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Melatonin prevents dopaminergic cell loss induced by lentiviral vectors expressing A30P mutant alpha-synuclein

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Summary. Two hallmarks of Parkinson's disease (PD) are dopaminergic cell loss and the presence of cytoplasmic inclusions (Lewy bodies). Different point mutations in alpha-synuclein, the main constituent of Lewy bodies, have been identified in familial PD. Alpha-synuclein also constitutes one of the main components of Lewy bodies in sporadic cases of PD. Moreover, oxidant stress and generation of free radicals from both mitochondrial impairment and dopamine metabolism are considered to play critical roles in PD etiopathogenesis. Melatonin, a known potent antioxidant secreted by the pineal gland, may protect against the effect of several Parkinsonogenic compounds that are associated with progressive impairment of mitochondrial function and increased oxidative damage. However, the neuroprotective effect of melatonin has never been tested in the newly available genetic models of PD based on the viral expression of mutated alpha-synuclein. Lentiviral vectors encoding A30P mutant human alpha-synuclein (lenti-A30P) were stereotactically injected into the right substantia nigra of adult male Sprague-Dawley rats and neuroprotection was examined by administration of melatonin or vehicle from two days before nigral administration of lenti-A30P until eight weeks after injection. It was found that lenti-A30P induced a significant TH^+ cell-loss both in the medial and lateral substantia nigra versus the contrallateral side injected with lenti-eGFP. However, melatonin administration showed a total neuroprotective effect in both regions of the substantia nigra. In conclusion, the data here show that melatonin is neuroprotective against mutant alpha-synuclein-induced injury in the *substantia nigra*.

Key words: Lentiviral vectors, Alpha-synuclein (A30P), Parkinson's disease, Melatonin, Neuroprotection

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Lang and Lozano, 1998). The causes of cell death in PD are still poorly understood, but a defect in mitochondrial oxidative phosphorylation and enhanced oxidative stress has been proposed (Ebadi et al., 2005). One role of alpha-synuclein (alpha-syn) in PD pathogenesis is demonstrated by cases of familial PD resulting from three point mutations (A53T, A30P and E46K) in the alpha-syn gene (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004) or overexpression of alpha-syn, as well as by the observation that SN neurons in mice with alpha-syn deletion are protected against the parkinsonian neurotoxins MPTP and 6-OHDA (Alvarez-Fischer et al., 2008; Dauer et al., 2002). Later, alpha-synuclein was identified as the major component of Lewy bodies and Lewy neurites, the neuropathological hallmarks of PD. The mechanisms by which alpha-

synuclein toxicity is mediated are not fully understood, but are thought to include free radical mediated damage, mitochondrial dysfunction and the promotion of cell death by apoptosis (Shapira, 2006). Moreover, alpha-syn has been directly involved in toxin-induced forms of parkinsonism (Moore et al., 2005). These findings have prompted the development of animal models based on the overexpression of human alpha-syn.

Melatonin (N-acetyl-5-methoxytryptamine), an indoleamine, is a highly conserved anti-oxidant molecule secreted from the pineal gland, gastrointestinal tract, ovaries, testes, bone marrow and eye lenses (Esposito and Cuzzocrea, 2010). It scavenges hydroxyl, carbonate and various organic radicals, peroxynitrite and other reactive nitrogen species (Bonnefont-Rousselot et al., 2011; Galano et al., 2011). It is also known to control the transcription, translation and catalytic activities of the preventive antioxidants, including glutathione peroxidase, superoxide dismutase and catalase (Barlow-Walden et al., 1995; Pablos et al., 1995; Rodriguez et al., 2004). The decline in melatonin production in aged individuals has been suggested as one of the primary contributing factors for the development of age-associated neurodegenerative diseases (Srinivasan et al., 2005) and experimental studies using 1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine (MPTP), 6-hydroxy-dopamine (6-OHDA), rotenone, maneb, methamphetamine/amphetamine, and paraquat have shown an enormous potential of melatonin in amelioration of toxin-induced oxidative stress and the symptomatic features of PD (Absi et al., 2000; Sharma et al., 2006; Sae-Ung et al., 2012; Singhal et al., 2011, 2012). However, there are conflicting reports suggesting that as melatonin elicits significant functional changes in the nigrostriatal dopamine system which may affect the entry of some neurotoxins into cells, it does not provide neuroprotection in these models (Itzhak et al., 1998; van der Schyf et al., 2000; Morgan and Nelson, 2001; Tapias et al., 2010).

The neuroprotective effect of melatonin has never been tested in the newly available genetic models of PD based on the viral expression of mutated (A30P)-synuclein. Brain delivery of human alpha-synuclein with viral vectors is an ideal model for assessing protection, because it does not rely on the dopamine transporter uptake to exert neurotoxicity. As oxidant stress is one of the intermediary risk factors that promote the degeneration of DA neurons, and the mechanism of synuclein cell entry is due to SN viral transduction, it was decided to study here whether the therapeutic potential of melatonin would also apply to synuclein-induced PD-models, rather than be limited to neurotoxins.

Materials and methods

Animals, stereotactic surgery and treatments

Two groups of seven male adult Sprague-Dawley (250-300 g) rats housed with free access to food and water at 12:12 dark-light cycle, 22±1°C temperature-controlled room, and 50-70% humidity were used. All animal experiments were approved by the bioethical committee of the University of La Laguna. After anesthesia, the animals were stereotactically injected with lentiviral vectors (LV) encoding A30P mutant human alpha-synuclein (lenti-A30P) into the right substantia nigra (SN). LV overexpressing the green fluorescent protein (lenti-eGFP) was injected into the left SN to determine the efficiency of transgene expression in the dopaminergic neurons of the rat SN. The coordinates used were: SN (target AP 3.0, L 2.0, DV 7.0, from lambda). Eight μl of concentrated vector (10^8-10^9 pg p24/ml) supplemented with 4 μg/ml polybrene at a rate of 0.25 μl/min were injected. After injection, the needle was left in place for an additional 10 minutes.

Possible neuroprotection was evaluated by i.p. injection, once a day, of melatonin (Sigma) (10 mg/kg) or vehicle (saline in ethanol 0.5%) from two days before nigral administration of lenti-A30P/lenti-eGFP until eight weeks after injection. Melatonin was freshly prepared each time and protected from light.

Lentiviral vector construction and production

The cDNA encoding human alpha-syn (A30P), obtained from Dr. Kelly Conway (Center for Neurologic Diseases, Boston, Mass.) was cloned into lentiviral pHR'-derived transfer plasmid containing a central polypurine tract sequence, the SIN-18 deletion, and the woodchuck hepatitis posttranscriptional regulatory element (Follenzi et al., 2000; Zennou et al., 2000; Zufferey et al., 1998, 1999; Baekelandt et al., 2002). The lentiviral vectors were produced as previously described (Baekelandt et al., 2000). A second generation attenuated packaging plasmid pCMVR8.91 lacking vif, vpr, vpu and nef genes was used in this study (Zufferey et al., 1997).

Histology

The animals were sacrificed eight weeks after melatonin administration. Tyrosine hydroxylase immunostaining was performed to visualize dopaminergic neurons in the SN. The survival of dopaminergic neurons in the SN pars compacta was investigated by counting the number of dopaminergic neurons in the SN.

The rats were deeply anaesthetized with pentobarbital and transcardially perfused with saline followed by ice-cold 4% paraformaldehyde in PBS for 15 min to assess lentiviral transduction. The brain was postfixed overnight in the same fixing solution, and cryopreserved in 30% sucrose in PBS. Thirty μm-thick coronal brain sections were cut with a freezing-microtome and stored at 4°C. First, sections were treated with 3% hydrogen peroxide and incubated overnight with the primary mouse anti-tyrosine hydroxylase

(Sigma, 1:12000), and rabbit anti-GFP (SySy, 1:1500) in 4% normal goat serum. The sections were then incubated in biotinylated goat anti-mouse and anti-rabbit secondary antibody respectively, followed by incubation with Strept-ABC-HRP complex (DAKO). Detection was with diaminobenzidine (DAB) using H_2O_2 as a substrate.

Cell Counting

Cells that were clearly stained for TH with a visible nucleus were counted. The SN was divided in two different regions: medial and lateral (in each one SN region a 134101, 63 um^2 cell-counting square per section was used), in every fifth 30 μm section, to determine the number of TH positive cells. All TH^+ cells were counted at a magnification of 200x by two independent observers, also using a microscope DM 4000 B (Leica) and the software Qwin V3 (Leica).

Statistical analysis

Statistical analysis was performed using the Statistica software package (StatSoft, Inc.). Results are expressed as means±SEM. Analysis of variance with post hoc Scheffé's test was used for intergroup comparisons.

Results

Lentiviral vectors to overexpress a clinical mutant of alpha-syn, A30P, in the rat right substantia nigra, were used in this study. Moreover, the ability of melatonin to prevent A30P-syn toxicity *in vivo* was evaluated.

Substantial eGFP-positive cells were evident in the SN on the eGFP injected side, and along the needle track at the site of injection (Fig. 1A). The eGFP carrying lentivirus had no effect on the number of TH expressing cells (Fig. 1B,C).The transduced cells displayed a predominantly neuronal morphology, confirming the strong tropism of LV for neuronal cells (Blömer et al., 1997). The next step was to determine whether lentiviral-mediated overexpression of mutant alpha-syn (lenti-A30P) induced nigral neuron degeneration eight weeks after viral injection. The results show a reduction of TH-positive neurons which is appreciable in both the medial and lateral regions of the SN (Fig. 1B,D) when compared to the lenti-eGFP injected contralateral side (Fig. 1B,C). This expression loss was restricted to the substantia nigra region in all the injected animals (Fig. 1B,D). Nissl staining confirmed the reduction of dopaminergic neurons in the SN of animals expressing the A30P mutant (Fig. 1F) compared to the SN of animals expressing eGFP (Fig. 1E). This neurodegeneration is not due to physical trauma since the number of dopaminergic neurons after injection with LV encoding eGFP did not differ, as mentioned above, between the injected and non-injected hemisphere. A stereological quantification of the number of dopaminergic neurons was also carried out in the medial and lateral SN to evaluate the lesion degree. A clear reduction of dopaminergic neurons in the injected hemisphere that varied between 20 and 40% with respect to the side injected with lenti-eGFP was observed in the lenti-A30P injected rats (Fig. 2).

A histological analysis was performed in the brains of rats injected with lenti-eGFP or lenti-A30P to evaluate the neuroprotective effect of melatonin. The results confirmed that the density of TH positive cells in the medial and lateral SN was similar in both groups (Fig. 1 G,H). Quantification of the percentage of nigral TH-IR neuron loss compared to the contralateral eGFP injected side showed that melatonin significantly prevents TH-IR cell loss in both the right medial and lateral SN (Fig. 2). Interestingly, the results here show that melatonin treatment rescued TH-IR neurons from A30P alpha-synuclein neurotoxicity.

Discussion

Along with their therapeutic potential for gene therapy of CNS diseases, LV mediates stable and loco-regional overexpression of disease-associated genes in the adult brain.

Neurodegeneration is a crucial feature of any *in vivo* model for PD. In contrast to alpha-synuclein transgene mouse models, expression of human alpha-syn with lentiviral or adeno-associated viral vectors induces a progressive degeneration of dopamine neurons in the substantia nigra (Lo Bianco et al., 2002; Klein et al., 2002; Kirik et al., 2002, 2003; Lauwers et al., 2003, 2007). Firstly, an LV was used in this study to overexpress a clinical mutant of alpha-syn, A30P, in the rat SN. According to previous data, the results here show that LV-mediated overexpression of human-alpha-syn in the SN reduces the viability of dopaminergic cells (Figs. 1, 2). Between 40% and 50% of dopaminergic neurons identified by immunohistochemistry for tyrosine hydroxylase are transduced in the SN of mice and rats injected with LV (Déglon et al., 2000; Bensadoun et al., 2000). The percentage of dopaminergic cell death in the rats in this study (20% in medial SN and 40% in lateral SN) (Fig. 2) is lower than that reported by other groups. Differences between studies may relate to the use of AAV vectors (Kirik et al., 2002; Klein et al., 2002) versus LV or the use of different promoters (PGK versus CMV) (Lo Bianco et al., 2002). However, the extent of neuronal loss reached 40%, a high proportion considering the limited degree of infection (Lo Bianco et al., 2004). An interesting fact in PD is that not all midbrain DA neurons show the same susceptibility to degeneration. Neurons in the ventrolateral and caudal regions of the SN (SNcv) are more vulnerable than those in the rostromedial and dorsal region (SNrm) (German et al., 1989; Damier et al., 1999). The higher percentage of alpha-syn A30P mediated-dopaminergic cell loss obtained in the lateral SN compared to medial SN is in agreement with that reported for PD.

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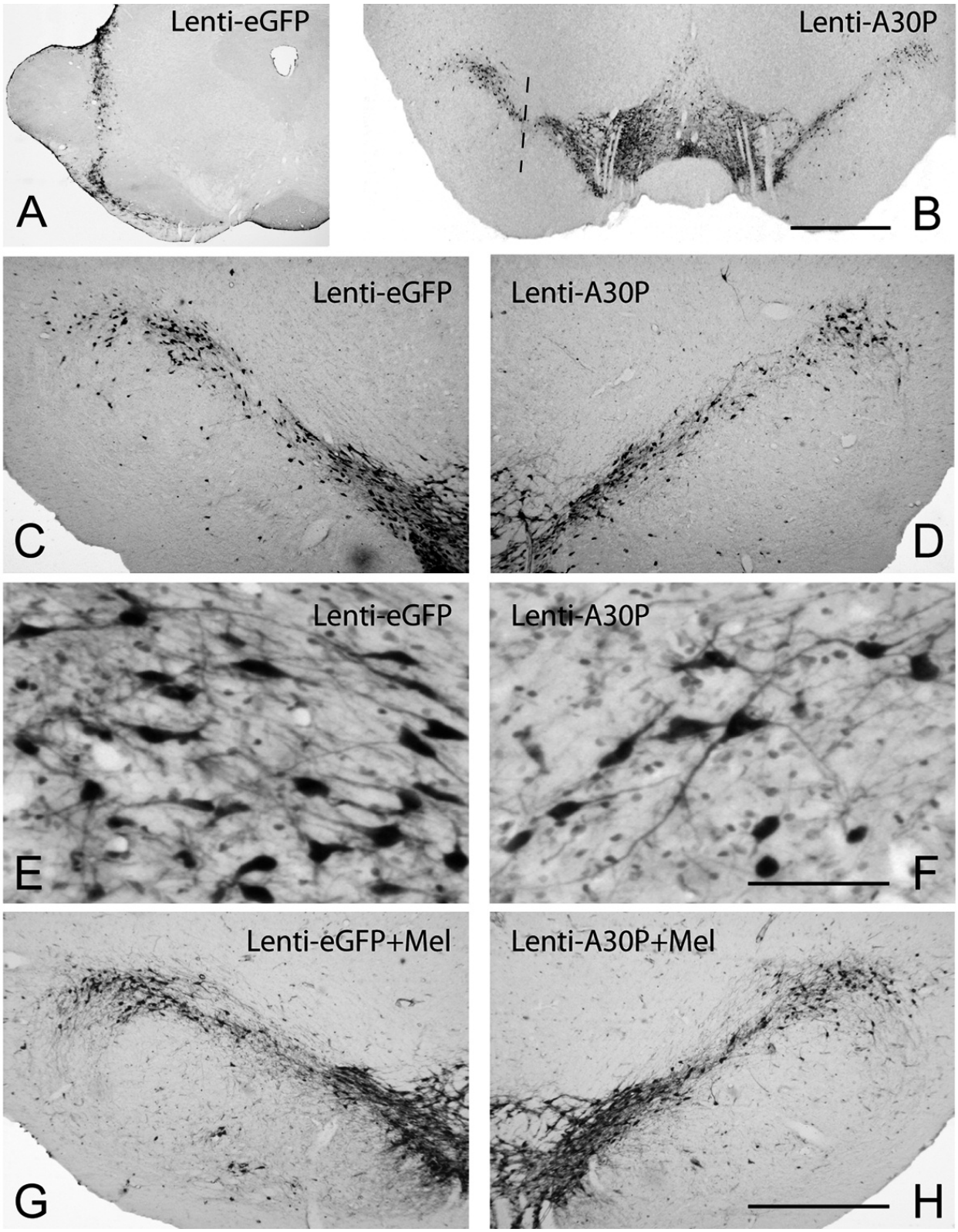

Fig. 1. Immunohistochemistry evidence showing SN cell loss induced by LV encoding A30P mutant human alpha-synuclein (lenti-A30P) and the neuroprotective effect of melatonin. **A.** GFP-immunohistochemistry after injection of lenti-eGFP into the left SN. **B.** TH-immunohistochemistry showing a panoramic view of the ventral midbrain after injections of lenti-A30P and lenti-eGFP into the right and left SN respectively. **C and D.** TH-immunohistochemistry at higher magnification with respect to **B**. TH immunohistochemistry and Nissl staining of the SN after injections of lenti-eGFP **(E)** and lenti-A30P **(F)** into the left and right SN respectively. TH-immunohistochemistry in the SN of lenti-eGFP **(G)** AND LENTI-A30P **(H)** injected animals and melatonin pre-treated. A, x 20; Scale bar: B, 1.2 mm; F (for E, F), 50 μm; H (for C, D, G, H), 700 μm.

important to point out that the possible involvement of oxidative stress as an etiological factor of PD is further supported by studies with specific neurotoxins which are potent inducers of Parkinsonism in humans and animals (Jenner, 1992; Coyle and Puttfarchen, 1993; Onyango, 2008; Singhal et al., 2012). The mechanisms by which alpha-synuclein toxicity is mediated are not fully understood, but are thought to include free radical mediated damage, mitochondrial dysfunction and the promotion of cell death by apoptosis (Schapira, 2006). Moreover, studies with cell and animal models of PD reveal an oxidative stress and alpha-synuclein aggregation induced by different toxins (Betarbet et al., 2000; Sherer et al., 2002; Uversky, 2004; Bove et al., 2005; Betarbet et al., 2006; Klongpanichapak et al., 2007, 2008; Ishido, 2007; Lin et al., 2007; Cannon et al., 2009; Chau et al., 2010)

The results here support previous data which have shown that over-expression of alpha-syn, and especially PD-causing mutant isoforms, exaggerate the vulnerability of neurons to dopamine-induced cell death through excess intracellular ROS generation (Junn and Mouradian 2002; Wersinger and Sidhu, 2003; Jiang et al., 2007; Qian et al., 2008; Parihar et al., 2009). Nigral degeneration was found eight weeks after lenti-A30P injection, and cytoplasmic alpha-synuclein inclusions into SN were not found in the present study. This is in accordance with previous data in which alpha-syn inclusions were detected in the SN ten months after injection (Lauwers et al., 2003), suggesting that oxidative stress induced by mutated synuclein may be an early event in the nigral degeneration process. Other findings also indicate that toxicity and aggregation are two distinct phenomena in alpha-synuclein-induced pathology. In fact, behavioral impairments linked to neuronal dysfunction without aggregate formation in transgenic mice expressing A53T human alpha-synuclein have been reported (Gispert et al., 2003). Additionally, toxicity induced by overexpression of human alpha-synuclein in primary midbrain cells is not associated with the presence of visible protein aggregates (Petrucelli et al., 2002). Thus, mutations of alpha-synuclein may lower the threshold to oxidative damage (Junn and Moradiam, 2002). However, a summation of effects and the possibility of participation of other factors involved in the differential vulnerability of SN DA-cells must also be considered (González-Hernández et al., 2009).

Because of its previously mentioned powerful antioxidant properties, melatonin has been proposed as a potential therapeutic agent in diseases in which oxidative stress is thought to be a major pathogenic factor. It is an ideal neuroprotective agent as it can easily cross the blood-brain barrier and enter the subcellular compartments, and it lacks toxicity when compared with other neuroprotective agents, and possesses effective combating efficacy against oxidative stress-related DA neuron degeneration (Zisapel, 2001; Gupta et al., 2003; Sharma et al., 2006; Capitelli et al., 2008; Singhal et al., 2011, 2012). Moreover, melatonin prevents toxin-induced DA-cell line and nigral degeneration, as well as alpha-synuclein aggregation (Ishido, 2007; Lin et al., 2007, 2008; Klongpanichapak et al., 2008; Singhal et al., 2012). However, there are few reports suggesting that melatonin does not provide neuroprotection in 6-OHDA and MPTP models of PD, because entry of both toxins into dopaminergic neurons occurs through the dopamine transporter (Itzhak et al., 1998; van der Schyf et al.,

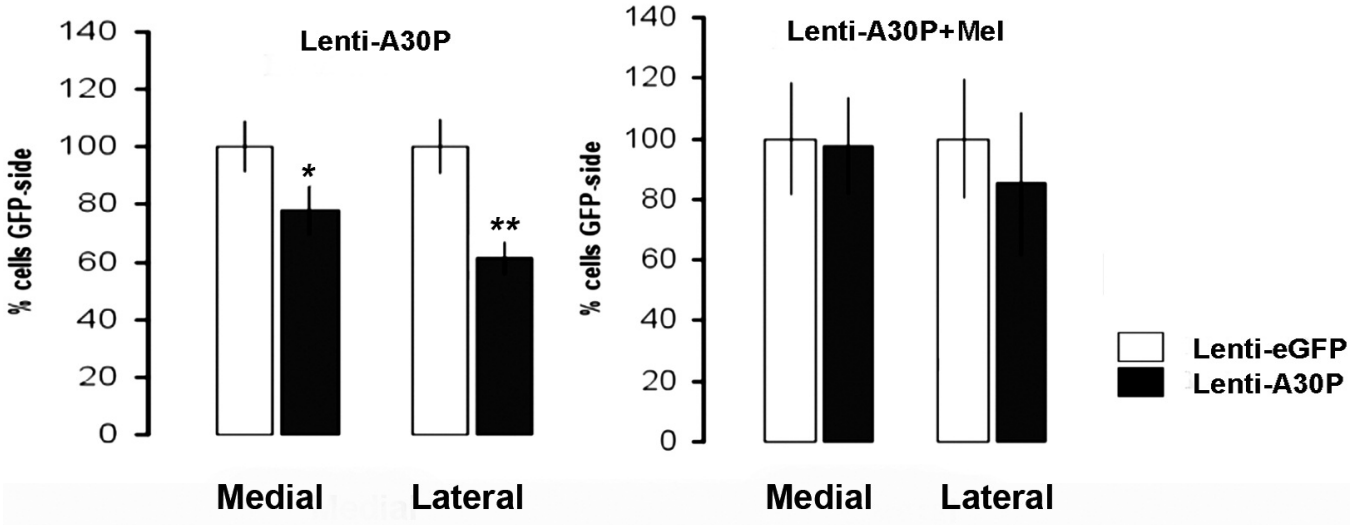

Fig. 2. LV-mediated mutant alpha-syn (A30P) injection in the right SN (lenti-A30P) induced a significant TH+ cell-loss in both the medial (20%) and lateral (40%) SN versus contrallateral side injected with lenti-eGFP. However, melatonin administration showed a total neuroprotective effect in both sides of the SN. Values refer to means ± SEM; n= seven animals per group; *p<0.01 and *p<0.001 vs lenti-eGFP-injected side.

2000; Morgan and Nelson, 2001; Tapias et al., 2010), a required event in producing selective dopaminergic neuron toxicity (Mayer et al., 1986; Schwarting and Huston, 1996; Gainetdinov et al., 1997). As melatonin down-regulates dopamine transporter expression (Lin et al., 2008) and alters DA signaling (Alexiuk and Vriend, 2007), protection may be partially mediated by alterations in neuronal toxin uptake. The next major goal was to determine whether melatonin exerted a neuroprotective effect on *in vivo* LV-mediated expression of alpha-syn in the SN. In agreement with previous neurotoxin studies, the major finding of this report is that melatonin also efficiently prevents PD-linked mutant (A30P)-synuclein-induced dopaminergic cell loss *in vivo* (Figs. 1 G,H, 2). These data indicate that melatonin administration showed a total neuroprotective effect in both regions of the SN. It is of interest that, with this LV-mediated alpha-syn A30P gene transfer approach, alpha-syn enters cells independently of transporters and, thus, is an ideal model for assessing protection, because as the mechanism of cell entry is due to viral transduction it does not rely on the dopamine transporter uptake to exert neurotoxicity. Moreover, the interpretation of results from experiments with neurotoxins is complicated by the fact that they may have pleiotropic pharmacological effects in DA neurons, effects on non-DA cell types, or both (Smeyne and Jackson-Lewis, 2005).

This is the first report suggesting that melatonin is neuroprotective against LV-alpha-syn induced toxicity in the rat SN and, therefore, melatonin does interfere with pathways affected by mutated-synuclein toxicity. Dissecting the molecular mechanism of MT protection against A30P alpha-synuclein toxicity should provide important clues about the unique vulnerability of dopamine neurons in PD. In conclusion, the data here suggest that melatonin may be clinically useful to combat mutant alpha-synuclein-induced oxidative injury in the CNS.

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