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Targeting Tyrosine Hydroxylase to Improve Bradykinesia

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1. Introduction

The product of the tyrosine hydroxylase (TH) catalyzed conversion of L-tyrosine, L-DOPA, has been the gold standard for treating Parkinson's disease (PD) for half a century (Birkmayer and Hornykiewicz, 1961; Calne and Sandler, 1970). While L-DOPA therapy can improve locomotor disability in PD, it does not arrest the course of PD progression. Furthermore, dyskinesia is a debilitating side-effect of L-DOPA use over time. There have been promising results from preclinical and clinical studies for PD treatment. In fact, preclinical work indicates that enhanced TH protein expression accompanies locomotor improvements. Furthermore, the fact that L-DOPA treatment alone can improve locomotor dysfunction in PD is, by itself, a testimony to the critical importance of TH function in the nigrostriatal pathway for maintaining the capacity for initiating and maintaining normal locomotor activity. Clearly the major loss of TH, the rate-limiting enzyme of DA biosynthesis, in PD diminishes the capacity for locomotor activity. Surprisingly, efforts to treat PD symptoms have not deliberately focused on restoration of TH protein or its activity. Nonetheless, the rationale to focus on improving TH function to reduce the motor deficits associated with PD has been developed serendipitously over the past decade. Notably, research studying the neurobiological basis of how growth factors can improve locomotor activity in both Parkinson's disease and aging-related bradykinesia has revealed that increasing TH protein and its activity, through enhanced phosphorylation, should be a central feature of therapies intended to restore locomotor function in PD and aging-related Parkinsonism. Furthermore, these studies have led to the prospect that increasing DA tissue content in the substantia nigra alone could improve locomotor activity. Taken together, the possibility that increasing TH protein and activity in the substantia nigra could, by itself, restore locomotor deficits must be explored and no longer ignored.

2. The importance of tyrosine hydroxylase in Parkinson's disease

Undoubtedly, research in Parkinson's disease (PD) is flourishing on multiple fronts and substantial progress has been made in understanding PD and determining rationale for its treatment. Numerous studies have produced intriguing results on PD etiology from environmental, genetic, and aging-related associations. There has also been considerable focus upon PD treatment, from restoration of nigrostriatal neuropil function by increasing

the expression of dopamine (DA)-regulating proteins with growth factors to understanding the impact of extrinsic forces (such as the physical activity and diet) on PD prevention or improvement of symptoms. There is also a rich literature on defining the role of not only DA regulation in PD, but also the involvement of other neurotransmitters, particularly glutamate, in PD progression. The awesome breadth and depth of this work precludes discussion for the purposes of this chapter. The goal of the chapter is to provide evidence that one way to understand PD and determine an accurate treatment is to examine post-translational events in the nigrostriatal pathway and its allied tissue during PD progression. Defining these cellular and biochemical processes could unravel a therapeutic avenue that would facilitate these post-translational events, which may already be at work to correct PD-related deficiencies in the nigrostriatal pathway. The brain is indeed a robust organ capable of plasticity to accommodate insults in order to maintain as normal of function that is possible. During PD progression, there is evidence of dopaminergic compensation which may maintain sufficient levels of DA necessary for normal locomotor activity until major loss of TH (>70%) occurs. Thus, a major theme of this article is to focus on how the dopamine-regulating proteins of the nigrostriatal pathway, in particular tyrosine hydroxylase (TH), are functioning during PD progression and to propose that targeting TH should be a major priority in treating the PD patient. It will be proposed that the insights gained, and still to be gained, from understanding TH regulation during PD progression or in aging-related Parkinsonism will yield molecular targets for accurate treatment of the locomotor dysfunction accompanying these conditions.

The theme of this chapter does not infer that PD can be simply resolved by targeting TH. Certainly there are a number of molecular events and deficiencies that occur in PD, including the devastating non-motor symptoms of PD, as highlighted in several recent and comprehensive reviews (Obeso et al., 2010; Lim and Lang, 2010). However, with regard to the goal of improving compromised locomotor activity and execution, it cannot be ignored that, at the very least, a partial restoration of the expression or enhancement of function of the dopamine-regulating proteins (TH, dopamine transporter, and vesicular monoamine transporter) is important to reclaim a normal locomotor phenotype in the PD patient. This should be obvious since DA has an established role in the modulation of locomotor activity (Rech et al., 1966; Calne and Sandler, 1970; Brown and Robbins, 1991).

The dopaminergic neuropil of the nigrostriatal pathway modulates the basal ganglia circuitry to affect locomotor activity. The modulation of DA signaling in the striatum, the terminal field region of the nigrostriatal pathway, has engaged most research efforts to define the role of DA in locomotor activity. Thus the current model of how DA modulates basal ganglia output to impact locomotor activity is based upon DA release and modulation of post-synaptic DA receptor-regulation of the activity of the striatal medium spiny neurons. Conversely, the attention paid to DA in the somatodendritic region has been spent on measures of viability of the nigrostriatal neuropil in post-mortem tissue from PD patients, models of PD, and in aging related Parkinsonism by quantification of TH protein levels in the substantia nigra (SN). In subsequent sections, it should become evident to the reader that measures of nigral TH should not be considered to be static index of nigrostriatal viability, but rather a possible measure of the remaining functional capacity of DA biosynthesis that could affect locomotor capacity. Indeed, a distinct minority of studies

have shown that interference with dopamine signaling in the somatodendritic region of the nigrostriatal pathway in the SN does impact locomotion.

This chapter is also intended to present and discuss research of TH, an extraordinary enzyme with regard its multiple phosphorylation sites, and what phosphorylation of TH does to modulate its activity. Tyrosine hydroxylase is a vastly studied enzyme and its function in the CNS has been of great interest in the neurosciences. It is well known that TH protein levels precipitously decrease in PD progression. However, this decline is considerable prior to the presentation of PD symptoms (Bernheimer et al., 1973). While this curious observation led to examination of TH function in PD models, it has been a rather neglected area of study since. It is a major intent of this chapter to re-energize interest in studying the importance of TH function, through its post-translational modifications in PD progression and in aging-related Parkinsonism or bradykinesia, and how agents such as growth factors mechanistically improve its activity. There is a caveat in these studies of TH protein and function in the CNS, largely because we simply do not know at the present time (2011) the extent that site-specific phosphorylation of TH affects its activity *in vivo*. However, strides have been made to identify the post-translational changes in TH function, through phosphorylation assessment, that are associated with behavioral outcomes. However, the role TH phosphorylation may play in compensating for TH loss occurring during PD progression is poorly understood. This is ultimately a vital question for accurate therapies for PD and aging-related Parkinsonism because it may define what phosphorylation site could be targeted to enhance synthesis capabilities. Certainly, increasing TH protein expression is also a vital goal, at least in PD. Again, the fact that L-DOPA, the product of TH catalysis of tyrosine, is an effective treatment for PD symptoms belies the critical importance of TH in maintaining dopamine in quantities sufficient for normal locomotor activity.

3. Site-specific tyrosine hydroxylase phosphorylation *in vivo*: current status

The phosphorylation of TH is a well-established mechanism of regulating its activity. It is unique in that there are three physiologically-regulated sites in brain, ser19, ser31, and ser40 (Haycock and Haycock, 1991). As to be discussed, there is plenty of evidence from cellular work that increased phosphorylation at ser40 can regulate L-DOPA biosynthesis. However, there is also evidence that ser31 also plays a role and furthermore, there is recent evidence that ser31 phosphorylation status has a significant role in regulating basal levels of DA in terminal field and somatodendritic regions of DA pathways (including the nigrostriatal pathway) in brain (Salvatore et al., 2009b). Yet, the question remains today as to how phosphorylation at each site can regulate its activity *in vivo*. The answer to this question will provide insight in how to most efficiently activate TH in the face of its progressive loss as seen in PD. Significant momentum and insight to answer this question has been gained from growth factor studies, as to be discussed in later sections. If TH activity can be enhanced from a treatment, then perhaps only partial restoration of TH protein in PD may be sufficient to maintain the levels of DA that are necessary for locomotion. However, again, the questions are 1) at which phosphorylation site can or should this be achieved and, 2) as recent work is telling us, where, neuroanatomically, should this restoration of DA biosynthesis capabilities be targeted: the terminal fields in striatum or somatodendritic region on the substantia nigra?

The discovery of cAMP-dependent protein kinase (PKA) in brain (Miyamoto et al., 1969) and that PKA could activate TH in brain homogenates (Morgenroth et al. 1975) set forth an explosion of research to identify TH-phosphorylating protein kinases and TH phosphorylation sites that were later characterized to be ser8, ser19, ser31, and ser40; with ser40 being a PKA-phosphorylation site (Haycock, 1990). However, the focus of TH activation has predominated around PKA-mediated ser40 phosphorylation, with the long-standing assumption that any increase in ser40 phosphorylation increases TH activity. One could argue that because of the dominating attention the PKA-targeted phosphorylation site received, it has become virtual dogma that this phosphorylation site is the most critical site for regulation of TH activity. Yet it was also apparent, as studies went forward, that TH activation could occur on one of these phosphorylation sites not associated with PKA-mediated phosphorylation. Shortly after the discovery of ser31 as a TH phosphorylation site (Haycock, 1990), evidence suggested its phosphorylation could affect TH activity (Haycock et al., 1992). This supported an earlier observation, before ser31 was identified, that the peptide fragment associated with TH activation had ser31 in the sequence (Tachikawa et al., 1987). Indeed, increased ser31 phosphorylation, alone from NGF treatment or in conjunction with depolarization-stimulated ser19 phosphorylation, enhances L-DOPA accumulation and is independent of any affect on ser40 phosphorylation (Mitchell et al., 1990; Harada et al., 1996; Salvatore et al., 2001).

There certainly is substantial evidence that ser40 phosphorylation can modulate TH activity from *in vitro* and *in situ* work. Phosphorylation at ser40 reduces catecholamine-influenced end-product inhibition of TH (Fitzpatrick et al., 1999). It was also shown that the temporal dynamics of VIP-stimulation of PKA-activation and the associated increase in TH phosphorylation and activation were matched in chromaffin cells (Waymire et al., 1991), one of several studies to show that PKA activity could enhance TH activity. The identification of ser40 phosphorylation as the PKA-targeted site (Haycock, 1990) then firmly established the notion that ser40 phosphorylation was the key regulatory site of TH. The notion that ser40 is the sole regulator of TH activity has expanded into *in vivo* and *in situ* studies, as there are numerous reports detailing phosphorylation assessment only at ser40, to the exclusion of ser31 and ser19. While evidence supports that ser40 phosphorylation can regulate TH activity, including *in vivo* (Leviel et al., 1991), a critical threshold of phosphorylation may be necessary for an impact on biosynthesis. In PC12 cells, a two-fold increase in ser40 phosphorylation has no affect on L-DOPA biosynthesis, whereas a three-fold increase is associated with an increase (Salvatore et al, 2001). However, in the case of ser31, a two-fold increase may be sufficient to increase L-DOPA biosynthesis (Salvatore et al., 2001). These observations are highly relevant when applied to interpreting the impact of changes in ser31 and ser40 phosphorylation observed *in vivo*. Indeed, the results obtained in PC12 cells may have direct relevance to the *in vivo* situation because the basal phosphorylation of TH at ser31 and ser40 in the PC12 cell line versus that in the CNS are very similar, ranging from a phosphorylation stoichiometry of 0.02-0.05 for ser40 compared to 0.05 to 0.35 for ser31 (Salvatore et al., 2001; 2004; 2005; 2009a; 2009b) (Table 1). Therefore, in order to definitively answer whether any change in ser40 phosphorylation observed *in vivo* is of any consequence to L-DOPA biosynthesis capabilities, we must first define how much phosphorylation is necessary at ser40 and ser31 to affect L-DOPA biosynthesis capabilities *in vivo*.

Phosphorylation site	PC12 cells	striatum	Substantia nigra
Ser19	0.049	0.02 – 0.10	0.08 - 0.25
Ser31	0.088	0.15 – 0.35	0.05 - 0.10
Ser40	0.033	0.01 – 0.03	0.02 - 0.04

Table 1. **Tyrosine hydroxylase phosphorylation stoichiometry *in situ* and *in vivo*.** A comparison of TH phosphorylation in PC12 cells (Salvatore et al., 2001) versus the ranges reported in the nigrostriatal pathway *in vivo* (Salvatore et al., 2000; 2004; 2005; 2009a; 2009b). The *in vivo* TH phosphorylation ranges in striatum and substantia nigra represents results from mice (C57B1/6) and rats (Sprague-Dawley, Fischer 344, and Brown-Norway/Fischer 344 F₁ hybrid).

The first indication that increased ser40 phosphorylation might not necessary for activation of TH came from a study wherein depolarizing stimulation produced increased L-DOPA biosynthesis, even though the cells expressed TH with leucine substitution at ser40 (Harada et al., 1996). This study was the first to challenge the notion that only ser40 phosphorylation was important for regulation of TH activity. Yet, this conclusion was supported by earlier evidence to show that enhanced ser31 phosphorylation (or the peptide later found to correspond to ser31 in tryptic digest) alone was associated with enhanced TH activity (Tachikawa et al., 1987; Haycock et al., 1992). After the study by Harada and colleagues, it was shown in a cell line wherein PKA could not be activated (the A126 PC12 cell line) that a depolarization stimulated increase in ser31 phosphorylation could increase L-DOPA biosynthesis. Specifically, in both the wild-type PC12 and A126 cell line, the inhibition of mitogen-activated protein kinase produced a selective decrease in depolarization-stimulated ser31 phosphorylation accompanied by a reduction in depolarization-stimulated L-DOPA biosynthesis (Salvatore et al., 2001). This effect was later observed in striatal slices (Lindgren et al., 2002).

The finding that an increase in ser31 phosphorylation alone could enhance L-DOPA biosynthesis has significant impact for understanding how growth factors may improve locomotor activity (as to be discussed in the next section). Nearly two decades earlier, Greene and colleagues reported that the PC12 cell line was responsive to nerve growth factor (NGF) (Greene and Tischler, 1976). Furthermore, treatment with NGF increased TH activity for out to 1 hour (Greene et al., 1984). Subsequent studies indicated that the effect of NGF on TH phosphorylation is solely due to enhanced ser31 phosphorylation (Haycock 1990; Salvatore et al., 2001). There is also evidence that NGF-signaling can increase TH promoter activity (Suzuki et al., 2004). These findings are tremendously relevant for the evidence that growth factors can improve deficient locomotor activity in both PD and aging models. The synthesis of these three findings that 1) ser31 phosphorylation alone can increase L-DOPA biosynthesis in response to depolarizing stimulation, 2) a growth factor could accomplish the same end result, and 3) a growth factor like NGF can induce TH gene expression all make it plausible that the mechanism by which growth factors increase locomotor activity could be related to enhanced ser31 TH phosphorylation and regulation of TH protein expression, both of which would serve to regulate DA levels in a quantity sufficient to enhance locomotor capabilities.

While there is evidence for ser31 and ser40 phosphorylation in regulating TH activity, the role for ser19 phosphorylation *in vivo* is yet unknown. However, insights from cellular work indicate ser19 phosphorylation may be a sentinel for increased depolarizing activity, thus signaling that increased TH activity would be required to replenish DA lost from release.

Indeed, phosphorylation of ser19 requires Ca^{2+} (Waymire et al., 1988; Salvatore et al., 2001). Although ser19 does not directly influence TH activity (Sutherland et al., 1993; Haycock et al., 1998), it has been shown to facilitate ser40 phosphorylation *in situ* (Bevilaqua et al., 2001). To date, there are no reports on how ser19 phosphorylation may influence ser31 phosphorylation.

To date, there is evidence that ser31 may regulate basal DA biosynthesis capabilities *in vivo* (Salvatore et al., 2009b). We have recently shown that the differences in ser31 phosphorylation, but not ser40 phosphorylation, co-vary with differences in DA tissue content (Figure 1).

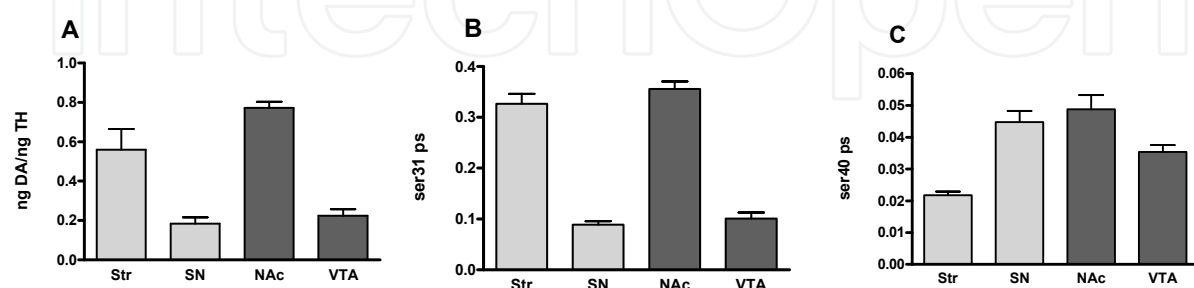


Fig. 1. Relationship of DA tissue content with tyrosine hydroxylase ser31 and ser40 phosphorylation stoichiometry *in vivo*. Four dopaminergic regions, striatum (Str), substantia nigra (SN), nucleus accumbens (NAc), and ventral tegmental area (VTA) were dissected from Brown-Norway/Fischer F344 F₁ hybrid rats and DA tissue content and TH protein inherent for each sample were analyzed from the same samples. **A)** The total recovered DA was normalized to total recovered TH protein to reveal differences in DA tissue content that could not be accounted for due to differences in TH protein recovery. To determine if phosphorylation at ser31 or ser40 could account for the differences in DA tissue content, we determined phosphorylation stoichiometry in each region. The differences in ser31 phosphorylation stoichiometry (**B**), but not ser40 (**C**), were similar to the differences in DA tissue content seen in **A**. *From Salvatore et al., 2009b*

In summary, there is no question that either increased ser31 or ser40 phosphorylation of TH can increase the biosynthesis of L-DOPA, leading to increased DA. There are still issues to be resolved. The first challenge lies in the acceptance of and practice of assessing ser31 phosphorylation in addition to ser40 phosphorylation in CNS studies of TH function or its role in behavioral paradigms. The second challenge is to ascertain how much phosphorylation at each site is necessary to produce an increase in L-DOPA biosynthesis.

4. Growth factors: dopamine & tyrosine hydroxylase

The objective in treating Parkinson's disease and aging-related Parkinsonism is to increase locomotor execution by increasing overall locomotor activity levels and the speed of execution. The discovery that glial cell line-derived neurotrophic factor (GDNF) delivery in CNS tissue can produce significant improvement in locomotor activity measures that are compromised in both aging and in PD models has revealed the critical importance of GDNF-signaling in maintenance of the nigrostriatal pathway (Hoffer et al., 1994; Gash et al., 1996; Gerhardt et al. 1999; Grondin et al 2003). There have also been successful outcomes to improve locomotor deficiencies from GDNF delivery via the use of viral-vectors. Delivery of GDNF using lentiviral vectors augments DA function in aged monkeys and reverses

functional deficits caused by MPTP (Kordower et al., 2000). In fact, the precise delivery of specific quantities of the protein in the sub-nanogram quantities has shown promise for optimal outcomes in locomotor function and TH expression (Eslamboli et al., 2005). In the clinic, the outcomes from GDNF delivery on PD patients have been mixed (Gill et al., 2003; Slevin et al., 2005; Lang et al., 2006). There is evidence that the manner in which GDNF is infused into the brain may have significant impact on the clinical outcome due to the volume of distribution obtained following infusion (Hamilton et al., 2001; Ai et al., 2003; Gash et al., 2005; Salvatore et al., 2006). Notwithstanding the critical technical issues involved with GDNF delivery into the brain, there is substantial evidence that the neurobiological events triggered by GDNF delivery can have a positive influence on compromised locomotor function in PD and aging models, and possibly PD patients.

Recent work clearly indicates that maintaining GDNF signaling in the nigrostriatal pathway is critical for maintaining the DA phenotype into and beyond adulthood, as well as normal locomotor activity (Pascual et al., 2008; Nevalainen et al., 2010). Furthermore, there is definite evidence that GDNF-signaling regulates the DA phenotype through its impact on TH regulation. The mechanism by which GDNF signaling maintains or, in the case of treating PD, restores the DA phenotype involves its impact on TH expression and even TH phosphorylation. The partial depletion of the GDNF gene (GDNF +/- genotype) leads to a locomotor deficit at an earlier age during the course of aging (Boger et al., 2006). Not only is this deficit produced in the GDNF heterozygotes, but it is also observed in GDNF receptor (GFR α -1) heterozygote mice (Zaman et al., 2008). In both cases, there is significantly greater loss of TH protein with advancing age. These data suggest that there is a definite relationship between GDNF-signaling and locomotor activity, in which TH protein expression plays a vital mechanistic link. Other trophic factors can also enhance locomotor activity and DA signaling. Neurturin, an analog of GDNF, also enhances DA signaling and can protect against PD-like lesion (Gasmi et al., 2007; Cass and Peters, 2010). Most recently, there is evidence that another trophic factor, brain-derived neurotrophic factor (BDNF), also influences dopaminergic function and locomotor functions, as BDNF heterozygote mice exhibit declines in striatal DA release but, notably, without impact on TH protein expression (Boger et al., 2011). The possibility remains that diminished BDNF-signaling could affect TH phosphorylation, however, as GDNF has such effects, particularly on ser31 phosphorylation (Salvatore et al., 2004; 2009a).

Clearly the GDNF-related increase in DA tissue content or release capabilities seen in the nigrostriatal pathway has implications for involvement of enhanced tyrosine hydroxylase phosphorylation or TH protein biosynthesis. Indeed, in neuroblastoma and primary mesencephalic neurons it was shown that GDNF could increase TH phosphorylation (Kobori et al., 2003). The first *in vivo* study of the impact of GDNF on TH phosphorylation showed significant increases in TH phosphorylation in both striatum and substantia nigra, but the impact on specific phosphorylation sites showed a dichotomous result (Salvatore et al., 2004). In the substantia nigra, there was a profound increase in ser31, and only ser31 phosphorylation, the magnitude of which exceeded all other changes in TH phosphorylation examined (Salvatore et al., 2004). In striatum, all phosphorylation sites exhibited an increase in phosphorylation (Salvatore et al., 2004). Notably, this treatment also reduced TH protein levels in the nigrostriatal pathway, an effect which has also been reported in other studies in *intact*, but not lesioned, tissue (Georgievska et al., 2004). There is also evidence that the impact of GDNF on TH protein and phosphorylation is dose-dependent (Aoi et al., 2000; Salvatore et al., 2009a).

It is particularly notable that increased locomotor activity produced by GDNF is accompanied by increased DA tissue content in the SN, but not in striatum, regardless of the model of locomotor dysfunction, be it aging or a PD model (Hoffer et al., 1994; Gash et al., 1996; Hebert and Gerhardt, 1997; Gerhardt et al. 1999; Grondin et al. 2003). However, the synaptic levels of striatal DA are affected following perturbations in GDNF-, BDNF-, or neurturin-related gene expression or delivery of these agents *in vivo* (Salvatore et al., 2004; Cass and Peters, 2010; Boger et al., 2011). These observations naturally raise the question of whether enhancement of locomotor activity by growth factors requires elevated DA-signaling in the striatum or SN. Furthermore, there is the question of how TH function in either region would ultimately affect DA signalling to affect locomotor activity. Bilateral improvement of locomotor activity after unilateral delivery of GDNF in striatum has been reported in clinical trials (Slevin et al., 2005) and bilateral effects on DA-regulating proteins like TH by unilateral GDNF have been shown to be limited to the SN (Salvatore et al., 2009a). Thus the increase in ser31 phosphorylation of TH, specifically in the SN, could be a critical molecular source to provide DA necessary for generating locomotor activity. This possibility leads to the necessity of asking how DA in striatum and the SN impact locomotor activity.

5. Tyrosine hydroxylase regulation in Parkinson's disease: role in dopaminergic compensation

It has been long noted that the pathological sequelae of PD include a major loss of TH protein in both the striatum and SN. There is evidence that TH activity may increase during PD progression, as increased DA release and TH activity occur in PD models (Snyder et al., 1990). TH activity may also be negatively affected by alpha-synuclein (to be discussed). Post-mortem analysis of PD brain tissue revealed the profound and yet unresolved finding that symptoms of PD were not apparent until the patient had at least 70% loss of the dopaminergic neuropil (Bernheimer et al., 1973). This observation led to formulating the concept of dopaminergic compensation, whereby locomotor functions continue normally in spite of loss of TH and other dopamine-regulating proteins until the 70-80% threshold is reached. One of the earliest observations to support dopaminergic compensation led to two hypotheses: one, that this mechanism was driving normal locomotion until the majority of the dopamine-regulating proteins were lost, but, two, at the same time could contribute to PD pathological progression (Agid et al., 1973). Other reports also suggest this compensatory mechanism may exacerbate toxicity to the nigrostriatal pathway (Zigmond et al., 2002). The human condition has been verified in MPTP-lesioned rhesus monkey in that the locomotor symptoms of PD are not present until there is nearly 80% loss of striatal DA (Bezard et al., 2001; Pifl et al., 2006). It is currently being debated as to whether or not increased striatal DA turnover can be observed during the asymptomatic stages of the disease or if, in fact, increased DA turnover is even a relevant index of dopaminergic compensation. There is support for dopaminergic compensation to maintain normal locomotion by evidence of enhanced striatal DA release (Perez et al., 2008). How TH activity is actually regulated by phosphorylation in a PD model is the subject of current investigation in this laboratory. Increased TH activity may be critical for ultimately maintaining normal locomotor activity during its progressive loss in PD. Infusion of the TH inhibitor AMPT hinders locomotor activity following nigrostriatal lesion (Leng et al., 2005), which argues that *de novo* DA biosynthesis is critical for dopaminergic compensation. As

such, insights into which signaling pathway is more or less active could be made by determining how TH phosphorylation changes at each phosphorylation site in a PD model. This information, such as a decrease in ser31 phosphorylation for example, could be a guide to determining where deficiencies in signaling exist and reveal a therapeutic target.

The regulation of TH during PD progression may also be affected by alpha-synuclein. Not only has this ubiquitous protein been well-studied in *in vitro*, *in situ*, and in animal models for potential involvement in PD pathogenesis, but there is evidence that it may control TH activity and expression. Indeed, perhaps the strongest evidence to date that implicates alpha-synuclein in PD vulnerability is a report that in both aged monkeys and humans, there is a strong correlation to nigral TH protein loss with aging-related accumulation of non-aggregated alpha-synuclein (Chu and Kordower, 2007). Under specific conditions, there is evidence that alpha-synuclein can act as a molecular chaperone to regulate TH activity through phosphorylation (Perez et al., 2002; Peng et al., 2005; Drolet et al., 2006). Therefore, the adverse impact of this protein on TH activity or its ability to be activated by phosphorylation may be considered when determining therapeutic options involving the targeting of TH.

These considerations must also include consideration of where TH could be best targeted, either in the terminal fields or somatodendritic region. As already mentioned in the discussion on growth factors, targeting TH may be best in the SN. There is evidence that the compensatory response is greater in the SN than in striatum, because extracellular DA levels in the SN are maintained despite 90% cell loss (Sarre et al., 2004). Furthermore, prevention of rotational behavior induced by L-DOPA can be blocked by intranigral infusion of a DA D₁ antagonist, and is more effective on blocking rotational behavior than striatal infusion (Robertson and Robertson, 1989). Thus, it may be possible that elevated TH activity in the SN could produce DA in quantities sufficient enough to sustain locomotion until a critical amount of TH protein is lost during PD progression.

6. Tyrosine hydroxylase regulation in aging-related Parkinsonism

Aging is a significant risk factor for developing Parkinson's disease. However, aging-related Parkinsonism is a much greater risk factor associated with aging, with up to 50% of the elderly developing bradykinesia after reaching age 80 (Bennet et al., 1996; Prettyman, 1998; Murray et al., 2004; Fleischman et al., 2007). However, unlike the loss of TH seen in PD, over the course of the lifespan the loss of striatal TH in humans is very minor and nowhere near the >70% loss seen in symptomatic PD (Haycock et al., 2003). It might be argued that there is a decrease in striatal TH phosphorylation during aging, which would support the evidence of striatal DA loss in human (Kish et al., 1992; Haycock et al., 2003), but the rodent models do not consistently support this possibility (Cruz-Muros et al., 2007; Salvatore et al., 2009b). Thus, the dominating hypothesis that >70% loss of striatal DA must be present for the emergence of locomotor symptoms associated with PD are challenged when viewed from the standpoint of striatal TH regulation during aging. In animal model studies of aging effects on DA regulation, no study has shown loss of DA or TH to reach that of the symptomatic threshold of striatal DA or TH loss. In fact, while some studies do show loss of DA or TH approaching that seen in PD, ~60% (Collier et al., 2007), many studies report much less, if any, loss of striatal DA or TH (Ponzio et al., 1982; Marshall and Rosenstein, 1990; Emerich et al., 1993; Irwin et al., 1994; Hebert and Gerhardt, 1998; Yurek et al., 1998; Gerhardt et

al., 2002; Haycock et al., 2003; Cruz-Muros et al., 2007; Salvatore et al., 2009). In fact, 60% striatal DA loss still does not produce bradykinesia in a PD model (Bezard et al., 2001).

Two fundamental observations should prompt us to pause and consider the prospect that nigral DA affects locomotor activity. First, GDNF enhances nigral DA tissue content in both aging and PD models in conjunction with increases locomotor activity. Second, aging work reveals that little or no striatal TH loss occurs with advanced age and there is a highly variable but consistently less than 60% DA loss in aging. A deficiency in nigral DA in either PD or in aging may contribute to decreased locomotor activity. Two aging studies have reported loss of both DA and ser31 TH phosphorylation of a 30-50% magnitude in the midbrain or SN (Cruz-Muros et al., 2007; Salvatore et al., 2009b). Loss of TH in the SN of aged non-human primates has been reported to be ~50% (Emborg et al., 1998). In a PD model, bradykinesia is present when nigral TH loss is at 50% (Bezard et al., 2001). Human data also indicate loss of DA neuropil of this magnitude in the SN in aging (Fearnley and Lees, 1991; Ross et al., 2004) or with PD (Marsden, 1990). Clinical studies of TH function in movement disorders is quite limited, but recent work in post-mortem tissue of Restless Leg Syndrome patients indicates that there is increased TH activity not only in putamen, but also in the SN (Connor et al., 2009). The possible importance of targeting the SN for treating PD has also been suggested for future work, as suggested in a recent report of a clinical trial involving the bilateral AAV-mediated gene delivery of neurturin in the putamen (Marks et al., 2010).

The abundance of GDNF-impact data has pointed to the possibility that DA regulation in the SN affects locomotor activity. In fact, a recent report has shown there is an aging-related decrease in the expression of the soluble isoform of the GDNF receptor, GFR α -1 only in the substantia nigra (Pruett and Salvatore, 2010). Taken together, these results all point to the possibility that TH regulation in the SN may be an important target for improving aging- or PD-related locomotor deficits, particularly bradykinesia. Thus, if deficits in nigral TH expression or phosphorylation in PD and aging are involved with locomotor dysfunction, an important question to ask is whether interference with DA signaling, specifically in the SN, could affect locomotor activity in otherwise normal rats.

7. Proposed role for nigral tyrosine hydroxylase function in locomotor activity

When it comes to defining how exactly DA modulates basal ganglia function, and hence locomotor activity, there is a wide consensus that DA function in the striatum is most critical. Yet, ever since somatodendritic DA release was reported (Cheramy et al., 1981), there have been reports from a variety of paradigms to suggest nigral DA alone can influence locomotor behavior. This possibility is a critical perspective to recognize if we are to understand how to successfully treat PD or aging-related bradykinesia. Specifically, from the perspective of striatal DA loss in PD and in aging, if striatal DA is most critical for bradykinesia arising from aging or PD, then there is a critical discrepancy at hand. That is, even though both conditions share a common symptom of bradykinesia, there is a starkly different magnitude of striatal DA loss in some cases and furthermore, no aging study has reported striatal DA to reach this critical threshold. Thus, if we are to accept that PD symptoms like bradykinesia are not present until there is 70% loss of TH or DA, which is supported by human PD pathology and PD models (Berheimer et al., 1973; Bezard et al., 2001), then how does bradykinesia come about in aging? Furthermore, do we dare ask if

70% striatal loss of TH or DA is really the threshold for symptom manifestation in PD, or is loss in another region like the SN more critical?

Observations in intact rats suggest that this question should be asked. Nigral application of DA modulates the output of the pars reticulata output (Waszczak and Walters, 1983; Kleim et al., 2007). Nigral infusion of a D₁ receptor antagonist suppresses operant behavior and open-field locomotion (Trevitt et al., 2001), and execution of motor performance (Bergquist et al., 2003). Depletion of nigral DA stores with tetrabenazine also hinders the ability to negotiate simple motor tasks (Andersson et al., 2006). We have most recently shown in longitudinally-characterized locomotor activity in rats that nigral DA correlates to lifetime locomotor activity initiation and maintenance, but not speed (Salvatore et al., 2009b).

These observations suggest that nigral TH function, as governed by TH protein expression and phosphorylation, may play a critical role in regulating the capacity for locomotor activity. The combination of phosphorylation at ser31 and the protein levels of TH may be a significant molecular source of producing DA that impacts the capacity for locomotor activity with regard to its initiation and frequency. Thus, the amount of local release of DA is proposed to be regulated by TH protein and phosphorylation. The released DA is proposed to act upon post-synaptic DA D₁ receptors. This local action in the SN increases GABA release from the striatonigral terminals, and disinhibits the inhibitory output neurons of the pars reticulata, thus facilitating locomotor activity. It is proposed that aging or PD-related deficits in locomotor activity may stem from deficiencies in either TH protein or TH phosphorylation at ser31 in the SN (Figure 2).

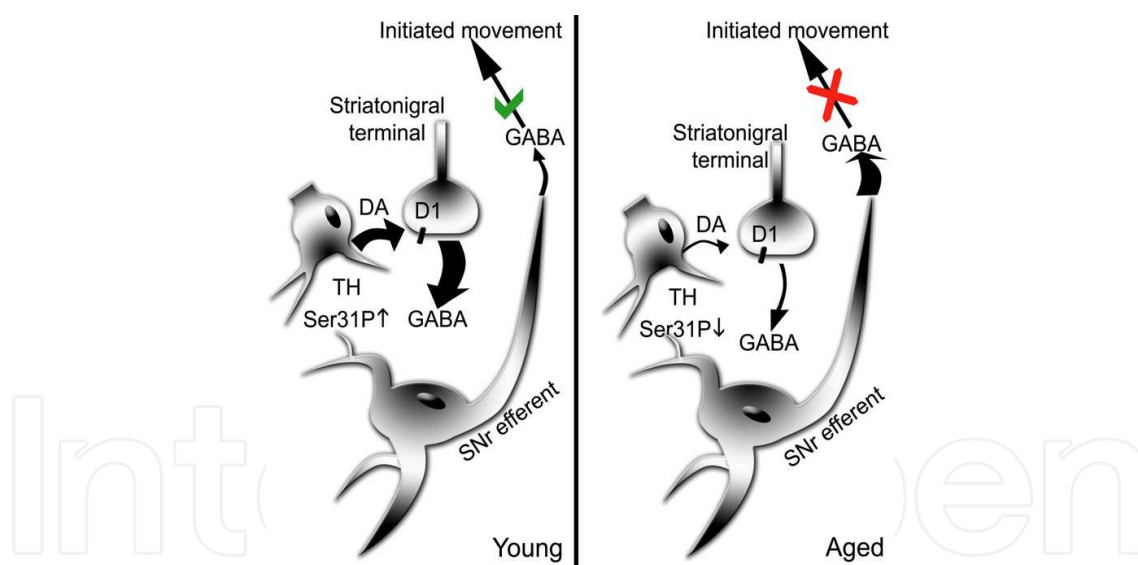


Fig. 2. Proposed role of nigral DA leading to bradykinesia in aging. It is proposed that inhibition of locomotion (bradykinesia) may occur by diminished local release of DA in the SN. Normally (as depicted in the young rat scenario on the left), released DA, acting upon post-synaptic D₁ receptors of the striatonigral terminals, promotes GABA release, which in turn reduces tonic release of GABA from the SNr efferent. This facilitates locomotor activity. When DA release capacity is deficient (as depicted in the aged rat scenario on the right), as proposed to be due to decreased TH protein or ser31 phosphorylation levels, the ability to promote GABA release from the striatonigral terminals is diminished, thereby removing an inhibition of GABA release from the SNr efferent, which promotes excess GABA release and inhibits locomotor activity.

8. Non-invasive approaches to target tyrosine hydroxylase function

The impact of exogenous sources of growth factors upon locomotor activity, DA tissue content and release, TH protein and phosphorylation is well-established. These observations allow us to question whether any non-invasive means exist to influence DA regulation *in vivo*. Increased production of growth factors from forced exercise, caloric restriction, and even components of the diet *in vivo* have been observed. Their impact on locomotor activity is an emerging and exciting topic as to the non-invasive measures we can take to improve locomotor deficits in PD or stave-off deficits produced by aging. Indeed, the enhancement of TH protein or its activity by phosphorylation may be a central mediator of the benefits to locomotor function realized by these non-invasive practices.

8.1 Exercise

Exercise is an activity that can be done at will with varying degrees of intensity, frequency, and longevity. Human and preclinical studies of exercise are revealing that these three variables associated with exercise have a significant impact upon our cognition and ability to move with advancing age and in the PD patient. For example in human studies, the volume of the hippocampus can increase and resist aging-related loss in volume as a result of aerobic exercise. Such changes are associated with improved memory function (Erickson et al., 2011). Exercise can prevent or reduce the risk of PD, as some longitudinal human studies support that regular exercise may lower the risk of PD (Chen et al., 2005). Other such studies do not support this hypothesis, with the caveat that study size was limited (Logroscino et al., 2006). The incidence of aging-related Parkinsonism and the disabilities arising from it may also be reduced from the quantity of physical activity that begins in midlife of healthy individuals (Savelle et al., 2010). In the PD patient, there are definite benefits of exercise, and the frequency and intensity of it may be critical for its benefit. Exercise can improve motor performance and the physical activities of daily living in PD patients (Crizzle and Newhouse, 2006) and improve the efficacy of L-DOPA to improve motor performance (Muhlack et al., 2007). Most recently, the results of a forced exercise paradigm in human PD patients has shown that patients choosing to exercise on a bike with a trainer at a rate 30% greater than their preferred voluntary rate had a 35% improvement in their Unified Parkinson's Disease Rating Scale motor scores (Ridgel et al., 2009). Indeed, there is evidence from human studies that the intensity, frequency, and longevity of exercise may influence our innate capacity to move normally. The exciting aspect of this work is that exercise can be beneficial over short or long-term, even in a motorically-compromised state as seen in PD and may diminish the incidence of aging-related Parkinsonism.

The molecular events triggered in the CNS from exercise have been the subject of much study and appear to be related to growth factor production. An increase in mild cellular stress and angiogenesis are also thought to initiate signaling cascades that can be protective of DA neurons (rev. Zigmond et al., 2009). It is certainly believed that a relationship between innate DA function and physical activity exists (Knab and Lightfoot, 2010), and a number of studies examining this relationship supports this idea. Perhaps the single most compelling observation is that exercise does show a positive correlation to locomotor capabilities, which have a well-established relationship with DA regulation in the nigrostriatal pathway. Thus, the impact of exercise on TH regulation in aging and in PD models has been studied in several exercise paradigms. In animal studies, exercise paradigms are divided into voluntary or forced paradigms. In voluntary exercise, test subjects are given free access

(within defined periods of time allowed for access) to an apparatus that permits and engages physical activity, most commonly a running wheel (Gerecke et al., 2010). In forced exercise, the test subjects are placed onto a treadmill on a near-daily basis and are coerced to run at a given rate of speed (12 – 20 meters/min) within a specific period of time that is typically much shorter than that used in voluntary (Tajiri et al., 2010).

Regardless of the exercise paradigm used in animal studies, there is strong evidence of enhanced growth factor production, notably brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Tajiri et al., 2010). In humans, there is a two- to three-fold increase in BDNF release from the human brain during exercise (Rasmussen et al., 2009) and endurance training also enhances the quantity of normal BDNF release compared to that seen at rest (Seifert et al., 2010). Given the well-established relationship of growth factors with TH and DA modulation, it would therefore be expected that exercise could affect TH and DA. Treadmill exercise in both PD and aging models does modulate DA tissue content and TH protein. In PD models, the impact of chemical lesions to the nigrostriatal pathway is lessened by treadmill exercise as evidenced by increased TH protein in the striatum and SN (Yoon et al., 2007). However, other reports show that despite locomotor activity improvements from exercise, no change in striatal DA or TH is observed (O'Dell et al., 2007; Petzinger et al., 2007), leaving open the possibility that enhancement of nigral TH function may be involved with locomotor activity effect. In fact, others have shown that extended periods of exercise (4-12 weeks), either treadmill (forced) or voluntary, increase nigral TH mRNA expression (Foley and Fleshner, 2008) or TH expression (Tumer et al., 2001; Gerecke et al., 2010; Tajiri et al., 2010). Thus, frequent exercise may enhance DA tone in the nigrostriatal pathway through enhancement of TH expression.

8.2 Diet: caloric restriction

The relationship of caloric intake with regulation of nigrostriatal DA function is becoming established. Seminal work showed aged rats that underwent calorie restriction (CR) had locomotor performances equal to that of younger adult rats. Furthermore, these CR aged rats had a 5-fold improvement in locomotor performance compared to age-matched controls, fed *ad libitum*. This work indicates that an innate molecular process associated with aging is hindered or diminished by CR. Calorie restriction increases striatal expression of GDNF in non-human primates (Maswood et al., 2004). Thus, if GDNF signaling is sufficiently active from CR, the impact of CR on locomotor activity could again, as in the case proposed in exercise, increase TH expression or activity enough to maintain DA signaling necessary for normal locomotor activity. Caloric restriction (CR) improves locomotor capabilities in PD models (Maswood et al., 2004) and preserves locomotor capabilities in aging models (Ingram et al., 1987; Weed et al., 1997; Kastman et al., 2010). Striatal DA loss from lesion is less severe in MPTP-treated monkeys on a 30% CR for about 6 months (Maswood et al., 2004) and amphetamine produces a marked enhancement locomotor activity in CR rats compared to rats fed *ad libitum* (Mamczarz et al., 2005; Marinkovic et al., 2007). An increase in DA release capacity from CR, as suggested by the Mamczarz study, strongly suggests an increase in DA biosynthesis capacity via increased TH protein or phosphorylation. The impact of CR on TH function has been studied sparingly. In fact, literature search yielded just one paper on the effect of 30 days CR on TH protein and ser40 phosphorylation (ser31 not studied). There was a trend toward increased TH protein in the SN. There was also an increase in striatal TH protein. There was no significant effect on ser40 phosphorylation of TH (Pan et al., 2006). However, through

enhancement of GDNF expression, CR could increase TH activity via ser31 phosphorylation, which is increased by exogenous GDNF delivery *in vivo* (Salvatore et al., 2004). This would improve locomotor activity by enhancement of DA signaling, as already demonstrated in the Mamczarz and Marinkovic studies.

8.3 Diet: nutritive substances

There is an emerging literature on the relationship of fatty acid and cholesterol intake and the risk of PD (Liu et al., 2010; Miyake et al., 2010). Inhibition of cholesterol synthesis can reduce the severity of L-DOPA-induced dyskinesia in 6-OHDA lesioned rats (Schuster et al., 2008). A high-fat diet has been recently shown to promote greater DA depletion in both the striatum and SN following 6-OHDA (Morris et al., 2010). Cholesterol metabolites (oxysterols) do cross the blood-brain barrier and thus could interact with nigrostriatal neurons, possibly through liver X receptors (Sacchetti et al., 2009). Indeed, there is evidence that these oxysterols can modulate TH expression in neuroblastoma cells (Rantham Prabhakara et al., 2008). However, there is also evidence that a high dietary intake of omega-3 polyunsaturated fatty acids (PUFA), as found in fish oil extracts, is reported to be effective to sparing MPTP-induced loss of dopaminergic neuropil in the somatodendritic region of the nigrostriatal pathway (Bousquet et al., 2008). Furthermore, while the high PUFA was without effect on preventing MPTP-induced loss of TH protein in the striatum, there was a significant effect of the high PUFA diet on protecting against striatal DA loss (Bousquet et al., 2008). This exciting result may signify that high PUFA diet can activate signaling pathways to increase TH phosphorylation when loss of TH protein is occurring. The high-PUFA diet can increase BDNF expression in the striatum (Bouquet et al., 2009). Given that BDNF can increase ERK activity (Jovanovic et al., 2004), an increase in ERK activity would increase TH phosphorylation at ser31. Thus, a common denominator in enhancement or protection against nigrostriatal DA loss in a PD model, once again, appears to be an enhancement of ERK-signaling, via increased growth factor production, which could ultimately increase TH activity.

9. Future directions

Certainly there is evidence that the non-invasive lifestyle habits of exercise, caloric restriction, and diet could achieve a desirable end result of activating nigrostriatal TH to amounts sufficient to produce levels of DA necessary for normal locomotor activity. However, it is clear that the impact of these strategies upon striatal DA and TH have yielded ambiguous results and their relative impact on nigral DA and TH has yet to be fully revealed. Nonetheless, these lifestyle strategies can enhance growth factor expression. Growth factors also can enhance DA signaling and TH expression or phosphorylation *in vivo*, in conjunction with their locomotor benefits. Thus, it is an exciting prospect that TH function could be regulated from a non-invasive approach. Still, it is likely that some motorically-compromised individuals would be incapable of performing the rigorous demands of exercise required to yield an improvement in locomotor capabilities; certainly this would be the case for one afflicted with moderate-severe stage PD or one who has a physical impairment that prevents exercise. Thus the therapeutic options for such individuals, when removing the prospect of surgical approaches, are currently non-existent. Therefore, the ultimate challenge to maintain locomotor activity to conduct normal daily

activities may be to take a pharmacological approach that targets TH and augments its expression and activity.

Clearly there is a critical battery of studies to support that augmenting nigral DA tissue content through enhancement of TH protein and phosphorylation could be the approach to improving the locomotor deficits seen in PD and aging. We have also known of the existence of somatodendritic DA release since the late 70s. There are handful of studies spanning nearly 30 years to support that nigral DA can influence aspects of locomotor activity. Clearly, there are still challenges in understanding the role of TH phosphorylation *in vivo*, notably, defining how much phosphorylation at TH is necessary at ser31 and ser40 to affect TH activity. Nonetheless, research efforts from a variety of angles that have been intended to improve locomotor impairment in PD and aging-related bradykinesia have shown, perhaps serendipitously, that targeting TH protein and its phosphorylation may be a promising molecular target to combat the locomotor deficits of PD and in aging.

10. References

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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