. Thy1-αSyn mice exposed to a novel environment had an increased stool frequency relative to wildtype mice; although, when habituated to the experimental environment Thy1-aSyn mice displayed reduced stool frequency (Wang et al., 2008) (see Table 2). Correspondingly, mice treated with MPTP have significantly higher stool frequency 2-3 days after treatment, which decreases to saline-treated animals' levels by 8-10 days after MPTP treatment (Anderson et al., 2007) (see Table 2). Similar results were observed in VMAT2-deficient mice (Taylor et al., 2009) (see Table 2).

Colon motility and innervation can be monitored in more detail using bead latency and isometric muscular force recording (Anitha et al., 2006; Anderson et al., 2007). In the bead latency test, mice are anesthetized and a glass bead is inserted into the colon; distal colon motility is assessed by monitoring the time required for the bead to be expelled (Wang et al., 2008). To evaluate enteric neuronal circuitry, sections of proximal colon are removed with their enteric innervation intact and suspended between electrodes in Krebs buffer. Myenteric neuron function can be interrogated using a combination of pharmacological agents and electrical field stimulation using muscular force as a readout (Anitha et al., 2006; Anderson et al., 2007). Bead latency was found to be unchanged in Thy1-αSyn mice, compared to wildtype littermates (Wang et al., 2008)

(see Table 2). In mice treated with MPTP, enteric dopamine neuron loss was found to impair relaxation of muscle from the proximal colon, indicating dysfunction in the inhibitory neurons in the enteric nervous system of MPTP-treated animals (Anderson et al., 2007) (see Table 2).

Other assays of total GI transit time and small intestine transit can also be considered. As with most non-motor symptoms, since the exact cause of GI dysfunction in PD patients remains unknown, interpretation of gastrointestinal dysfunction and response to therapeutic interventions in mouse models should be cautious.

Anxiety and Depression

Anxiety and depression affect approximately 40% of patients with PD; however, the reason for such a high frequency of these two disorders in PD is poorly understood (Lemke et al., 2004; Remy et al., 2005; Poewe, 2007). The rate of severe depression in PD has been found to be twice that of other equivalently disabled patients, but because of overlapping clinical symptoms, diagnosis of anxiety and depression is subjective (Lemke et al., 2004; Remy et al., 2005). Moreover, even though there is a significant association between phobic anxiety scores, depressive episodes and risk of developing PD, neither disorder parallels PD pathogenesis or progression (Brown and Jahanshahi, 1995; Weisskopf et al., 2003; Remy et al., 2005). Although depressed PD patients have corresponding loss in monoaminergic (dopaminergic, noradrenergic, and serotonergic) projections, mood fluctuations have been found to occur independently of motor fluctuations and are often improved by anti-PD medications (Maricle et al., 1995; Czernecki et al., 2002; Poewe, 2007). Clinical features of depression in PD include increased levels of dysphoria, irritability, feelings of failure, but low suicide rates and ideations of suicide (Cummings, 1992; Lemke et al., 2004; Poewe, 2007). Even though anxiety and depression are under-diagnosed and undertreated in PD, they have a major impact on quality of life in PD (Schrag et al., 2000; Lemke et al., 2004; Poewe, 2007).

Anxiety-like behaviors can be measured effectively in mice by using the elevated plus maze (EPM), open field testing, and light-dark exploration. The EPM apparatus consists of two

open arms and two enclosed arms arranged in a plus-sign orientation. Because rodents naturally prefer dark, enclosed compartments, a greater willingness to explore the open, well-lit arms is believed to represent a decrease in the animal's anxiety (Pellow et al., 1985; File et al., 1988; Paine et al., 2002). DJ-1 knockout mice were found not to exhibit an anxiety-like phenotype as measured by EPM, showing no significant difference in exploration times compared to wildtype littermates (Chandran et al., 2008) (see Table 2). Alpha-synuclein (A53T) transgenic mice displayed reduced anxiety-like behaviors on the EPM at younger ages (George et al., 2008) (see Table 2). Most notable, the α-synuclein transgenic mice demonstrated significantly higher time spent in the open arms of the EPM compared to wildtype and α-synuclein knockout mice (George et al., 2008). Interestingly, younger VMAT2-deficient animals spend a greater percentage of their time in the closed arms of the elevated plus maze as compared to age-matched WT animals, exhibiting an anxiety-like phenotype (Taylor et al., 2009) (see Table 2).

In the open field test, mice are placed into an open behavioral chamber, usually during the light cycle, where horizontal and vertical activities can be monitored by photobeam breaks (Fukui et al., 2007). Moreover, the animal's behavior can be videotaped and analyzed for time spent in the center of the chamber versus along the perimeter (Treit and Fundytus, 1988). Since rodents have a natural inclination to stay near the perimeter of a chamber (thigmotaxis), increased thigmotaxis is indicative of anxiety-like behavior (Treit and Fundytus, 1988). Although, the A53T α-synuclein transgenic mice did not have an anxiety-like phenotype in the EPM, the α-synuclein transgenics did display a selective anxiety-like phenotype in the open field test (George et al., 2008) (see Table 2). This was indicated by reduced habituation and increased thigmotaxis compared to α-synuclein knockout and wildtype mice (George et al., 2008). Parkin-deficient mice also exhibit increased thigmotaxic behavior, coupled with decreased horizontal travel distance, compared to wildtype mice (Zhu et al., 2007) (see Table 2).

The light-dark exploration test is frequently used to further analyze anxiety-like phenotypes in mice. The test is conducted in a behavioral chamber partitioned into two

compartments. One compartment is clear without a lid and illuminated by an overhead lamp, while the other compartment is enclosed with black cloth and covered with a black Plexiglas lid. Mice are placed in the illuminated side, and, after a period of time, are allowed access to the darkened side of the chamber. Latency to first enter the darkened side, time spent on each side, head pokes into each side, and number of transitions between chambers can all be used to assess anxiety-like behavior (Fukui et al., 2007; Zhu et al., 2007). Both younger and older parkin mice were found to spend significantly less time in the illuminated side of the light-dark chamber and made fewer transitions between the two compartments than wildtype littermates (Zhu et al., 2007) (see Table 2). Taken together, these results are indicative of an anxiety-like phenotype that does not appear to be age-dependent (Zhu et al., 2007). However, mice treated with MPTP were not found to exhibit an anxiety-like phenotype as measured by the light-dark exploration test (Vuckovic et al., 2008) (see Table 2).

Depression is one of the most difficult behaviors to assess in mouse models, and most tests are actually tests of antidepressant efficacy. The forced swim test and tail suspension tests are acute measures of antidepressant efficacy that rely on immobility or behavioral despair to determine depressive-like behavior in mice. This immobility may be related to variations in stress-induced behavioral depression and can be correlated with the psychological construct of entrapment seen in clinical depression (Dixon, 1998; Lucki et al., 2001). In the forced swim test, mice are placed individually in glass cylinders with 6 inches of water, and their behavior is videotaped from the side of the cylinder for 6 minutes. After the first two min, the total duration of time spent immobile is recorded during a 4 minute test period (Porsolt et al., 1979; Fukui et al., 2007). VMAT2 heterozygous mice, which have a 50% decrease in VMAT2 expression, have significantly increased immobility times compared to wildtype animals, suggesting a depressive-like phenotype (Fukui et al., 2007). When given the antidepressant imipramine, immobility times were reduced to those of wildtype mice (Fukui et al., 2007). Similarly, VMAT2-deficient mice, which have a 95% decrease in VMAT2 expression, display an age-dependent depressive-like

phenotype demonstrated by an increased immobility time, which is decreased to WT levels when dosed with desipramine (Taylor et al., 2009). In the tail suspension test, mice are individually suspended by the tail from a horizontal ring stand bar (distance from the floor = 30 cm) using adhesive tape. A six minute test session is then videotaped and scored by a trained observer for escape-oriented behavior and bouts of immobility; mice are excluded from the test if they climb up their tail during the test session (Cryan et al., 2004). As in the forced swim test, VMAT2 heterozygous mice exhibit increased immobility times compared to wildtype littermates, which are reduced to wildtype levels when dosed with fluoxetine, reboxetine or bupropion (Fukui et al., 2007). VMAT2-deficient mice have an age-dependent increase in immobility time in the tail suspension test compared to wildtype littermates, which is ameliorated with desipramine (Taylor et al., 2009). However, mice exposed to MPTP did not demonstrate a significant difference in immobility time from saline-treated animals in the tail suspension test (Vuckovic et al., 2008) (see Table 2).

To study anhedonia associated with depression in PD, the sucrose preference test can be employed. This test is conducted in the animal's home cage and the mouse is habituated to consume a palatable, weak sucrose solution. Prior to the test, mice are deprived of water overnight (Fukui et al., 2007; Vuckovic et al., 2008). During the test period, two bottles, one containing water and one containing the sucrose solution, are presented to the animal. Consumption is measured by weighing the bottles before and after the test period, and preference for the sucrose solution is determined by dividing the volume of sucrose consumed by the total liquid (water and sucrose) consumed (Skalisz et al., 2002; Fukui et al., 2007). The concentration of sucrose can also be varied over consecutive test days to assess the degree of sucrose preference, and therefore, the degree of anhedonic phenotype (Fukui et al., 2007). Reserpinized mice display a significant decrease in sucrose preference compared to vehicle treated mice (Skalisz et al., 2002) (see Table 2). Since reserpine is known to elicit locomotor deficits, total liquid consumption was also measured; no differences were observed in total liquid consumption

between vehicle and reserpine-treated animals (Skalisz et al., 2002). VMAT2-heterozygous mice were also found to have reduced preferences for various concentrations of sucrose solution compared to wildtype littermates, suggesting an anhedonic phenotype when VMAT2 expression is decreased (Fukui et al., 2007). Alternatively, MPTP treated mice do not display any differences in sucrose preference compared to vehicle treated mice (Vuckovic et al., 2008) (see Table 2).

Cognitive deficits in PD

PD patients experience a myriad of cognitive deficits, including, but not limited to, impairments in executive functions, language, memory, visuospatial skills, and dementia (Fuchs et al., 2004; Caballol et al., 2007; Tadaiesky et al., 2008). To test for these deficits in mouse models of PD, primarily memory and general cognitive tests are used as it is hard to measure executive function in mice. The radial arm maze functions to assess the mouse's ability to remember a set of spatial locations based on memory and not response patterns. The maze consists of eight arms and a central platform, with food rewards located randomly among the 8 arms (Olton, 1987). To increase motivation in learning the maze correctly, a 15% reduction in daily food intake is enforced. During the test sessions, four randomly selected arms are baited with one pellet of food each; the baited arms are kept unchanged throughout the experiment (Olton, 1987; Heneka et al., 2006). After each test session, the mice are evaluated for three parameters: working memory errors, reference memory errors, and total arm entries. Working memory errors are classified as reentries into baited arms that had been previously entered during the test session, and reference memory errors are classified as entry into non-baited arms (Schwegler et al., 1991; Heneka et al., 2006). The mice were considered to have learned the task when the number of working memory errors reached zero and the number of reference memory errors is one (Olton, 1987; Schwegler et al., 1991).

The modified Morris water maze is an analogous test using visual cues to measure spatial learning and memory in mouse models. Spatial learning is evaluated using a circular water tank

filled with water made opaque by the addition of milk or non-toxic paint to obscure an escape platform (Prediger et al., 2006; Zhu et al., 2007; Prediger et al., 2009). The tank is divided into 4 quadrants, with designated start positions in each quadrant. During training sessions, the escape platform is made visible by the attachment of a flag; the mice were subsequently trained to swim to the submerged platform without the flag (Prediger et al., 2006; Zhu et al., 2007). Mice are allowed one minute to locate the escape platform, and escape latencies are recorded. On test day, the escape platform is removed, and the swimming path is recorded while the mouse searches for the missing platform (Zhu et al., 2007). Parkin-deficient mice have been found to exhibit mild cognitive impairment, indicated by longer escape latencies and failure to selectively cross into the quadrant that formerly contained the escape platform (Zhu et al., 2007) (see Table 2).

Conclusions

In addition to motor deficits, there are a variety of non-motor symptoms associated with PD. These symptoms may precede the onset of motor symptoms, sometimes by years, and include anosmia, problems with gastrointestinal motility, sleep disturbances, cognitive deficits, anxiety, and depression. As research in the field advances, non-motor symptoms associated with Parkinsonism have been illuminated, demonstrating that PD is not exclusive to dopaminergic disturbances; norepinephrine (NE), serotonin (5-HT), and non-monoaminergic transmitter systems may significantly contribute to disease progression as well. To complement the expanding definition of Parkinson's disease, new animal models of the disease should encompass more than motor deficits. Most studies of widely-accepted mouse models of PD only explore motor deficits; very few begin to assess non-motor behaviors that are highly prevalent within the disease (see Table 2). Mouse models that display overt pathology but lack behavioral deficits must be interpreted with caution. It is very important to acknowledge the possible relationships between neurodegeneration, behavioral outcome, and potential therapeutic interventions. By testing potential mouse models of PD for a broader behavioral phenotype, conceivably the

etiology of PD can be better understood, leading to earlier diagnosis and improved therapeutic strategies.

Mouse Model	General Activity (open field, novelty-induced locomotor activity)	Coordination (pole test, challenging beam traversal, rotarod)	Gait (stride length, grid test, treadmill)	Other (AIMS)
MPTP	↓locomotion (Fredriksson and Archer, 1994)	↓pole test (Ogawa et al., 1985), ↓rotarod performance (Rozas et al., 1998) +L-DOPA	↓stride length, performance on grid test (Tillerson et al., 2002) +L-DOPA	↑ catalepsy, akinesia, tremor (Gupta et al., 1986; Mitra et al., 1992; Mohanakumar et al., 2000)
Rotenone	↓locomotion (Richter et al., 2007; Rojo et al., 2007)	↔rotarod (Richter et al., 2007)	↓stride length (Richter et al., 2007)	↑ catalepsy (Richter et al., 2007)
Paraquat	↓locomotion (Li et al., 2005; Rojo et al., 2007)	↓ pole test (Li et al., 2005)	N.P.	N.P.
Paraquat + Maneb	↓locomotion (Thiruchelvam et al., 2003)	↓ challenging beam performance, inverted screen performance (Thiruchelvam et al., 2003)	N.P.	N.P.
Reserpine	↓locomotion (Schneider, 1954)	N.P.	↓stride length (Fernagut et al., 2002) +L-DOPA	↑ akinesia (Fernagut et al., 2002)
A53T Synuclein Transgenics	↑ locomotion (Unger et al., 2006)	↔rotarod (Giasson et al., 2002)	↓stride length (Gispert et al., 2003)	N.P.
A30P Synuclein Transgenics	↓locomotion(Yavich et al., 2005; Oksman et al., 2009) -L-DOPA	↓challenging beam performance (Plaas et al., 2008), ↔rotarod (Yavich et al., 2005)	↓stride length (Plaas et al., 2008)	↑dystonia, rigidity (Gomez-Isla et al., 2003) -L-DOPA
Thy1-aSyn	N.P.	↓challenging beam performance , pole test (Fleming et al., 2006) -L-DOPA	↔ stride length (Fleming et al., 2006), ↔ grid test (Fleming et al., 2004) -L-DOPA	↔ rearing, grooming (Fleming et al., 2006) -L-DOPA
Parkin	↔locomotion (Goldberg et al., 2003; Zhu et al., 2007)	↔rotarod (Goldberg et al., 2003; Zhu et al., 2007), ↔ pole test, challenging beam (Goldberg et al., 2003; Perez and Palmiter, 2005)	↔ stride length, grid test (Perez and Palmiter, 2005)	↔ catalepsy test (Perez and Palmiter, 2005)
LRRK2	↓locomotion (Li et al., 2009) +L-DOPA	N.P.	N.P.	↓rearing (Li et al., 2009) +L-DOPA
UCH-L1	N.P.	N.P.	N.P.	N.P.
PINK1	↓locomotion (Gispert et al., 2009)	↔rotarod (Zhou et al., 2007; Gispert et al., 2009)	N.P.	N.P.
DJ-1	↓locomotion (Chen et al., 2005; Chandran et al., 2008)	↔pole test (Chandran et al., 2008), ↔rotarod (Chen et al., 2005)	↓stride length (Chandran et al., 2008), ↑foot faults in grid test (Manning-Bog et al., 2007) -L-DOPA	N.P.
Pitx3-aphakia	↔locomotion, ↓exploratory activity (Hwang et al., 2005) +L-DOPA	↔rotarod (Ardayfio et al., 2008), ↓challenging beam traversal and pole test performance (Hwang et al., 2005) +L-DOPA	N.P.	N.P.
MitoPark	↓locomotion, exploratory behavior (Ekstrand et al., 2007) +L-DOPA	N.P.	N.P.	↑ tremor, rigidity (Ekstrand et al., 2007)
VMAT2-deficient	↓locomotion (Caudle et al., 2007) +L-DOPA	↓challenging beam performance (Mooslehner et al., 2001)	↓stride length* +L-DOPA	N.P.

+/- L-DOPA: L-DOPA efficacy or inefficacy in rescuing motor phenotype, respectively, N.P.: test not performed
*Taylor and Miller, unpublished observations

Table 1. L-DOPA responsive motor phenotypes in parkinsonian mouse model

Mouse Model	Olfactory Disturbances	Sleep Abnormalities	GI Dysfunction	Anxiety	Depression	Cognitive Decline
MPTP	**+**, buried pellet (Schintu et al., 2009)	**+**, polysomnography (Laloux et al., 2008a; Laloux et al., 2008b)	**-**, gastric emptying, **+** stool frequency (Anderson et al., 2007) **+** colonic motility (Anderson et al., 2007)	**-**, light-dark exploration (Vuckovic et al., 2008)	**-**, TST, sucrose preference (Vuckovic et al., 2008)	**+**, T-maze (Tanila et al., 1998)
Rotenone	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
Paraquat	N.P.	N.P.	N.P.	**+**, open field (Litteljohn et al., 2009)	N.P.	N.P.
Paraquat + Maneb	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
Reserpine	N.P.	N.P.	N.P.	N.P.	**+** sucrose preference (Skalisz et al., 2002)	**+**, discriminative avoidance task (Silva et al., 2002)
A53T Synuclein Transgenics	N.P.	N.P.	N.P.	**-**, EPM; **+** thigmotaxis, open field (George et al., 2008)	N.P.	N.P.
A30P Synuclein Transgenics	N.P.	N.P.	N.P.	N.P.	N.P.	**+**, Morris water maze, fear conditioning (Freichel et al., 2007)
Thy1-aSyn	**+**, block test, habituation/dishabituation (Fleming et al., 2008)	N.P.	**+** colonic transport, stool frequency (Wang et al., 2008)	N.P.	N.P.	N.P.
Parkin	N.P.	N.P.	N.P.	**+**, light-dark exploration, **-** open field (Zhu et al., 2007) **-**, EPM (Perez and Palmiter, 2005)	**-**, FST, TST (Perez and Palmiter, 2005)	**+**, Morris water maze (Zhu et al., 2007), T-maze (Itier et al., 2003); **-**, novel-object recognition (Perez and Palmiter, 2005)
LRRK2	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
UCH-L1	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
PINK1	N.P.	N.P.	N.P.	-, open field (Gispert et al., 2009)	N.P.	N.P.
DJ-1	N.P.	N.P.	N.P.	**-**, EPM (Chandran et al., 2008)	N.P.	N.P.
Pitx3-aphakia	N.P.	N.P.	N.P.	N.P.	N.P.	**+**, T-maze (Ardayfio et al., 2008)
MitoPark	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
VMAT2-deficient	**+**, novel scent test, block test (Taylor et al., 2009)	**+**, sleep latency (Taylor et al., 2009)	**+**stool frequency, gastric emptying (Taylor et al., 2009)	**+**, EPM (Taylor et al., 2009)	**+**, FST, TST (Taylor et al., 2009)	N.P.

Table 2. Behavioral phenotypes in mouse models of Parkinson's disease
+, evidence of symptom; -, no evidence of symptom, N.P., not performed

PD symptom	Preliminary Behavioral Assays	Comprehensive Behavioral Assays
Hyposmia/Anosmia	Buried pellet test, novel scent test, social olfactory discrimination (block test)	habituation/dishabituation
Sleep Abnormalities	sleep latency	polysomnography, EEG
GI Dysfunction	stool frequency, solid/liquid gastric emptying	colonic motility, bead latency, isometric muscular force recording
Anxiety	elevated plus maze, open field, light-dark exploration	novelty suppressed feeding
Depression	forced swim test, tail suspension test, sucrose preference	learned helplessness
Cognitive Decline	novel object recognition, radial arm maze, T-maze	Morris water maze, fear conditioning, discriminative avoidance task
Motor abnormalities	open field, novelty-induced locomotor activity, stride length	challenging beam traversal, pole test, grid test, catalepsy test

Table 3. Summary of parkinsonian behavioral analyses

CHAPTER 3

NON-MOTOR SYMPTOMS OF PARKINSON'S DISEASE REVEALED IN AN ANIMAL MODEL WITH REDUCED MONOAMINE STORAGE CAPACITY

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Vernon, AC. Mice with reduced vesicular monoamine storage content display nonmotor features of Parkinson's disease. J Neuroscience 29(41):12842-4, 2009.

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the loss of dopamine neurons in the substantia nigra pars compacta, culminating in severe motor symptoms, including: resting tremor, rigidity, bradykinesia, and postural instability. In addition to motor deficits, there are a variety of non-motor symptoms associated with PD. These symptoms generally precede the onset of motor symptoms, sometimes by years, and include anosmia, problems with gastrointestinal motility, sleep disturbances, sympathetic denervation, anxiety, and depression. Previously, we have shown that mice with a 95% genetic reduction in vesicular monoamine transporter expression (VMAT2-deficient, VMAT2-deficient) display progressive loss of striatal dopamine, L-DOPA responsive motor deficits, α-synuclein accumulation, and nigral dopaminergic cell loss. We hypothesized that since these animals exhibit deficits in other monoamine systems (norepinephrine, serotonin), which are known to regulate some of these behaviors that the VMAT2-deficient mice may display some of the non-motor symptoms associated with PD. Here, we report that the VMAT2-deficient mice demonstrate progressive deficits in olfactory discrimination, delayed gastric emptying, altered sleep latency, anxiety-like behavior, and age-dependent depressive behavior. These results suggest that the VMAT2-deficient mice may be a useful model of the non-motor symptoms of PD. Furthermore, monoamine dysfunction may contribute to many of the non-motor symptoms of PD and interventions aimed at restoring monoamine function may be beneficial in treating the disease.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease that has long been considered to be a disorder of the dopamine (DA) system. Pathophysiologically, PD is believed to be caused by the death of neuromelanin-containing DA neurons in the substantia nigra pars compacta (SNpc) and the appearance of proteinaceous intracellular inclusions known as Lewy bodies (Olanow and Tatton, 1999; Jenner and Olanow, 2006). Motor disturbances do not present clinically until approximately 70-80% of striatal dopamine has already been lost; however, other non-motor symptoms are evident before the onset of motor disturbances. These include hyposmia/anosmia, gastrointestinal disturbances, sleep abnormalities, autonomic dysfunction, anxiety, depression, and, at later stages, impaired cognition (Braak et al., 2003; Langston, 2006). Moreover, a careful reading of James Parkinson's description of his patients indicates that many of the non-motor symptoms that are of great interest today were observed nearly 200 years ago (Parkinson, 1817). It is possible that other neurotransmitters such as norepinephrine (NE) and serotonin (5-HT) may significantly contribute to these symptoms, as the locus coeruleus (LC) and raphe nucleus have also been shown to degenerate in PD (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Rommelfanger and Weinshenker, 2007). This is supported by studies that show neurodegeneration in the LC, the primary source of NE, in addition to the SNpc, and evidence that perturbation of the noradrenergic system can lead to serious alteration in DA neurotransmission (Antelman and Caggiula, 1977; Gesi et al., 2000; Marien et al., 2004; Rommelfanger and Weinshenker, 2007). In contrast to DA and NE, 5-HT has been understudied from PD research, even though the raphe nuclei has been shown to undergo degeneration in PD patients (Braak et al., 2002; Braak et al., 2003). Although the degeneration of the raphe is not as prominent as that of the SNpc or LC, 5-HT is widely recognized in the development of many other diseases such as depression, psychiatric, and sleep disorders, all of which have been observed in PD patients (Halliday et al., 1990; Jellinger, 1991; Murai et al., 2001).

Recently, we have characterized a new potential model of PD based on reduced vesicular storage of monoamines. Animals expressing 5% of normal vesicular monoamine transporter 2 (VMAT2-deficient) exhibit increased oxidative stress, progressive loss of DA terminals and cell bodies in the SNpc, as well as α-synuclein accumulation (Caudle et al., 2007). Monoaminergic dysfunction in the VMAT2-deficient animals arises from reduced expression of VMAT2, resulting in severely diminished levels of DA, NE, and 5-HT (Mooslehner et al., 2001; Caudle et al., 2007). Because VMAT2-deficient mice demonstrate a significant reduction in NE and 5-HT levels in multiple brain regions, our laboratory has characterized the behavioral manifestations of reduced monoaminergic innervation in the VMAT2-deficient mice. In this study, we report that the VMAT2-deficient mice display many of the non-motor symptoms of PD.

Materials and Methods

Animals. Male and female VMAT2-deficient mice were generated as previously described (Mooslehner et al., 2001; Caudle et al., 2007). Briefly, the mouse VMAT2 locus was cloned from the 129/Sv genomic library and a 2.2 kb *Pvu*II fragment from the third intron of the VMAT2 gene, and cloned into the bluntended *Not*I site of the construct. The targeting vector was introduced into 129/Ola CGR 8.8 embryonic stem (ES) cells and injected into blastocytes of C57BL/6 mice. Highly chimeric males were bred with C57BL/6 females; genotype was confirmed by Southern blot analysis. A recent report uncovered that the C57BL/6 inbred strain of mice originally used to establish the VMAT2-deficient mouse line contained a spontaneous chromosomal deletion spanning the α-synuclein gene locus (Specht and Schoepfer, 2001a), which was confirmed in the original strain (Mooslehner et al., 2001; Patel et al., 2003b; Colebrooke et al., 2006). Through backcrossing, we eliminated all traces of this mutation from our strain of mice and routinely verify the presence of α-synuclein via Southern blot analysis. This report represents the second set of data on VMAT2-deficient mice with a normal α-synuclein background. All mice were generated through redundant breeding of mice that were heterozygous for the VMAT2 allele and wild type (WT) for the α-synuclein allele. The genotype of all mice

was confirmed by PCR of DNA extracted from tail samples. For all behavioral tests, WILDTYPE littermate controls were used. All procedures were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and previously approved by the Institutional Animal Care and Use Committee at Emory University.

HPLC determination of monoamines and metabolites. HPLC-electrochemical analysis of mouse neurochemistry was performed as previously described (Richardson and Miller, 2004; Caudle et al., 2006; Caudle et al., 2007). Briefly, dissected right striata, cortex, and hippocampus were sonicated in 0.1 M perchloric acid containing 347 μM sodium bisulfate and 134 μM EDTA. Homogenates were centrifuged at 10,000 x g for 10 min at 4°C; the supernatant was removed and filtered through a 0.22 μm filter by centrifugation at 5,000 x g for 3 min. The supernatants were then analyzed for levels of DA, DOPAC, homovanillic acid (HVA), NE, 5-HT, and 5-HIAA. Levels were measured using HPLC with an eight-channel coulometric electrode array (ESA Coularray; ESA Laboratories, Chelmsford, MD). Quantification was made by reference to calibration curves made with individual standards.

Olfactory discrimination. Olfactory experiments were adapted from previous work from our laboratory (Tillerson et al., 2006). Briefly, wooden blocks (1.8 cm^3) were placed individually in 50 mL conical tubes containing 1 g of animal bedding from test animals' cages for 12 h. The animal was presented with a block scented with its own bedding and a block scented with another mouse's bedding (of the same sex). The time spent sniffing (nose less than 1 cm away from the block) or in contact with each block was recorded for a 2-min trial (Tillerson et al., 2006). A single cohort of mice was tested at each time point; however, naïve mice were introduced each month to ensure that the original cohort of mice was not learning the task. Data from male and female mice was combined, since there were no detectable sex differences after testing 20 mice per genotype.

To measure non-social olfactory acuity, methods were modified from previous methods used in our laboratory (Tillerson et al., 2006). Glass plates with 25 μL of either a novel scent

(lemon, peppermint, or vanilla) or water were presented simultaneously to the animals. Time spent sniffing each glass plate was recorded for a 3 minute session.

Non-olfactory sensory tests. To assess non-olfactory sensory function in wildtype and VMAT2-deficient mice, we examined the responses of these animals in several sensory tests using the paradigm of Tillerson and colleagues (Tillerson et al., 2006). Responsiveness to tactile stimulation was measured as latency to contact or remove a 113.1 mm^2 (1.3 cm in diameter) adhesive dot in a 2 minute session (Avery Office International) (Schallert et al., 2000; Tillerson et al., 2006). Animals were removed from their home cage, and the dot placed between the ears, on top of the head. Animals were then put back into their home cage and the cage returned to its normal position on the rack to reduce external distractions (Tillerson et al., 2006).

Quinine is often used in taste aversion paradigms, due to its unpalatable bitterness (Tillerson et al., 2006). The tip of a cotton swab was placed into either an aliquot of 2 mg/mL quinine or water and then into the animals' mouths. Presentation of clean versus quinine stimuli was counterbalanced between animals. Latency to groom and/or drag the jaw along the ground was recorded during a 1 minute test session (Grill and Norgren, 1978; Schallert and Whishaw, 1978; Tillerson et al., 2006).

The trigeminal nerve innervates areas of the olfactory epithelium and nasal mucosa, and is responsible for non-odor sensations such as mild irritation and burning (Tillerson et al., 2006). In this test, we assessed the function of the trigeminal nerve by exposing the mice to either ammonia, known to exert a trigeminal response, or water. A glass plate with water or ammonia (counterbalanced between animals) was placed in the animal's cage. Time sniffing (as defined above) was recorded for a 2 minute session (Tillerson et al., 2006).

Sleep latency. Wildtype and VMAT2-deficient animals were individually housed in large Plexiglas cages and allowed to acclimate for 4 hours. Saline (0.9%) was then administered intraperitoneally and mice were observed by a trained experimenter for behavioral signs of sleep.

Sleep was defined as 2 minutes of uninterrupted sleep behavior, and 75% of the next 10 minutes spent asleep (Mitchell et al., 2008). A single cohort of mice was tested at all time points.

Gastric emptying. Following a 12-hour fast, wildtype and VMAT2-deficient mice were allowed free access to food for 1 hour. The amount of food consumed was calculated based on food weight before and after access. Two hours after food removal, animals were sacrificed and the stomach contents were weighed (wet and dry). The percentage of food remaining in the stomach was measured (Whited et al., 2006). Since this is a terminal procedure, separate cohorts of wildtype and VMAT2-deficient mice were aged and subjected to analysis for each time point.

One hour stool collection. Each mouse was placed in a separate clean cage and observed throughout the 60 min collection period. Fecal pellets were collected immediately after expulsion and placed in sealed (to avoid evaporation) 1.5 ml tubes. Tubes were weighed to obtain the wet weight of the stool, this was then dried overnight at 65°C and reweighed to obtain the dry weight (Li et al., 2006).

Forced swim test. These studies were conducted on mice using a modified method of Porsolt and coworkers (Porsolt et al., 1979). All mice were injected intraperitoneally with 100 µL of 5 mg/kg desipramine (Sigma-Aldrich, St. Louis, MO) or saline 30 minutes before testing. Mice were placed individually in glass cylinders (24 x 16 cm) with 15 cm of water maintained at 25°C. The mice were left in the cylinder and their behavior was videotaped from the side of the cylinder for 6 minutes. After the first 2 minutes, the total duration of time spent immobile was recorded during a 4 minute test. The mouse was deemed immobile when it was floating passively; subtle movement of feet or tail required to keep the head above the surface of the water were excluded as immobility. Immobility time refers to the time that the animal spent floating for at least 3 seconds (Porsolt et al., 1979; Xu et al., 2000; Fukui et al., 2007). A single cohort of mice was tested at each time point; however, naïve mice were introduced each month to ensure that the original cohort of mice was not learning the task.

Tail suspension test. These experiments were conducted using the methods of Cryan and colleagues (Cryan et al., 2004). All mice were injected intraperitoneally with 100 µL of 5 mg/kg desipramine (Sigma-Aldrich, St. Louis, MO) or saline 30 minutes before testing. Mice were individually suspended by the tail to a horizontal ring stand bar (distance from the floor = 30 cm) using adhesive tape. A 6-minute test session was videotaped and scored by a trained observer for escape-oriented behavior and bouts of immobility. The time spent immobile was recorded for each mouse. Mice were excluded from the study if they were able to climb on top of the ring stand. A single cohort of mice was tested at each time point; however, naïve mice were introduced each month to ensure that the original cohort of mice was not learning the task.

Elevated Plus Maze. Male and female wildtype and VMAT2-deficient mice were individually housed in a reversed light cycle room (lights on at 1900 h, lights off at 700 h), and were allowed a minimum of two weeks to habituate to the new lighting conditions. Food and water were available *ad libitum* throughout the course of the study. Data from male and female mice was combined, since there were no detectable sex differences.

The elevated plus maze (EPM) paradigm was adapted from Schank and colleagues (Schank et al., 2007). The EPM apparatus consisted for two open arms and two enclosed arms arranged in a plus sign orientation. The arms were elevated 30 inches above the floor, with each arm projecting 12 inches from the center. Because rodents naturally prefer dark, enclosed compartments, a greater willingness to explore the open, well-lit arms is believed to represent a decrease in the animal's anxiety (Pellow et al., 1985; File et al., 1988; Paine et al., 2002).

No drugs were administered prior to behavioral testing. To begin each test, mice were placed in the EPM, facing an open arm and allowed to freely explore the apparatus during a videotaped, 5 minute trial. Videotapes were later scored by an observer who was blind to genotype. The measure used for analysis is the percentage of time spent exploring the open arms, which was calculated by dividing the time spent in the open arms by the combined time spent in open and closed arms (Pellow et al., 1985). Entry into an arm of the EPM was defined as the

animal placing all four paws in that particular part of the maze. All tests were run during the dark cycle, between 1400 and 1600 h. Mice were excluded from data analysis if they jumped or fell off the maze after the test began.

Grid test. The grid test was performed using the methods of Tillerson and colleagues (Tillerson and Miller, 2003). Mice were placed horizontally on the center of the grid and supported until they grasped the grid with their forepaws and hindpaws. The grid was then inverted and the mice were videotaped while hanging upside-down for latency to release their grip on the grid.

Electroretinalgraphy (ERG). Wildtype and VMAT2-deficient mice were dark-adapted for at least 12 hrs and anesthetized with a ketamine (60mg/kg) (Hospira, Inc., Lake Forest, IL)/xylazine (7.5mg/kg) (Sigma Aldrich, St. Louis, MO) mixture for recording the scotopic flash ERG. Pupils were dilated with 1% cyclopentolate/1% tropicamide solutions. A DTL-fiber working electrode was placed on the cornea of the eye, while the reference needle electrode was placed on the cheek just below the eye. A ground electrode was attached to the tail of the mouse and a rectal probe was inserted to monitor and control the temperature of the mouse. After recording a flat baseline, the mouse was exposed to multiple flashes of light with increasing intensities (.111, .228, .576, 1.16, 2.325, 5.85, 119.6 $cd*s*m^{-2}$). The responses to 3-10 flashes at each intensity were averaged to determine a-wave amplitude, b-wave amplitude, a-wave implicit time, and b-wave implicit time.

Statistical analysis. Data from male and female mice was combined, since there were no detectable sex differences. All data were analyzed using unpaired independent samples Student's t-test (gastric emptying), completely randomized two-factor ANOVA followed by Bonferroni post hoc analysis (odor discrimination, olfactory acuity, sleep latency, gastric emptying, forced swim test, tail suspension test), or repeated measures two-factor ANOVA (ERGs). Analyses were completed using Graph Pad Prism 5.0 for Windows, and for all tests all *post hoc* measures were error-corrected to keep the overall error rate per group at 0.05.

Results

VMAT2-deficient mice have diminished levels of monoamines at 12-15 months of age, accompanied by increased turnover.

Previously, our laboratory has shown that VMAT2-deficient animals have age-dependent reductions in levels of striatal dopamine, accompanied by increased turnover (Caudle et al., 2007). To determine if the reduction in VMAT2 affects the other major monoamines in addition to DA, the striatum, cortex, and hippocampus were isolated from wildtype and VMAT2-deficient mice and analyzed by HPLC for neurochemical levels. At 12-15 months of age, the VMAT2-deficient mice had severely diminished levels of all three monoamines in all brain regions tested, although in general, DA and NE depletion were more severe than 5-HT depletion. In the striatum, DA and NE were decreased by 91%, while 5-HT was decreased by 81%. Similarly, in the cortex there was a 92% and 94% decrease in DA and NE, respectively, with only a 78% decrease in 5-HT. However, in the hippocampus, a 99% decrease in DA was seen, while NE and 5-HT were decreased by 87% and 86%, respectively (Figure 1A). VMAT2-deficient animals also had significantly increased DA and 5-HT turnover; a two-way ANOVA revealed a significant brain region by genotype interaction for both DA ($F_{(2,38)}$=80.91; $p<0.0001$) and 5-HT turnover ($F_{(2,44)}$=26.09; $p<0.0001$) (Figure 1B, C).

VMAT2-deficient mice display progressive olfactory discrimination deficits.

One of the earliest manifestations of PD is the loss of the sense of smell. Olfactory abnormalities have been found in nearly all PD patients and can precede neurological deficits by decades (Braak et al., 2002; Braak et al., 2003; Langston, 2006). To determine whether VMAT2-deficient animals exhibit comparable olfactory dysfunction, both wildtype and VMAT2-deficient animals were subjected to a battery of olfactory discrimination tests. First, wildtype and VMAT2-deficient animals were subjected to odor discrimination tests, using wooden blocks scented with either home cage bedding or bedding from the cage of a foreign mouse of the same sex. No differences were seen between the behavior of wildtype or VMAT2-deficient animals at

2 and 4 months of age shown as the investigatory index (percentage of time spent investigating self – percentage of time spent investigating other; n=12, Figure 2). However, at 5, 6, and 12 months of age, VMAT2-deficient animals were unable to discriminate between the two blocks, and consequently displayed no preferential exploration of either block (2 months: % self=28.19, % other=71.81; 12 months: % self=44.63, % other=55.37; interaction between effects of scent and age: $F_{(4,95)}$=2.41, p<0.05, n=12, Figure 2). In contrast, wildtype mice show marked preferential exploration of the block scented with foreign bedding (n=12-15, main effect of scent: $F_{(1,110)}$=546.44, p<0.0001), with no change in behavior as they aged (2 months: % self=28.69, % other=71.31, 12 months: % self=13.14, % other= 86.86; main effect of age: $F_{(4,110)}$=0.36, p=0.8379). To ensure that these results were not due to differences in motivation, the total investigatory time was calculated for both genotypes of mice at all ages. Investigatory times were similar at 4 months (WT: 5.36±0.6505, n=11; LO: 4.50±1.167, n=10; t_{19}=0.6627, p=0.5155), 5 months (WT: 6.17±0.5618, n=12; LO: 5.00±0.9309, n=10; t_{20}=1.114, p=0.2785), and 6 months of age (WT: 4.36±0.8232, n=11; LO: 2.80±0.4667, n=10; t_{19}=1.608, p=0.1244); only at 12 months of age did the VMAT2-deficient animals display a significant reduction in total investigatory time relative to age-matched wildtype littermates (WT: 9.11±0.8219, n=15; LO: 5.52±1.418, n=11; t_{24}=2.325, p=0.0288). The olfactory deficit is not corrected by L-DOPA treatment in human PD patients, nor is it effective in our mice. Acute administration of L-DOPA and the peripheral aromatic acid decarboxylase inhibitor, benserazide did not affect the performance of either genotype (data not shown) (Muller et al., 2002). Two-way ANOVA exposed a significant scent/genotype interaction; VMAT2-deficient mice also had deficits in non-social olfactory acuity using the scents of lemon (interaction between effects of genotype and age: $F_{(1,32)}$=3.69, p=0.0637), vanilla (interaction between effects of genotype and age: $F_{(1,34)}$=10.04, p=0.0032), and peppermint (interaction between effects of genotype and age: $F_{(1,36)}$=4.91, p=0.0331), beginning at 3 months of age (data not shown) and persisting until 18 months of age (Figure 3).

Mice were tested for non-olfactory sensory deficits to ensure that there was not a problem in general sensory perception. VMAT2-deficient animals showed no deficits in response to tactile stimulation (interaction between effects of genotype and age: $F_{(1,45)}$=0.03, p=0.8679), quinine-induced taste aversion (main effect of genotype: $F_{(1,44)}$=0.00, p=0.9830), or to mild irritation/burning sensations stimulated by ammonia (main effect of genotype: $F_{(1,38)}$=0.14, p=0.7136) as compared to age matched WT controls (Figure 4). Collectively, these results demonstrate that although the VMAT2-deficient animals have normal tactile and gustatory sensory perception, they do exhibit progressive deficits in olfactory discrimination, which is a common phenotype seen in PD.

VMAT2-deficient animals display altered latency to behavioral signs of sleep.

Many PD patients experience sleep disturbances, including excessive sleepiness and insomnia (Comella, 2003; Langston, 2006; Ziemssen and Reichmann, 2007). In order to begin investigating behavioral sleep disturbances in the VMAT2-deficient mice, we conducted sleep latency tests in wildtype and VMAT2-deficient mice during their circadian nadir. Beginning at 2 months of age, VMAT2-deficient mice show a shorter latency to behavioral signs of sleep, which is most prevalent at 4-6 months of age, than age-matched wildtype controls (interaction effects of genotype and age: $F_{(4,73)}$=2.82, p=0.0310). A Bonferroni *post hoc* analysis compared the two genotypes at each age; the difference in sleep latency between VMAT2-deficient and WT animals becomes increasingly less pronounced, and is absent at 24 months of age (Figure 5A). While wildtype animals display an age-dependent decrease in sleep latency, no age-related changes in sleep latency occur in VMAT2-deficient mice. The circadian activity of VMAT2-deficient animals is also significantly lower than that of age-matched WT controls at 4-6 months of age (main effect of genotype: $F_{(1,156)}$=37.19, p<0.0001) , but not at 12-15 (main effect of genotype: $F_{(1,228)}$=2.62, p=0.1072) or 18 months of age (main effect of genotype: $F_{(1,168)}$=1.16, p=0.2827), due to a decline in WT circadian activity at the latter ages (Figure 5B-E). These results suggest

that VMAT2-deficient mice develop premature changes in sleep latency and are displaying behavioral phenotypes reminiscent of older WT animals.

VMAT2-deficient mice have delayed gastric emptying.

Gastrointestinal dysfunction in PD occurs in over 70% of PD patients and has been attributed to lack of activity, inadequate hydration, or autonomic and enteric neuronal dysfunction (Langston, 2006; Ziemssen and Reichmann, 2007). To study this non-motor symptom in VMAT2-deficient mice, WT and deficient animals were behaviorally examined for gastric emptying at 2, 6, 12, and 18 months of age. Student's t-test revealed that solid gastric emptying was significantly delayed overall in VMAT2-deficient mice (WT: 21.98 ± 2.605 n=16, LO: 30.27 ± 2.101 n=16, t_{28}=2.478, p=0.0195), and was most apparent at 2, 6, and 12 months of age, although not significant as shown by two-way ANOVA with Bonferroni *post hoc* analysis (n=16 per genotype, interaction effects of age and genotype: $F_{(3,21)}$=0.63, p=0.6043) (Figure 6A). Two-way ANOVA revealed stool frequency was also found to be altered in VMAT2-deficient mice, with a significant increase in frequency at 2 and 6 months of age, compared to wildtype animals (n=4 per genotype, interaction effects of age and genotype: $F_{(3,24)}$=8.79, p=0.0004, Figure 6B).

VMAT2-deficient mice display anxiety-like and depressive phenotypes.

One of the most prevalent non-motor symptoms of PD is depression, with signs of anxiety co-morbid in patients with major depression (Zimmerman et al., 2002; Fukui et al., 2007). Anxiety-like behavior was measured in wildtype andVMAT2-deficient animals using the elevated plus maze. Younger VMAT2-deficient mice (4-6 mo) spent significantly more time in the closed arms of the maze as compared to age-matched controls (n=11-12, interaction effects of age and genotype: $F_{(1,38)}$=3.45, p<0.05, Figure 7A), while older VMAT2-deficient mice (12-15 months) did not display an anxiety-like phenotype compared to age-matched WT controls. Similar to what we observed in the sleep latency test, the behavior of wildtype animals in this test changed over time, while that of VMAT2-deficient mice did not, indicating possible premature development of symptoms normally associated with older animals.

The VMAT2-deficient animals were tested for altered behavior in the forced swim (FST) and tail suspension (TST) tests. At 4-6 months of age, there was no difference found between the immobility times of wildtype andVMAT2-deficient animals in both tests, and neither genotype responded to an acute low dose of desipramine (5 mg/kg administered 20 minutes prior to testing) (n=4-6, interaction effects of drug treatment and genotype: $F_{(1,13)}$=0.53, p=0.4807 (FST); $F_{(1,13)}$=0.08, p=0.7827 (TST); Figure 7B, C). However, at 12-15 months of age, immobility times were higher in VMAT2-deficient animals compared to age-matched WT controls (n=4-5, main effect of genotype: $F_{(1,15)}$=9.72, p=0.0071 (FST); $F_{(1,16)}$=11.12, p=0.0042 (TST), Figure 7D, E). The differences in immobility times across age groups in VMAT2-deficient animals suggests that there is an age-dependent development of depressive behavior, which correlates with the progressive neurochemical deficits previously characterized (Mooslehner et al., 2001; Caudle et al., 2007). Initially, both wildtype andVMAT2-deficient mice were exposed to 20 mg/kg desipramine, which was found to decrease immobility times in both genotypes (data not shown). However, when treated with an acute low dose of desipramine (5 mg/kg administered intraperitoneally 20 minutes prior to testing), the immobility times of VMAT2-deficient mice decreased to WT levels (n= 4-5, interaction effects of treatment and genotype: $F_{(1,15)}$=5.55, p=0.0325 (FST); $F_{(1,16)}$=7.95, p=0.0123 (TST); Figure 7D, E); the immobility times of wildtype animals were not affected by desipramine at 12-15 months of age.

VMAT2-deficient mice display normal vision and muscle strength.

While the phenotypic behaviors help define the disease, the lack of deficits in other systems are also important in showing the relative selectivity. As in PD, other sensory systems appear to be functioning normally in the VMAT2-deficient mice (Figure 3, 4); moreover, vision and muscle strength have also been found to be normal in patients. Although VMAT2-deficient mice had significantly decreased DA (64%) and DOPAC (45%) (interaction effects of neurotransmitter and genotype: $F_{(1,20)}$=16.28, p=0.0006; Figure 8A), the magnitude of the decrease was substantially less than that found in the brain regions examined and was coupled

with increased DA turnover (WT: 0.2434 ± 0.039 n=6, LO: 0.4017 ± 0.056 n=6, t_{10}=2.317, p=0.0430). Consequently, visual function appeared to be normal. Visual function was quantified by use of electroretinography (ERG). The ERG can detect abnormalities in electrical responses of the photoreceptor cells (rods and cones) and inner retinal cells (bipolar, amacrine, and ganglion cells). Compared to wild type littermates, VMAT2-deficient mice displayed no differences in A-wave amplitude (main effect of genotype: $F_{(1,4)}$=0.04, p=0.8569) or implicit time (main effect of genotype: $F_{(1,4)}$=0.04, p=0.8480) or B-wave amplitude (main effect of genotype: $F_{(1,4)}$=0.28, p=0.6237) or implicit time (main effect of genotype: $F_{(1,4)}$=0.02, p=0.8895) when their retinas were stimulated with light flashes as revealed by repeated measures two-way ANOVA with Bonferroni *post hoc* analyses (Figure 8B-E). In addition, *Adcy1* mRNA expression, which is regulated in retina by DA D_4 receptors (Jackson et al., 2009), was not different between VMAT-deficient (2.450±0.4204, n=11) and wild type mice (2.112±0.4775, n=10; t_{10}=0.5324, p=0.8019). General muscle strength was assessed by subjecting wildtype andVMAT2-deficient animals to the grid test. VMAT2-deficient mice showed no difference in latency to fall on the grid test apparatus compared to age-matched WT animals (data not shown).

Discussion

Although Parkinson's disease (PD) has been traditionally viewed as a neurodegenerative motor disorder, the increasing recognition of non-motor symptoms, including hyposmia, sleep abnormalities, anxiety, depression, and gastric dysfunction, suggests the disease is more multi-faceted than commonly thought. Moreover, these symptoms imply that more general monoaminergic dysfunction is occurring in concert with dopamine (DA) degeneration. Evidence for degeneration of the locus coeruleus (LC) and dorsal raphe (DR) in PD patients highlights the importance of looking beyond the nigrostriatal system in order to illuminate the deficits in other neurotransmitter systems (Braak et al., 2002; Braak et al., 2003; Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Lemke et al., 2004; Rommelfanger and Weinshenker, 2007). In this study, we hypothesized that general monoamine dysfunction may recapitulate many of the non-

motor symptoms of PD. We observed a reduction in monoamines that was correlated with behavioral dysfunction.

Previous neurochemical analysis of VMAT2-deficient animals shows general age-dependent reductions in brain tissue levels of DA, norepinephrine (NE), epinephrine, and serotonin (5-HT) by approximately 80-90% (Mooslehner et al., 2001; Colebrooke et al., 2006; Caudle et al., 2007), which is accompanied by increased dopamine and serotonin turnover in multiple regions of the brain (Figure 1) (Colebrooke et al., 2006; Caudle et al., 2007). Loss of VMAT2 function causes gradual neurodegeneration in the SNpc, LC, and DR of aged VMAT2-deficient mice (Taylor and Miller, unpublished observations). In contrast, other pharmacological *in vivo* models of PD, such as 6-OHDA lesions, cause sudden and profound loss of cell bodies. Although the precise mechanisms of cell death remain unclear, this progressive degeneration may be caused by oxidant species produced from improper monoamine storage within the neuron (Caudle et al., 2008). Catecholamines have the intrinsic ability to auto-oxidize, yielding reactive oxygen species and cysteinyl adducts; likewise, serotonin has an oxidizable indole ring (Graham, 1978; Jenner, 1998, 2003; Guillot and Miller, 2009). Presumably, improper storage of the monoamines contributes to progressive cellular injury.

Braak and colleagues have proposed that PD pathology actually begins in the lower brainstem and olfactory bulb, revealed by the presence of α-synuclein positive Lewy bodies (Braak et al., 2002; Langston, 2006). Olfaction abnormalities are present in 100% of PD patients, many noticing a decline in the sense of smell long before the onset of motor symptoms (Muller et al., 2002). Alterations in olfactory function are typically stable over time, non-responsive to traditional PD therapeutics, and unrelated to disease stage or duration (Doty et al., 1992; Ziemssen and Reichmann, 2007), and have been suggested to be an excellent pre-clinical marker of PD. VMAT2-deficient mice demonstrate a progressive loss in olfactory discrimination beginning at 5 months of age, stabilizing at 6 months, and remaining unchanged at 12 months that

appears to correlate to the loss of striatal DAT (Caudle et al., 2007), similar to that seen in patients (Siderowf et al., 2005; Ross et al., 2008).

Sleep disturbances have been shown to occur prior to the clinical presentation of motor deficits (Braak et al., 2003; Langston, 2006). Nocturnal sleep disruptions occur in 60-98% of PD patients, and are correlated with disease severity (Comella, 2003; Ziemssen and Reichmann, 2007). Sleep disturbances are manifested through a variety of syndromes including REM sleep behavioral disorder (RBD), excessive daytime sleepiness, sleep fragmentation, and deficiencies in sleep latency (Friedman, 1980; Comella, 2007; Ziemssen and Reichmann, 2007). VMAT2-deficient mice demonstrate a shorter latency to behavioral signs of sleep; however, more in depth studies using EEGs must be performed to better understand the underlying sleep architecture in these mice.

Gastrointestinal dysfunction is certainly one of the most common and possibly one of the earliest symptoms of PD (Edwards et al., 1993; Abbott et al., 2001; Pfeiffer, 2003). Dopamine depletion in the colon and Lewy bodies throughout the enteric nervous system suggest GI symptoms are a manifestation of the primary disease process, rather than an epiphenomenon related to motor dysfunction (Qualman et al., 1984; Kupsky et al., 1987; Wakabayashi et al., 1988; Edwards et al., 1993; Singaram et al., 1995; Abbott et al., 2001; Langston, 2006). Unfortunately, treatment of PD with L-DOPA may exacerbate GI dysfunction by slowing gastrointestinal motility, even though increased colonic transport time may derive from the same processes that cause motor abnormalities (Abbott et al., 2001; Ziemssen and Reichmann, 2007). The VMAT2-deficient mice exhibit some disturbances in GI dysfunction, including delayed gastric emptying and altered stool frequency that declines with age (Figure 6), indicating that reduced vesicular monoamine storage impacts GI function.

Disruptions in DA, NE, and 5-HT neurotransmission have been found in PD patients with anxiety and/or depression, including degeneration of the LC and DR (Lemke et al., 2004; Remy et al., 2005; Ziemssen and Reichmann, 2007). Depression in PD has been difficult to study since

it differs from major depression alone; only about 6% of PD patients suffer from major depression (Tandberg et al., 1996; Ziemssen and Reichmann, 2007). Moreover, a reliable rating scale does not currently exist to measure depressive symptoms in PD patients (Tandberg et al., 1996; Ziemssen and Reichmann, 2007). However, antidepressants such as nortriptyline and paroxetine have proven effective in depressed PD patients without worsening motor symptoms (Andersen et al., 1980; Ceravolo et al., 2000). The role of VMAT2 in anxiety and depression was discovered decades ago and is well characterized; reserpine, a VMAT2 inhibitor, has been shown to precipitate depressive-like symptoms in humans (Freis, 1954). Recently, Wetsel and colleagues have shown that mice with a 50% reduction in VMAT2 display depressive behavior in the forced swim and tail suspension test that is ameliorated by acute antidepressant therapy (Fukui et al., 2007). However, these VMAT2 heterozygous knockouts do not display anxiety-like behavior (Fukui et al., 2007). We found that the severe reduction of VMAT2 expression in the VMAT2-deficient mice does trigger both anxiety and progressive depressive behavior. VMAT2-deficient mice showed a significant increase in percent open arm time in the elevated plus maze at 4-6 months of age, while the increased immobility time in the forced swim and tail suspension tests did not occur until 12 months of age; suggesting that anxiety precedes depressive symptoms in VMAT2-deficient animals. Additionally, a low dose of desipramine that had no effect in wild-type animals was able to normalize immobility times in VMAT2-deficient mice. It is also important to note that many of the behaviors that are normal in PD patients (sensory, vision, strength) are also normal in the VMAT2-deficient mice, demonstrating a selective deficit in monoamine-related behaviors.

Data from the VMAT2-deficient mice suggest that all monoamine transmitter systems, not just DA, play a role in the clinical manifestations of PD. Classical descriptions/hypotheses about the pathogenesis of PD suggest the degeneration or loss of DA neurons is the major contributor to the development of the disease. However, it is not implausible to couple NE and 5-HT with DA in these classically established hypotheses for PD genesis. NE and 5-HT share

structural similarities with DA; NE shares a catechol ring, and all three species have the ability to oxidize within the cell yielding deleterious effects (Guillot and Miller, 2009). Similarly, the qualities that predispose the nigrostriatal system to oxidative damage may not be unique (Ahlskog, 2007); rather, these factors may be present in all monoaminergic neurons to varying degrees. Although the cause of the non-motor symptoms associated with PD remains unknown, it is clear that they are not caused purely by dopaminergic deficits; our data suggest that NE and 5-HT may contribute as well. When combined with the previously reported nigrostriatal degeneration in this model, the observed non-motor symptoms suggest that the VMAT2-deficient mice represent an excellent model of the monoaminergic deficits that manifest in human PD.

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Figure 1. VMAT2-deficient mice display widespread reductions in monoamines at 12-15 months of age. A, All monoamine levels are significantly lower than age-matched wildtype controls at 12-15 months of age in the striatum, cortex, and hippocampus. Results represent the mean ratios of raw values (ng/mg tissue) ± SEM for 8 animals per genotype. B, C, Ratio of DOPAC/DA and 5-HIAA/5-HT in the striatum, cortex, and hippocampus of wildtype and VMAT2-deficient mice. Results represent the mean ratios of raw values (ng/mg tissue) ± SEM for 4 animals per genotype. ***$p<0.001$

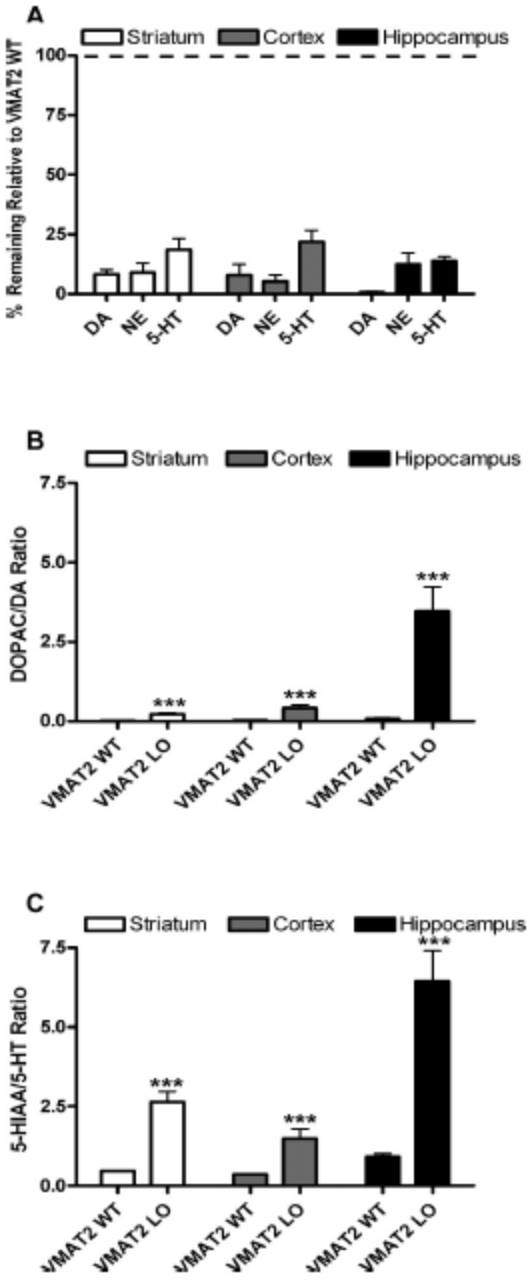
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Figure 2. VMAT2-deficient animals display progressive deficits in olfactory discrimination. When given the choice between a wooden block scented with a foreign animal's bedding and a block scented with the animal's own bedding, wildtype animals show preferential exploration of the block scented with the foreign animal's bedding at all ages, as shown by the investigatory index. The investigatory index represents the percentage of time the animal spent sniffing the block scented with a foreign animal's bedding minus the percentage of time the animals spent sniffing the block scented with their own bedding. B, Conversely, VMAT2-deficient animals exhibit a preferential exploration at 2 and 4 months of age, with a significant decrease at 5 months of age, worsening at 6 and 12 months of age. Results represent the investigatory index ± SEM for 10-15 animals per genotype. ***$p<0.001$. **$p<0.01$, *$p<0.05$

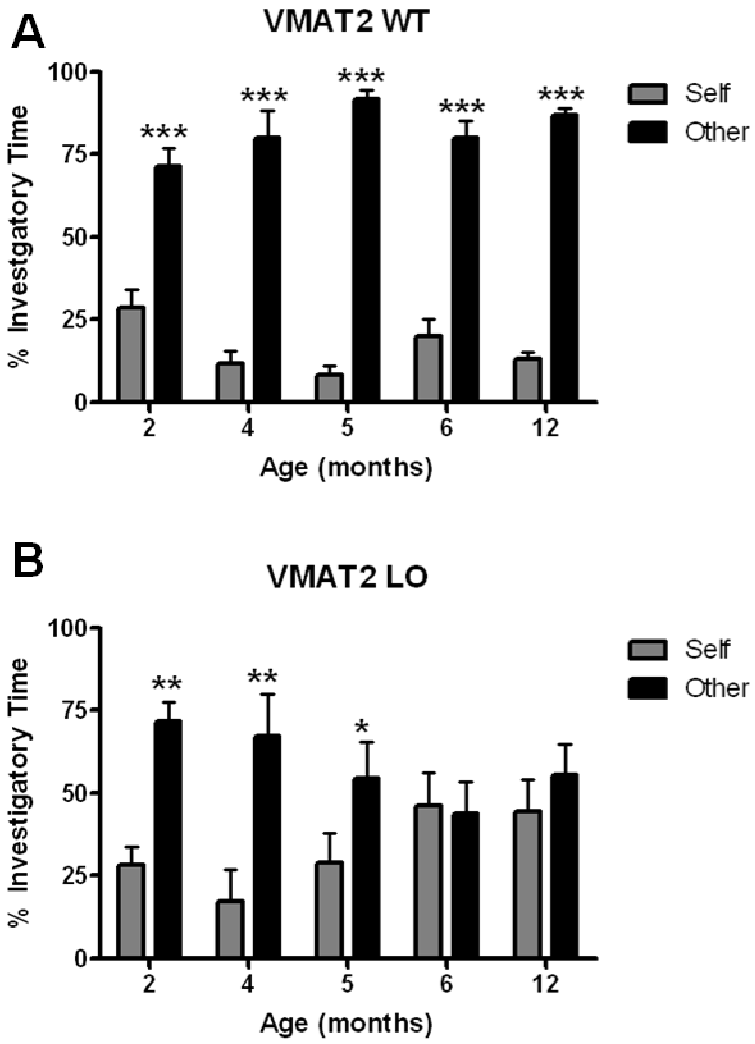
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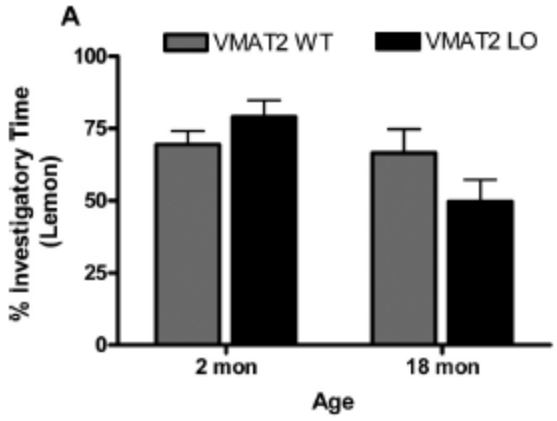
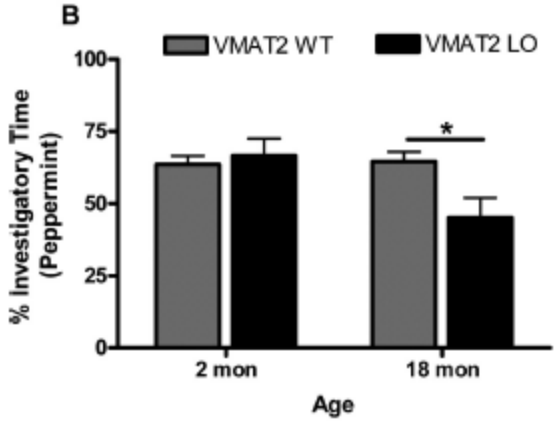
Figure 3. VMAT2-deficient mice display age-dependent deficits in non-social olfactory acuity. A, When given the choice between a novel odor (lemon) and water, both genotypes show a preferential exploration of the novel scent at 2 months of age, but the VMAT2-deficient mice lose the ability to discriminate between lemon and water by 18 months of age. B, C, VMAT2-deficient animals display similar behaviors at 18 months of age when presented with the novel odors of peppermint (B) and vanilla (C). Results represent the time spent investigating each scent ± SEM for 10 animals per genotype. ***$p<0.001$. **$p<0.01$


A
VMAT2 WT
VMAT2 LO
% Investigatory Time (Lemon)
100
75
50
25
0
2 mon
18 mon
Age


B
VMAT2 WT
VMAT2 LO
% Investigatory Time (Peppermint)
*
100
75
50
25
0
2 mon
18 mon
Age

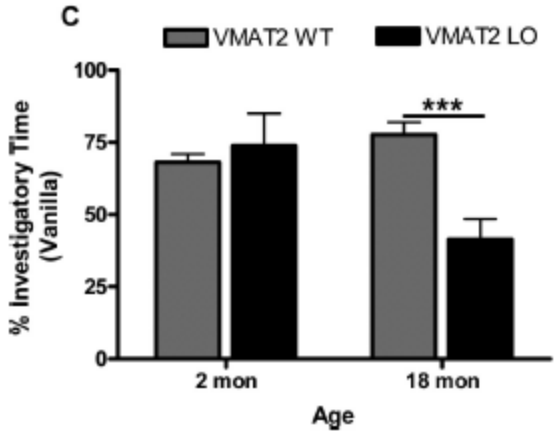

C
VMAT2 WT
VMAT2 LO
% Investigatory Time (Vanilla)
***
100
75
50
25
0
2 mon
18 mon
Age

Figure 4. VMAT2-deficient mice do not display deficits in general sensory behavioral tests. A, At 6 months and 12 months of age VMAT2-deficient mice display similar response latencies to tactile stimulation in the dot test. C, D. Trigeminal nerve function in VMAT2 wild-type and LO animals was tested by response to ammonia. Like the wildtype mice, VMAT2-deficient mice displayed preferential exploration of water compared to ammonia at both 6 (B) and 12 (C) months of age. Gustatory function was also found to be normal in VMAT2-deficient mice at 6 (D) and 12 (E) months of age as measured by the quinine taste aversion test.

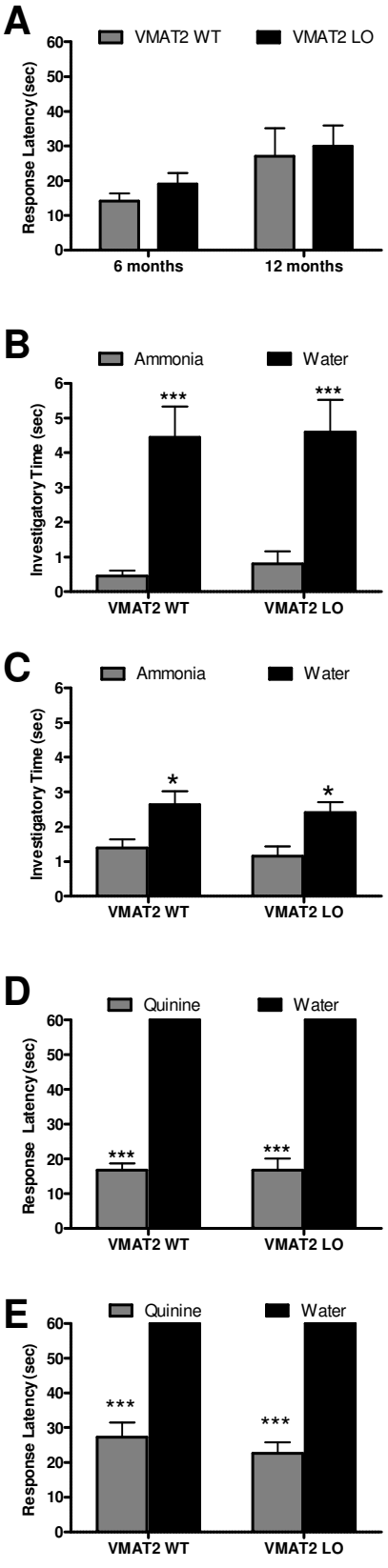

A
VMAT2 WT
VMAT2 LO
Response Latency (sec)
60
50
40
30
20
10
0
6 months
12 months
B
Ammonia
Water
Investigatory Time (sec)
6
5
4
3
2
1
0
***
***
VMAT2 WT
VMAT2 LO
C
Ammonia
Water
Investigatory Time (sec)
6
5
4
3
2
1
0
*
*
VMAT2 WT
VMAT2 LO
D
Quinine
Water
Response Latency (sec)
60
50
40
30
20
10
0
***
***
VMAT2 WT
VMAT2 LO
E
Quinine
Water
Response Latency (sec)
60
50
40
30
20
10
0
***
***
VMAT2 WT
VMAT2 LO

Figure 5. VMAT2-deficient animals display normal circadian activity but a premature shortened latency to behavioral signs of sleep. A, VMAT2-deficient mice at 4-6 months of age display shorter latency to behavioral signs of sleep as compared to WT controls. wildtype and LO animals have similar latencies to behavioral signs of sleep at 12-15 and 24 months of age. Results represent the time (min) passed until the animal achieved 2 minutes of uninterrupted sleep ± SEM for 8 animals per genotype. *$p<0.05$ B-E, VMAT2-deficient animals display normal circadian activity levels at 2, 4-6, 12-15, and 18 months of age, as compared to age-matched WT controls.

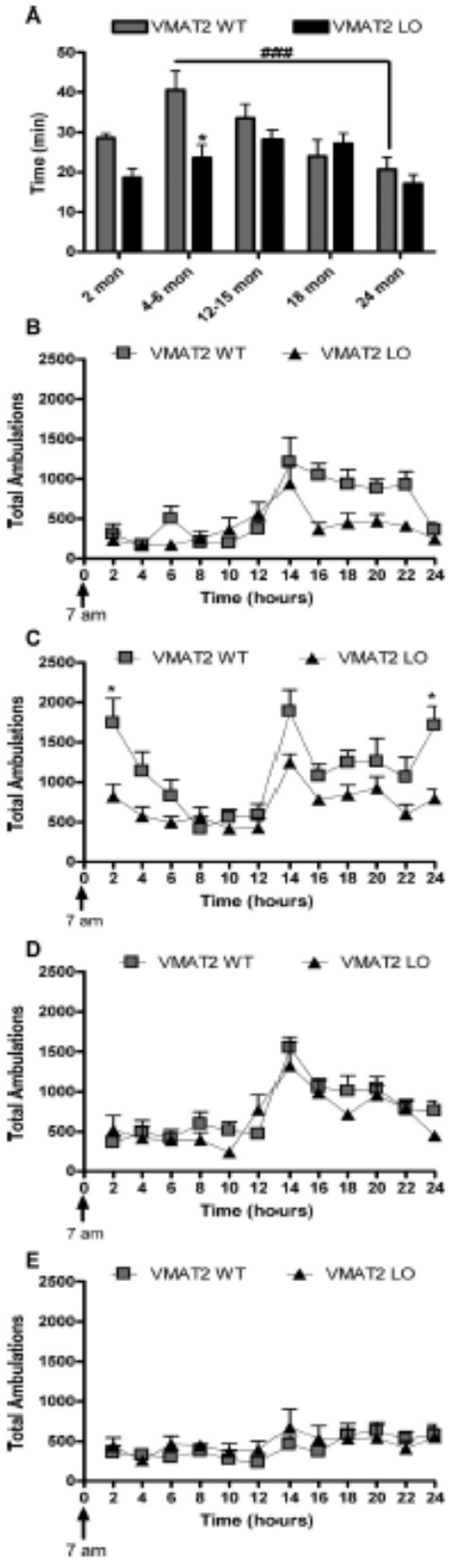

A
VMAT2 WT
VMAT2 LO
###
*
Time (min)
2 mon
4-6 mon
12-15 mon
18 mon
24 mon
B
VMAT2 WT
VMAT2 LO
Total Ambulations
Time (hours)
7 am
C
VMAT2 WT
VMAT2 LO
*
*
Total Ambulations
Time (hours)
7 am
D
VMAT2 WT
VMAT2 LO
Total Ambulations
Time (hours)
7 am
E
VMAT2 WT
VMAT2 LO
Total Ambulations
Time (hours)
7 am

Figure 6. VMAT2-deficient animals have delayed gastric emptying. **A**, Data broken down by age reveals a potential age-dependence of the effect. **B**, VMAT2-deficient mice have increased stool frequency compared to age-matched wildtype animals at 2 and 6 months of age. Results represent average stool frequency for two trials ± SEM for 4 animals per genotype. **p<0.01, ***p<0.001

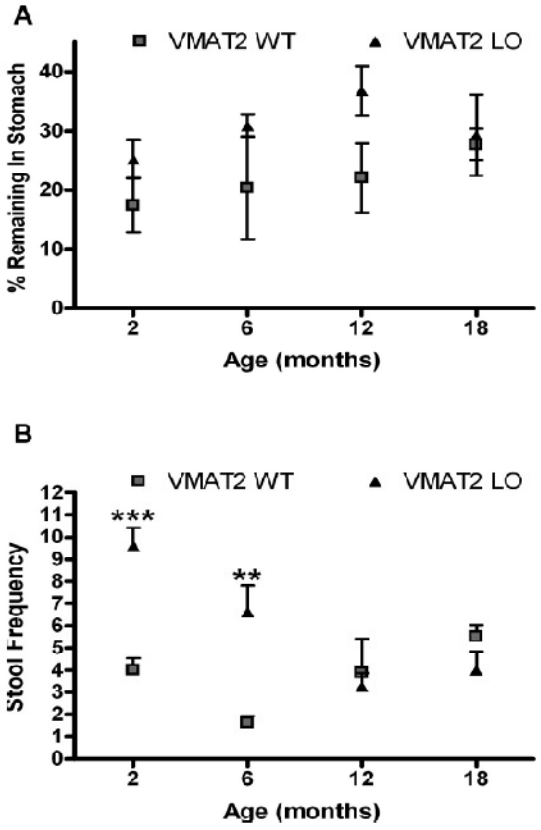
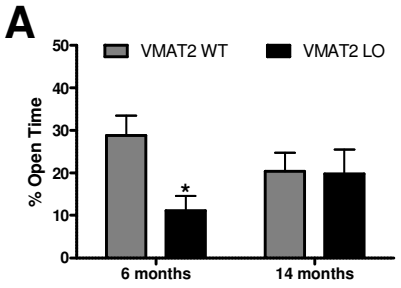
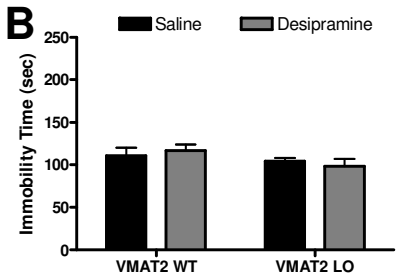
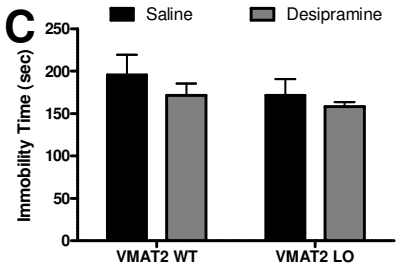
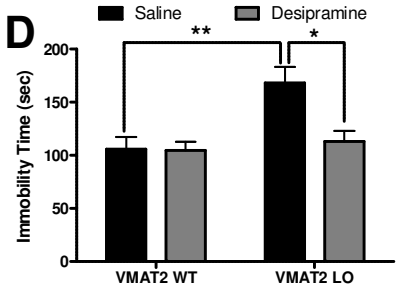

A
VMAT2 WT
VMAT2 LO
% Remaining In Stomach
0
10
20
30
40
2
6
12
18
Age (months)
B
VMAT2 WT
VMAT2 LO
Stool Frequency
***
**
0
1
2
3
4
5
6
7
8
9
10
11
12
2
6
12
18
Age (months)

Figure 7. VMAT2-deficient animals display an anxiety-like and a progressive depressive-like phenotype. A, Four to six-month-old VMAT2-deficient animals spend less time in the open arms and more time in the closed arms of the elevated plus maze as compared to age-matched WT animals over the 5 min test period. At 12-15 months of age, wildtype and LO animals display similar amounts of time in both the closed and open arms of the EPM. Results represent the mean time (seconds) ± SEM for 7 mice per genotype. *$p<0.05$ B and C, Four to six-month-old VMAT2-deficient animals have similar immobility times in the forced swim test (B) and tail suspension test (C) as compared to age-matched WT animals; the immobility times of both genotypes is not affected by 100 µL of 5 mg/kg desipramine 30 minutes prior to testing given intraperitoneally. D, At 12-15 months of age, wildtype animals remain non-responsive to desipramine administration. However, VMAT2-deficient mice have an increased immobility time, which is decreased to WT levels when dosed intraperitoneally with 100 µL of 5 mg/kg desipramine 30 minutes prior to testing. Results represent the mean time (seconds) ± SEM for 4-5 mice per genotype. *$p<0.05$, **$p<0.01$ E, At 12-15 months of age, VMAT2-deficient mice display a significant increase in immobility time compared to WT control animals, which is ameliorated by 5 mg/kg desipramine. **$p<0.01$, n=4-5 mice per genotype.


A
VMAT2 WT
VMAT2 LO
% Open Time
50
40
30
20
10
0
*
6 months
14 months


B
Saline
Desipramine
Immobility Time (sec)
250
200
150
100
50
0
VMAT2 WT
VMAT2 LO


C
Saline
Desipramine
Immobility Time (sec)
250
200
150
100
50
0
VMAT2 WT
VMAT2 LO


D
Saline
Desipramine
**
*
Immobility Time (sec)
200
150
100
50
0
VMAT2 WT
VMAT2 LO

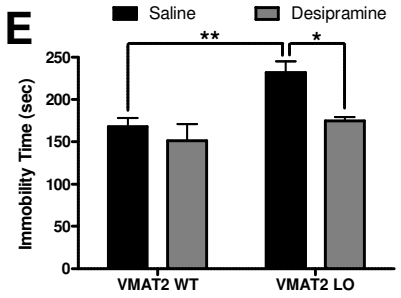
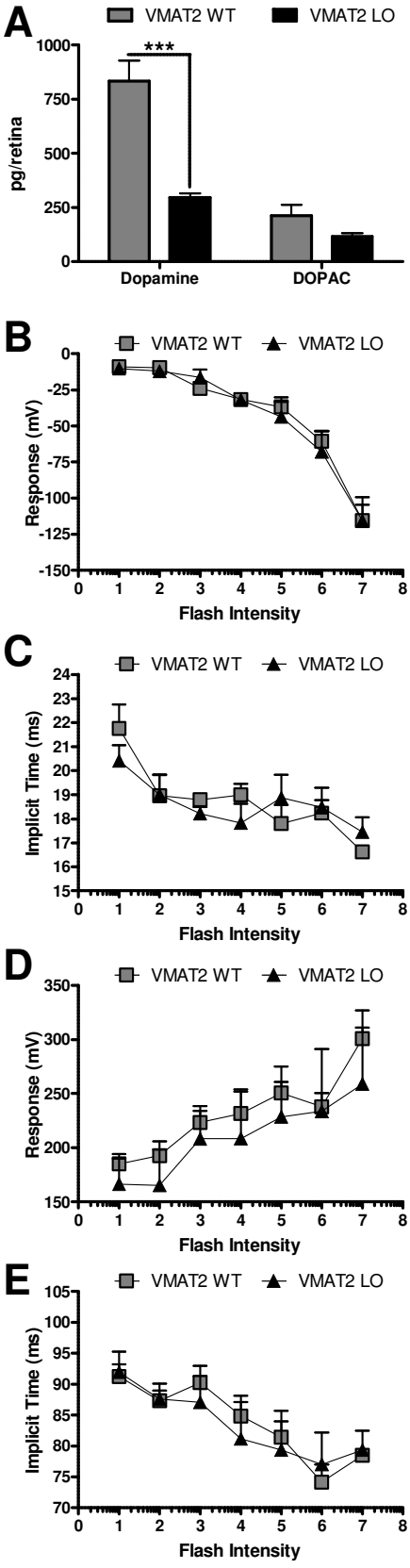

E
Saline
Desipramine
**
*
Immobility Time (sec)
250
200
150
100
50
0
VMAT2 WT
VMAT2 LO

Figure 8. VMAT2-deficient mice have decreased retinal DA but normal vision. A, VMAT2-deficient animals have a 64% decrease in retinal DA, concurrent 45% decrease in retinal DOPAC, and increased DA turnover. **B-E,** No changes were observed in A-wave amplitude (**B**) and implicit time (**C**) or in B-wave amplitude (**D**) and implicit time (**E**) in VMAT2-deficient mice compared to age-matched wildtype mice.


A
VMAT2 WT
VMAT2 LO
***
pg/retina
1000
750
500
250
0
Dopamine
DOPAC
B
VMAT2 WT
VMAT2 LO
Response (mV)
0
-25
-50
-75
-100
-125
-150
0 1 2 3 4 5 6 7 8
Flash Intensity
C
VMAT2 WT
VMAT2 LO
Implicit Time (ms)
24
23
22
21
20
19
18
17
16
15
0 1 2 3 4 5 6 7 8
Flash Intensity
D
VMAT2 WT
VMAT2 LO
Response (mV)
350
300
250
200
150
0 1 2 3 4 5 6 7 8
Flash Intensity
E
VMAT2 WT
VMAT2 LO
Implicit Time (ms)
105
100
95
90
85
80
75
70
0 1 2 3 4 5 6 7 8
Flash Intensity

CHAPTER 4

PROGRESSIVE NORADRENERGIC DEGENERATION PRECEDES NIGRAL CELL LOSS IN A MOUSE MODEL OF PD

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disease that is thought to primarily affect the motor processes of the brain. Pathophysiologically, PD has been characterized by the death of neuromelanin-containing dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and the appearance of proteinaceous intracellular Lewy bodies. We have previously reported a mouse model of neurodegeneration based on reduced vesicular storage of monoamines. These animals have a 95% reduction in the vesicular monoamine transporter (VMAT2; VMAT2-deficient); previously we have shown that these mice display behavioral deficits associated with PD, including L-DOPA responsive motor deficits, olfactory disturbances, and depressive-like behaviors. Here, we examine the resulting extranigral pathology associated with reduced vesicular storage in the VMAT2-deficient mice. Using immunohistochemical and unbiased stereological methods, we observe severe, progressive noradrenergic neurodegeneration in the locus coeruleus (LC) that precedes nigral degeneration. Neurodegeneration is accompanied by α-synuclein accumulation in the major catecholaminergic centers in the VMAT2-deficient animals. This pattern of progressive loss (noradrenergic > dopaminergic) has previously not been seen in an animal model of PD; however, this pattern of degeneration has been observed in human PD patients (German et al., 1992). To investigate the underlying mechanisms of degeneration, SNpc and LC primary neurons were cultured and exposed to physiological concentrations of DA and norepinephrine; those from VMAT2-deficient mice undergo increased catecholamine-mediated oxidant injury via formation of H_2O_2 and other oxidative species, whereas neurons from wildtype mice were unaffected. These data suggest that aberrant vesicular catecholamine storage can produce concomitant damage in norepinephrine and dopamine neurons. Since a similar pattern of degeneration is observed in human PD, the VMAT2-deficient mice appear to represent a model of PD that more faithfully reproduces the breadth of symptoms that result from multisystem dysfunction.

Introduction

Parkinson's disease (PD) is a multi-faceted neurodegenerative disease that affects millions of people, second only to Alzheimer's disease. Although PD is classically depicted as disorder of the midbrain dopamine (DA) system, the repository of affected brain regions has expanded beyond the nigrostriatal tract (Langston, 2006). The pathological processes underlying idiopathic PD take years to manifest themselves symptomatically (Braak et al., 2002; Dauer and Przedborski, 2003; Olanow, 2007). PD pathology lies, in part, with the appearance of α-synuclein positive inclusions in select neuronal populations (Duda et al., 2000; Hawkes et al., 2007). Lewy bodies occur in both clinically symptomatic patients and presymptomatic patients who never display the motor phenotype in life (Hawkes et al., 2007). Moreover, sporadic PD has recently been demonstrated to involve an extended prodromal phase during which several non-motor features, including hyposmia, sleep disturbances, and depression, develop (Hawkes et al., 2009), pointing to the participation of systems outside of the dopaminergic system. Neuronal damage and Lewy body formation have been noted in noradrenergic, serotonergic, and cholinergic systems among others; however, clinical correlates of these lesions are not as well characterized (Mann, 1983; Dauer and Przedborski, 2003; Zarow et al., 2003). Degeneration of the locus coeruleus has been shown in some cases to precede nigral loss and be more severe (German et al., 1992; Rye and DeLong, 2003; Benarroch, 2009). The temporal relationship of damage to specific neurochemical systems has not been well established (Dauer and Przedborski, 2003).

Reports have highlighted the importance of shifting the focus of PD research from the nigrostriatal system and underscoring the deficits seen in other neurotransmitter systems. The chemical models take advantage of dopamine's heightened susceptibility to oxidative damage to induce parkinsonian pathology (Olanow, 2007; Lim and Ng, 2009). Currently, no animal model fully replicates the constellation of symptoms in PD, despite the advent of transgenic models that utilize genes known to be involved in familial PD. Genetic models have the opportunity to not

only reproduce the progressive chronic nature of PD, but also involve multiple brain regions. Neurodegeneration in PD frequently involves other areas of the brain such as the locus coeruleus (LC), olfactory nucleus, and dorsal vagus, which questions the notion that nigral pathology is the exclusive hallmark of PD. Many studies have shown that norepinephrine (NE), in addition to DA, could play a prominent role in disease progression; degeneration has been shown in the LC, with Lewy body formation (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Rommelfanger and Weinshenker, 2007). Moreover, it has long been known that DA and NE share an intimate relationship; NE has been shown to exhibit a modulatory influence on DA systems, regulating their function (Antelman and Caggiula, 1977; Rommelfanger et al., 2004). Loss of LC neurons have been shown to exacerbate dopaminergic injury in animal models of PD (Marien et al., 1993; Rommelfanger et al., 2007), further demonstrating that initial loss of noradrenergic innervation could play an important role in disease pathogenesis. The VMAT2-deficient mice represent a unique opportunity to study catecholaminergic pathology in concert, due to the ubiquitous 95% reduction (Mooslehner et al., 2001; Caudle et al., 2007). In this study, we report distinct noradrenergic parkinsonian pathology that parallels nigral degeneration in the VMAT2-deficient mice, indicative of a mouse model which closely mimics PD observed in humans.

Materials and Methods

Animals. Male and female VMAT2-deficient mice were generated as previously described (Caudle et al., 2007; Taylor et al., 2009). Briefly, the mouse VMAT2 locus was cloned from the 129/Sv genomic library and a 2.2 kb *Pvu*II fragment from the third intron of the VMAT2 gene, and cloned into the bluntended *Not*I site of the construct. The targeting vector was introduced into 129/Ola CGR 8.8 embryonic stem (ES) cells and injected into blastocytes of C57BL/6 mice. Highly chimeric males were bred with C57BL/6 females; genotype was confirmed by Southern blot analysis. A recent report uncovered that the C57BL/6 inbred strain of mice originally used to establish the VMAT2-deficient mouse line contained a spontaneous chromosomal deletion spanning the α-synuclein gene locus (Specht and Schoepfer, 2001a), which

was confirmed in the original strain (Mooslehner et al., 2001; Patel et al., 2003b; Colebrooke et al., 2006). Through breeding, we eliminated all traces of this mutation from our strain of mice and routinely verify the presence of α-synuclein via Southern blot analysis. This report represents the fourth set of data on VMAT2-deficient mice with a normal α-synuclein background. All mice were generated through redundant breeding of mice that were heterozygous for the VMAT2 allele and wild type (WT) for the α-synuclein allele. The genotype of all mice was confirmed by PCR of DNA extracted from tail samples. For all behavioral tests, wildtype littermate controls were used. All procedures were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and previously approved by the Institutional Animal Care and Use Committee at Emory University.

Neuropathology. Tissue staining was completed as previously described (Miller et al., 1997; Miller et al., 1999b; Caudle et al., 2006). Briefly, VMAT2 wildtype and deficient mice were perfused transcardially with phosphate buffered saline (pH=7.4) and then with 4% paraformaldehyde, removed and placed in 4% paraformaldehyde for 24 h, and finally cryoprotected in 30% sucrose for 48 h. The brains were then cut to a thickness of 40 µm (silver staining) or 50 µm (immunohistochemistry and stereological counts) on a freezing microtome (Microm, Kalamazoo, MI, USA). Sections were incubated with a polyclonal anti-TH (1:2000; Chemicon) or monoclonal anti-tryptophan hydroxylase (1:250; Sigma-Aldrich) antibody overnight and then incubated in a biotinylated goat anti-mouse secondary antibody for 1 hour at room temperature. Visualization was performed using 3, 3´-diaminobenzidine (DAB) for 2 min at room temperature. After DAB, all sections were counterstained in hematoxylin, mounted on slides, dehydrated, and coverslipped using Permount (Fisher Scientific) and sections were viewed using a light microscope (Olympus).

Detection of neurodegeneration. Silver staining (FD Neurosilver kit: FD NeuroTechnologies, Ellicott City, MD) for degenerating neurons was completed according to the manufacturer's protocol.

Histopathological analysis. Stereological sampling was performed using the Stereo Investigator software (MicroBrightField, Colchester, VT). Tissue staining was performed as described previously (McCormack et al., 2002; Reveron et al., 2002). Whole brains were removed and processed for frozen sections as described above, and serially sectioned at 50 µm (final mounted thickness of 26 µm) for systematic analysis of randomly placed counting frames (size, 50 × 50 µm) on a counting grid (size of 120 × 160 µm) and sampled using an 22 µm optical dissector with 2 µm upper and lower guard zones. The boundaries of substantia nigra pars compacta (SNpc) and locus coeruleus (LC) were outlined under magnification of the 4X objective as per the atlas of Paxinos and Franklin (2001). Cells were counted with a 40X objective (1.3 numerical aperture) using a Nikon Eclipse e800 microscope. Guard zones of 2 µm ensured the exclusion of lost profiles on the top and bottom of the section sampled. A dopaminergic or noradrenergic neuron was defined as an in-focus tyrosine hydroxylase immunoreactive (TH-IR) cell body with a TH-negative nucleus within the counting frame. For the SNpc, every other section was processed for TH-IR, resulting in 20-25 sections sampled per mouse. For the LC, every section was stained for TH-IR and counterstained with hematoxylin, resulting in 15 sections sampled per mouse. The number of neurons in the SNpc and LC was estimated using the optical fractionator method, which is unaffected by changes in the volume of reference of the structure sampled. The estimated total number of TH-IR neurons in the substantia nigra and the LC was calculated based on the following formula: $N = Q^- \times 1/ssf \times 1/asf \times t/h$ (West et al., 1991), where N is the estimate of the total number of cells, Q^- is the number of objects counted, ssf is the section sampling fraction, asf is the area sampling fraction, and t/h is the actual section thickness divided by the height of the dissector. Between 50 (LC) and 200 objects (SNpc) were counted to generate the stereological estimates. Gundersen (m=1) coefficients of error were less than 0.1.

Primary culture. Methods used to generate substantia nigra (SN) and locus coeruleus (LC) neuron-glia co-cultures were previously described, with slight modifications (Cardozo,

1993; Smeyne and Smeyne, 2002; Guillot et al., 2008). Briefly, brains from postnatal days 0–6 wildtype andVMAT2-deficient mice were removed and placed in dissociation media (DM) pH 7.4. Under a dissecting microscope, a 0.8–1.0 mm coronal slice of the mesencephalon was obtained; regions containing the SN and LC were isolated, placed in fresh DM, and minced into small pieces. The SN and LC were digested in DM containing papain and DNAase (Worthington Biochemical, Freehold, NJ) and incubated at 37°C (twice for 30 min). The SN and LC sections were then triturated and the cell suspension was layered over PM and centrifuged for 8 min at 1400 x g. The supernatant was removed, and the pellet resuspended in PM with 2% rat serum (RS). Cells were counted using Trypan Blue, and plated at a density of 350,000 cells/cm^2 in Lab-Tek™ four-well Permanox chamber slides coated with laminin and poly-D-lysine (Collaborative Biomedical Products, Bedford, MA). Cells were maintained at 37°C, 5% CO_2, and fed with PM containing 2% RS. At 24 h post-plating, the cultures were fed with complete feeding media.

After 7 days in culture, neurons were treated with either dopamine (DA) or norepinephrine (NE) (10-50 μM dissolved in feeding media) or feeding media alone (controls). Seventy-two hours post-administration, cultures were rinsed twice for 5 min with TBS, fixed for 10–15 min in 4% buffered paraformaldehyde, and rinsed three times with TBS. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 10 min. Cells were permeabilized with 0.1% Triton X-100, 5% goat serum in TBS for 10 min. Catecholaminergic neurons were visualized with a rabbit polyclonal tyrosine hydroxylase (TH) antibody (1:500; Chemicon). Cultures were incubated with primary antibody overnight at 4°C, rinsed twice with TBS, incubated in secondary antibody (goat anti-rabbit or goat anti-mouse, 1:200; Jackson ImmunoResearch) and amplified with avidin–biotin-HRP (Vector Laboratories). Final visualization of the immunoreactive neurons was made using 0.03% DAB for 3 min, followed by three washes with TBS. All TH-positive cells from each culture were counted on a light microscope at magnification of 200x (Olympus), and quantified by counting the processes from a total of 30 neurons in four random fields as previously described (Radad et al., 2008). The

total number of processes counted was divided by the number of neurons counted; values are expressed as number of TH processes per neuron. The data reflect two separate experiments and were combined to yield a final sample size of 4 per treatment per genotype.

2,7 dichlorofluorescein diacetate (DCF) assay. Detection of oxidative stress in cultures was accomplished by adding 2, 7-dichlorofluorescein diacetate (DCF; Invitrogen) (5 μM) to cultures 72 h after treatment with 10 μM DA or NE (Larsen et al., 2002). DCF will be prepared in dimethylsulfoxide at 10 mM and diluted to a concentration of 1 μM in minimal essential medium. Neuronal cultures will be incubated in 5uM DCF for 30 minutes, and rinsed with oxygenated saline. The 96-well plate containing cultures will be placed in a Victor3 1420 multi-label counter (Perkin Elmer) with a Fluorescein filter set at 485, and emission filter set at 535. The amount of oxidative stress in cultures was expressed and quantified as relative fluorescein units (RDU). The cultures were then placed at 37°C for 1 h. Cells were washed twice, coverslipped, and viewed using an epifluorescence microscope (Olympus).

Statistical analysis. Data from male and female mice were combined, since there were no detectable sex differences. All data were analyzed using unpaired independent samples completely randomized two-factor ANOVA followed by Bonferroni post hoc analysis. Analyses were completed using Graph Pad Prism 5.0 for Windows, and for all tests all *post hoc* measures were error-corrected to keep the overall error rate per group at 0.05.

Results

VMAT2-deficient mice display exacerbated catecholaminergic degeneration in the SNpc and LC over time.

VMAT2-deficient mice have previously been shown to display nigral degeneration and loss of dopamine transporter (DAT) expression in the striatum beginning at 6 months of age, and the VMAT2-deficient animals develop evidence of nigral degeneration at 18 months of age, accompanied by reductions in striatal tyrosine hydroxylase (TH) expression (Caudle et al., 2007), presumably caused by the effects of reduced vesicular dopamine (DA) storage. To further study

the patterns of degeneration in these mice, the substantia nigra pars compacta (SNpc) and locus coeruleus (LC) of VMAT2-deficient mice were assessed immunohistochemically. At 12 months of age, there was no appreciable reduction in TH expression or evidence of neurodegeneration, as measured by silver deposition, in the SNpc or LC of the VMAT2-deficient mice compared to age-matched controls (data not shown). Beginning at 18 months of age, the VMAT2-deficient displayed a mild reduction in TH staining in the SNpc, which increased moderately with age (Figure 1). More dramatic reductions in TH staining were observed in the locus coeruleus (LC) at 18, 24, and 30 months of age (Figure 2), suggesting that the LC undergoes a much more severe degeneration than the SNpc in the VMAT2-deficient mice. Previous, we observed degeneration in the SNpc of aged VMAT2-deficient mice as measured by Fluorojade B staining and presence of silver deposition (Caudle et al., 2007). The reduction in TH expression in both the SNpc and LC were accompanied by neuronal silver deposits in these regions in VMAT2-deficient mice at 22 months of age (Figure 3B) (Caudle et al., 2007). Silver staining was not observed at 12 months of age in the VMAT2-deficient mice (data not shown) or in age-matched wildtype controls at any age.

Noradrenergic loss in the LC precedes nigral neuronal loss in VMAT2-deficient mice

To quantify the extent of neuronal loss in the SNpc and LC in the VMAT2-deficient mice, unbiased stereological cell counts were performed on TH/hematoxylin stained cells. Previously, it was established that the decline in TH-positive neurons was not a result of downregulation of TH in the SNpc (Caudle et al., 2007). At 6 months of age, no change was seen in neuronal number between wildtype and VMAT2-deficient animals in either the SNpc or LC (Figure 4). At 12 months of age, no change in the number of TH-positive (wildtype, 6081.96±572.21; VMAT2-deficient 5146.30±498.09; p=0.47) or hematoxylin-stained (wildtype, 7551.53±1124.98; VMAT2-deficient 7596.12±426.61; p=0.18) cells was observed between wildtype and VMAT2-deficient mice in the SNpc. However, the 12 month VMAT2-deficient mice did have a significant 25% decrease in TH-positive LC neurons compared to 6 months of

age (Figure 4C) (VMAT2-deficient 6 mon: 2682.98±406.99, VMAT2-deficient 12 mon: 2001.34±208.23, $F_{(4,23)}$=60.46, p<0.01). A similar decrease was observed in hematoxylin-stained cells at 12 months of age in the VMAT2-deficient animals (Figure 4D). As noted before, VMAT2-deficient display a 25% loss of TH-positive cells in the SNpc at 18 months of age compared to 6 months of age (VMAT2-deficient 6 mon: 5691.24±286.16, VMAT2-deficient 12 mon: 4247.74±593.34, $F_{(4,22)}$= 8.36, p>0.05), with a similar reduction in hematoxylin-stained cells compared to (Figure 4A, B) (Caudle et al., 2007). However, the VMAT2-deficient mice did not demonstrate a significant loss in nigral neurons until 24 months of age (VMAT2-deficient 6 mon: 5691.24±286.16, VMAT2-deficient 24 mon: 3347.36±330.64, $F_{(4,22)}$= 8.36, p<0.001). Overall, the VMAT2-deficient mice have a 54% loss in nigral neurons from 6-30 months of age, while the LC undergoes a loss of 72% of TH-positive neurons (Figure 4 A,C). Although the VMAT2 wildtype mice undergo 30% decrease in TH-positive neurons in both the SNpc and LC by 30 months of age, this loss can be attributed to the advanced age of the animal and was not enough of a loss to create a behavioral deficit (Figure 4A, C).

Cell loss in the LC did not follow the same pattern as the SNpc, and appears to precede degeneration of the SNpc. At 12 months of age, unlike in the SNpc, there was a 25.7% decrease in LC TH-positive neurons (wildtype, 2695.45±255.03; VMAT2-deficient, 2001.34±104.12; p<0.01), with a similar 20.8% decrease in hematoxylin-positive cells (Figure 4C,D). The LC of VMAT2-deficient mice undergoes a much more rapid decline from 12 to 18 months of age; there is a 52.4% decrease in VMAT2-deficient mice compared to age-matched wildtype animals (wildtype, 2273.6±107.01; VMAT2-deficient, 1080.26±54.73; p<0.001) (Figure 4C). At 24 and 30 months of age, the cell loss within the LC of VMAT2-deficient mice is more moderate, showing only a 17.9% and 15.9% decrease, respectively compared to 18 months of age (Figure 4C).

VMAT2-deficient mice are more sensitive to catecholamine-mediated oxidative stress.

Previously, it has been shown that VMAT2 plays an important factor in mitigating neuronal susceptibility to endogenous and exogenous oxidative stress (Uhl, 1998; Miller et al., 1999a; Caudle et al., 2008). Normally, VMAT2 acts to sequester monoamines before they can auto-oxidize into quinone species or cysteinyl adducts (Graham, 1978). Similarly, VMAT2 has been shown to protect against oxidative stress and neuroinflammation induced by MPTP and methamphetamine exposure in dopaminergic populations (Takahashi et al., 1997; Gainetdinov et al., 1998; Fumagalli et al., 1999; Guillot et al., 2008). Specifically, oxidative stress has been identified in the VMAT2-deficient animals in the form of increased protein carbonyl and cysteinyl-adduct formation (Caudle et al., 2007). To further explore the role of VMAT2 in monoaminergic-induced oxidative stress, primary SNpc and LC neuronal cultures were exposed to physiological concentrations of dopamine and norepinephrine, respectively. Compared to wildtype primary cultures, VMAT2-deficient neurons undergo increased oxidative stress via formation of ROS as measured by DCF fluorescence when exposed to dopamine ($n=6$, $p<0.05$, Figure 5A, C-D). Similar trends were observed when VMAT2-deficient neurons were exposed to norepinephrine; however, neither had the same severity of effect as dopamine (Figure 5B-C).

VMAT2-deficient mice display increased α-synuclein accumulation with age.

The association of the ubiquitous protein α-synuclein with the pathogenesis of parkinsonian pathology has long been known as the role of the protein is being elucidated via work with transgenic models and *in vitro.* Moreover, catecholamines have been shown to inhibit deleterious α-synuclein fibrilization, but not in their fully oxidized form (Conway et al., 2001; Norris et al., 2005). The VMAT2-deficient mice could be uniquely sensitive to α-synuclein accumulation due to their severely diminished catecholamine levels and reduced expression of VMAT2 resulting in increased oxidized cytosolic dopamine and norepinephrine (Norris et al., 2005; Taylor et al., 2009). Previously, we have shown that at 18 and 22 months of age, the VMAT2-deficient mice have increased α-synuclein accumulation in the SNpc compared to age-

matched WT animals (Caudle et al., 2007). Immunohistochemical analysis of 30-month wildtype and LO mice revealed α-synuclein accumulation in both the SNpc and LC of only VMAT2-deficient animals (Figure 6).

Discussion

Parkinson's disease (PD) has been traditionally viewed as a neurodegenerative movement disorder; the increasing prominence of non-motor symptoms suggests the disease is more multi-faceted than commonly thought (Langston, 2006). Moreover, these symptoms imply that more dysfunction is occurring in concert with dopamine (DA) degeneration. Evidence for degeneration of the locus coeruleus (LC) in humans highlights the importance of shifting the focus of PD research from the nigrostriatal system in order to expose the deficits in other neurotransmitter systems (Braak et al., 2002; Braak et al., 2003; Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Lemke et al., 2004; Rommelfanger and Weinshenker, 2007). Clinically, PD is often not diagnosed until motor deficits appear which is when 80% of DA neurons in the brain have died. Because of this fact, it is very difficult to identify the early factors that contribute to the pathology of the disease.

Pathophysiologically, PD is believed to be caused by the death of neuromelanin-containing DA neurons in the substantia nigra pars compacta (SNpc) and the appearance of Lewy bodies (Olanow and Tatton, 1999; Jenner and Olanow, 2006). Motor disturbances do not present clinically until approximately 70-80% of dopaminergic neurons have been lost; however, other non-motor symptoms are evident before the onset of motor disturbances. In the VMAT2-deficient mice it has been observed that the major motor deficits do not appear until 28 months of age, coinciding with the most severe nigral cell loss (Taylor and Miller, unpublished observations, Figure 3, 4A). Therefore the preceding phenotypes seen in the VMAT2-deficient mice can perhaps be attributed to extra-dopaminergic changes (Taylor et al., 2009). The temporal relationship between noradrenergic and dopaminergic degeneration established in this study suggests that NE could play a significant role in PD-development, ranging from olfactory

disturbances observed at 4 months of age to the depressive-like behaviors seen at 12-15 months (Taylor et al., 2009). Similarly in humans, it is possible that other neurotransmitters such as NE may significantly contribute to these symptoms, as the LC has been shown to degenerate as well (Greenfield and Bosanquet, 1953; Zarow et al., 2003; Tong et al., 2006).

Degeneration of the noradrenergic LC and other non-dopaminergic centers have been noted in post-mortem PD brains, these monoaminergic centers are not directly related to the manifested motor phenotype, yet contribute significantly to disease progression (Halliday et al., 1990; Gesi et al., 2000; Murai et al., 2001; Braak et al., 2003; Rommelfanger and Weinshenker, 2007). As an alternative hypothesis, Braak and colleagues propose that PD actually begins in the lower brainstem and olfactory bulb (Braak et al., 2002; Braak et al., 2003). This is in contrast to the theory that degeneration begins with dopaminergic neurons in the substantia nigra (Braak et al., 2002; Chaudhuri et al., 2006). Braak staging allows for the incorporation of the non-motor symptoms and other neurotransmitter systems in PD pathogenesis. More recently, laboratories have begun to explore the specific contributions of noradrenergic dysfunction to the progression of PD. NE has also been theorized to compensate for dopaminergic loss, sustaining the dopaminergic system until it is overwhelmed by neuronal loss (Gesi et al., 2000; Rommelfanger and Weinshenker, 2007). When a dopaminergic neurotoxin is given in concert with a noradrenergic toxin, such as DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride), nigrostriatal damage is exacerbated and vice versa, revealing parkinsonian behavioral deficits (Mavridis et al., 1991; Dauer and Przedborski, 2003; Marien et al., 2004; Rommelfanger et al., 2004). Moreover, mice lacking NE have been shown to have parkinsonian motor deficits, despite their normal striatal dopamine content; this suggests that both catecholamines play a significant role in the pathogenesis of PD (Rommelfanger et al., 2007; Rommelfanger and Weinshenker, 2007). However, if NE, and even 5-HT, neurons have a similar susceptibility to oxidative stress, using an approach that targets all monoaminergic neurons may more faithfully recapitulate the constellation of symptoms associated with PD.

Current PD treatments focus on restoring the dopamine system; however, drugs that act on the DA system aid only in the relief of classical motor symptoms (Lang and Obeso, 2004). By focusing on monoamine systems as a whole and not just the dopaminergic system, non-motor symptoms that are classically thought of as early-stage markers may become signs of the disease's end stages. Since the VMAT2-deficient mice have reduced levels of DA, NE, and 5-HT, mice with altered VMAT2 expression may represent a new model of PD that encompasses many of the motor and non-motor symptoms, as well as the neurochemical pathophysiology (Mooslehner et al., 2001; Caudle et al., 2007; Fukui et al., 2007; Taylor et al., 2009). These mice provide a unique opportunity to study monoamine dysfunction, perhaps expanding PD to a monoaminergic disorder rather than merely a disease of the dopamine system. Parkinson's disease, as a field, needs to be expanded to acknowledge the parallel degeneration of the SNpc with other monoaminergic nuclei, including the LC, and extra-nigral pathology. Even in the earlier reports of parkinsonian pathology, investigators recorded loss of many LC cells and severe degenerative changes including neurofibrillary tangles and spherical inclusions (Hassler, 1938; Beheim-Schwarzbach, 1952; Greenfield and Bosanquet, 1953) Moreover, it is essential to note that the first neurons affected in PD are non-dopaminergic; it is only in the latter stages of the disease that the SNpc and other dopaminergic nuclei are affected (Braak et al., 2003; Ahlskog, 2007). Until this point, the use of dopamine-centric animal models has proven to be invaluable; however, most have limitations as they give little insight into PD beyond dopamine deficiency states leading to biased treatments (Langston, 2006; Ahlskog, 2007). Continuing to focus only on the dopaminergic system may hinder the explication core mechanisms of neurodegeneration and the development of novel therapeutic approaches.

Acknowledgments

I thank Dr. Kennie Shepherd for performing the analysis on primary nigral and locus coeruleus cultures. I thank Dr. W. Michael Caudle for conducting the silver staining of the locus coeruleus and for his critical reading and helpful suggestions regarding the preparation of this manuscript. Additionally thank Dr. Min Wang for her excellent technical skills in breeding and aging the VMAT2-deficient mice.

Figure 1. Immunohistochemical analysis of tyrosine hydroxylase in substantia nigra of aged VMAT2-deficient mice. TH immunoreactivity was slightly reduced in VMAT2-deficient mice at 18, 24, and 30 months of age. Analysis was performed on 5 animals per genotype at each age. Representative sections are shown. Scale bars, 500 μm.

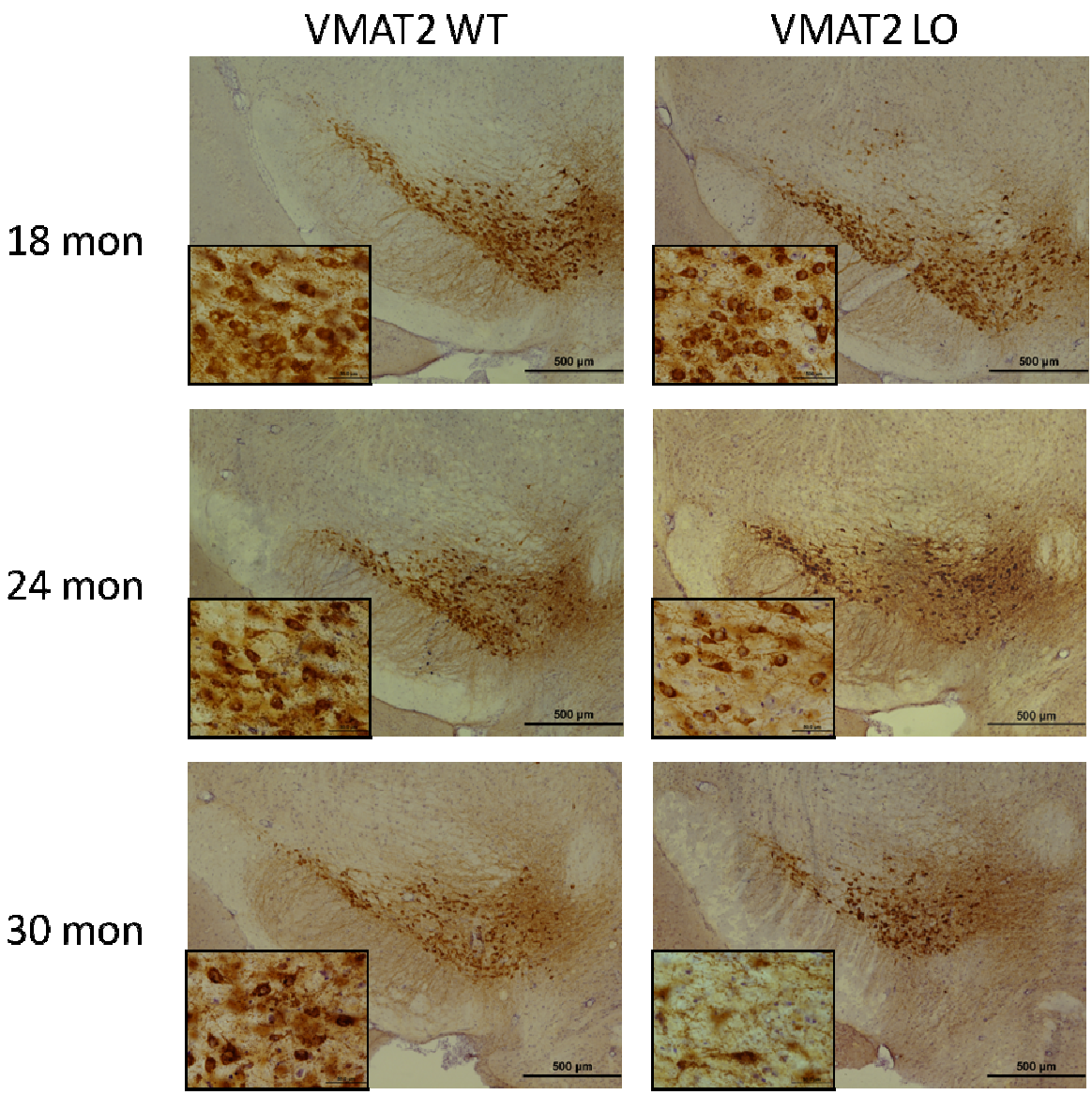

VMAT2 WT
VMAT2 LO
18 mon
24 mon
30 mon
500 µm

Figure 2. Immunohistochemical analysis of tyrosine hydroxylase in locus coeruleus of aged VMAT2-deficient mice. TH immunoreactivity was significantly reduced in VMAT2-deficient mice at 18, 24, and 30 months of age, preceding loss observed in the substantia nigra. Analysis was performed on 5 animals per genotype at each age. Representative sections are shown. Scale bars, 500 µm.

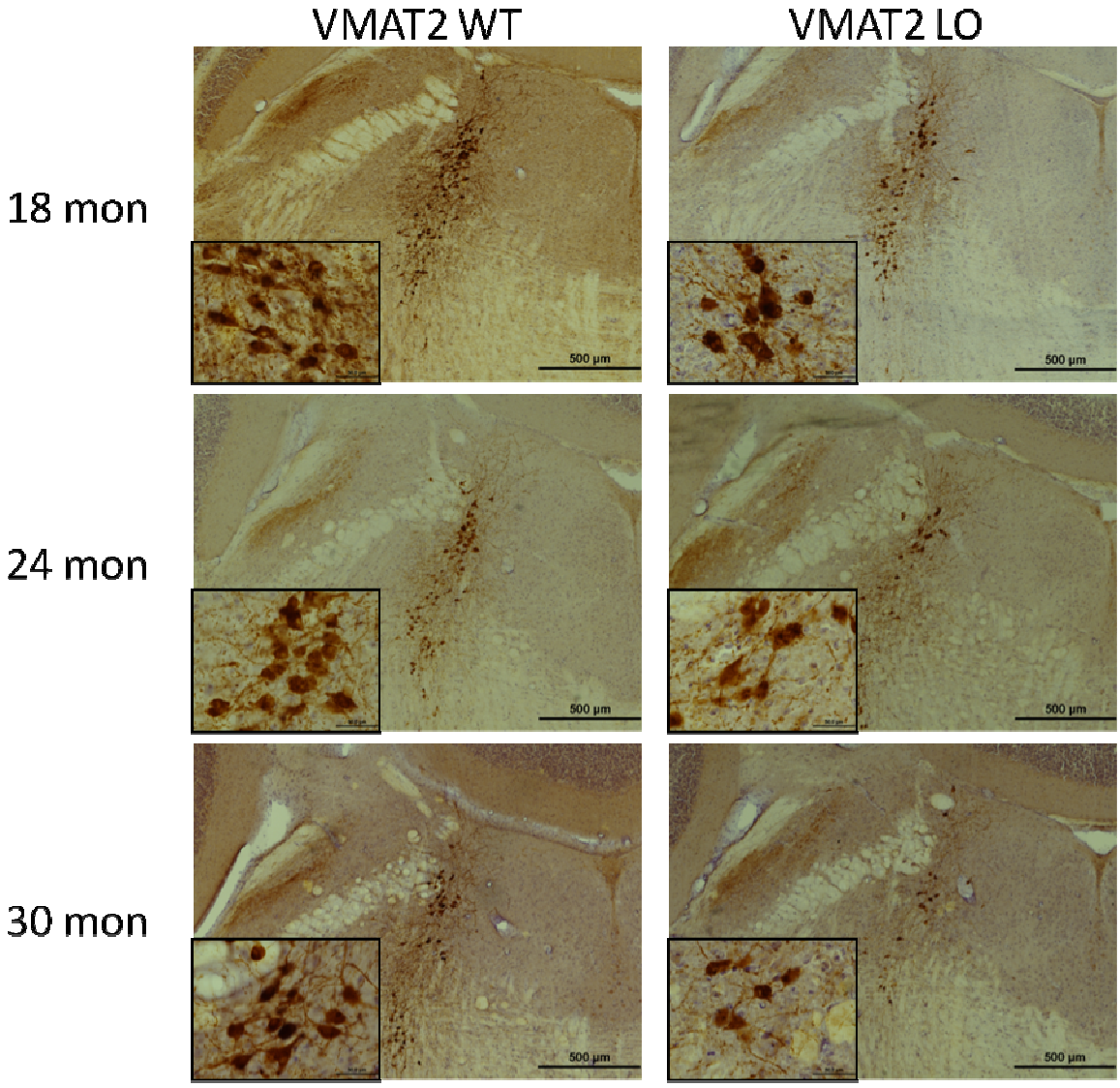

VMAT2 WT
VMAT2 LO
18 mon
24 mon
30 mon
500 µm

Figure 3. VMAT2-deficient mice show evidence of noradrenergic degeneration at 24 months of age. Accumulation of silver deposits in the LC of 22 month old wildtype(A) andVMAT2-deficient (B) mice. Arrows denote silver deposits in degenerating neurons. Analysis was performed in 3 animals per genotype; representative sections are shown. Scale bar: 50 µm.

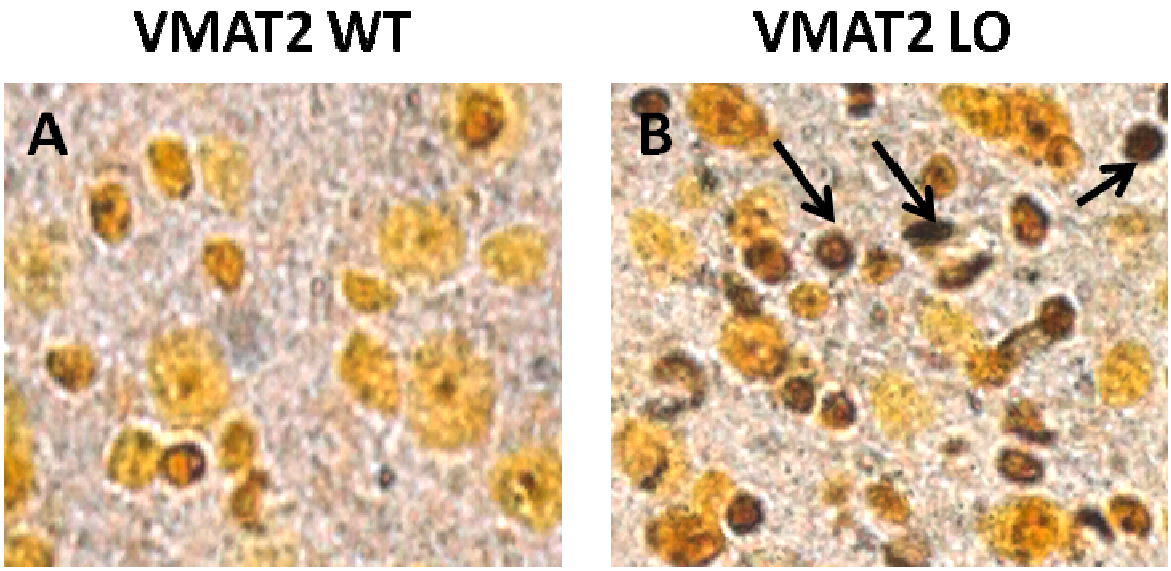

VMAT2 WT
VMAT2 LO
A
B

Figure 4. TH and hematoxylin cell counts in the substantia nigra (SNpc) and locus coeruleus (LC) of 18, 24, and 30 month wildtype andVMAT2-deficient mice. **A**, A significant reduction in TH cells in the SNpc was observed in the 18-month-old VMAT2-deficient mice compared with WT. In addition, a greater reduction in TH cells was seen in 30-month-old VMAT2-deficient mice compared with WT. **B**, Furthermore, a similar reduction in hematoxylin cells in the SNpc was seen at 18 months and 30 months between wildtype and LO mice. **C**, A significant reduction in TH+ cells in the LC was observed at all ages in the VMAT2-deficient mice compared to WT littermates. **D**, A significant reduction was also seen in hematoxylin cells in the LC at 18, 24, and 30 months of age in VMAT2-deficient mice compared to wildtype in the LC. Results represent the mean SEM for 5 animals per genotype at each age. *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

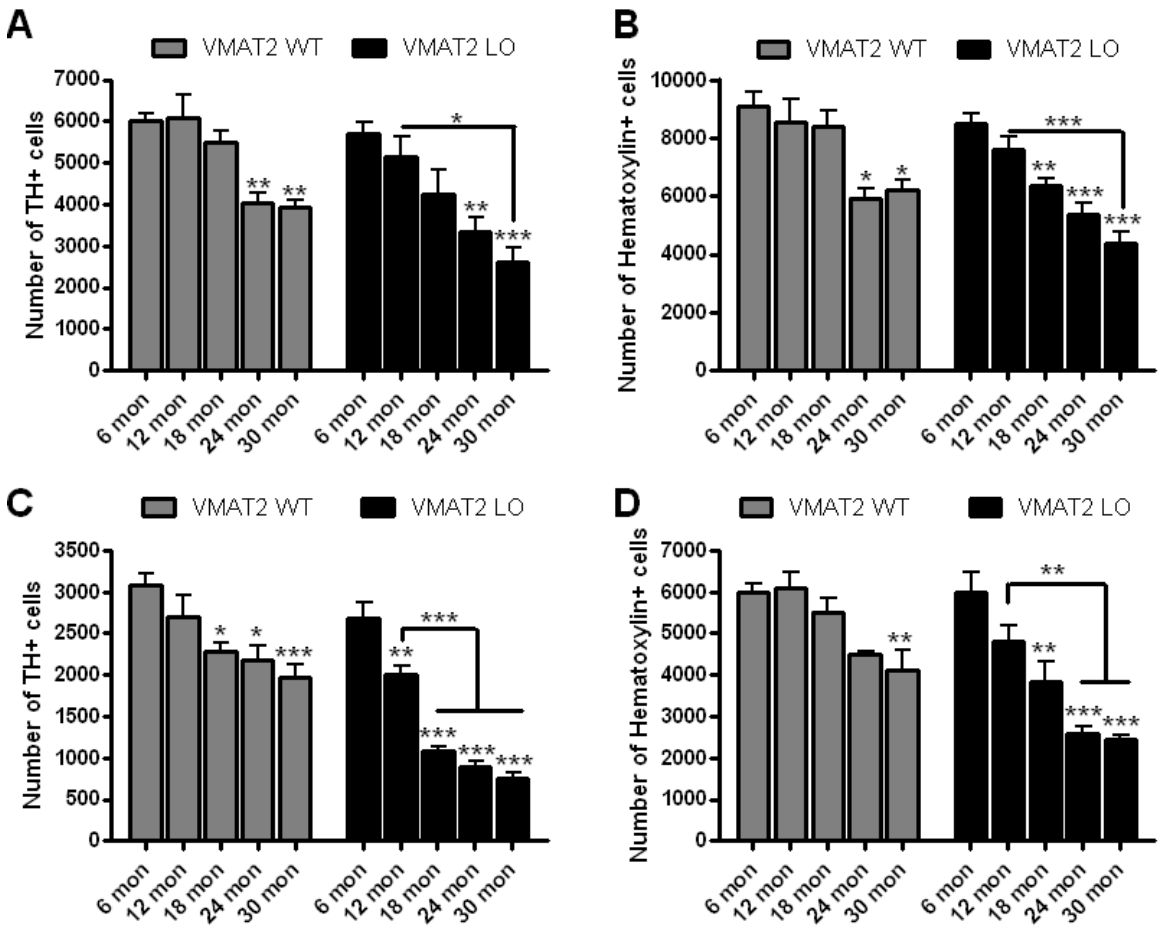

A
VMAT2 WT
VMAT2 LO
Number of TH+ cells
7000
6000
5000
4000
3000
2000
1000
0
*
**
**
**
***
6 mon
12 mon
18 mon
24 mon
30 mon
6 mon
12 mon
18 mon
24 mon
30 mon
B
VMAT2 WT
VMAT2 LO
Number of Hematoxylin+ cells
10000
8000
6000
4000
2000
0
***
*
*
**
***
***
6 mon
12 mon
18 mon
24 mon
30 mon
6 mon
12 mon
18 mon
24 mon
30 mon
C
VMAT2 WT
VMAT2 LO
Number of TH+ cells
3500
3000
2500
2000
1500
1000
500
0
***
*
*
**
***
***
***
6 mon
12 mon
18 mon
24 mon
30 mon
6 mon
12 mon
18 mon
24 mon
30 mon
D
VMAT2 WT
VMAT2 LO
Number of Hematoxylin+ cells
7000
6000
5000
4000
3000
2000
1000
0
**
**
**
***
***
6 mon
12 mon
18 mon
24 mon
30 mon
6 mon
12 mon
18 mon
24 mon
30 mon

Figure 5. Evidence of dopaminergic-induced oxidative stress in VMAT2-deficient SNpc primary neurons. A, B. Increased DCF labeling was seen in postnatal SNpc neurons from VMAT2-deficient mice (B) exposed to 10 μM dopamine compared to wildtype primary neurons (A). VMAT2-deficient nigral neurons undergo dose-dependent increases oxidative stress as measured by DCF fluorescence relative to wildtype primary neurons (C). D, Similarly, nigral neurons from VMAT2-deficient animals undergo increased cell death when exposed to 10 and 50 μM dopamine. n=6, **p<0.01, ***p<0.001

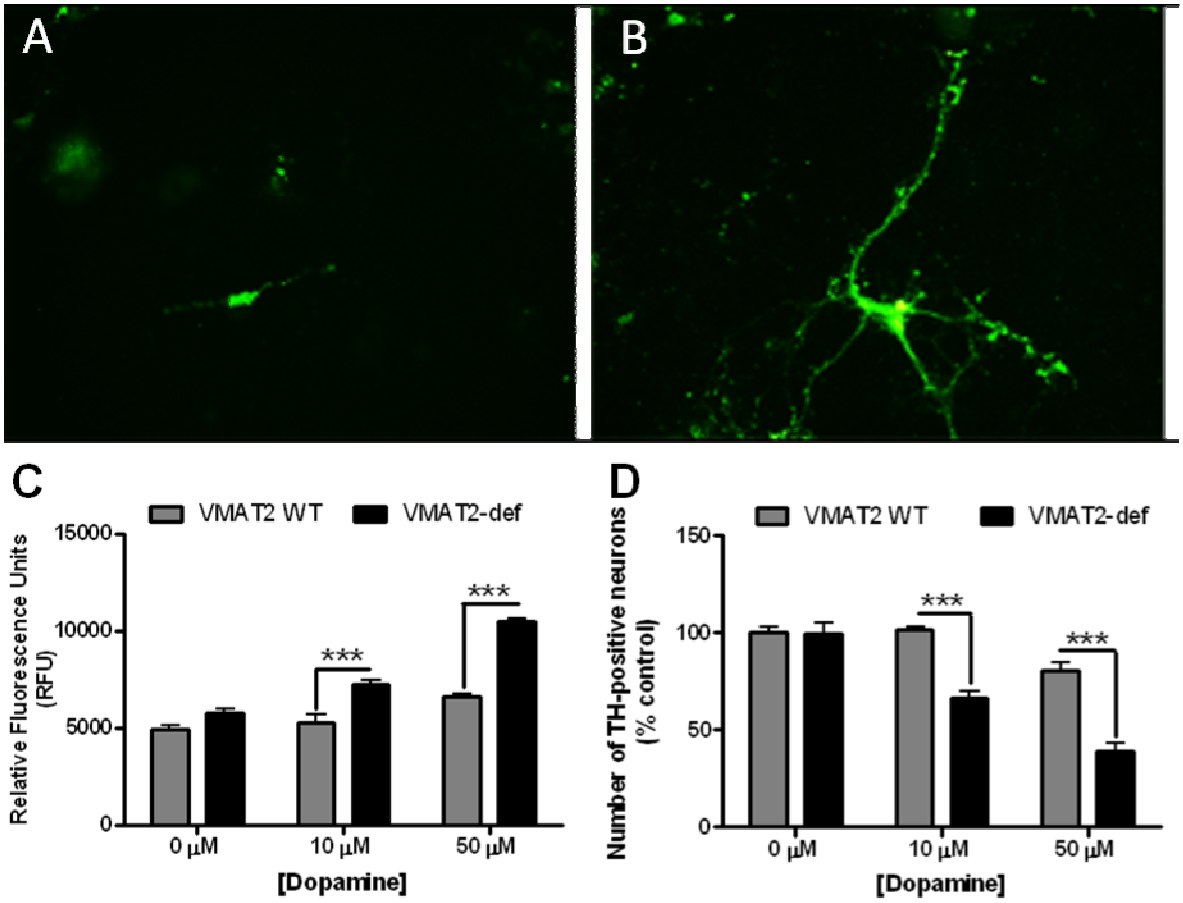

A
B
C
VMAT2 WT
VMAT2-def
Relative Fluorescence Units (RFU)
15000
10000
5000
0
***
***
0 µM
10 µM
50 µM
[Dopamine]
D
VMAT2 WT
VMAT2-def
Number of TH-positive neurons (% control)
150
100
50
0
***
***
0 µM
10 µM
50 µM
[Dopamine]

Figure 6. Evidence of noradrenergic-induced oxidative stress in VMAT2-deficient LC primary neurons. A, B. Increased DCF labeling was seen in postnatal LC neurons from VMAT2-deficient mice (B) exposed to 10 μM norepinephrine compared to wildtype primary neurons (A). VMAT2-deficient LC neurons undergo dose-dependent increases oxidative stress as measured by DCF fluorescence relative to wildtype primary neurons (C). D, LC neurons from VMAT2-deficient animals undergo increased cell death when exposed to 50 μM norepinephrine. n=6, **p<0.01, ***p<0.001

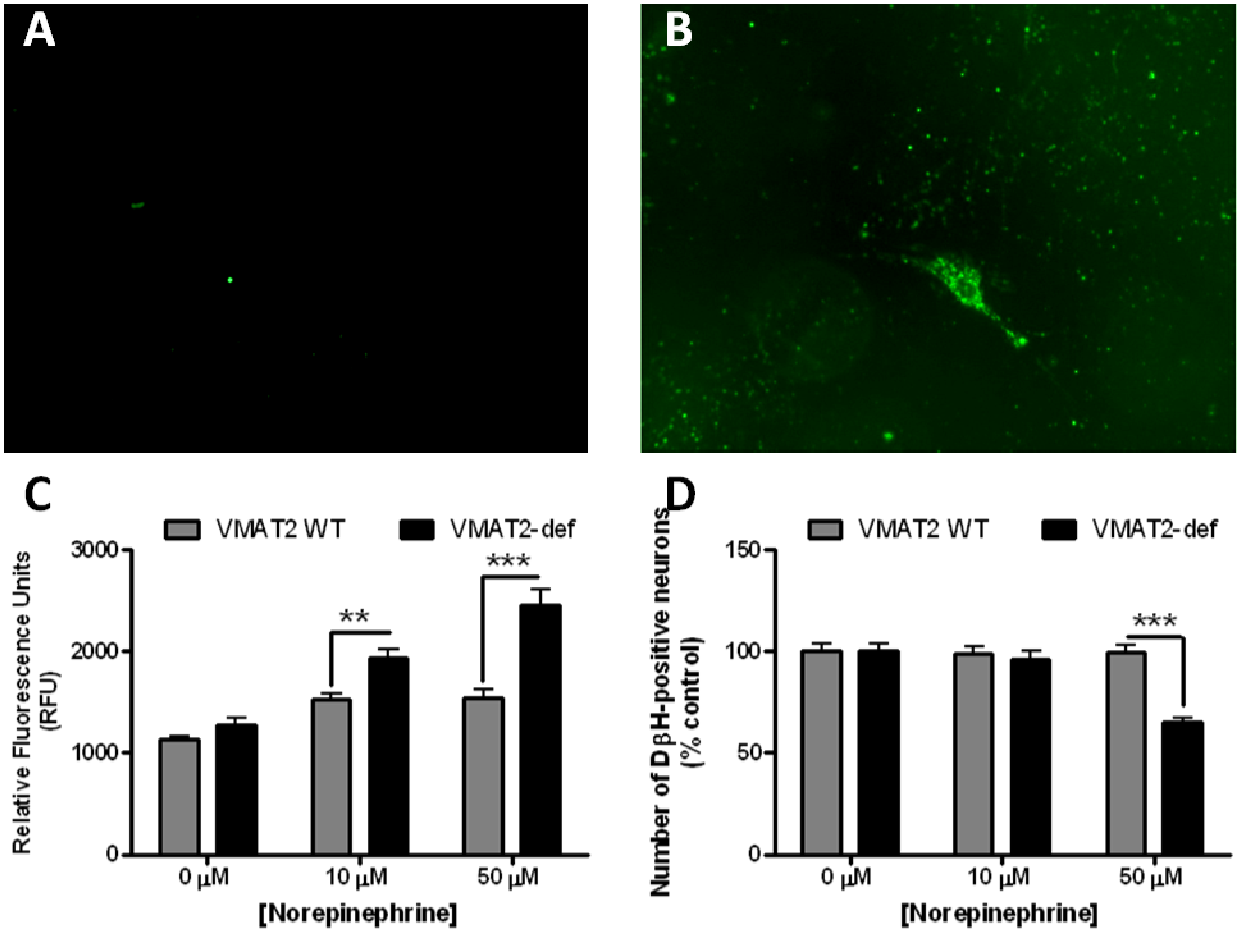
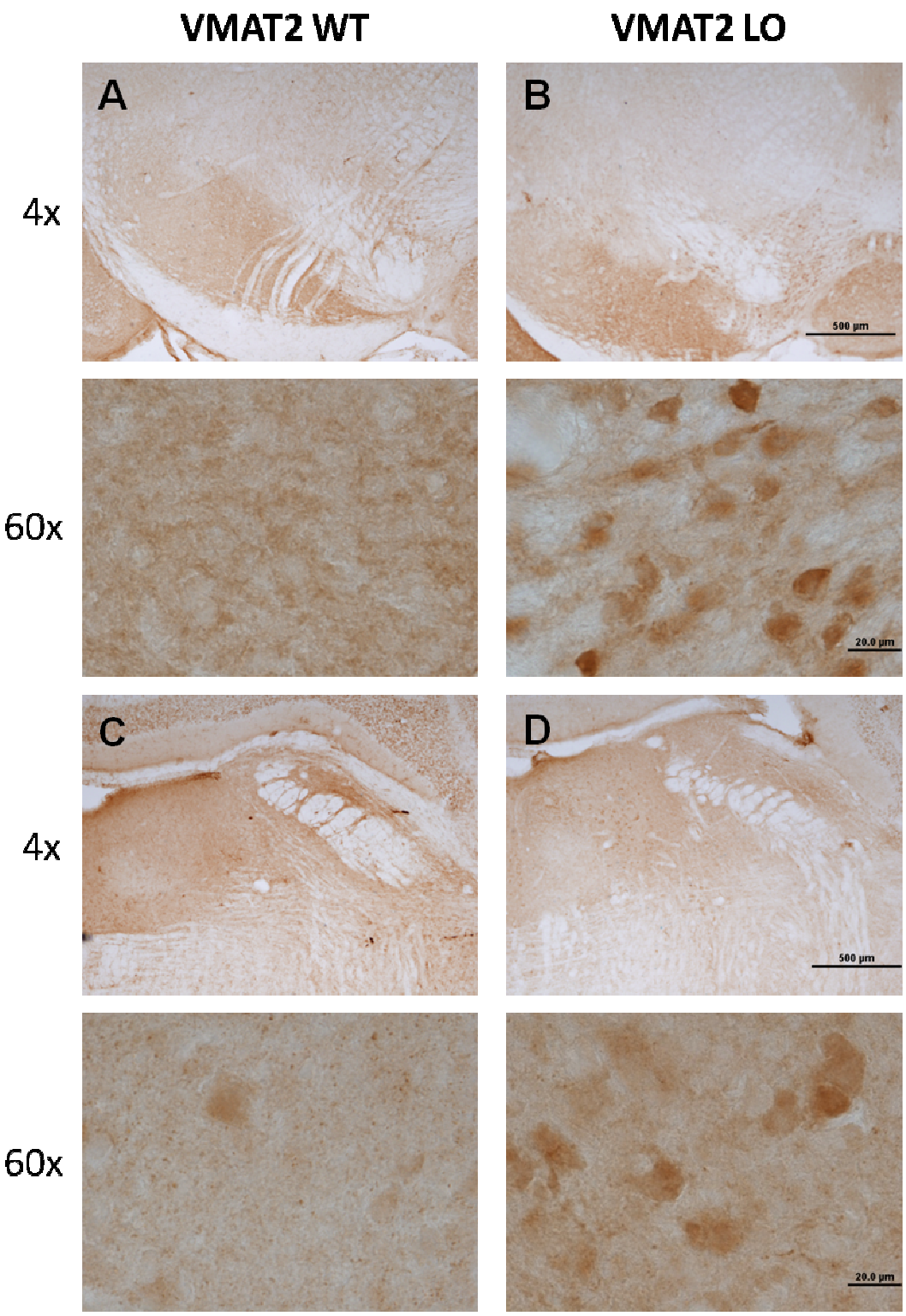

A
B
C
VMAT2 WT
VMAT2-def
Relative Fluorescence Units (RFU)
3000
2000
1000
0
**
***
0 μM
10 μM
50 μM
[Norepinephrine]
D
VMAT2 WT
VMAT2-def
Number of DβH-positive neurons (% control)
150
100
50
0
***
0 μM
10 μM
50 μM
[Norepinephrine]

Figure 7. Immunohistochemical analysis of α-synuclein in substantia nigra (A, B) and locus coeruleus (C, D) of aged VMAT2-deficient mice. Synuclein immunoreactivity was visibly increased in VMAT2-deficient mice at 30 months of age, demonstrating increased α-synuclein accumulation in catecholaminergic neurons. Analysis was performed on 3 animals per genotype at each age. Representative sections are shown. Scale bars, low magnification (4x): 500 µm; high magnification (60x): 20 µm.


VMAT2 WT
VMAT2 LO
A
B
4x
500 µm
60x
20.0 µm
C
D
4x
500 µm
60x
20.0 µm

CHAPTER 5

SEROTONERGIC DYSFUNCTION IN A MOUSE MODEL OF PARKINSON'S DISEASE

Abstract

Previously, we have shown that mice with low levels of VMAT2 (SLC18a2) display both motor and nonmotor symptoms of Parkinson's disease (PD) (Caudle et al., 2007; Taylor et al., 2009). These behavioral phenotypes are accompanied by degeneration in the substantia nigra pars compacta (SNpc) and in the locus coeruleus (LC) (Caudle et al., 2007) (see Chapter 4). Here, we report the neuropathological, neurochemical, and behavioral symptoms associated with the serotonergic system in VMAT2-deficient mice. Neurochemical analysis by our lab has demonstrated that VMAT2-deficient mice have reduced levels of serotonin (5-HT) coupled with increased 5-HT turnover (Taylor et al., 2009). Silver-staining reveals marked raphe degeneration in 24 month old VMAT2-deficient mice in contrast to age-matched wildtype animals; immunocytochemistry at 30 months reveals increased α-synuclein accumulation in the raphe of VMAT2-deficient mice. Moreover, cultured post-natal VMAT2-deficient raphe neurons exhibit increased sensitivity to serotonergic-induced oxidative stress as evidenced by increased DCF reactivity (60% over wildtype) and decreased immunostaining against tryptophan hydroxylase (50% decrease from wildtype). VMAT2-deficient mice demonstrate a significant increase in immobility time during forced swim tests that is rescued by treatment with the selective serotonin reuptake inhibitor, fluoxetine. These findings of serotonergic dysfunction in the VMAT2-deficient mice are reminiscent of the loss of serotonergic neurons in human PD.

Introduction

Parkinson's disease (PD) is a debilitative neurodegenerative disease affecting the monoaminergic processes of the brain. The contribution of serotonin (5-HT) to the development of PD has not been extensively studied. Biologically derived from tryptophan, 5-HT possesses an indole ring and, like the catecholamines, has the ability to auto-oxidize in the cytosol unless sequestered into vesicles via the vesicular monoamine transporter 2 (VMAT2) (Graham, 1978; Guillot and Miller, 2009). Serotonin is traditionally highlighted in PD in relation to dopamine (DA) neurotransmission; additionally, 5-HT has been shown to degenerate in PD patients with decreases in serotonergic markers in the striatum (Chase, 1972; Halliday et al., 1990; Kish et al., 2008). Serotonin neurons stemming from the dorsal and median raphe innervate basal ganglia circuitry possibly imposing an inhibitory regulatory tone on DA function (Jenner et al., 1983). Pathologically, there has been evidence indicating intracellular changes leading to protein aggregation and formation of Lewy neurites and Lewy bodies possibly originate in the caudal raphe nucleus, among other non-dopaminergic nuclei, before these changes occur in the SNpc (Del Tredici et al., 2002; Braak et al., 2003).

Serotonergic dysfunction appears to play a role in a number of parkinsonian symptoms, including: mood, psychosis, motor function, and L-DOPA-induced dyskinesias (Fox et al., 2009). Serotonin's role in PD has traditionally involved hypotheses highlighting its ability to act as a false neurotransmitter during L-DOPA administration, inducing dyskinesias (Carlsson et al., 2007). However, in this report we show that 5-HT may have a unique contribution to the pathogenesis of PD. The VMAT2-deficient animals demonstrate a significant decrease in 5-HT levels in multiple brain regions, increased 5-HT turnover, and behavioral deficits traditionally associated with serotonergic dysfunction (Taylor et al., 2009). In this study, we report that serotonergic nuclei play a role in both pathology and behavioral manifestations of PD as a consequence of reduced vesicular storage.

Materials and Methods

Animals. Male and female VMAT2-deficient mice were generated as previously described (Caudle et al., 2007; Taylor et al., 2009). Briefly, the mouse VMAT2 locus was cloned from the 129/Sv genomic library and a 2.2 kb *Pvu*II fragment from the third intron of the VMAT2 gene, and cloned into the bluntended *Not*I site of the construct. The targeting vector was introduced into 129/Ola CGR 8.8 embryonic stem (ES) cells and injected into blastocytes of C57BL/6 mice. Highly chimeric males were bred with C57BL/6 females; genotype was confirmed by Southern blot analysis. All mice were generated through redundant breeding of mice that were heterozygous for the VMAT2 allele and wild type (WT) for the α-synuclein allele. The genotype of all mice was confirmed by PCR of DNA extracted from tail samples. For all behavioral tests, wildtype littermate controls were used. All procedures were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and previously approved by the Institutional Animal Care and Use Committee at Emory University.

Neuropathology. Tissue staining was completed as previously described (Miller et al., 1997; Miller et al., 1999b; Caudle et al., 2006). Briefly, wildtype and VMAT2-deficient mice were perfused transcardially with phosphate buffered saline (pH=7.4) and then with 4% paraformaldehyde, removed and placed in 4% paraformaldehyde for 24 h, and finally cryoprotected in 30% sucrose for 48 h. The brains were then cut to a thickness of 40 µm (silver staining) or 50 µm (immunohistochemistry) on a freezing microtome (Microm, Kalamazoo, MI, USA). Sections were incubated with a monoclonal anti-tryptophan hydroxylase (1:250; Sigma-Aldrich) antibody overnight and then incubated in a biotinylated goat anti-mouse secondary antibody for 1 hour at room temperature. Visualization was performed using 3, 3´-diaminobenzidine (DAB) for 2 min at room temperature. After DAB, all sections were counterstained in hematoxylin, mounted on slides, dehydrated, and coverslipped using Permount (Fisher Scientific) and sections were viewed using a light microscope (Olympus).

Detection of neurodegeneration. Silver staining (FD Neurosilver kit: FD NeuroTechnologies, Ellicott City, MD) for degenerating neurons was completed according to the manufacturer's protocol.

Primary culture. Methods used to generate dorsal raphe (DR) neuron-glia co-cultures were previously described, with slight modifications (Cardozo, 1993; Smeyne and Smeyne, 2002; Guillot et al., 2008). Briefly, brains from postnatal days 0–6 wildtype and VMAT2-deficient mice were removed and placed in dissociation media (DM) pH 7.4. Under a dissecting microscope, a 0.8–1.0 mm coronal slice of the mesencephalon was obtained; regions containing the DR were isolated, placed in fresh DM, and minced into small pieces. The DR was digested in DM containing papain and DNAase (Worthington Biochemical, Freehold, NJ) and incubated at 37°C (twice for 30 min). The sections were then triturated and the cell suspension was layered over PM and centrifuged for 8 min at 1400 x g. The supernatant was removed, and the pellet resuspended in PM with 2% rat serum (RS). Cells were counted using Trypan Blue, and plated at a density of 350,000 cells/cm^2 in Lab-Tek™ four-well Permanox chamber slides coated with laminin and poly-D-lysine (Collaborative Biomedical Products, Bedford, MA). Cells were maintained at 37°C, 5% CO_2, and fed with PM containing 2% RS. At 24 h post-plating, the cultures were fed with complete feeding media.

After 7 days in culture, neurons were treated with serotonin (5-HT) (10-50 μM dissolved in feeding media) or feeding media alone (controls). Seventy-two hours post-administration, cultures were rinsed twice for 5 min with TBS, fixed for 10–15 min in 4% buffered paraformaldehyde, and rinsed three times with TBS. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 10 min. Cells were permeabilized with 0.1% Triton X-100, 5% goat serum in TBS for 10 min. Serotonergic neurons were visualized with monoclonal tryptophan hydroxylase (TrpH) (1:500; Sigma-Aldrich). Cultures were incubated with primary antibody overnight at 4°C, rinsed twice with TBS, incubated in secondary antibody (goat anti-rabbit or goat anti-mouse, 1:200; Jackson ImmunoResearch) and amplified

with avidin–biotin-HRP (Vector Laboratories). Final visualization of the immunoreactive neurons was made using 0.03% DAB for 3 min, followed by three washes with TBS. All TH and TrpH-positive cells from each culture were counted on a light microscope at magnification of 200x (Olympus), and quantified by counting the processes from a total of 30 neurons in four random fields as previously described (Radad et al., 2008). The total number of processes counted was divided by the number of neurons counted; values are expressed as number of TrpH processes per neuron. The data reflect two separate experiments and were combined to yield a final sample size of 4 per treatment per genotype.

2,7 dichlorofluorescein diacetate (DCF) assay. Detection of oxidative stress in cultures was accomplished by adding 2, 7-dichlorofluorescein diacetate (DCF; Invitrogen) (5 μM) to cultures 72 h after treatment with 10 μM 5-HT (Wang and Joseph, 1999; Larsen et al., 2002). DCF will be prepared in dimethylsulfoxide at 10 mM and diluted to a concentration of 1 μM in minimal essential medium. Neuronal cultures will be incubated in 5 μM DCF for 30 minutes, and rinsed with oxygenated saline. The 96-well plate containing cultures will be placed in a Victor3 1420 multi-label counter (Perkin Elmer) with a fluorescein filter set at 485, and emission filter set at 535. The amount of oxidative stress in cultures was expressed and quantified as relative fluorescein units (RDU). The cultures were then placed at 37°C for 1 h. Cells were washed twice, coverslipped, and viewed using an epifluorescence microscope (Olympus).

Forced swim test. These studies were conducted on mice using a modified method of Porsolt and coworkers, as previously described (Porsolt et al., 1979; Taylor et al., 2009). Briefly, all mice were injected intraperitoneally with 100 μL of 5 mg/kg fluoxetine (Sigma-Aldrich, St. Louis, MO) or saline 30 minutes before testing. Mice were placed individually in glass cylinders (24 x 16 cm) with 15 cm of water maintained at 25°C. The mice were left in the cylinder and their behavior was videotaped from the side of the cylinder for 6 minutes. After the first 2 minutes, the total duration of time spent immobile was recorded during a 4 minute test. The mouse was deemed immobile when it was floating passively; subtle movement of feet or tail

required to keep the head above the surface of the water were excluded as immobility. Immobility time refers to the time that the animal spent floating for at least 3 seconds (Porsolt et al., 1979; Xu et al., 2000; Fukui et al., 2007). A single cohort of mice was tested at each time point; however, naïve mice were introduced each month to ensure that the original cohort of mice was not learning the task.

Tail suspension test. These experiments were conducted using the methods of Cryan and colleagues, as previously described (Cryan et al., 2004; Taylor et al., 2009). All mice were injected intraperitoneally with 100 µL of 5 mg/kg fluoxetine (Sigma-Aldrich, St. Louis, MO) or saline 30 minutes before testing. Mice were individually suspended by the tail to a horizontal ring stand bar (distance from the floor = 30 cm) using adhesive tape. A 6-minute test session was videotaped and scored by a trained observer for escape-oriented behavior and bouts of immobility. The time spent immobile was recorded for each mouse. Mice were excluded from the study if they were able to climb on top of the ring stand. A single cohort of mice was tested at each time point; however, naïve mice were introduced each month to ensure that the original cohort of mice was not learning the task.

Statistical analysis. Data from male and female mice were combined, since there were no detectable sex differences. All data were analyzed using unpaired independent samples completely randomized two-factor ANOVA followed by Bonferroni post hoc analysis. Analyses were completed using Graph Pad Prism 5.0 for Windows, and for all tests all *post hoc* measures were error-corrected to keep the overall error rate per group at 0.05.

Results

VMAT2-deficient neurons are more sensitive to serotonergic toxicity

Previously, it has been shown that VMAT2 plays an important factor in mitigating neuronal susceptibility to oxidative stress by sequestering monoamines before they can auto-oxidize into quinones or cysteinyl adducts (Graham, 1978; Uhl, 1998; Miller et al., 1999a; Caudle et al., 2008; Guillot and Miller, 2009) . Specifically, evidence of oxidative damage has been

identified in the VMAT2-deficient animals in the form of increased protein carbonyl and cysteinyl-adduct formation (Caudle et al., 2007). To further explore the role of VMAT2 in monoaminergic-induced oxidative stress, primary DR neuronal cultures were exposed to physiological doses of serotonin. Compared to wildtype primary cultures, VMAT2-deficient neurons undergo increased oxidative stress via formation of H_2O_2 and other oxidant species as measured by DCF fluorescence when exposed to serotonin ($n=6$, $p<0.05$, Figure 1).

VMAT2-deficient mice have evidence of degeneration at 24 months of age in the dorsal raphe

VMAT2-deficient mice have previously been shown to display nigral and locus coeruleus degeneration beginning between 6-12 months of age (Caudle et al., 2007) (see Chapter 4), presumably caused by the effects of reduced vesicular catecholamine storage. To study if the serotonergic system follows similar patterns of damage in these mice, the DR of VMAT2-deficient mice was assessed immunohistochemically. At 12 months of age, there was no appreciable reduction in TrpH expression or evidence of neurodegeneration, as measured by silver deposition, in the DR of the VMAT2-deficient mice compared to age-matched controls (data not shown). Beginning at 18 months of age, the VMAT2-deficient displayed a mild reduction in TrpH staining in the DR, which increased moderately with age (Figure 2A, B). Reduction in TrpH expression was accompanied by neuronal silver deposits in the raphe in VMAT2-deficient mice at 24 months of age (Figure 2C, D). Silver staining was not observed in age-matched wildtype controls at any age.

VMAT-deficient mice display increased α-synuclein accumulation at 30 months in DR

The relationship between α-synuclein and parkinsonian pathology has long been known; furthermore, catecholamine cytosolic oxidation has been shown to be associated with deleterious α-synuclein fibrilization (Conway et al., 2001; Norris et al., 2005). Although the effects of cytosolic 5-HT have not been extensively studied, 5-HT possesses an indole-ring, which has the potential to auto-oxidize in the cytosol (Guillot and Miller, 2009). Previously, we have shown at older ages, VMAT2-deficient mice have increased α-synuclein accumulation in the SNpc and LC

compared to age-matched WT animals (Caudle et al., 2007) (see Chapter 4). Similarly, immunohistochemical analysis of 30-month mice revealed α-synuclein accumulation in DR of only VMAT2-deficient animals (Figure 3).

Evidence for the involvement of the serotonergic system in the depressive-like phenotype of VMAT2-deficient mice

The VMAT2-deficient animals were previously tested for depressive-like behavior using the forced swim (FST) and tail suspension (TST) tests. At younger ages, no difference was observed between the immobility times of wildtype andVMAT2-deficient animals in either test, with no change in behavior after an acute dose of the noradrenergic specific antidepressant, desipramine (Taylor et al., 2009). However, at 12-15 months of age, immobility times were higher in VMAT2-deficient animals compared to age-matched WT controls (Figure 4) (Taylor et al., 2009). Initially, both wildtype andVMAT2-deficient mice were exposed to desipramine, which was found to decrease immobility times in VMAT2-deficient animals but not wildtype littermates (Taylor et al., 2009). When treated with an acute low dose of the serotonin selective reuptake inhibitor, fluoxetine, (5 mg/kg administered intraperitoneally 30 minutes prior to testing), the immobility times of VMAT2-deficient mice decreased to wildtype levels (n= 4-5, interaction effects of treatment and genotype: $F_{(1,12)}$=6.56, p=0.0249 (FST); $F_{(1,9)}$=2.80, p=0.1288 (TST); Figure 4); the immobility times of wildtype animals were not affected by fluoxetine at 12-15 months of age.

Discussion

The role of 5-HT dysfunction in the pathogenesis of PD has thus far proven to be elusive, which may be due to DA and 5-HT systems being extensively interconnected (Hajdu and Hassler, 1973; Marsden and Guldberg, 1973). In PD, the death of nigrostriatal DA neurons may or may not induce a hyperinnervation of 5-HT, potentially masking the true extent of 5-HT loss (Karstaedt et al., 1994). Alternatively, reductions in 5-HT in PD patients could compensate for reduced DA levels in the striatum (Scholtissen et al., 2006). However, serotonergic

compensation has the potential to intensify DA deregulation by stimulating DA release via striatal serotonergic neurons (Ng et al., 1970; Tanaka et al., 1999). Non-physiological release of DA by serotonergic neurons may exacerbate PD dysfunction, causing swings in DA levels, producing dyskinesias (Carlsson et al., 2007; Kish et al., 2008). The VMAT2-deficient mouse may prove to be a useful tool in delineating these nuances in serotonergic function.

Postmortem analysis of PD patients has shown substantially reduced levels of 5-HT in the raphe nuclei and the basal ganglia, accompanied by Lewy body pathology (Hornykiewicz, 1966; Scatton et al., 1983; Halliday et al., 1990; Braak et al., 2002). In the VMAT2-deficient animals, reduced levels of 5-HT coupled with increased serotonergic turnover cause an accumulation of oxidative species within the cytosol (Figure 1). Over the course of a lifetime, these oxidative products could eventually lead to degeneration of the cell, which is evidenced through increase silver deposition at 24 months of age in these animals (Figure 2). Hypotheses implicating oxidized forms of catecholamines in the fibrillization of α-synuclein could be extended to 5-HT, as it has the ability to auto-oxidize in the cytosol, and metabolism by monoamine oxidase also yields reactive oxygen species (Conway et al., 2001; Guillot and Miller, 2009). To fully assess the pathogenic potential of the 5-HT system in the VMAT2-deficient animals, more factors must be analyzed including: loss of striatal innervation by the raphe nuclei, the activity of the 5-HT transporter (SERT), and the extent of raphe nuclei loss, in addition to its temporal relationship with neuronal loss in the SNpc and LC.

There is evidence that depression in PD is dependent upon 5-HT dysfunction, which could ultimately aid in an earlier diagnosis of PD. This is seen through reduced 5-HT metabolites in the cerebrospinal fluid and 5-HT precursor depletion observed in depressed PD patients (Mayeux et al., 1986; McCance-Katz et al., 1992). However, as of yet, no relationship has been demonstrated between the degree of 5-HT reduction and the overall severity of parkinsonian deficit (Jenner et al., 1983). The VMAT2-deficient mice display an age-dependent depressive phenotype that is responsive to serotonergic drugs; however, it is not specific to the 5-HT system,

as noradrenergic drugs have been shown to ameliorate the phenotype as well (Figure 4) (Taylor et al., 2009). Taken together, these data suggest that the decreased 5-HT content caused by reduced vesicular storage, possibly leading to serotonergic dysfunction and the development of non-motor features associated with PD. If the structural and functional changes in the serotonergic loci of the brain can be better elucidated, perhaps 5-HT's contribution to PD can be more fully understood.

Acknowledgments

I thank Dr. Kennie Shepherd for performing the analysis on primary raphe cultures. Additionally thank Dr. Min Wang for her excellent technical skills in breeding and aging the VMAT2-deficient mice.

Figure 1. Evidence of serotonergic-induced oxidative stress in VMAT2-deficient DR primary neurons. A, B. Increased DCF labeling was seen in postnatal DR neurons from VMAT2-deficient mice (B) exposed to 10 μM serotonin compared to wildtype primary neurons (A). VMAT2-deficient raphe neurons undergo dose-dependent increases oxidative stress as measured by DCF fluorescence relative to wildtype primary neurons (C). D, Similarly, raphe neurons from VMAT2-deficient animals undergo increased cell death when exposed to 10 and 50 μM 5-HT. n=6, **p<0.01, ***p<0.001

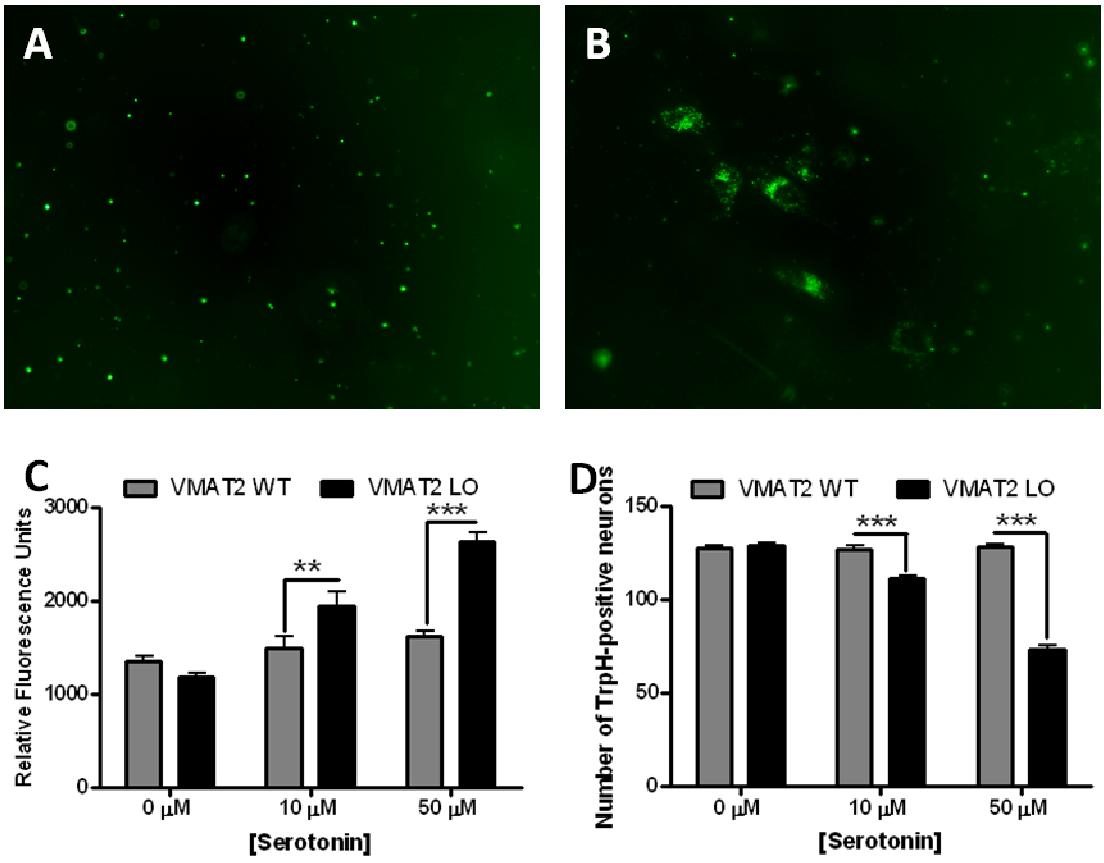

A
B
C
VMAT2 WT
VMAT2 LO
Relative Fluorescence Units
3000
2000
1000
0
**
***
0 µM
10 µM
50 µM
[Serotonin]
D
VMAT2 WT
VMAT2 LO
Number of TrpH-positive neurons
150
100
50
0
***
***
0 µM
10 µM
50 µM
[Serotonin]

Figure 2. VMAT2-deficient mice show evidence of monoaminergic degeneration at 24 months of age. **A, B**, TrpH staining in the DR of wildtype (A) and VMAT2-deficient (B) mice at 24 months of age. Scale bars, 500 µm. **C, D**, Accumulation of silver deposits in the DR of 24 month old wildtype(C) andVMAT2-deficient (D) mice. Arrows denote silver deposits in degenerating neurons, with evidence of altered cell bodies and processes. Scale bars, 50 µm. Analysis was performed in three animals per genotype; representative sections are shown.

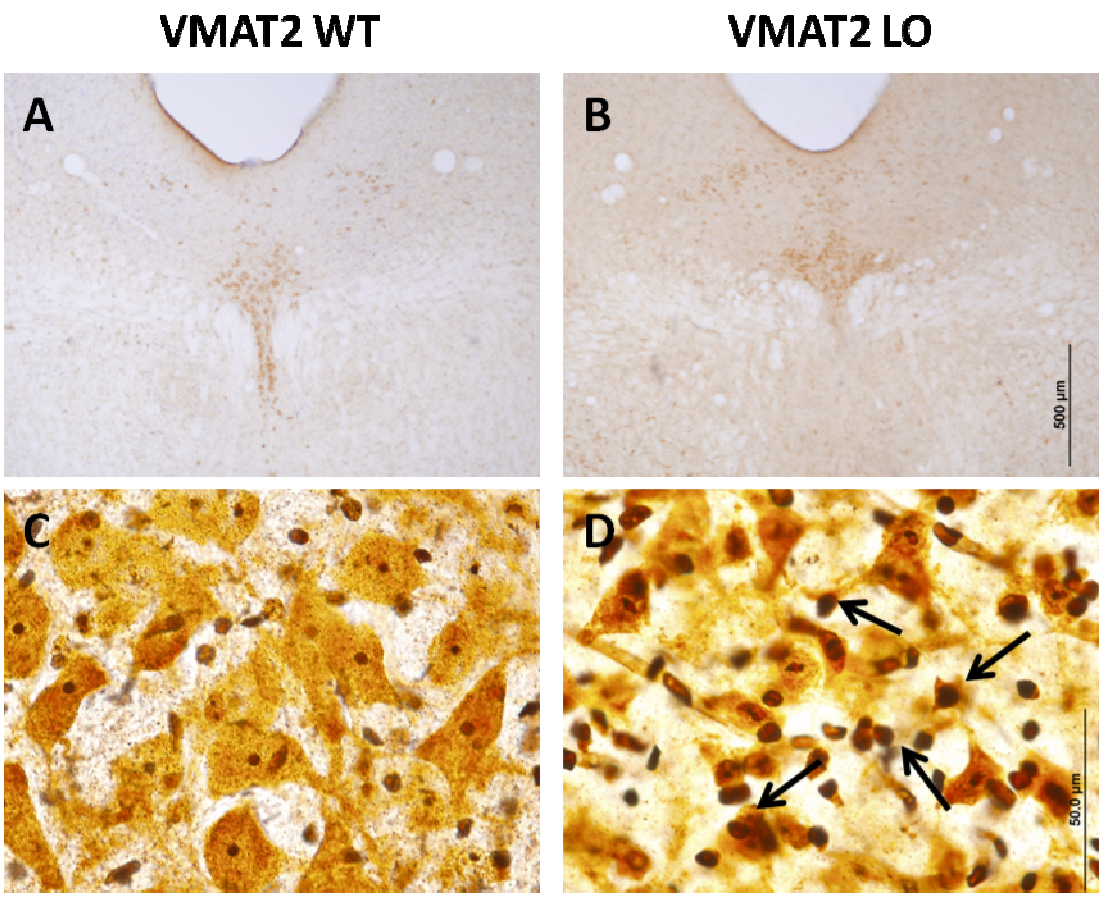

VMAT2 WT
VMAT2 LO
A
B
500 µm
C
D
50,0 µm

Figure 3. Immunohistochemical analysis of α-synuclein in dorsal raphe of aged VMAT2-deficient mice. Synuclein immunoreactivity was visibly increased in VMAT2-deficient mice at 30 months of age (B), demonstrating increased α-synuclein accumulation in serotonergic neurons. Analysis was performed on 3 animals per genotype at each age. Representative sections are shown. Scale bars, low magnification (4x): 500 µm; high magnification (inset) (60x): 20 µm.

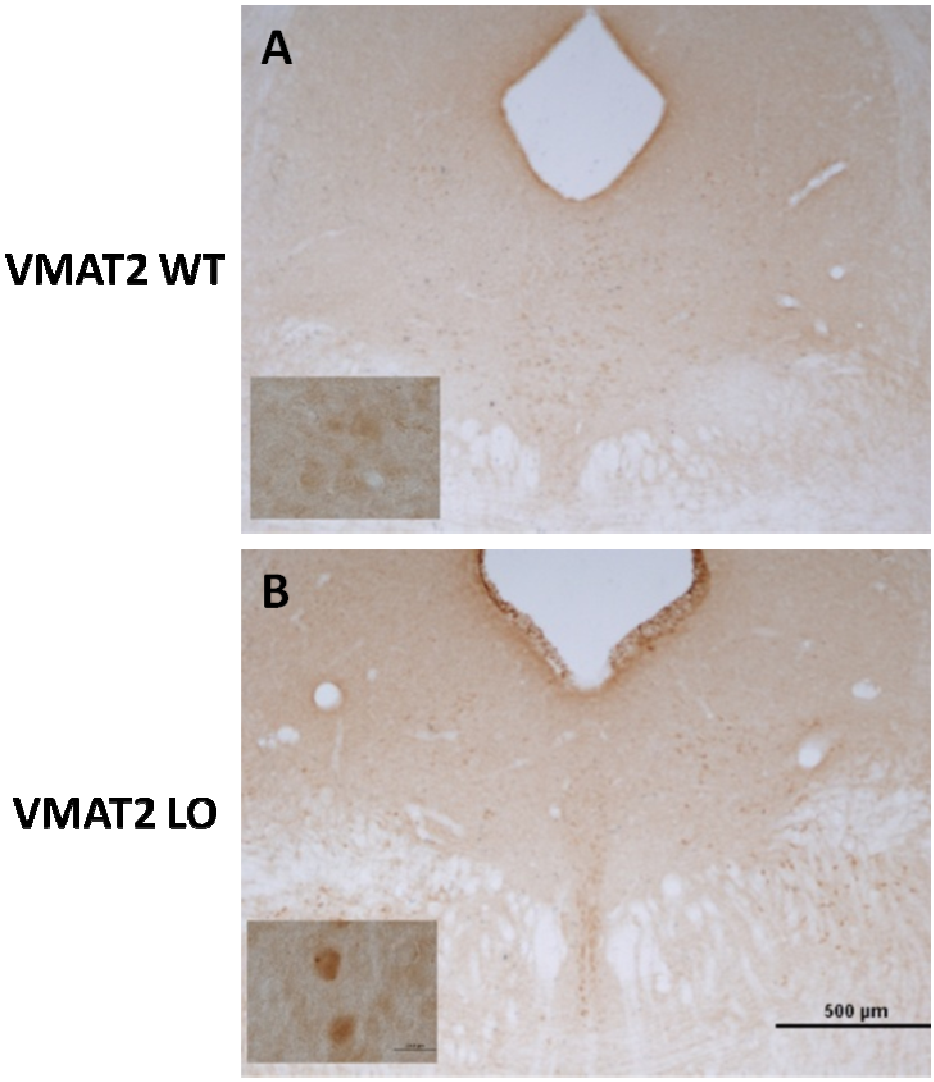
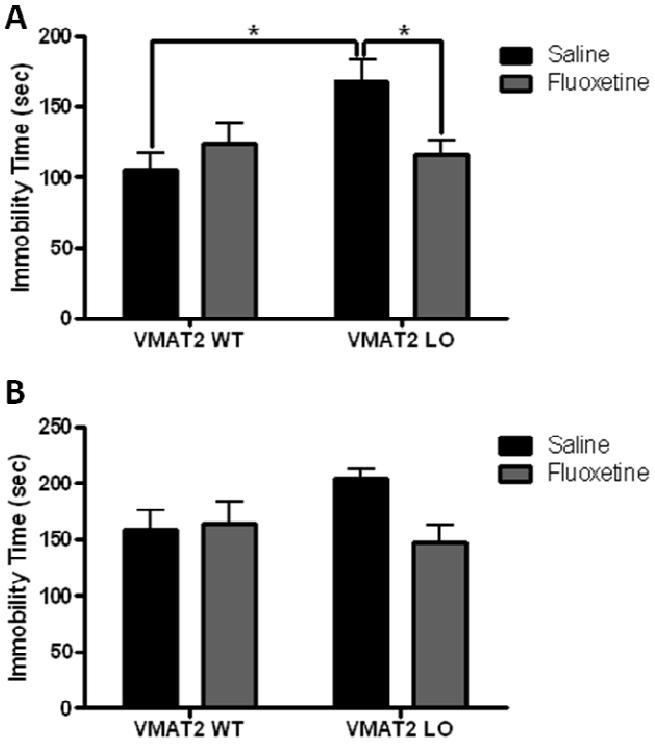

A
VMAT2 WT
B
VMAT2 LO
500 µm

Figure 4. VMAT2-deficient animals display a progressive depressive-like phenotype that is sensitive to fluoxetine. **A**, Four to six-month-old VMAT2-deficient animals have similar immobility times in the forced swim test (A) and tail suspension test (B) as compared to age-matched WT animals; the immobility times of both genotypes is not affected by fluoxetine. **C, D**, At 12-15 months of age, WILDTYPE animals are non-responsive to fluoxetine administration in the FST (C) and TST (D). However, VMAT2-deficient mice have an increased immobility time, which is decreased to WT levels when dosed with fluoxetine. Results represent the mean time (seconds) ± SEM for 4-5 mice per genotype. *$p<0.05$, **$p<0.01$


A
Immobility Time (sec)
200
150
100
50
0
*
*
Saline
Fluoxetine
VMAT2 WT
VMAT2 LO
B
Immobility Time (sec)
250
200
150
100
50
0
Saline
Fluoxetine
VMAT2 WT
VMAT2 LO

CHAPTER 6

SUMMARY AND CONCLUSIONS

This work is part of an invited review:
Taylor, TN, Caudle WM, Miller GW. Vesicular monoamine transporter deficient mice as a model of Parkinson's disease. *Parkinson's Disease*.

Introduction

Parkinson's disease (PD) is a devastating neurodegenerative disease that selectively affects the monoaminergic processes of the brain. Abnormalities with monoaminergic handling and neurotransmission are associated with a number of neurodegenerative diseases, in addition to PD, as well as neuropsychiatric disorders including schizophrenia, depression, and drug addiction. With respect to PD, many researchers have proposed that DA has the ability to induce cytotoxicity with age; however, the long term toxicity of DA *in vivo* has never been firmly established. Many chemical models of PD manipulate the predisposition of DA to induce oxidative damage as a primary mechanism of action, and this is one of the current working hypotheses for the slow, progressive nature of PD. The endogenous generation of reactive oxygen species (ROS), resulting from both metabolism of monoamines in the cytosol and auto-oxidation of monoamines, has also been implicated as a mediator in the pathophysiology of PD (Jenner, 2003; Caudle et al., 2007). However, physiologically neurons have many safeguards to maintain neuronal health and protect against degeneration. The vesicular monoamine transporter 2 (VMAT2) is one such custodian that functions to regulate the cytosolic environment of the neuron, protecting it from endogenous and exogenous toxins. Originally VMAT2 was partly identified via its ability to confer resistance to the dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP+), which is commonly used to induce a parkinsonian phenotype in mice (Liu et al., 1992). Molecularly, VMAT2 is a 12-transmembrane domain H^+-ATPase antiporter, which uses an electrochemical gradient to drive transport; two protons are exchanged for one monoamine (Rudnick, 1986; Forgac, 1989; Erickson et al., 1992). Phylogenetically, VMAT2 is a member of the solute carrier protein family and the toxin-extruding antiporter (TEXAN) gene family, which includes bacterial resistance genes (Schuldiner et al., 1995; Eiden et al., 2004). Moreover, VMAT2 contains sequence homology and functional similarities to the major facilitator superfamily of drug resistance transporters; many researchers have hypothesized that VMAT2 has evolved to serve an analogous role in eukaryotic systems by providing a mechanism

to sequester and clear toxins from the cell (Miller et al., 1999a; Vardy et al., 2004). Thus vesicular sequestration serves a dual purpose: preventing the interaction of toxins with molecular machinery and limiting exposure of neighboring cells to the toxin. The level of VMAT2 expression is essential to proper monoaminergic handling, as it regulates both the size of the vesicular monoamine pool and influences the availability of monoamines in the cytosol, influencing cellular susceptibility to oxidation (Liu et al., 1992). The monoamines, particularly dopamine (DA) and norepinephrine (NE) have the ability to spontaneously oxidize in the cytosol, potentially damaging cellular machinery (Graham, 1978).

Historically, VMAT2 has long been involved with modeling of PD, beginning with administration of reserpine to mice. Reserpine, an inhibitor of VMAT2, has been shown to inhibit motor function and precipitate depressive symptoms in rodents (Colpaert, 1987; Skalisz et al., 2002). Theoretically, the loss of VMAT2 function within the neuron would be associated with a reduction in vesicular monoamines and a concomitant accumulation of cytosolic monoamines, resulting in striatal monoaminergic depletion and the development of a parkinsonian phenotype. It is thought that, together with the dopamine transporter (DAT), VMAT2 may be able to modulate susceptibility to neurodegeneration (Miller et al., 1999a). There has been much speculation about the role of VMAT2 in mediating efficient clearance of DA in those populations vulnerable to neurodegeneration (Liu and Edwards, 1997; Uhl, 1998). To this end, a relationship exists between VMAT2 expression levels and regions of the brain spared from parkinsonian degeneration. *In vivo* imaging and post mortem binding studies displayed marked reductions in VMAT2 immunoreactivity in the caudate, putamen, and nucleus accumbens of PD brains (Kilbourn et al., 1993; Miller et al., 1999b). Additionally, a gain of function haplotype of VMAT2 was found to be protective against the development of PD in humans (Glatt et al., 2006).

VMAT2 has also been directly implicated with a pathological hallmark of PD: α-synuclein. This key component of Lewy bodies has been found to bind and permeabilize

vesicles, potentially causing leakage of monoamines into the cytosol (Lotharius and Brundin, 2002). This has been hypothesized to be mediated via a direct interaction between VMAT2 and α-synuclein and VMAT2, disrupting synaptic vesicle dynamics (Guo et al., 2008). Moreover, overexpression of α-synuclein causes the downregulation of VMAT2 protein *in vitro*, triggering increases in DA and ROS (Lotharius and Brundin, 2002; Mosharov et al., 2006). Taken together with evidence from oxidative stress studies, these data demonstrate the perturbation of VMAT2 can create an environment conducive to PD-related cell damage and pathology.

Genetic manipulation of VMAT2

In order to investigate the exact role of VMAT2 in the CNS, many groups created a VMAT2 knockout (VMAT2 KO) mouse. Manipulating VMAT2 could potentially unlock the role of exocytotic release of monoamines in signaling. Creation of a complete deletion of the VMAT2 gene resulted in an animal that moved little, fed poorly, and died within a few days after birth (Fon et al., 1997; Wang et al., 1997). Amphetamine administration promotes feeding and movement, but only prolongs survival until three weeks of age (Fon et al., 1997). Neurochemically, the VMAT2 KO mice were found to be completely devoid of monoamines; however, even though the absence of proper monoaminergic signaling ultimately leads to the death of VMAT2 KO mice, the absence of exocytotic monoamine release had no discernible effect on brain development (Fon et al., 1997; Wang et al., 1997). Because the VMAT2 KO mice are not fully viable, most of the meaningful data obtained from these mice are from primary neuronal cultures. From these data, it is possible that vesicular sequestration of monoamines is not essential for fetal development. It is only during postnatal development, when the monoamines are subjected to enhanced degradation and increased monoamine synthesis can no longer compensate, that the VMAT2 KO mice fail to thrive (Wang et al., 1997). Physiologically, neuronal cell groups and projections develop normally, but the presynaptic storage and release of monoamines is a vital determinant of postsynaptic receptor responsiveness (Fon et al., 1997; Wang et al., 1997). Further studies demonstrated that specialized cells that express VMAT2 in

the periphery do not release monoamines in the VMAT2 KO mice (Travis et al., 2000). From this, it was ascertained that vesicular monoamine transport is also a key determinant of quantal size in monoaminergic cells (Fon et al., 1997; Travis et al., 2000). Even though the VMAT2 KO mice are not fully viable, studies with the mice and neurons isolated from them definitively reveal that VMAT2 expression regulates monoamine storage and release (Fon et al., 1997; Wang et al., 1997).

Although the VMAT2 KO mice do not survive into adulthood, their creation also yielded mice that are heterozygous for VMAT2 (VMAT2 HT). Unlike the VMAT2 KO mice, the VMAT2 HTs are fully viable into adulthood and display a 50% reduction in VMAT2 expression and were physiologically similar to their wildtype littermates (Takahashi et al., 1997). Although reports have varied, overall the VMAT2 HTs appear to have a significant reduction in monoamines, perceived to be a consequence of reduced vesicular storage capacity (Takahashi et al., 1997; Wang et al., 1997; Gainetdinov et al., 1998; Reveron et al., 2002). Thus, using the VMAT2 HT mice as a model of altered vesicular storage has implications for studies involving both monoaminergic and drug function or toxicity. Behaviorally, the VMAT2 HT mice perform normally in passive avoidance and locomotor activity tests, but display a depressive-like phenotype including: anhedonia, locomotor retardation, and sensitivity to stress (Takahashi et al., 1997; Fukui et al., 2007). This phenotype is ameliorated with the administration of antidepressants such as imipramine, fluoxetine, and bupropion, suggesting a combined involvement of all three monoamine neurotransmitters (Fukui et al., 2007). Moreover, even though anxiety is co-morbid with most cases of depression in humans, the VMAT2 HT mice were found to be devoid of an anxiety-like phenotype (Fukui et al., 2007).

When challenged with various exogenous toxins, the VMAT2 HTs begin to manifest deficits due to reduced vesicular storage. Methamphetamine causes greater neurotoxicity in the VMAT2 HT mice compared to wildtype animals, with significant reductions in DA, DA metabolites, and DAT (Fumagalli et al., 1999). These findings were coupled with a less

pronounced increase in extracellular DA, suggesting that cytosolic DA is the prevailing factor in the potentiation of methamphetamine toxicity observed in the mice (Fumagalli et al., 1999). Behaviorally, amphetamine produced enhanced locomotor activity but reduced reward as measured by conditioned place preference (Takahashi et al., 1997). In addition to the amphetamines, VMAT2 HT mice were also found to be acutely more sensitive to the effects of the parkinsonian drug MPTP. VMAT2 HT mice undergo twice the dopaminergic cell loss observed in wildtype animals, accompanied by markers of striatal damage such as reductions in DA, DAT and increased glial fibrillary acidic protein (GFAP) mRNA (Takahashi et al., 1997; Gainetdinov et al., 1998). Although the VMAT2 HT mice did not display any overt signs of Parkinsonism or PD-like neuropathology, they do exhibit an increased susceptibility to MPTP toxicity and thus, researchers postulated that the mice may be useful in teasing out the mechanisms of L-DOPA toxicity. It was found that primary DA neurons harvested from VMAT2 HTs were more vulnerable to L-DOPA than wildtype neurons; decreased VMAT2 activity might attenuate L-DOPA efficacy by augmenting endogenous dopaminergic toxicity (Kariya et al., 2005). However, these results were not observed *in vivo* (Reveron et al., 2002). Despite the absence of a clear link between L-DOPA induced dopaminergic dysfunction, manipulating VMAT2 still produces an increased sensitivity to parkinsonian toxins and replicates one of the most prevalent non-motor symptoms associated with PD.

The VMAT2 Hypomorph mouse

As investigators continued to ponder the role of VMAT2 in the pathogenesis of PD, further perturbation of the gene was necessary to produce a more profound disruption of monoamine storage that previously achieved with the VMAT2 HT mice. This perturbation was manifested in a line of mice that expressed only 5% of the VMAT2 protein. A fortuitous accident, the VMAT2 hypomorph (KA1) mice were generated when one arm of a gene-targeting construct inserted into the VMAT2 locus, severely interfering with transcription (Mooslehner et al., 2001). It is important to note that unlike the previous VMAT2 KO and HT mice, the KA1

line was created through gene targeting using a completely differently strain of mouse, which unfortunately was found to be α-synuclein null (Specht and Schoepfer, 2001b). Unlike the previously made VMAT2 KO mice, the KA1 mice are fully viable into adulthood with the absence of gross physical defects (Mooslehner et al., 2001). The survival of these KA1 mice finally allowed the examination of the effects of reduced vesicular storage over a lifetime, in addition to the study of the nuances of vesicular uptake mechanisms; whereas, both VMAT2 KO and chronically reserpinized animals are not amenable to studying the effects of aging on monoamine packaging defects.

Although no VMAT2 protein can be detected in these mice through immunohistochemistry or in situ hybridization, residual VMAT2 was detected using Western blotting approximating a 95% reduction (Mooslehner et al., 2001). Consequently, there were general reductions in tissue levels of the major monoamines, DA, NE, and 5-HT reduced by 92%, 87%, and 82%, respectively, which became progressively worse with age, accompanied by increased monoamine turnover and reduced DA availability in terminal and cell body regions of the SNpc and ventral tegmental area (VTA) (Mooslehner et al., 2001; Colebrooke et al., 2006). In addition to the reduction of monoamines, the KA1 mice were also found to have altered striatal neurotransmission and signaling. Although levels of DAT mRNA, protein, and activity and D_1/D_2 receptor expression remained unchanged, electrically stimulated DA release was dramatically reduced by approximately 70% compared to age-matched wildtype animals (Patel et al., 2003a; Colebrooke et al., 2007). A decrease in striatal DA release is indicative of smaller vesicular DA stores (Patel et al., 2003a). Considering that electrically stimulated DA release is absent in VMAT2 KO neurons, these data suggest considerable intraneuronal compensation for the 95% deficit in VMAT2 (Wang et al., 1997). Moreover, due to the disproportionate decrease in DA release compared to the reduction in VMAT2 expression, it is possible to conceive that in wildtype neurons, not all VMAT2 protein is required to fill vesicles for exocytotic release; many transporters may, in fact, act as a reserve (Patel et al., 2003a). Additionally, even though no

compensation was seen through changes in DA receptor expression, ablating VMAT2 by 95% did induce a supersensitization of the D_2/D_3 autoreceptors and downregulated phosphorylation of tyrosine hydroxylase (TH) at a residue critical for catechol feedback inhibition (Patel et al., 2003a). Finally, the KA1 mice were found to downregulate substance P while upregulating enkephalin, allowing for the possibility of abnormalities in organization of DA-mediated signaling via both the direct and indirect pathways (Mooslehner et al., 2001; Colebrooke et al., 2007). Taken together, these data show that VMAT2 expression plays a vital role in regulating the size of both vesicular and cytosolic DA pools within the CNS, thus influencing extracellular neurotransmission (Patel et al., 2003a; Colebrooke et al., 2006).

With the abundance of changes in striatal neurotransmission, the KA1 mice were tested for the presence of a behavioral phenotype that correlated with PD. As in reserpinized animals, reductions in VMAT2 in the KA1 mice cause a general decrease in locomotor activity (Mooslehner et al., 2001). However, they exhibit normal reactivity in novelty place preference task (Mooslehner et al., 2001). At an early age, the KA1 mice demonstrated a significant impairment in motor coordination, independent of motivational factors, as measured by the challenging beam traversal and rotarod, which becomes progressively more severe with age (Mooslehner et al., 2001; Colebrooke et al., 2006). As expected, the KA1 are exquisitely sensitive to acute doses of MPTP and amphetamine, and exhibit locomotor hyperactivity and amelioration of deficits in motor coordination and balance when L-DOPA is administered (Mooslehner et al., 2001; Colebrooke et al., 2006). Interestingly, despite the presence of both striatal dopamine deficiency and a motor phenotype, when assessed for signs of parkinsonian degeneration, no evidence of DA cell loss was found at any age (Colebrooke et al., 2006). The KA1 mouse models several aspects of PD: lowered brain content of monoamines, specifically DA, presence of motor complications, and a similar pattern of peptide alterations associated with striatal pathways (Mooslehner et al., 2001). However, as mentioned above, these mice contain a spontaneous chromosomal deletion spanning the α-synuclein gene locus (Mooslehner et al., 2001;

Specht and Schoepfer, 2001b; Patel et al., 2003a; Colebrooke et al., 2006). The lack of this noteworthy gene in PD pathogenesis may account for the absence of degeneration as dopamine and other monoamines have been proposed to inhibit α-synuclein fibrillization (Conway et al., 2001; Norris et al., 2005). Assuming that protofibrils are the pathogenic species, a 95% decrease of VMAT2 in neurons should have lethal implications, causing the cytosolic autooxidation of catecholamines to increase, amplifying protofibril concentration. To answer the question more fully, the α-synuclein gene must be introduced into mice with low VMAT2 expression.

VMAT2-deficient mice

Although the availability of the VMAT2 KA1 mice provided an extremely useful model with which to further examine the importance of DA handling, the complete ablation of such a ubiquitous protein such as synuclein severely limited the utility of these mice. Through genotyping it was discovered that the KA1 mice contained mice that were wildtype, heterozygous, and null for α-synuclein; by diligent breeding, all traces of the α-synuclein null mutation were eliminated from the KA1 mice yielding the VMAT2-deficient mice (Caudle et al., 2007). Reduced expression of VMAT2 diminishes the neuron's ability to package DA and dopaminergic neurotoxicants; as a result, DA accumulates in the cytosol with the ability to induce oxidative stress independent of any oxidative stress caused by exogenous toxicants. Consistent with previous reports of genetic and pharmacological reductions of VMAT2, DA levels were reduced by 85% in VMAT2-deficient mice with a concomitant reduction in the metabolites, DOPAC and HVA; VMAT2-deficient mice also exhibited an age-dependent decline in DA (Caudle et al., 2007). Striatal DA levels in VMAT2 wildtype mice remained constant throughout most of their life (Caudle et al., 2007). Several intraneuronal compensatory mechanisms were also observed in the VMAT2-deficient mice including, an increase in TH activity, increased DA turnover, and an age-dependent decline in DAT expression and activity (Caudle et al., 2007). Additionally, several markers of oxidative stress and damage were observed in the VMAT2-deficient mice. Although cysteinyl-DA was undetectable due to the reduced basal levels of DA

and increased DA turnover, free cysteinyl-DOPA and DOPAC adducts were significantly increased at both 2 and 12 months of age; protein carbonyls and 3-nitrotyrosine did not manifest until 12 months of age, demonstrating that neuronal oxidative stress became progressively worse with age (Caudle et al., 2007). The chronic dysregulation of DA within VMAT2-deficient neurons began to contribute to neuronal degeneration in older animals, as evidence of cell death was seen through silver deposition and a progressive loss of TH-positive neurons within the SNpc (Caudle et al., 2007).

Behaviorally, VMAT2-deficient mice exhibit many of the parkinsonian motor phenotypes. Beginning at 2 months of age, VMAT2-deficient mice have general deficits in novelty-induced locomotor activity, which is L-DOPA responsive (Table 1) (Caudle et al., 2007). Interestingly, in the VMAT2-deficient mice it has been observed that the major motor deficits do not appear until 28 months of age, coinciding with the most severe nigral cell loss (see Chapter 4). Compared to age-matched wildtype littermates, VMAT2-deficient mice do not demonstrate a deficit in forepaw stride length until 28 months of age; this behavior is thought to mimic the shuffling gait observed in PD patients (Tillerson et al., 2002) (Figure 1). This behavior is also L-DOPA responsive, establishing that the motor phenotype is due to dopamine insufficiency. Combined with the dopaminergic characterization of these mice, these data reveal that reduced vesicular storage of DA is enough to induce parkinsonian neurodegeneration.

Mounting evidence for degeneration of the locus coeruleus (LC) in human PD highlights the importance of expanding the focus of research from the nigrostriatal system in order to expose the deficits in other neurotransmitter systems (Braak et al., 2002; Braak et al., 2003; Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Lemke et al., 2004; Rommelfanger and Weinshenker, 2007). Beginning at 18 months of age, the VMAT2-deficient mice displayed a mild reduction in TH staining in the SNpc and striatum, which increased moderately with age (see Chapter 4)(Caudle et al., 2007). More dramatic reductions in TH staining were observed in the locus coeruleus (LC) at 18, 24, and 30 months of age (see Chapter 4). This pattern of neuronal loss was

verified using unbiased stereological counts, demonstrating that neuronal loss in the LC precedes nigral loss in the VMAT2-deficient mice (see Chapter 4). The LC of VMAT2-deficient mice undergoes a much more rapid decline from 12 to 18 months of age, with an overall 72% neuronal loss from 6-30 months of age (see Chapter 4). The SNpc of the VMAT2-deficient mice does not start to degenerate until 18 months of age, with an overall 59% cell loss, similar to the loss observed in humans (see Chapter 4). Taken together, these data suggest that, unlike other chemical and genetic models of PD, the LC undergoes a much more severe degeneration than the SNpc in the VMAT2-deficient mice.

In classical PD, motor disturbances do not present clinically until approximately 70-80% of striatal dopamine and 40-50% of nigral cell bodies have already been lost; however, other non-motor symptoms are evident before the onset of motor disturbances. These include, but are not limited to, hyposmia/anosmia, gastrointestinal disturbances, sleep abnormalities, autonomic dysfunction, anxiety, and depression (Braak et al., 2003; Langston, 2006). It is probable that other neurotransmitters such as NE and 5-HT significantly contribute to these symptoms, as both the LC and raphe nucleus have also been shown to degenerate in PD, in addition to the SNpc (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Rommelfanger and Weinshenker, 2007). With the pathology observed in the major monoaminergic systems of the VMAT2-deficient mice, the presence of non-motor phenotypes would not be unlikely.

Olfactory disturbances are one of the first non-motor symptoms observed in PD; patients have demonstrated impairments in odor detection, differentiation, and identification (Ward et al., 1983; Doty et al., 1992; Tissingh et al., 1998). Moreover, this non-motor symptom is not responsive to traditional dopaminergic therapies (Kranick and Duda, 2008). When subjected to a battery of olfactory discrimination tests at various ages, VMAT2-deficient animals were unable to discriminate between two blocks (one scented with bedding from their home cage and one scented from the cage of a foreign animal of the same sex), and consequently displayed no preferential exploration of either block (Taylor et al., 2009). VMAT2 wildtype animals displayed

preferential exploration of the foreign-scented block at all ages tested (Taylor et al., 2009). When challenged in a similar test of olfactory acuity using scents commonly used on the University of Pennsylvania Smell Identification Test (UPSIT), VMAT2-deficient mice again showed no preferential exploration of the novel scent as compared with water, whereas VMAT2 wildtype animals spent more time investigating the novel scent (Taylor et al., 2009). The olfactory deficit is not corrected by L-DOPA treatment in human PD patients, nor is it effective in our mice (Table 1). To ensure there was not a problem in general sensory perception, mice were tested for non-olfactory sensory deficits. VMAT2-deficient animals showed no deficits in response to tactile stimulation, quinine taste aversion, trigeminal nerve function, muscle strength, or visual acuity (Taylor et al., 2009).

In order to investigate behavioral sleep disturbances in the VMAT2-deficient mice, sleep latency tests were conducted in VMAT2 wildtype andVMAT2-deficient mice during their circadian nadir. Beginning at 2 months of age, VMAT2-deficient mice show a shorter latency to behavioral signs of sleep compared to age-matched wildtype controls, which is responsive to an acute dose of L-DOPA (Table 2) (Taylor et al., 2009). The circadian activity of VMAT2-deficient animals is also significantly lower than that of age-matched wildtype controls at younger ages, but follows normal patterns compared to wildtype animals (Taylor et al., 2009). VMAT2-deficient animals were next behaviorally examined for gastric emptying at 2, 6, 12, and 18 months of age, as gastrointestinal dysfunction in PD occurs in over 70% of PD patients (Langston, 2006; Ziemssen and Reichmann, 2007). Solid gastric emptying was significantly delayed overall in VMAT2-deficient mice, with an increased stool frequency, indicating a fair amount of gastrointestinal dysfunction in the VMAT2-deficient animals (Taylor et al., 2009). As in humans, an acute dose of L-DOPA did not ameliorate the gastrointestinal dysfunction observed in these animals (Figure 2).

Disruptions in DA, NE, and 5-HT neurotransmission, including degeneration of the LC and DR, have been found in PD patients with anxiety and/or depression; similar pathology has

been observed in the VMAT2-deficient mice indicating the possibility for both anxiety-like and depressive-like phenotypes (Lemke et al., 2004; Ziemssen and Reichmann, 2007; Taylor et al., 2009). Moreover, the VMAT2 HT mice have been previously found to display a depressive-like phenotype (Fukui et al., 2007). Severe reduction of VMAT2 expression in the VMAT2-deficient mice was found to trigger both anxiety and progressive depressive behavior. VMAT2-deficient mice showed a significant increase in percentage of open arm time in the elevated plus maze at 4-6 months of age, while the increased immobility time in the forced swim and tail suspension tests did not occur until 12 months of age; suggesting that anxiety precedes depressive symptoms in VMAT2-deficient animals and that the depressive-like phenotype is progressive (Taylor et al., 2009). Additionally, a low dose of desipramine that had no effect in wild-type animals was able to normalize immobility times in VMAT2-deficient mice; similarly, an acute dose of L-DOPA was also able to ameliorate depressive-like symptoms in the VMAT2-deficient mice (Table 2) (Taylor et al., 2009).

Conclusions

As the VMAT2-deficient mice have reduced levels of DA, NE, and 5-HT, L-DOPA responsive motor deficits, and almost the full constellation of non-motor symptoms, mice with altered VMAT2 expression may represent a new model of PD that encompasses many of the motor and non-motor symptoms, as well as the neurochemical pathophysiology (Figure 2) (Mooslehner et al., 2001; Caudle et al., 2007; Fukui et al., 2007; Taylor et al., 2009). Moreover, most current models of PD, genetic and chemical, represent a relatively short disease progression. The average lifespan of a mouse is two years; disease progression must reflect this because sporadic PD, like Alzheimer's disease, is a disease of aging. The VMAT2-deficient mice exhibit a high age-dependency coupled with a progressive behavioral decline (Figure 2). The mice show that it is possible that PD pathogenesis represents more than altered DA homeostasis, and that a global disruption of monoamine storage and handling is necessary to fully invoke the pathology associated with the disease. Utilizing the VMAT2-deficient mice as a new model of PD, could

potentially lead to new adjunct therapeutic strategies, which complements current dopamine replacement therapy.

Future Directions

This thesis has largely focused on the behavioral and neuropathological characterization of the VMAT2-deficient mice. The VMAT2-deficient mice have the potential to be very valuable to the PD field because of their progressive pathophysiology and multi-neurotransmitter dysfunction. Moreover, since the pathology of the VMAT2-deficient mice occurs over the course of their lifetime as opposed to developing rapidly, these mice present a great opportunity to study mechanisms of slow degeneration. Current research suggests that PD primarily affects the dopaminergic and noradrenergic centers of the brain, with damage to the serotonergic nuclei being secondary (Jellinger, 1991; Fahn and Sulzer, 2004). To this end, future research should focus on mechanisms of catecholaminergic degeneration and the consequences of altered neurotransmission. I hypothesize that altered vesicular storage in catecholaminergic neurons is sufficient to trigger the pathological changes associated with PD. To examine this hypothesis, studies should be conducted to directly measure how reduced vesicular storage impacts catecholaminergic neurotransmission. The specific aims of this project would be:

Specific Aim 1: To determine the effect of reduced vesicular storage on catecholamine release and neurotransmission in the VMAT2-deficient mice. In this aim I would test the hypothesis that reduced vesicular storage negatively affects neurotransmitter release in the VMAT2-deficient mice. Although it is currently known that the VMAT2-deficient animals have severely diminished levels of DA, NE, and 5-HT, it is unknown if the reduced levels are due to reduced synthesis or release (Caudle et al., 2007; Taylor et al., 2009). Using fast-scan cyclic voltammetry (FSCV), DA and NE release can be measured in the SNpc/striatum and LC/nucleus accumbens/bed nucleus of the stria terminalis, respectively and compared with neurotransmitter release in wildtype animals. FSCV is currently the only way to directly detect neurotransmitter release and can be done in living tissue and freely moving animals (Wightman et al., 1991).

Wildtype and VMAT2-deficient animals at various ages will be used for this study to determine how neurotransmission is affected and how it changes over time with neurodegeneration.

Specific Aim 2: To determine if noradrenergic innervation of dopaminergic areas affects pathological outcomes in the VMAT2-deficient mice. In this aim, I would test the hypothesis that NE plays a significant role in the initiation of dopaminergic dysfunction and degeneration. Using the knowledge that the LC degenerates 6-12 months before the SNpc in the VMAT2-deficient mice (see Chapter 4), noradrenergic innervation of dopaminergic areas would be assessed by immunohistochemical analysis of the NE transporter (NET). Additionally, microdialysis can be used to measure how much NE is released into DA areas and how this changes with age in the VMAT2-deficient mice. Finally, if the NE system negatively affects the VMAT2-deficient animals, treatment with the noradrenergic precursor, L-DOPS, may be beneficial in these animals and rescue dopaminergic dysfunction.

Specific Aim 3: To determine if reduced vesicular storage in catecholamine regions specifically induces parkinsonian pathology and phenotype. In this aim, I would use a TH-specific VMAT2-deficient animal to test this hypothesis. To create an animal that only has reduced vesicular storage in catecholaminergic areas I would cross the VMAT2-deficient mice with mice engineered to overexpress VMAT2 in serotonergic neurons using a TrpH promoter. Without serotonergic involvement, the contributions of DA and NE can be more accurate assessed and it can be determined if catecholamine dysregulation is enough to trigger parkinsonian pathology. Alternatively, to fully elucidate the role of NE in the development of PD and its contribution to DA degeneration, a DβH-specific VMAT2-deficient mouse could be developed using similar techniques to generate the TH-specific VMAT2-deficient animal. These mice would be helpful in elucidating the mechanisms behind noradrenergic neurons' heightened susceptibility to death over DA neurons, which appear more vulnerable due to factors such as their increased oxidant capacity and sensitivity to neuroinflammation.

Final Thoughts

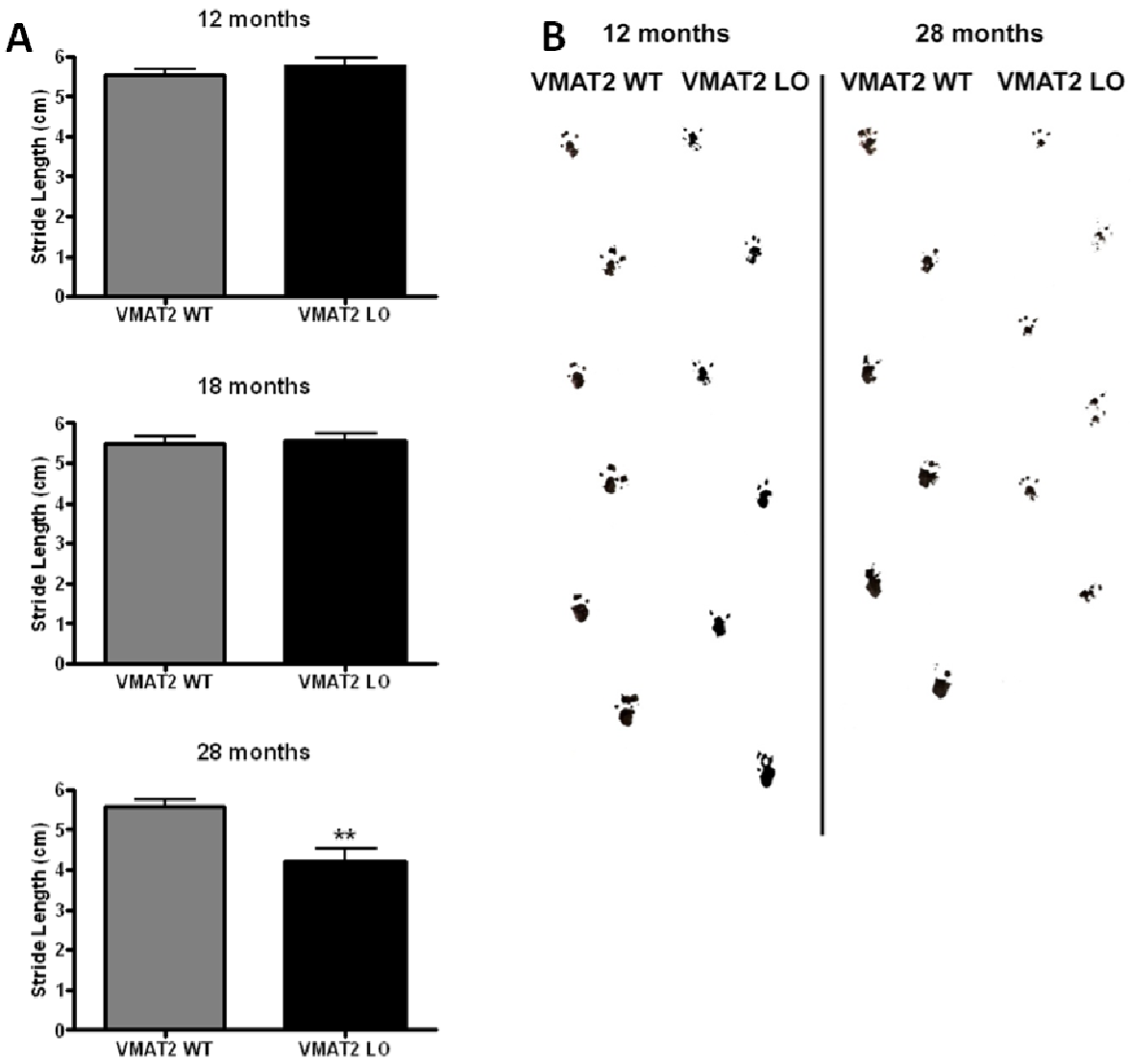
The goals of this dissertation were to examine the contribution of reduced vesicular storage of catecholamines to the pathogenesis of Parkinson's disease and illuminate the relative contribution of norepinephrine, and to a lesser extent, serotonin, to the symptomatology of PD. Despite the many strides that have been made, therapeutic research has failed to produce a lasting cure for PD which could be due to the lack of a sufficient animal model of the disease. Moreover, most current animal models of PD focus mainly on dopaminergic degeneration; whereas, parallel degeneration of the substantia nigra and locus coeruleus/raphe nuclei has been shown in post-mortem PD patients. Even though VMAT2 has not been directly implicated in PD, alterations in vesicular storage appear to play a role in disease pathogenesis. Many of the PARK loci, even though their function is not known, have been hypothesized to disrupt or affect vesicular storage or trafficking, implicating vesicular storage as a potential key mediator in PD. The VMAT2-deficient mice manifest almost the full assortment of parkinsonian behavioral phenotypes, including the presymptomatic non-motor behaviors, with nigral and locus coeruleus pathology. This suggests, disruption of vesicular storage may prove to be a point-of-convergence for the many factors contributing to the human disease; targeting animal models with defects in vesicular storage or trafficking may yield a more efficacious treatment for PD. Similarly, searching for defects in vesicular packaging in patients may aid in an earlier diagnosis. With the VMAT2-deficient animals, we have demonstrated that the catecholamines, and not just dopamine, play a significant role in both pathology and development of motor and non-motor symptoms associated with PD.

Behavior	L-DOPA responsive in VMAT2-Deficient?	L-DOPA responsive in humans?
Olfactory Discrimination	No	No
Sleep Latency	Yes	No
Anxiety	Suggested	Variable
Depression	Yes	Variable
Gastrointestinal Dysfunction	No	No
Locomotor Activity	Yes	Yes*
Forepaw Stride Length	Yes	Yes*

* Falling, freezing of gait, and postural instability are all L-DOPA unresponsive

Table 1. Summary of L-DOPA responsive parkinsonian symptoms observed in VMAT2-deficient mice.

Figure 1. VMAT2-deficient animals display impaired stride length at older ages. **A**, No deficits in forepaw stride length were apparent at 12 or 18 months of age in VMAT2-deficient mice. At 28 months of age, VMAT2-deficient mice display motor deficits as measured by inked paw stride length. Results represent average stride length (cm) ± SEM for 4-6 animals per genotype, **p<0.01. **B**, Representative forepaw stride lengths for wildtype andVMAT2-deficient mice at 12 and 28 months of age.


A
12 months
Stride Length (cm)
VMAT2 WT
VMAT2 LO
18 months
Stride Length (cm)
VMAT2 WT
VMAT2 LO
28 months
Stride Length (cm)
**
VMAT2 WT
VMAT2 LO
B
12 months
28 months
VMAT2 WT
VMAT2 LO
VMAT2 WT
VMAT2 LO

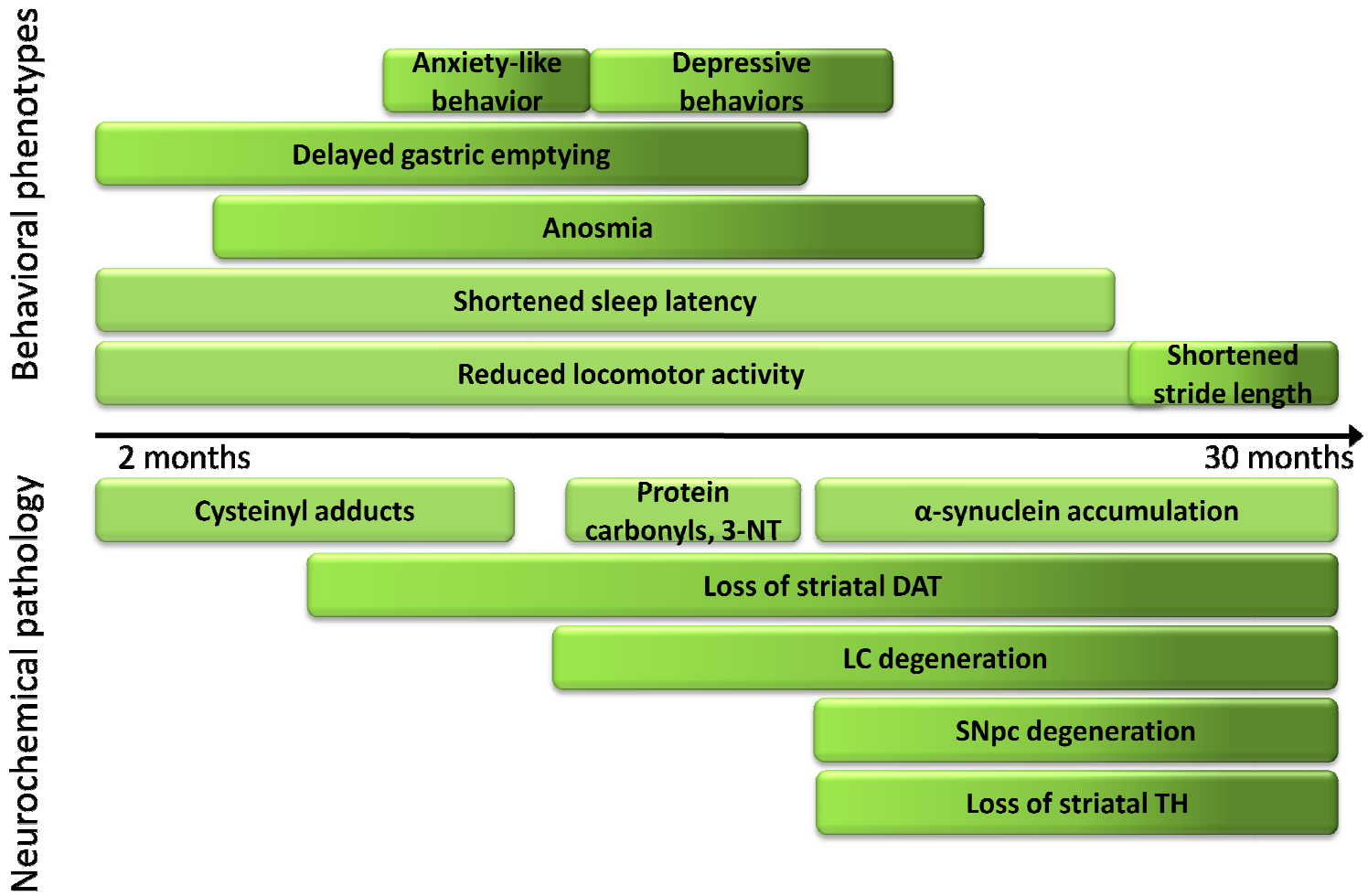


Figure 2. Timeline of parkinsonian features observed in the VMAT2-deficient mice.

APPENDIX A

THE EFFECT OF INCREASED CYTOSOLIC CATECHOLAMINES ON CELL TOXICITY

Summary

The following data demonstrate that some *in vitro* techniques are not appropriate for studying the intricacies of the intracellular oxidative environment when VMAT2 is severely diminished. In order to determine the role of catecholamines in oxidant-induced neurodegeneration in the VMAT2-deficient mice, *in vitro* methods were used to explore how genetically manipulating VMAT2 affects the cytosolic environment of the cell. Catecholamines have the ability to auto-oxidize and are metabolized by monoamine oxidase, yielding reactive oxygen species, potentially exacerbating neurotoxicity. To test this hypothesis, human embryonic kidney (HEK) cells were exposed to varying levels of exogenous DA, NE, and their precursors. The production of oxidative species within the cell was measured via DCF fluorescence; however, using this technique it is impossible to distinguish cytosolic oxidative damage from mitochondrial-induced damage. The DAT:VMAT2 ratio was manipulated by stably transfecting HEK cells with DAT, VMAT2 or both transporters to see if their presence affected the cells response to increased catecholaminergic concentration. Overall, addition of exogenous catecholamines regardless of the DAT:VMAT2 ratio was not toxic to these cell lines (data not shown). Enhanced production of hydrogen peroxide and other oxidant species, as measured by DCF fluorescence, was not observed in any scenario, which could be attributed to the use of a non-neuronal cell line (Figure 1).

To more accurately recreate the neuronal environment of the VMAT2-deficient mice, experiments were repeated using SN4741 cells, derived from mouse embryonic SNpc (Son et al., 1999). These cells make DA at a level of 4 pmol/mg, with functional DA uptake, and express other dopaminergic markers such as aromatic acid decarboxylase, brain-derived neurotrophic factor, DAT, and the D_2 receptor. Unfortunately, administration of the catecholamines or their precursors were neither deleterious to cell viability (Figure 2), nor did they cause an increase in DCF fluorescence (Figure 3) compared to cells dosed with media alone. The inability to ascertain

any meaningful results from these data may be due to the artificiality of the *in vitro* system. In the VMAT2-deficient mice, many factors including the presence of glial support cells and cross-communication with other neurotransmitter systems contribute to the viability of monoaminergic neurons. To more accurately replicate *in vivo* conditions, experiments were ultimately completed using primary neurons extracted from postnatal 2-5 day VMAT2 wildtype and VMAT2-deficient mice grown on a glial feeder mat (see Chapters 4 and 5).

Figure 1. Manipulation of DAT and VMAT2 do not enhance intracellular oxidative damage after exposure to exogenous catecholamines *in vitro*. HEK cells, stably transfected with DAT and/or VMAT2, were pre-loaded with DCF and exposed to dopamine (A), norepinephrine (B), L-DOPA (C), or L-DOPS (D) for 90 minutes. Compared to control HEK cells the addition of DAT and/or VMAT2 did not change induce or inhibit oxidative damage within the cell as measured by DCF fluorescence.

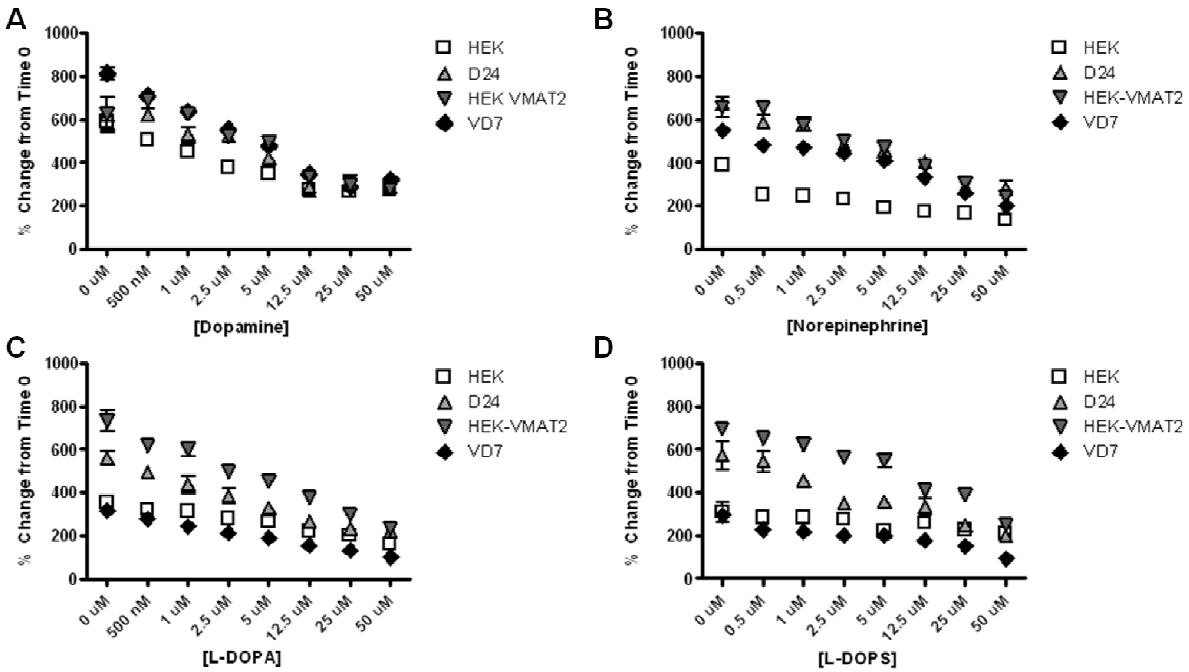

A
% Change from Time 0
1000
800
600
400
200
0
0 uM
500 nM
1 uM
2.5 uM
5 uM
12.5 uM
25 uM
50 uM
[Dopamine]
HEK
D24
HEK VMAT2
VD7
B
% Change from Time 0
1000
800
600
400
200
0
0 uM
0.5 uM
1 uM
2.5 uM
5 uM
12.5 uM
25 uM
50 uM
[Norepinephrine]
HEK
D24
HEK-VMAT2
VD7
C
% Change from Time 0
1000
800
600
400
200
0
0 uM
500 nM
1 uM
2.5 uM
5 uM
12.5 uM
25 uM
50 uM
[L-DOPA]
HEK
D24
HEK-VMAT2
VD7
D
% Change from Time 0
1000
800
600
400
200
0
0 uM
0.5 uM
1 uM
2.5 uM
5 uM
12.5 uM
25 uM
50 uM
[L-DOPS]
HEK
D24
HEK-VMAT2
VD7

Figure 2. Toxicity of catecholamines and their precursors to SN4741 cells. SN4741 cells were exposed to dopamine (A), norepinephrine (B), L-DOPA (C), or L-DOPS (D) for 48 hours. Exogenous catecholamines did not decrease cell viability as compared to cells exposed to media alone.

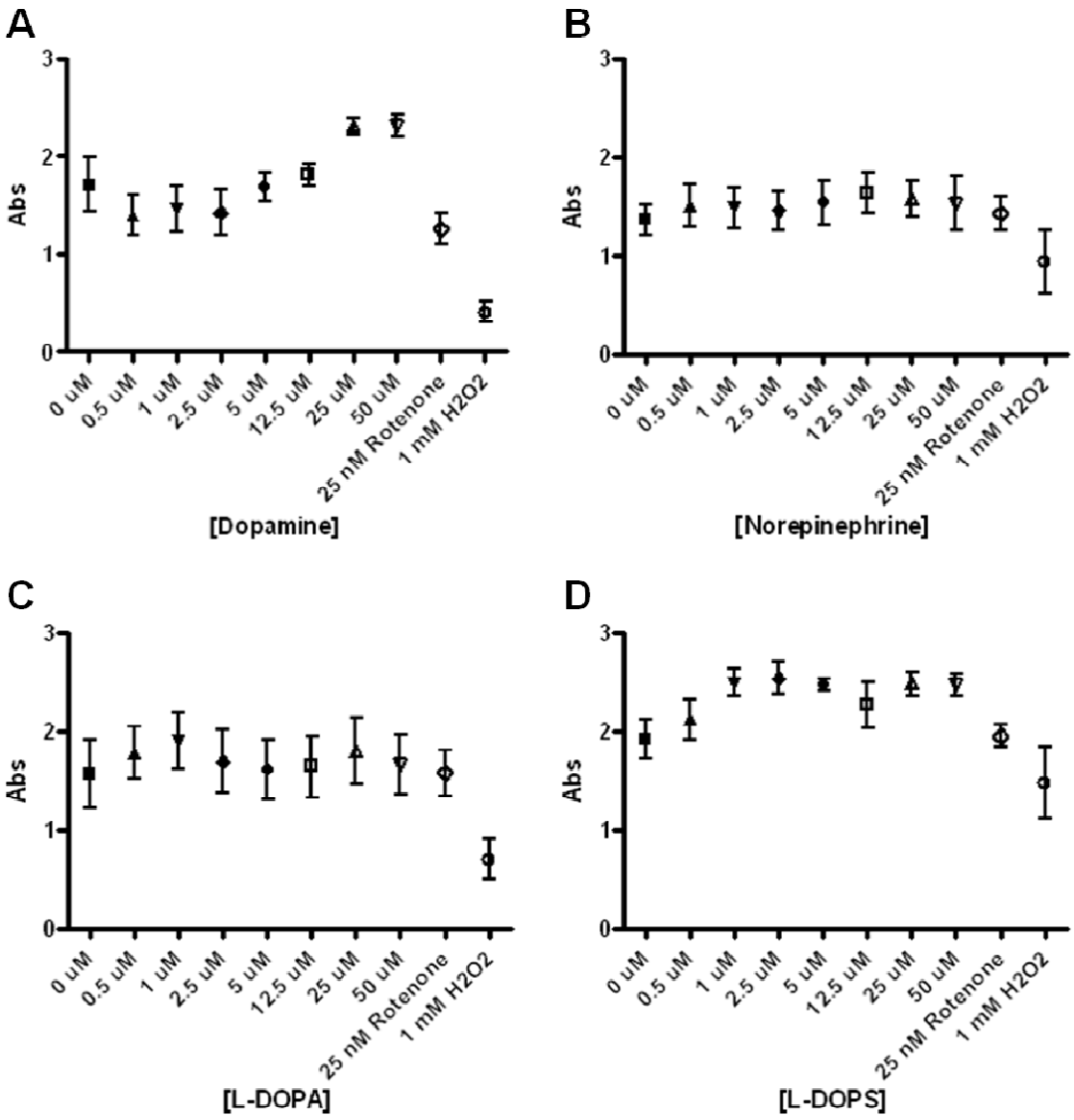
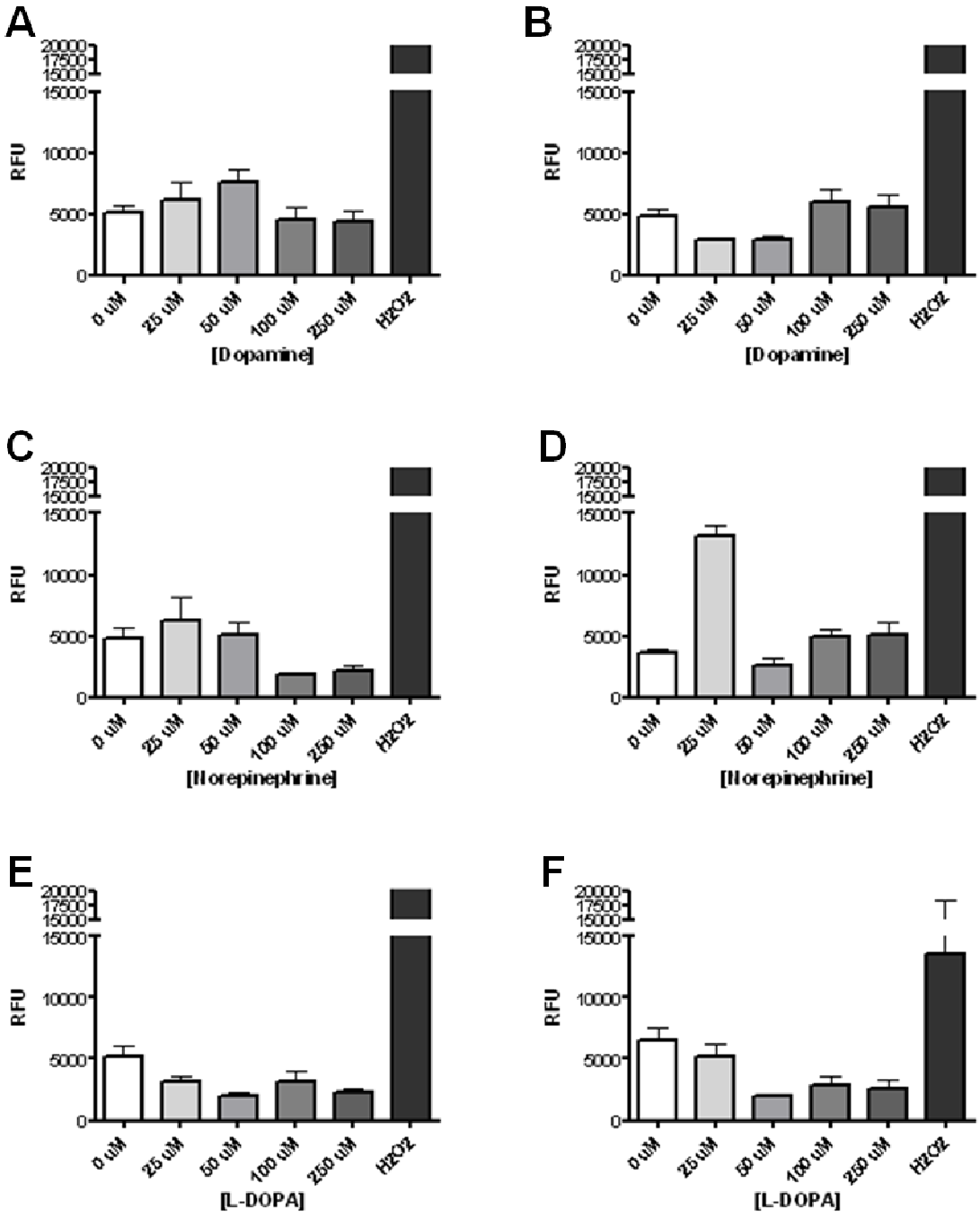

A
B
C
D
Abs
0
1
2
3
0 uM
0.5 uM
1 uM
2.5 uM
5 uM
12.5 uM
25 uM
50 uM
25 nM Rotenone
1 mM H2O2
[Dopamine]
[Norepinephrine]
[L-DOPA]
[L-DOPS]

Figure 3. SN4741 cells exposed to various concentrations of catecholamines do not exhibit increased oxidative stress as measured by DCF. Untransfected cells were pre-treated for 90 minutes with various concentrations of DA (A), NE (C), or L-DOPA (E), rinsed, and loaded with DCF; only H_2O_2 produced a significant increase in DCF fluorescence. Similar results were obtained when cells transiently transfected with DAT (B, D, F) were exposed to identical concentrations of DA, NE, and L-DOPA, with only H_2O_2 causing a significant increase in DCF fluorescence. NE was able to increase oxidant species formation in DAT-SN4741 cells at the 25 μM concentration; however, this was the only concentration of any catecholamine or precursor to have an effect.


A
B
C
D
E
F
RFU
20000
17500
15000
10000
5000
0
0 uM
25 uM
50 uM
100 uM
250 uM
H2O2
[Dopamine]
[Norepinephrine]
[L-DOPA]

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