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Uncovering novel actors in astrocyte–neuron crosstalk in Parkinson's disease: the Wnt/ β -catenin signaling cascade as the common final pathway for neuroprotection and self-repair

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by progressive loss of dopaminergic (DAergic) neuronal cell bodies in the substantia nigra pars compacta and gliosis. The cause and mechanisms underlying the demise of nigrostriatal DAergic neurons are ill-defined, but interactions between genes and environmental factors are recognized to play a critical role in modulating the vulnerability to PD. Current evidence points to reactive glia as a pivotal factor in PD pathophysiology, playing both protective and destructive roles. Here, the contribution of reactive astrocytes and their ability to modulate DAergic neurodegeneration, neuroprotection and neurorepair in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rodent model of PD will be discussed in the light of novel emerging evidence implicating wingless-type mouse mammary tumor virus integration site (Wnt)/ β -catenin signaling as a strong candidate in MPTP-induced nigrostriatal DAergic plasticity. In this work, we highlight an intrinsic Wnt1/frizzled-1/ β -catenin tone that critically contributes to the survival and protection of adult midbrain DAergic neurons, with potential implications for drug design or drug action in PD. The dynamic interplay between astrocyte-derived factors and neurogenic signals in MPTP-induced nigrostriatal DAergic neurotoxicity and repair will be summarized, together with recent findings showing a critical role of glia–neural stem/progenitor cell (NPC) interactions aimed at overcoming neurodegeneration and inducing neurorestoration. Understanding the intrinsic plasticity of nigrostriatal DAergic neurons and deciphering the signals facilitating the crosstalk between astrocytes, microglia, DAergic neurons and NPCs may have major implications for the role of stem cell technology in PD, and for identifying potential therapeutic targets to induce endogenous neurorepair.

Keywords

dopaminergic neurons; neurodegeneration; neurogenesis; neuroprotection Parkinson's disease; reactive astrocytes

Introduction

Dopamine-synthesizing [dopaminergic (DAergic)] neurons in the ventral midbrain (VM) constitute a pivotal neuronal population controlling motor behaviors, and cognitive and affective brain functions. In Parkinson's disease (PD), DAergic cell bodies within the substantia nigra pars compacta (SNpc) progressively degenerate, from causes and mechanisms that are poorly understood. The main hallmark of PD, the second most frequent neurodegenerative disorder after Alzheimer's disease, is the selective loss of DAergic neurons in the SNpc and their projections into the caudate nucleus, leading to substantial decreases in dopamine (DA) levels, which manifest as resting tremor, bradykinesia, rigidity, and gait dysfunction, accompanied by progressive impairment of autonomic, cognitive and mood functions (Di Monte & Langston, 1995; Olanow *et al.*, 2003; Langston, 2006).

Although most cases of PD are observed later in life, there is evidence that the disease has progressed at the point at which it is diagnosed (Olanow *et al.*, 2003). In fact, the clinical symptoms appear only following a > 70–80% loss of midbrain DAergic neurons in the SNpc, suggesting that compensatory mechanisms are established while the neurodegeneration progresses (Hornykiewicz, 1993; Bezard & Gross, 1998).

Current DAergic treatments improve the motor symptoms and quality of life for patients during the early stages of PD, but do not prevent the progression of the disease associated with disabling side-effects (Olanow *et al.*, 2003).

So far, several scenarios regarding the mechanisms by which DAergic neurons degenerate have been suggested, including oxidative stress, deficits in mitochondrial function, excitotoxicity, accumulation of aberrant or misfolded proteins, and impairment of anti-oxidant and neuroprotective mechanisms (Olanow *et al.*, 2003; Abou-Sleiman *et al.*, 2006). In particular, current evidence from epidemiological and post-mortem studies, as well as from experimentally induced PD rodent models, including the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the rotenone and the 6-hydroxydopamine (6-OHDA) models of basal ganglia injury, point to reactive glia as a pivotal factor in PD, albeit a dual, detrimental/neuroprotective, influence is presently recognized (McGeer *et al.*, 1988; Langston *et al.*, 1999; Olanow *et al.*, 2003; Chen *et al.*, 2005; Marchetti & Abbracchio, 2005; Whitton, 2007; Gao & Hong, 2008; McGeer & McGeer, 2008; Hirsch & Hunot, 2009; Przedborski, 2010; L'Episcopo *et al.*, 2010a).

Astrocytes and microglia normally play neuroprotective roles, but, when chronically activated (i.e. under inflammatory/neurotoxic exposure, or upon brain injury), glial cells produce a wide range of cytotoxic mediators, including reactive oxygen species and reactive nitrogen species, and a panel of proinflammatory cytokines and chemokines that may perpetuate/exacerbate glial activation, thereby increasing neuronal vulnerability, and/or promoting DAergic cell death (Morale *et al.*, 2004; Hu *et al.*, 2008; Hoang *et al.*, 2009; Streit, 2010; Marchetti *et al.*, 2011).

Notably, several genes that cause certain forms of inherited PD (< 10% of cases) have been identified; however, the majority of cases (> 90%) appear to be sporadic, and probably represent an interplay between genetic and environmental influences (Warner & Schapira, 2003), whereby the hormonal background appears to be a critical risk factor (Morale *et al.*, 2004, 2006, 2008; Marchetti *et al.*, 2005a,b,c). Hence, glia may represent a common pathway mediating both genetic and environmental influences on DAergic neurodegeneration/neuroprotection (Marchetti *et al.*, 2005a,b,c; Morale *et al.*, 2006;

Marchetti, 2007; Frank-Cannon *et al.*, 2008; Gao & Hong, 2008; Hirsch & Hunot, 2009; L'Episcopo *et al.*, 2010a; Marchetti *et al.*, 2011; Kim *et al.*, 2012; Gillardon *et al.*, 2012).

Recently, the wingless-type mouse mammary tumor virus integration site (Wnt) 1 pathway has emerged as an essential signaling cascade that regulates multiple processes in developing and adult tissues, including differentiation, neuronal survival, axonal extension, synapse formation and plasticity, neurotrophin transcription, neurogenesis, and neuroprotection (Patapoutian & Reichardt, 2000; Ciani & Salinas, 2005; Lie *et al.*, 2005; Maiese *et al.*, 2008; Inestrosa & Arenas, 2010; Zhang *et al.*, 2011; Harrison-Uy & Pleasure, 2012). The interactions of Wnts (Wnt1–Wnt19) with their sevenpass transmembrane receptors of the frizzled (Fzd) family (Fzd1–Fzd10) trigger several signaling pathways, such as the so-called 'canonical' Wnt/ β -catenin pathway, and the 'non-canonical' Wnt/planar cell polarity (PCP) and Wnt/ Ca^{2+} pathways (Clevers, 2006; Clevers & Nusse, 2012; Salinas, 2012).

Compelling evidence clearly indicates that the Wnt/ β -catenin signaling pathway plays a central role in midbrain DAergic neurodevelopment (Castelo-Branco *et al.*, 2003, 2004, 2006, 2010; Prakash & Wurst, 2006; Rawal *et al.*, 2006; Joksimovic *et al.*, 2009; Tang *et al.*, 2009, 2010; Inestrosa & Arenas, 2010; Kele *et al.*, 2012). Importantly, mounting evidence points to dysregulation of Wnt/Fzd signaling in major neurodegenerative disorders (Toledo *et al.*, 2008; Inestrosa & Arenas, 2010; Kim *et al.*, 2011; Shruster *et al.*, 2011; Purro *et al.*, 2012), but only recently has the expression of Wnt/ β -catenin ligands and Wnt signaling components been characterized in the adult intact and MPTP-injured midbrain. Hence, dysfunctional Wnt/ β -catenin signaling was shown to play a causal role in the pathophysiology of nigrostriatal DAergic neurons and PD experimental models (L'Episcopo *et al.*, 2011a,b). In particular, Wnt1/ β -catenin signaling and MPTP-reactive astrocytes were uncovered as candidate components of neurorescue pathways involved in nigrostriatal DAergic plasticity (L'Episcopo *et al.*, 2011a,b). Of special interest, emerging evidence indicates that proteins encoded by *PARK* genes can modify Wnt signaling (Berwick & Harvey, 2012a,b).

Wnt/ β -catenin signaling critically contributes to the regulation of adult neurogenesis (Lie *et al.*, 2005; Adachi *et al.*, 2007; Kalani *et al.*, 2008; Wexler *et al.*, 2008; Kuwabara *et al.*, 2009; Shruster *et al.*, 2011; Zhang *et al.*, 2011 for extensive review). In particular, activated astrocytes promote neurogenesis from adult neural stem/progenitor cells (NPCs) (Lim & Alvarez-Buylla, 1999; Jiao & Chen, 2008), also via activation of the Wnt/ β -catenin pathway (Lie *et al.*, 2005; Kazanis, 2009; Kuwabara *et al.*, 2009; Zhang *et al.*, 2011). Additionally, reactive VM astrocytes express Wnt1 and promote neurogenesis and DAergic neurogenesis as a function of a specific 'inflammatory milieu' *in vitro* (L'Episcopo *et al.*, 2011a), and efficiently protect adult NPCs of the subventricular zone (SVZ) against MPTP-induced neurogenic impairment, again via Wnt/ β -catenin activation (L'Episcopo *et al.*, 2012a,b).

In this review, after a brief summary of neuron–glia interactions in PD, the Wnt signaling cascade and astrocyte–DAergic neuron interactions for neuroprotection and neurorepair will be highlighted. In particular, the evidence gathered on the Wnt/ β -catenin signaling pathway as a novel actor at both the neuron–glia and glia–neuroprogenitor interfaces in mice affected by MPTP-induced nigrostriatal degeneration will be discussed in the light of emerging evidence implicating the Wnt signaling cascade in major inflammatory and neurodegenerative diseases, with potential therapeutic implications for PD.

Astrocytes and microglia are key mediators of neuroinflammatory responses

The MPTP-lesioned mouse model of basal ganglia injury recapitulates many of the pathogenetic processes operative in PD (Jackson-Lewis & Przedborski, 2007). The neurotoxin MPTP, converted into its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP⁺), in astrocytes, is selectively transported into striatal DAergic terminals via the DA transporter (DAT), where it induces oxidative stress, the opening of mitochondrial permeability transition pores, the release of cytochrome *c*, and the activation of caspases (Vila *et al.*, 2001). In synergy with these early events, which account for ~10% of DAergic neuronal death (Wu *et al.*, 2002), glial inflammatory mechanisms are thought to contribute to nigrostriatal DAergic degeneration (Gao *et al.*, 2003; Hu *et al.*, 2008; Hirsch & Hunot, 2009; Hoang *et al.*, 2009; L'Episcopo *et al.*, 2010a,b; Przedborski, 2010).

Reactive astrocytes, microglial cells and infiltrating monocyte-derived macrophages are the key actors playing both detrimental and neuroprotective roles via an array of growth/neurotrophic factors, proinflammatory/anti-inflammatory cytokines, chemokines, and neurogenic transcription factors (Miller & Streit, 2007; Whitton, 2007; L'Episcopo *et al.*, 2010a,b; Marchetti *et al.*, 2011; Depboylu *et al.*, 2012; Njie *et al.*, 2012) (Fig. 1). The neuroinflammatory reaction also modulates the regenerative capacity of the adult brain, in both positive and negative ways (Butovsky *et al.*, 2006; Pluchino *et al.* 2008; Ekdahl *et al.* 2009; Martino *et al.*, 2011; Ekdahl, 2012).

Activated glia may benefit the host partly by producing cytotoxic molecules that kill pathogens, virally infected cells, or tumor cells, but they may also be detrimental by killing host cells, particularly neurons. Once activated, microglia display conspicuous functional plasticity, and ultimately transform into a macrophage-like phenotype that involves morphological changes, proliferation, increased expression of cell surface receptors, and the production of neurotrophic and neurotoxic factors. Astrocytes respond to injury with hyperplasia and hypertrophy of cell bodies and cell processes, and increased expression of the major astrocytic cytoskeletal protein glial fibrillary acidic protein (GFAP). Importantly, in response to brain injury, astrocytes and microglia play roles that are very dynamic and cell type-dependent, in that they may have 'harmful' effects, or they can turn into highly protective cells, and exert anti-inflammatory, neuroprotective and pro-regenerative ('beneficial') functions, thereby facilitating neuronal recovery and repair, posing the 'to be or not to be inflamed' dilemma (Marchetti & Abbracchio, 2005) (Fig. 1).

Importantly, peripheral immune responses also can trigger inflammation and exacerbation of central nervous system (CNS) degeneration in several neurodegenerative diseases, including PD (Cunningham *et al.*, 2005; Hu *et al.*, 2008; Pott Godoy *et al.*, 2008; L'Episcopo *et al.*, 2011c). Indeed, increasing inflammation and breakdown of the blood-brain barrier upregulates communication between the CNS and peripheral immune systems, with potential harmful consequences for neuronal survival.

Among the cytotoxic molecules produced by activated microglia, nitric oxide, produced by inducible nitric oxide synthase (iNOS), and superoxide, produced by the plasma membrane NADPH oxidase, are two key harmful mediators. iNOS is not normally expressed, but is induced as a part of the activation state in microglia, by cytokines [particularly interferon (IFN)- γ], tumor necrosis factor- α , or interleukin (IL)-1 β], bacterial cell wall components [particularly lipopolysaccharide (LPS)], and oxidative stress. Of particular interest is the fact that, if iNOS and NADPH oxidase are active at the same time, then microglia might produce peroxynitrite (ONOO⁻), a potent toxin, which may promote the nitration of tyrosine, producing hydroxyl radicals (Fig. 1). Hence, the generation of the free radical nitric oxide

followed by production of peroxynitrite may be implicated in neuronal cell death (Gao *et al.*, 2003; Gao & Hong, 2008; Gao *et al.* 2008; Hirsch & Hunot, 2009) .

Reactive astrocytes are characterized by the upregulation of several molecules, including GFAP and S100, and express receptors involved in innate immunity (e.g. Toll-like receptors), participating in the regulation of astrocyte response to injury. In addition, reactive astrocytes express receptors for growth factors, chemokines, and hormones, and produce a wide array of chemokines and cytokines that act as immune mediators in cooperation with those produced by microglia (Gennuso *et al.*, 2004; L'Episcopo *et al.*, 2010a).

Astrocytic reaction represents a pivotal feature of the neuroinflammation accompanying PD. Prolonged activation of astrocytes stimulated by cytokines released from microglia and damaged neurons is implicated in chronic neurodegenerative diseases such as PD, but the crosstalk between damaged neurons and reactive astrocytes still remains poorly understood. Given the cardinal role of astrocytes in the maintenance of brain homeostasis, energy metabolism, and, in particular, the defense against oxidative stress, impairment of astrocyte–neuron crosstalk may contribute to disease progression and impair the recovery process (see McNaught & Jenner, 1999). Dysfunction and/or degeneration of astrocytes may critically reduce their neuroprotective functions, and impair neurogenesis, causing a further delay in the recovery from neurodegeneration. Thus, disturbed and/or insufficient astrocytic function in the face of a highly activated microglial phenotype might represent a critical vulnerability factor compromising the self-repair ability of DAergic neurons.

Wnt signaling cascades, receptors, coreceptors, and endogenous regulators

In the CNS, the Wnt glycoprotein ligands control cell fate during embryonic development, self-renewal in adult neurogenic niches, and neuronal integrity and homeostasis in adult brain (Harrison-Uy & Pleasure, 2012; Salinas, 2012; Willert & Nusse, 2012 and references therein). The *Wnt* genes encode secretory glycoproteins that activate the Wnt signaling pathway. Wnt signals are context-dependently transduced to the canonical and non-canonical pathways, depending on the expression profiles of Wnt ligands, Wnt antagonists, the Fzd family receptors, coreceptors, and the activity of cytoplasmic Wnt signaling regulators. Wnt proteins share molecular and structural characteristics involving the sequence identity of 39–46-kDa lipid-modified secreted glycoproteins containing 350–400 amino acids with a highly conserved pattern of 23–24 cysteines and several asparagine-linked glycosylation sites (Willert & Nusse, 2012).

Wnt proteins (Wnt homepage: <http://www.stanford.edu/~rnusse/wntwindow.html>) are generally classified into functional groups according to their ability to induce a secondary body axis in *Xenopus* embryos and to activate specific signaling cascades, essentially described as the Wnt1 (including Wnt2, Wnt3, Wnt3a, Wnt8, and Wnt8a) and the Wnt5a (including Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and Wnt11) classes, involving intracellular signaling pathways specifying Wnt signal transduction. Generally, members of the Wnt1 class signal into the cell via the ‘canonical’ Wnt/β-catenin pathway, whereas members of the Wnt5a class signal via the ‘non-canonical’ Wnt/PCP and Wnt/Ca²⁺ pathways. Common to all three pathways is binding of the Wnt ligand to the seven-pass transmembrane receptors of the Fzd family, recently reviewed by Janda *et al.* (2012). The hallmark of the Wnt/β-catenin pathway is the stabilization of cytosolic β-catenin. In the absence of Wnt, β-catenin is constantly phosphorylated by a destruction complex consisting of, besides other components, glycogen synthase kinase-3β (GSK-3β), thereby targeting it for ubiquitination

and degradation by the proteasome (Aberle *et al.*, 1997; Gordon & Nusse, 2006; Jope *et al.*, 2007).

Wnt signaling inhibits GSK-3 β activity, thus increasing the amount of β -catenin, which enters the nucleus, and associates with T-cell factor/lymphoid enhancer-binding factor transcription factors, leading to the transcription of *Wnt* target genes involved in cell survival, proliferation, and differentiation (Gordon & Nusse, 2006).

The Wnt/ β -catenin pathway is involved in several aspects of neural development (Harrison-Uy & Pleasure, 2012), and has been reported to play an important role in midbrain DAergic neuron development, as recently reviewed by Inestrosa & Arenas (2010). Although this article will concentrate on Wnt signaling in the adult midbrain and adult NPCs, it seems crucial to recall that Wnt1, the prototypical ligand of the Wnt/ β -catenin pathway, regulates midbrain development, neurogenesis, proliferation of DA progenitors, and differentiation (see Introduction). Additionally, the low-density lipoprotein receptor-related protein (Lrp)6 receptor is important for the onset of midbrain DA differentiation and morphogenesis (Castelo-Branco *et al.*, 2010), and β -catenin is necessary for the integrity of the VM neurogenic niche and the progression from progenitors to DA neurons (Tang *et al.*, 2009, 2010).

Wnt pathways linked to intracellular Ca²⁺ release are defined as non-canonical or Wnt/Ca²⁺ pathways (Angers & Moon, 2009). The PCP pathway controls the remodeling of the cytoskeleton via a β -catenin-independent mechanism and controls tissue polarity, coordinated cell migration, and axon guidance. The Wnt5a class binds to Fzd receptors on the cell surface, resulting in several cellular processes that involve stimulation of heterotrimeric G proteins, increased intracellular Ca²⁺ release, decreased cGMP levels, and activation of the two kinases Ca²⁺/calmodulin-dependent protein kinase II and calcineurin, and protein kinase C (recently reviewed by van Amerongen, 2012).

A large number of antagonists can modulate the Wnt/ β -catenin signaling pathway. These include the Dkkopf (Dkk) family, Wnt inhibitory factor, Fzd-related proteins, and the cerberus and sclerostin families. The Dkk family, composed of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and soggy, is a group of secreted glycoproteins, with one of the best characterized members being Dkk1. Fzd receptors can also bind to proteins from other protein families, such as R-spondin and norrin. Additionally, Wnt-Fzd binding and cooperation with particular coreceptors, such as Lrp5/6, receptor tyrosine kinase-like orphan receptor 1/2, or receptor-like tyrosine kinase, can define downstream signal specificity (van Amerongen *et al.*, 2008; Glinka *et al.*, 2011; Clevers & Nusse, 2012).

The multiplicity of potential interactions between Wnts, their receptors and downstream effectors has exponentially increased the complexity of the signal transduction network. Signaling through each of the Wnt pathways, and crosstalk between them, together play critical roles in all facets of nervous system development, and probably also contribute to adult CNS homeostasis (Ciani & Salinas, 2005; Lie *et al.*, 2005; Prakash & Wurst, 2006; Inestrosa & Arenas, 2010; Marchetti & Pluchino, 2013). Not surprisingly, interruption of Wnt signaling leading to either hypo-functioning or aberrant functioning may promote diverse pathogenic outcomes. Hence, dysregulation of the Wnt/Fzd system is associated with a variety of human hereditary diseases, and modulation of Wnt signaling is actively targeted for cancer, regenerative medicine, stem cell therapy, bone growth, and wound healing (Clevers & Nusse, 2012).

Of specific importance, novel evidence indicates that the leucine-rich repeat kinase 2 (LRRK2), encoded by *PARK8* (whose mutations have been linked to PD), has a functional role in canonical Wnt signaling pathway (Berwick & Harvey, 2012a). Hence, LRRK2

interacts with key Wnt signaling proteins of the β -catenin destruction complex and Dvl proteins, *in vivo* (Berwick & Harvey, 2012a). Following Wnt stimulation, LRRK2 is recruited to membranes, where it binds to the Wnt co-receptor Lrp6 (Berwick & Harvey, 2012a). Pathogenic LRRK2 mutations were shown to reduce Wnt signaling strength and LRRK2-Lrp6 interactions, suggesting that dysregulated Wnt signaling might represent a new pathomechanism leading to *PARK8*-PD (Berwick & Harvey, 2012a,b).

During the last decade, within the adult CNS, a ‘pro-survival role’ for the Wnt/ β -catenin signaling pathway has emerged, and dysregulation of the Wnt/Fzd cascade has been defined as a critical determinant in major neurodegenerative disorders (De Ferrari *et al.*, 2003; Li *et al.*, 2007; Inestrosa & Toledo, 2008; Maiese *et al.*, 2008; Inestrosa & Arenas, 2010; Maiese *et al.*, 2012; Purro *et al.*, 2012; Shruster *et al.*, 2012; and references therein). In this article, we focus on adult midbrain DAergic neurons, and herein trace a candidate role for the Wnt/ β -catenin signaling system in healthy DAergic neurons and upon MPTP-induced DAergic plasticity brought about by astrocyte–neuron crosstalk.

Wnt/ β -catenin signaling and astrocyte–neuron crosstalk in the MPTP-injured midbrain: an intrinsic self-defense mechanism for DAergic neurorepair

A body of evidence suggests that astrocytes play a vital role in the response of SNpc DAergic neurons to injury or inflammation, by scavenging excess neurotoxic factors, removing dying cells and cellular debris, and stimulating repair processes, whereas impairment of astrocyte function, as a result of aging or exacerbated inflammation, may critically influence neurodegeneration and neurorepair (Morale *et al.*, 2004, 2006; Marchetti & Abbracchio, 2005; Hu *et al.*, 2008; McGeer & McGeer, 2008; Sandhu *et al.*, 2009; L’Episcopo *et al.*, 2010a, 2011a,b,c). Indeed, after injury, many adaptive changes occurring within the astroglial cell compartment may serve to increase the defense against oxidative stress, to reduce inflammation, to improve mitochondrial performance, to increase neurotrophic support, and/or to activate adult neurogenesis (Horner & Palmer, 2003; Barkho *et al.*, 2006; Chen *et al.*, 2009; Sandhu *et al.*, 2009; Voskuhl *et al.*, 2009; Sofroniew & Vinters, 2010).

Hence, astrocytes are known to secrete both inflammatory and anti-inflammatory and neurotrophic and survival factors, and may play a role in modulating microglial activity. Importantly, GFAP-expressing astrocytes can contribute to cell genesis, both as stem cells and as important cellular elements of the neurogenic microenvironment, with implications for self-recovery/neurorepair (Wagner *et al.*, 1999; Alvarez-Builla *et al.*, 2001; Song *et al.*, 2002).

Of special importance is that, in response to injury, the nigrostriatal DAergic system shows compensatory mechanisms, but the degree of plasticity becomes reduced with age (Ricaurte *et al.*, 1987; Hornykiewicz, 1993; Bezard & Gross, 1998; Ho & Blum, 1998). Interestingly, different conditions, including an enriched environment and exercise, by the activation of signaling cascades by neurotrophic factors such as glial cell line-derived neurotrophic factor, can induce neurorestoration in rodent PD models (see Zigmond *et al.*, 2009, 2012).

The idea that astrocytes might harbor Wnt1/ β -catenin signaling mechanisms to induce neurorepair arose from gene expression experiments conducted in the MPTP mouse model of basal ganglia injury (L’Episcopo *et al.*, 2011a). Hence, using wide gene expression analysis of 92 mRNA species involved in inflammation, immunity, stemness, self-renewal, DAergic development, and DA metabolism, we discovered major upregulation of certain proinflammatory chemokines and Wnt1 (but not Wnt3a or Wnt5a) during MPTP-induced

DAergic degeneration and self-recovery, suggesting a potential involvement of Wnt signaling as an intrinsic response to midbrain injury.

Indeed, the temporal correlation of *Wnt1* mRNA expression with Wnt1 protein appeared to be of interest in the light of recent evidence indicating that the Wnt signaling system may be reinduced in the adult CNS after injury (Osakada *et al.*, 2007) and the studies indicating that Wnt/ β -catenin activation reduces neurodegeneration in mouse models of Alzheimer's disease (Chacón *et al.*, 2008; Toledo *et al.*, 2008). The finding that Wnt components are expressed in adult astrocytes (Lie *et al.*, 2005; Cahoy *et al.*, 2008; Kuwabara *et al.*, 2009) prompted us to investigate a possible astrocyte source of Wnt1. Accordingly, *in situ* hybridization histochemistry revealed MPTP-reactive astrocytes as candidate sources of Wnt1 within the injured VM. In addition, increased transcription of *Wnt1* mRNA was observed in astrocytes derived *ex vivo* from the MPTP-injured VM, raising the possibility that astrocyte-derived Wnt signals might directly and/or indirectly participate in the DAergic neuroplasticity observed upon MPTP exposure (L'Episcopo *et al.*, 2011a).

Earlier studies implicated astrocyte-derived factors in the survival and growth of several types of neuron, including DAergic neurons (Engle & Bohn, 1991; Takeshima *et al.*, 1994; Gallo *et al.* 1995; Gallo *et al.*, 2000; Castelo-Branco *et al.*, 2006; Blackburn *et al.*, 2009). Additionally, *in vitro* astrocyte–neuron co-culture paradigms have underlined the ability of astrocyte-secreted glial cell-derived neurotrophic factor and the glutathione antioxidant system to protect DAergic neurons against 6-OHDA cytotoxicity (Sandhu *et al.*, 2009). Our studies further demonstrated the contribution of the Wnt/ β -catenin pathway to VM astrocyte-induced DAergic neuroprotection against MPP⁺, 6-OHDA, and growth factor deprivation-induced toxicity. Accordingly, inhibition of DAergic neuron survival was obtained by directly blocking Wnt/Fzd-1/ β -catenin signaling with the prototypical antagonist of the Wnt canonical pathway, Dkk1 (L'Episcopo *et al.*, 2011a,b), raising the possibility that endogenously secreted Wnt agonists/inhibitors might play a role in directing astrocyte-promoted DAergic cell survival/death (L'Episcopo *et al.*, 2011a,b; Marchetti *et al.*, 2012). Interestingly, exogenous activation of Wnt signaling in MPP⁺-treated astroglia–neuron co-cultures with a specific GSK-3 β antagonist sharply magnified astrocyte-induced DAergic neuroprotection. Moreover, addition of glial inserts or direct application of Wnt1 to purified DAergic neurons just prior to MPP⁺ insult afforded a significant degree of neuroprotection, an effect counteracted by the addition of Wnt1 blocking antibody, or the Wnt antagonist Fzd-1-cysteine-rich domain, thus supporting the critical role of Wnt1 in DAergic neuron survival (L'Episcopo *et al.*, 2011a).

Cytotoxic insults trigger beneficial astrocyte–DAergic neuron crosstalk: astroglial-born Wnt1 as a critical paracrine protective actor in neuroprotection

That Wnt1 might be the critical Wnt molecule was next corroborated by different lines of evidence. Besides the demonstration of the lack of effect upon tyrosine hydroxylase (TH)-positive neuroprotection induced by VM astrocytes in the presence of a specific Wnt1 antibody (L'Episcopo *et al.*, 2011a), depleting Wnt1 in VM astrocytes by introducing a small interfering RNA targeting *Wnt1* resulted in a significant decrease in TH-positive neuron survival upon serum deprivation (SD), 6-OHDA or MPP⁺ treatment, as compared with neurons co-cultured with Astro^{Ct} (L'Episcopo *et al.*, 2011b). Inhibition of TH-positive neuron survival was associated with a marked reduction in β -catenin protein levels, and activation of caspase-3, a recognized apoptotic cell death marker.

Within this context, and of particular interest, co-culture with VM astrocytes markedly increased the Fzd-1 immunofluorescent signal within the rescued TH-positive neurons, at the neurites and growth cones, as opposed to the dramatic downregulation of Fzd-1 receptor observed in purified neurons, either *in vitro* or *in vivo*, after the neurotoxic insult. Coupled with the finding that Wnt1 induced upregulation of Fzd-1 receptors in purified DA neurons upon cytotoxic challenge (L'Episcopo *et al.*, 2011b), these findings further suggested that Wnt1 signaling is required to maintain the expression of Wnt signaling components in DA neurons, corroborating the presence of a paracrine astrocyte–neuron autoregulatory loop. It seems important to recall the potential involvement of Fzd receptors localized at growth cones in regenerating neurites, together with the recently characterized role of Fzd-1 receptor in the presynaptic differentiation and function of hippocampal neurons (see Chacón *et al.*, 2008; Toledo *et al.*, 2008, and references herein), and further studies are in progress to clarify the role of Fzd-1 ligands and Fzd-1 receptors localized at the growth cones in TH-positive neurons in neurite outgrowth, maintenance and regeneration in conjunction with astroglia-derived factors.

Overall, besides a wide panel of astroglia-derived growth and neurotrophic factors, astrocyte-born Wnt1, via Fzd-1/ β -catenin signaling activation, was identified as a chief component of the self-protective machinery of DA neurons, thus prompting us to propose a candidate regulatory autoprotective circuit controlling midbrain DA neuron–astrocyte crosstalk via astroglial Wnt1 (Fig. 2).

Wnt/ β -catenin signaling and DAergic neuron integrity in the adult midbrain: building-up a 'Wnt/ β -catenin-linked hypothesis'

In vitro findings

Canonical Wnt signaling has been associated with the control of apoptosis during injury in various cell systems. Although little is known about the receptors that mediate Wnt effects in the adult midbrain, in the hippocampus Fzd-1 represents a potential target, as it is expressed at high levels and mediates the neuroprotective effect of Wnt3a against A β toxicity (Chacón *et al.*, 2008; Inestrosa & Arenas, 2010). In addition, the synaptic localization of Fzd-1 receptor in mammalian neurons was recently shown, and it was suggested to mediate the synaptic effects of the Wnt signaling pathway (Inestrosa & Arenas, 2010). We thus verified the expression of Fzd1–10 in developing DAergic neurons *in vitro*, and, in analogy to studies carried out in primary hippocampal neurons (Chacón *et al.*, 2008), we found that Fzd-1 receptors are expressed in mesencephalic DAT-expressing neurons in primary culture (L'Episcopo *et al.*, 2011b).

Midbrain DAergic neurons are exquisitely sensitive to oxidative stress and growth factor withdrawal, and significant changes indicative of mitochondrial dysfunction, oxidative stress and inflammation, proteasomal deficits and apoptosis have been identified in the human parkinsonian brain (Abou-Sleiman *et al.*, 2006). The ability of exogenous Wnt1 to afford TH neuroprotection *in vitro*, via β -catenin as a downstream effector, was of specific interest in the light of previous studies reporting the protective abilities of exogenous Wnts against a variety of cytotoxic insults, including SD-induced, A β -induced or tumor necrosis factor- α -induced apoptosis (see Chong and Maiese, 2007; Maiese *et al.*, 2008). Both β -catenin-dependent and β -catenin-independent mechanisms have been recognized to play a role, whereas the presence of Wnt antagonists has been largely linked to the occurrence of apoptosis (Maiese *et al.*, 2008). In our studies, by using specific antagonists for the canonical Wnt pathway, or silencing experiments with small interfering RNA to deplete β -catenin, as well as antisense oligonucleotides to knock down *Fzd-1*, we showed the failure of Wnt1 ligand to efficiently protect TH-positive neurons against SD-induced or 6-OHDA-

induced cytotoxicity, identifying for the first time a canonical Wnt1/Fzd-1/ β -catenin signaling pathway as a novel potential neuroprotective pathway (L'Episcopo *et al.*, 2011b). In different systems, the anti-apoptotic effects of Wnt signaling were shown to involve β -catenin/Tcf transcription-mediated pathways, the prevention of cytochrome *c* release from mitochondria, and the subsequent inhibition of caspase-9 activation, by activating nuclear factor- κ B, increasing the level of insulin-like growth factor, and inhibiting GSK-3 β (see Maiese *et al.*, 2008).

Interestingly, several previous studies defined active GSK-3 β as a critical mediator of neuronal apoptosis induced by oxidative stress caused by specific PD mimetics, including 6-OHDA, rotenone, and MPTP/MPP⁺ (Chen *et al.*, 2004; Wang *et al.*, 2007; Duka *et al.*, 2009; Petit-Paitel *et al.*, 2009). In fact, in the absence of Wnt activity, increased active GSK-3 β is known to phosphorylate β -catenin at serine or threonine residues of the N-terminal region, encouraging degradation of β -catenin through ubiquitination (Aberle *et al.*, 1997). It should be mentioned that GSK-3 β is activated by phosphorylation of Tyr216 located in the kinase domain, and inactivated by phosphorylation of the N-terminal Ser9. A potential role of active GSK3 β during cytotoxic injury was then verified in mesencephalic neurons *in vitro*, where we showed the ability of Wnt1 pretreatment to efficiently reverse SD-induced, 6-OHDA-induced and MPP⁺-induced GSK-3 β activation (i.e. increase in the amount of phosphorylated Ser216). Moreover, by the use of specific GSK-3 β inhibitors, it was possible to reverse the neurotoxin-induced TH-positive neuron demise, further showing the participation of the Wnt1/Fzd/ β -catenin signaling cascade in DAergic neuron death/survival (L'Episcopo *et al.*, 2011b). These findings supported the reports showing the ability of 6-OHDA, rotenone and MPTP/MPP⁺ to induce neuronal apoptosis in a GSK-3 β -dependent manner in SH-SY5Y cells, PC12 cells, cerebellar granule neurons, and primary mesencephalic neurons (Chen *et al.*, 2004; Wang *et al.*, 2007; Duka *et al.*, 2009; Petit-Paitel *et al.*, 2009), and further implicated β -catenin as a chief downstream component.

It is of note that two functional single-nucleotide polymorphisms have been reported in PD brains (see Kwok *et al.*, 2005; Duka *et al.*, 2009). In addition, post-mortem striata from patients diagnosed with PD revealed increased GSK-3 β activity as compared with age-matched controls (Duka *et al.*, 2009). It seems also important to recall that the Wnt pathway also uses protein kinase B (Akt) to promote cell survival (see Maiese *et al.*, 2008). Of particular interest is the fact that Akt inhibits the activity of GSK-3 β through phosphorylation of this protein to promote cell survival (Chong & Maiese, 2007; Nair & Olanow, 2008), thus anticipating the potential participation of Akt in Wnt1-induced DAergic neuroprotection.

All together, these results indicated the ability of Wnt1 to activate β -catenin via Fzd-1, and also via the inactivation of GSK-3 β , thereby blocking the phosphorylation of β -catenin and its proteosomal degradation. This, in turn, results in the stabilization of β -catenin in the cytoplasm, the consequent translocation of β -catenin in the nucleus, followed by β -catenin-mediated transcription of Wnt target genes involved in cell survival/protection. Together, these informations pointed to Wnt1/Fzd/ β -catenin transcriptional activation as a critical downstream pro-survival effector for mesencephalic DAergic neurons (Fig. 3). It is of note that stabilizing neuronal β -catenin was recently shown to render neurons 'anti-apoptotic' in cell cultures and transgenic mouse models (Li *et al.*, 2007), and recent *in vivo* studies have emphasized that downregulation of Wnt/ β -catenin signaling results in hippocampal neurodegeneration (Kim *et al.*, 2011).

In vivo findings

Mis-regulation of Wnt/ β -catenin signaling has been implicated in the pathology of Alzheimer's disease (De Ferrari *et al.*, 2003; Inestrosa & Toledo, 2008; Toledo *et al.*, 2008

and Refs therein). Wnt cascades have recently been linked to early-stage PD. Hence, downregulation of β -catenin in DAergic neurons of the substantia nigra (SN) (Cantuti-Castelvetri *et al.*, 2007) and upregulation of active GSK-3 β in the striatum (Duka *et al.*, 2009) have been reported in PD. Consistently, genetic screens have revealed GSK-3 β polymorphisms with altered transcription and splicing in PD (Kwok *et al.*, 2005). Other studies have revealed mutations in the Wnt/ β -catenin signaling-activated transcription factor Nurr1 (Sleiman *et al.*, 2009), the orphan nuclear receptor involved in DA neurodevelopment and neuroprotection (Kitagawa *et al.*, 2007). Gene expression profiling in progressively MPTP-lesioned macaques indicated downregulation of β -catenin and dysregulation of key components of Wnt signaling (Ohnuki *et al.*, 2010). In particular, mutations in *PARK8*, encoding LRRK2, which constitute a major cause of PD (Healy *et al.*, 2008), were linked to Wnt signaling (Berwick & Harvey, 2012a,b). On the other hand, parkin, an E3 ubiquitin ligase linked to familial PD, regulates β -catenin protein levels *in vivo* (Rawal *et al.*, 2009). Additionally, α -synuclein, a presynaptic protein that is causal in PD, contributes to GSK-3 β -catalyzed phosphorylation of tau (a protein linked to tauopathies, such as Alzheimer's disease) in rodent models of PD, and the importance of this kinase in the genesis and maintenance of neurodegenerative changes associated with PD was recently recognized (Duka *et al.*, 2009).

In our studies, we addressed the physiological relevance of the Fzd-1/ β -catenin pathway in the maintenance of adult midbrain DAergic neurons by investigating Fzd receptor and β -catenin expression, and the effect of blocking Fzd/ β -catenin signaling *in vivo*. Previous studies at the mRNA level, using *in situ* hybridization, indicated that *Fzd-1* is expressed in the cortex and in the hilus of the dentate gyrus (see Chacón *et al.*, 2008). In addition, Fzd-1 protein in the adult rodent brain was recently shown to be localized in the cortex and hippocampus (Chacón *et al.*, 2008). Our previous studies documented Fzd-1 receptor expression in the adult VM by real-time polymerase chain reaction and western blot analysis; however, it was not clear which cell type (neurons or glia) might harbor Fzd-1 receptor (L'Episcopo *et al.*, 2011a). We next showed that Fzd-1 receptors and β -catenin colocalize with TH-positive and DAT-positive cells, but not in GFAP-positive cells. Intracerebral infusion of Dkk1, or of inactivating lentiviral vectors expressing Wnt inhibitor/stimulator, was used as a tool to investigate the potential role of the canonical Wnt pathway in physiopathological conditions, including the regulation of hippocampal neurogenesis in intact mice (Lie *et al.*, 2005), or ischemia-induced striatal neurogenesis (Lei *et al.*, 2008), and estrogen-induced neuroprotection in global cerebral ischemia (Zhang *et al.*, 2009). In our hands, unilateral infusion of Dkk1 caused a time-dependent decrease in TH-positive neuron numbers in the ipsilateral-infused, but not in the contralateral-uninfused, SN, whereas unilateral infusion of saline within the SN did not change TH-positive neuron numbers in either the ipsilateral or the contralateral SNpc. That the Dkk1-induced loss of TH-positive neurons was caused by Wnt/ β -catenin antagonism, and not by a non-specific effect, was further demonstrated by at least two other lines of evidence: first, early and sharp downregulation of Fzd-1 receptor and β -catenin proteins was revealed in the ipsilateral as opposed to the contralateral SN; second, such β -catenin downregulation preceded and accompanied the tempo of TH-positive neuron degeneration, as revealed by FluoroJade-C staining, in the face of marked upregulation of active GSK-3 β , which showed, *in vivo*, a critical role for a paracrine canonical Wnt/ β -catenin tone as an endogenous pathway linked to the survival/maintenance of adult midbrain DAergic neurons. This acute decrease in cell number showed an initial return by 7 days post-Dkk1, suggesting the possible activation of repair mechanisms within the SN microenvironment. Interestingly, a marked increase in the number of reactive astrocytes within the ipsilateral Dkk1-infused SN was observed, with hypertrophic GFAP-positive cells abundantly covering the ipsilateral SN, as compared with the saline-infused SN, and longer time-course studies, which are in progress, will verify both astrocyte and TH-positive neuron responses with time. The fact that the preventive

activation of β -catenin signaling by pharmacological inhibition of active GSK-3 β efficiently promoted TH-positive neuron protection in either the Dkk1-lesioned or the MPTP-lesioned SN thus implied a causative link between the interruption of Wnt signaling and the acute degeneration of SNpc TH-positive neurons. Together with the dysregulation of Wnt signaling in the VM of aging mice being associated with downregulation of β -catenin upon MPTP insult and lack of TH-positive cell recovery (L'Episcopo *et al.*, 2011a; Marchetti *et al.*, 2012), the present findings further provides a new mechanistic insight into the regulation of the pro-survival/anti-apoptotic processes regulating the life and death of adult midbrain DA neurons (Fig. 3).

Activated astrocytes of the VM and Wnt/ β -catenin signaling are key players in promoting adult midbrain neurogenesis *in vitro*: role of a specific inflammatory milieu

Reactive astrocytes express receptors for growth factors, cytokines, chemokines, and hormones, and produce a wide array of neurotrophic and neuroprotective mediators together with those produced by microglia (L'Episcopo *et al.*, 2010a; Sofroniew & Vinters, 2010). In addition, astrocytes are known to release various region-specific signaling molecules, such as Shh and Wnts, which may interact with each other to dictate neurogenic behavior in the adult CNS (Alvarez-Builla *et al.*, 2001; Song *et al.*, 2002; Lie *et al.*, 2005; Barkho *et al.*, 2006; Jiao & Chen, 2008). Indeed, canonical β -catenin-regulatory mechanisms are known to be required for activation of adult neurogenesis both *in vitro* and *in vivo* (Lie *et al.*, 2005; Yu *et al.*, 2006; Adachi *et al.*, 2007). Astrocytes have pivotal roles in glia–neuron interactions and in defining the stem cell niche. Hence, embryonic day 13.5 VM astrocytes, but not cortex astrocytes, express Wnt1 and Wnt5a and different DA-specific transcription factors, such as Pax-2, En-1, and Otx-2, and increase the differentiation of VM embryonic precursors into TH-positive neurons *in vitro*, suggesting that VM astrocytes constitute a part of the neurogenic niche that plays a key role in VM DA neurogenesis (Wagner *et al.*, 1999; Castelo-Branco *et al.*, 2006). In our *ex vivo* and *in vitro* studies, we found that MPTP injury and certain proinflammatory chemokines induce the expression of Wnt1 in astrocytes of the VM, indicating not only region specificity but also a defined inflammatory milieu in the modulation of Wnt1 induction in astrocytes (L'Episcopo *et al.*, 2011a).

By culturing NPCs derived from the adult midbrain/hindbrain (MB) (Hermann *et al.*, 2006), using the neurosphere culturing technique, similar to that used for propagation of NPCs derived from the SVZ (Pluchino *et al.*, 2003, 2005), we found that the phenotype of these MB NPCs was similar to that of those derived from the SVZ (Papanikolaou *et al.*, 2008). Accordingly, nestin, a marker for precursor cells in the adult brain, and bromodeoxyuridine, a marker for cell proliferation, were expressed during *in vitro* clonal expansion of NPCs from the adult MB (L'Episcopo *et al.*, 2011a). In NPC cultures from either the MB or the the SVZ grown in N2 medium, and in the absence of exogenous factors, only rare Tuj1-positive neurons could be detected after only 3 days *in vitro*, as opposed to direct co-culture of NPCs with astrocytes, which produced a significant increase in the number of Tuj1-positive neuroblasts, in accordance with earlier studies carried out with adult SVZ precursors cultured on type 1 astrocyte monolayers (Lim & Alvarez-Buylla, 1999). Our studies further indicated the ability of VM astrocytes to promote neurogenesis from NPCs derived from the adult midbrain. In addition, chemokine-activated astrocytes significantly increased the proportion of new neurons as compared with co-culture with untreated astrocytes, indicating the ability of a specific inflammatory milieu to sharply increase the neurogenic potential of NPCs. By contrast, blocking Wnt/Fzd signaling with the Wnt antagonist Dkk-1, applied to NPCs just prior to starting co-culture with astrocytes, resulted in significant reductions in both proliferative and neuron differentiation potential, thus suggesting that Wnt/ β -catenin

signaling was required for activated astrocyte-induced neurogenesis from multipotent NPCs of the MB. Finally, when NPCs isolated from the MB were co-cultured with activated VM astrocytes in differentiating conditions for 7 days *in vitro*, we found that a certain, albeit small, proportion co-expressed Tuj1 and TH, and extended long and branched TH-positive processes with numerous DAergic varicosities. In sharp contrast, Dkk1 treatment induced a significant reduction in the proportion of TH-positive cells among the total number of Tuj1-positive cells (see L'Episcopo *et al.*, 2011a).

These findings are in line with previous studies suggesting that IL-1 β and IL-6 contribute to astroglial modulation of adult neurogenesis (Barkho *et al.*, 2006). Additionally, certain specifically activated microglial cells can induce neural cell renewal in the adult CNS (Butovsky *et al.*, 2006). Hence, microglia pretreated with IL-4 or IFN- γ induced neurogenesis and oligodendrogenesis in NPCs derived from the SVZ, whereas LPS-pretreated microglial cells blocked both processes in adult NPCs (Butovsky *et al.*, 2006), in line with reports that inflammation associated with LPS blocks adult neurogenesis (Butovsky *et al.*, 2006; Ekdahl *et al.*, 2009; Schwartz *et al.*, 2009). Therefore, according to a certain inflammatory microenvironment, factors derived from astrocytes and chemically activated astrocytes, including Wnt/ β -catenin signaling, probably contribute to the neuronal and dopaminergic differentiation of adult MB progenitors *in vitro*, suggesting a critical role for a specific inflammatory milieu in dictating the promotion or inhibition of adult neurogenesis, probably via Wnt (L'Episcopo *et al.*, 2011a,b,c, 2012a,b, 2013; Marchetti & Pluchino, 2013).

Our results, together with data in the literature, suggest a model in which adult VM astrocytes may, under specific controlled conditions, re-express region-specific factors, including Wnt1, contributing to the regulation of diverse aspects of DAergic neuron homeostasis in the injured VM, including amelioration of the impaired nigral milieu, reducing the amount of DAergic neuron death, and/or enhancing the survival, expansion and differentiation of DA progenitors. Although the presence of DA neurogenesis within the SN, either under physiological conditions or in PD animal models, is currently debated (Borta & Holinger, 2007; Hermann & Storch, 2008; Winner *et al.*, 2011), the presence of multipotent clonogenic neural stem cells in the adult mouse MB with functional neurogenic and DAergic potential *in vitro* was recently reported (see Hermann *et al.*, 2006, 2009; Hermann & Storch, 2008; for review), and further studies are clearly needed to determine whether: (i) NPCs might reside in the MB *in vivo*, during MPTP-induced nigrostriatal injury; (ii) they can be activated by endogenous astrocyte-derived factors and Wnt/ β -catenin signaling activators; and (iii) by manipulating the local microenvironment, it might be possible to boost such endogenous mechanisms to induce the promotion of a DAergic neuronal phenotype in the adult MB (Ourednik *et al.*, 2009; L'Episcopo *et al.*, 2012a,b, 2013).

The inflammatory milieu governs the plasticity of NPCs of the adult SVZ in response to MPTP: involvement of Wnt/ β -catenin signaling

The generation of new neurons in the brain SVZ continues throughout adult life, and may contribute to endogenous repair mechanisms after brain damage and/or disease (Curtis *et al.*, 2007; Winner *et al.*, 2011; Curtis *et al.*, 2012). In PD, neurogenesis is impaired. The decreased proliferation of NPCs in the SVZ of human PD brains and in non-human primate and rodent MPTP and 6-OHDA models has been attributed to the loss of the neurotransmitter DA (Baker *et al.*, 2004; Hoglinger *et al.*, 2004; Freundlieb *et al.*, 2006; Borta & Holinger, 2007; O'Keeffe *et al.*, 2009; Winner *et al.*, 2011; Hoglinger *et al.*, 2012). Other studies have proposed DA-independent effects of MPTP, but the cellular contributors and signaling pathways through which MPTP may exert its direct action on SVZ cells have not yet been identified (Hu *et al.*, 2006; Shibui *et al.*, 2009).

In our studies, we decided to focus on glia, and dissected the influence of MPTP, astrocytes and microglia on the SVZ response to PD: first, because NPCs are in intimate contact with the surrounding glia, forming the so-called SVZ ‘stem cell niche’, which can influence NPC proliferation and differentiation (Lim & Alvarez-Buylla, 1999; Alvarez-Buylla *et al.*, 2001; Song *et al.*, 2002; Kazanis, 2009); second, because, in the striatum bordering the SVZ of PD experimental models, astrocytes and microglia exhibit remarkable morphological and functional changes, as reported in the previous sections; and third, because although the inflammatory modulatory role on adult neurogenesis was previously reported (see Ekdahl *et al.*, 2009), whether inflammatory glial cells together with their signaling pathways might regulate neurogenesis within the SVZ niche of MPTP-injured mice was ill-defined.

Hence, using *in vivo*, *ex vivo* and *in vitro* experiments, different glia–NPC co-culture paradigms, and pharmacological antagonism/RNA silencing experiments coupled with functional studies, we provided evidence supporting an active and concerted role of reactive astrocytes and microglia in the remodeling of the SVZ niche upon MPTP/MPP⁺ injury, regulated, at least in part, by crosstalk between inflammation and Wnt/ β -catenin signaling cascades, with potential consequences for DAergic neuroprotection and self-repair (L’Episcopo *et al.*, 2012a). We thus showed that, in addition to DA, the PD neurotoxin MPTP/MPP⁺, directly and/or in conjunction with astrocyte-derived and microglial-derived mediators, may contribute to regulate SVZ plasticity in this PD mouse model. We found that the unfavorable conditions of the SVZ niche during the early degenerative phase resulting from decreased DAergic innervation and MPTP/MPP⁺-dependent striatal oxidative and nitrosative status probably inhibits the survival and/or the expansion/differentiation and/or migration of endogenous NPCs, at least in part via disruption of β -catenin-mediated Wnt signaling in the SVZ, characterized by increased active GSK-3 β associated with β -catenin depletion, and impaired survival/proliferation and neuroblast formation in NPCs of the SVZ (L’Episcopo *et al.*, 2012a,b), in line with studies of Sirerol-Piquer *et al.* (2011), showing that GSK-3 β overexpression induces neuronal death and depletion of the neurogenic niches in the dentate gyrus.

Interestingly, with time, a shift towards a less reactive microglial ‘harmful’ phenotype probably permitted the mitigation of the niche microenvironment, the return of astrocyte ‘beneficial’ expression of growth factors and neurogenic signals, including Wnts, and progressive striatal re-innervation, probably contributing to NPC recovery (L’Episcopo *et al.*, 2012a). In keeping with these findings, exogenous activation of β -catenin signaling or pharmacological mitigation of microglia over-activation upregulated β -catenin in the SVZ and successfully rescued NPC proliferation and neuroblast formation (L’Episcopo *et al.*, 2012a) (Fig. 4).

Concluding remarks and future directions

We have herein summarized different studies and presented several lines of evidence suggesting that activation of the Wnt/Fzd-1/ β -catenin pathway might play a determinant role in the maintenance of a normal complement of TH-positive neurons in the adult midbrain. Fascinatingly, Wnt1-induced neuroprotection is closely integrated with the astroglial response to oxidative stress and inflammation upon injury, and requires stabilization of Fzd-1 receptor and β -catenin, whose expression probably underlies the observed neuroprotection, to convey pro-survival signals to the nucleus. Then, microglia–astrocyte crosstalk may influence the expression/release of Wnt modulators *in vivo*, thereby modulating Wnt signaling components. Although the Wnt pathway itself is protective in different tissues, receptor context and a particular inflammatory setting may influence the regulation of canonical Wnt signaling by agonist or antagonists, suggesting that a specific receptor component coupled to a specific ligand in the absence or the presence of

endogenous inhibitors/activators finally mediates the tissue-specific/cell-specific response. Accordingly, manipulating Wnt expression in normal and injured VM glia might provide an insight into the role of Wnts in midbrain TH-positive neurorecovery (Marchetti & Pluchino, 2013).

It should also be underlined that upstream and downstream modulation of the astroglial Wnt1/Fzd-1/ β -catenin pathway may tip the balance between apoptosis and the programming of cell survival/neurorescue in these models (Fig. 3), and an in-depth understanding of the molecular pathways and their crosstalk underlying midbrain neuroprotection will be crucial to identify new avenues for pharmacological and cell replacement therapies for PD (Marchetti & Pluchino, 2013).

Given that Wnt/ β -catenin signaling controls the expression of a variety of target genes, mis-regulation of this signaling cascade may be involved in various diseases, particularly neurodegenerative disorders associated with impaired neurogenesis (He & Shen, 2009; Inestrosa & Arenas, 2010; L'Episcopo *et al.*, 2011a,b,c; Marchetti & Pluchino, 2013). Consistently, the signaling mechanisms involved in neuronal and NPC impairment observed in PD appear to target the Wnt/ β -catenin signaling pathway (L'Episcopo *et al.*, 2011a,b, 2012a,b).

As far as midbrain DAergic neurogenesis is concerned, further studies are actually in progress: (i) to verify whether the observed *in vitro* events may also occur *in vivo* in the MPTP-injured MB; (ii) to ascertain the presence of quiescent and/or activated DAergic NPCs; and (iii) to define the conditions that limit their progression towards the DAergic phenotype as opposed to those promoting acquisition of the mature TH-positive phenotype. Because restoration of tissue integrity and homeostasis is the ultimate goal of next-generation therapies, the reviewed evidence suggests new directions for therapeutic strategies, among which targeted pharmacological interventions by changing environmental signals *in situ* may have important implications for the design of novel treatments for neurodegenerative diseases (Marchetti & Pluchino, 2013). An understanding of the intracellular signaling networks that dictate the intrinsic beneficial Wnt response is critical for the identification of new potential therapeutic targets and the development of pharmacological and cellular approaches for neurodegenerative diseases, including PD (Cusimano *et al.*, 2012; Hoglinger *et al.*, 2012; L'Episcopo *et al.*, 2012b; Ribeiro *et al.*, 2012; Rueger *et al.*, 2012; Sakata *et al.*, 2012; Shruster *et al.*, 2012; Wallenquist *et al.*, 2012; Marchetti & Pluchino, 2013).

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Abbreviations

CNS	central nervous system
DA	dopamine
DAergic	dopaminergic

DAT	DA transporter
Dkk	Dkkopf
Fzd	frizzled
GFAP	glial fibrillary acid protein
GSK-3β	glycogen synthase kinase-3 β
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
Lrp	low-density lipoprotein receptor-related protein
LRRK2	leucine-rich repeat kinase 2
MB	midbrain/hindbrain
MPP⁺	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NPC	stem/progenitor cell
6-OHDA	6-hydroxydopamine
PCP	planar cell polarity
PD	Parkinson's disease
SD	serum deprivation
SN	substantia nigra
SNpc	substantia nigra pars compacta
SVZ	subventricular zone
TH	tyrosine hydroxylase
VM	ventral midbrain
Wnt	wingless-type mouse mammary tumor virus integration site

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Fig. 1.

A schematic representation of glia-mediated detrimental and beneficial inflammatory pathways in PD. Injury to nigrostriatal DAergic neurons, as a result of specific chemical insults (e.g. MPTP), and/or a combination of genetic and environmental factors, leads to astrocyte and microglia activation. MPTP is metabolized by astrocytes to MPP⁺, which is concentrated in DAergic neurons. Microglia-derived cytotoxic mediators further exacerbate inflammation and oxidative stress. Astrocytes and microglia can protect neurons by scavenging radicals and glutamate, by harboring receptors for endogenous anti-inflammatory molecules, by providing energy support, trophic factors, and 'protective' cytokines, and by stimulating repair/neurogenesis by expressing neurogenic factors, including Wnt1. Under conditions of chronic inflammatory stress, activated astrocytes and microglia may become dysfunctional and over-express a variety of cytotoxic mediators, eventually resulting in DAergic neuron demise via a synergistic action of reactive oxygen species (ROS) and reactive nitrogen species (RNS), with generation of the potent cytotoxic peroxynitrite (ONOO⁻). GF, Growth factor; MPO, myeloperoxidase-producing reactive species; NOX, nitric oxide-producing reactive species. Modified from L'Episcopo *et al.* (2010a).

Fig. 2.

Schematic illustration of Wnt1/β-catenin signaling as a key player in neuroprotection via DAergic neuron–astrocyte crosstalk and promoting neurogenesis via neuroprogenitor–astrocyte crosstalk. This is a simplified scheme linking reactive astrocytes and Wnt/β-catenin signaling to nigrostriatal injury and repair in the MPTP mouse model of PD. Astrocyte-derived Wnts, particularly Wnt1, control the integrity of DA neurons via blockade of GSK-3β-induced phosphorylation and proteosomal degradation of the neuronal pool of β-catenin. Activation of Wnt/β-catenin signaling can also promote neurogenesis from adult midbrain progenitors. Neurotoxic injury or increased oxidative load as a result of aging may antagonize Wnt/β-catenin signaling in DAergic neurons by upregulating active GSK-3β, leading to β-catenin degradation and increased DAergic neuron vulnerability. Neuronal injury also triggers reactive astrocyte expression of a panel of growth and neurotrophic factors, anti-oxidant and neuroprotective mechanisms, among which astrocyte Wnt1 may function as a vital component of the self-protective machinery of DAergic neurons, shifting the balance towards the programming of cell survival/neurorescue on the one hand, and promoting neurogenesis on the other. From L'Episcopo *et al.* (2011a) with permission.

Fig. 3.

Schematic illustration of Wnt1/Fzd-1/β-catenin signaling as a candidate regulatory circuit controlling mesencephalic DAergic neuron–astrocyte crosstalk. In the intact unlesioned midbrain, crosstalk between astrocytes and DA neurons represents a cardinal neuroprotective mechanism against inflammation, oxidative stress, and growth factor deprivation. This function appears to be of paramount importance for DAergic neurons, which are known to be particularly vulnerable to increased inflammation and oxidative stress. Astrocyte-derived Wnts, particularly Wnt1, via activation of Fzd-1 receptors, may contribute to maintain the integrity of DAergic neurons via blockade of GSK-3β-induced phosphorylation (P) and proteosomal degradation of the neuronal pool of β-catenin. Stabilized β-catenin can translocate into the nucleus, associate with a family of transcription factors, and regulate the expression of Wnt target genes involved in DAergic neuron survival. β-Catenin may also function as a pivotal defense molecule against oxidative stress, and can act as a co-activator for several nuclear receptors involved in the maintenance/protection of DA neurons. Crosstalk with upstream survival pathways converging to β-catenin stabilization can also be envisaged. Neurotoxic injury or increased oxidative load as a result of aging may antagonize Wnt/β-catenin signaling in DA ergich neurons by upregulating active GSK-3β, leading to β-catenin degradation and increased DAergic neuron vulnerability. Neuronal injury also triggers reactive astrocyte expression of a panel of growth and neurotrophic factors, anti-oxidant and neuroprotective mechanisms, among which astrocyte Wnt1 may function, via Fzd-1 receptors, as a vital component of the self-protective machinery of DAergic neurons, shifting the balance towards the programming of cell survival/neurorescue. From L'Episcopo *et al.* (2011b), with permission.

Fig. 4.

Crosstalk between inflammatory and Wnt/ β -catenin signaling pathways in MPTP-induced SVZ plasticity. This is a simplified scheme summarizing MPTP-induced neuroinflammation and SVZ plasticity via modulation of Wnt/ β -catenin signaling. During the early degeneration phase of MPTP toxicity, hyper-activated microglia contributes to the impairment of SVZ neurogenesis at different levels. By increasing oxidative and nitrosative stress and in synergy with MPTP/MPP⁺ direct toxicity, microglia-derived mediators [plasma membrane NADPH oxidase (PHOX)-derived reactive oxygen species (ROS) and iNOS-derived nitric oxide (NO) and peroxynitrite] may act as a molecular switch for cell signaling pathways that are critically involved in the physiological control of NPC homeostasis, with harmful consequences for astrocyte and NPC physiology, at least in part through GSK-3 β activation, followed by phosphorylation and consequent degradation of β -catenin. By contrast, pharmacological mitigation of inflammation and oxidative stress with Apo, L-Nil or HCT1026 upregulate β -catenin and successfully rescue NPC proliferation and neuroblast formation, a process associated with striatal DAergic neuroprotection, with further positive modulation of SVZ proliferation via DA subtype 2 receptor (D2R)-activated mechanisms. The role of astrocyte–microglia interactions in the plasticity of the SVZ response to MPTP is exemplified by the astrocyte’s ability to overcome microglial inhibitory effects, also via crosstalk with Wnt/ β -catenin signaling. EGF, epidermal growth factor; GF, growth factor; RNS, reactive nitrogen species. From L’Episcopo *et al.* with permission (2012a,b).