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GIRK2 expression in dopamine neurons of the substantia nigra and ventral tegmental area

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Abbreviated title: Substantia nigra GIRK2 distribution

Key words: GIRK2, human, mouse, substantia nigra

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ABSTRACT

G-protein regulated inward-rectifier potassium channel 2 (GIRK2) is reported to be expressed only within certain dopamine neurons of the substantia nigra (SN), although very limited data are available in humans. We examined the localization of GIRK2 in the SN and adjacent ventral tegmental area (VTA) of humans and mice using either neuromelanin pigment or immunolabeling with tyrosine hydroxylase (TH) or calbindin. Serial transverse 50µm sections were cut from formalin-fixed brainstems of five brain donors free from significant abnormalities (aged 85±3 years old) and compared with 40µm sections from paraformaldehyde-fixed brains from six adult C57BL6/J mice. GIRK2 immunoreactivity was found in nearly every human pigmented neuron or mouse TH-immunoreactive neuron in both the SN and VTA, although considerable variability in the intensity of GIRK2 staining was observed. The relative intensity of GIRK2 immunoreactivity was determined using Image J software grayscaling and the proportion of TH-immunoreactive neurons containing strong GIRK2 immunoreactivity determined (>50% of comparator intensity). In both species, nearly all SN TH-immunoreactive neurons had strong GIRK2 immunoreactivity compared with only 50-60% of VTA neurons. Most paranigral neurons also contained calbindin immunoreactivity and approximately 25% of these and nearby VTA neurons also had strong GIRK2 immunoreactivity. These data show that high amounts of GIRK2 protein are found in most SN neurons as well as in a proportion of nearby VTA neurons, and that previous studies suggesting GIRK2 is located only in limited neuronal groups within the SN of humans and mice have erroneously included VTA regions as part of the SN.

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INTRODUCTION

Part of the dopaminergic substantia nigra pars compacta (SNC) degenerates in Parkinson's disease leaving neighbouring dopamine neurons in the ventral tegmental area (VTA) and retrorubral fields relatively preserved (reviewed in (Double et al., 2010)). The degeneration within the SNC occurs in a distinct pattern, with specific cell clusters of the ventral tier rapidly disappearing while neighbouring dorsal cell clusters remain relatively immune until late in the disease. Hassler (1938) was the first to identify that certain dense clusters of neurons in the ventral tier of the SNC are most affected in PD, a finding confirmed in all subsequent studies (Bernheimer et al., 1973; Braak and Braak, 1986; Braak et al., 2003; Damier et al., 1999; Fearnley and Lees, 1991; Gibb and Lees, 1991; Greenfield and Bosanquet, 1953; Halliday et al., 1996; Hirsch et al., 1989). This selective loss of the ventral cell tier suggests significant differences between these SNC cells and those in the more resistant dorsal tier of the SNC.

The differential expression of cellular proteins within the SNC is thought to contribute to cell vulnerability, although there is considerable speculation on the most relevant proteins involved (reviewed in (Double et al., 2010)). The G-protein inward rectifier potassium 2 (GIRK2) channel, an ion channel modulating neuronal excitability (Lacey et al., 1987), has been suggested to be one such factor to explain differential vulnerability of ventral tier SNC neurons in Parkinson's disease. In rodents the expression of GIRK2 differs between SNC and VTA neurons, with higher mRNA expression in the SNC (Chung et al., 2005; Schein et al., 1998). A GIRK2 mutation in weaver mice causes developmental loss of most of the SNC (particularly the ventral tier) as well as cerebellar Purkinje cells (Kofuji et al., 1996; Navarro et al., 1996; Slesinger et al., 1996), supporting the concept that the differential expression of GIRK2 relates to cell vulnerability. In adult humans and mice, GIRK2 protein is reported to

be localized only within ventral tier SNC neurons (Mendez et al., 2005; Thompson et al., 2005), although the information in humans is very limited. Of interest, mRNA expression appears to be more widespread occurring in both SNC and lateral VTA dopamine neurons (Chung et al., 2005; Eulitz et al., 2007; Schein et al., 1998). The discrepancy between the localization of GIRK2 protein and mRNA in dopamine neurons and the limited data available in humans warrants further examination of the localization of this protein in the SNC and VTA to verify whether this protein may mark neurons most vulnerable to Parkinson's disease.

MATERIAL AND METHODS

Human brain tissue and preparation

The upper part of the brainstem from five brain donors (three males, two females) without neuropsychiatric, neurological, or neurodegenerative disease was obtained from the Sydney Brain Bank (tissue collection approved by the Human Research Ethics Committee of The University of New South Wales, Sydney, Australia) following approvals by the Australian Brain Donors Programmes (ABBN) Scientific Advisory Committee (study approval number 0118) and the Human Research Ethics Advisory Committee of The University of New South Wales, Sydney, Australia (ethics approval number 090030). The mean age (and standard deviation) at death of the brain donors was 85±3 years, their mean postmortem delay was 16±8 hours, and the mean time in fixative prior to this study was 68±47 months. Individual demographic details for all cases are given in **Table 1**. Tissue blocks of the upper brainstem were cryoprotected in 30% buffered sucrose, frozen in mounting medium, and sectioned transversely through their entire rostro-caudal axis at 50μm on a cryostat into 16 spaced series of sections (sections sampled every 800μm).

***** Table 1 about here *****

Mice brain tissue and preparation

Six C57BL/6J mice (8–10 weeks, 21–25 g, three male, three female) were obtained from the Animal Resource Centre, Perth, Australia and the study protocols approved by the Animal Care and Ethics Committee of The University of New South Wales, Sydney, Australia (ACEC 08/48B). Following transcardial perfusion of the mice with 4% paraformaldehyde (4°C, pH7.4), the brains were post-fixed in the same fixative for four hours and then cryoprotected in 30% sucrose-buffer. Each brain was cut into 40 µm sections on a freezing microtome in the coronal plane and consecutive sections of the SN were harvested from 2.30-4.60mm posterior to Bregma.

Antibody Characterization

GIRK2 antibody (**Table 2**) was characterised by the preabsorption of the antibody with the addition of an excess of the corresponding control peptide (3:1), which resulted in the complete abolition of immunostaining. No positive signal was detected. Specificity of the antibody in the tissues was determined by comparison with previously published localization studies (Eulitz et al., 2007; Schein et al., 1998), including those using the same Alomone GIRK2 antibody (Mendez et al., 2005; Thompson et al., 2005), and by substituting the GIRK2 antibody with normal serum. GIRK2 immunoreactivity has been previously identified in pontine and cerebellar neurons, neurons of the SNC but not its main projection region (neither neurons or neuropil immunostaining in the striatum), in lower layer neurons and throughout the neuropil of the cerebral cortex and hippocampus, as well as neurons and fibres in the basal forebrain and diencephalon (Eulitz et al., 2007; Schein et al., 1998). A similar distribution was observed in the present study, as indicated in the results. No positive reactions were observed in any sections in which GIRK2 antibody was not included.

***** Table 2 about here *****

The TH and calbindin antibodies (**Table 2**) were selected because of their common use as markers of dopamine neurons (for TH) and calbindin-containing neurons respectively. The specificity of the antibody in the tissues was determined by comparison with previously published localization studies (McRitchie and Halliday, 1995) and by substituting the TH or calbindin antibodies with normal serum. No positive reactions were observed in any sections in which TH and calbindin antibodies were not included.

Free-floating immunohistochemistry

For single immunolabeling, the series of serial sections from all 5 human cases were processed in the one immunorun, as were the series of serial sections from all 6 mouse brains. This was to avoid experimental variability. Human tissue sections were pretreated for 20 mins with 3% hydrogen peroxide (H_2O_2) in 0.1M phosphate-buffered saline (PBS) while mouse tissue sections were pretreated with 1% H_2O_2 in 50% ethanol. Sections were blocked in 10% normal serum (horse or goat, Sigma, St Louis, USA), washed in PBS, then incubated overnight at room temperature in 5% normal serum in PBS with 0.5% Triton X-100 and rabbit anti-GIRK2 at a dilution of 1:300 (for mouse tissue) or 1:400 (for human tissue). Additional series of tissue were also immunolabeled in the same method with rabbit anti-TH (1:1000) and mouse anti-calbindin (1:2000) for human, and mouse anti-TH (1:2000) for mouse. After washing with PBS, sections were incubated for two hours in 2% normal serum containing biotinylated goat anti-rabbit IgG, biotinylated goat anti-mouse IgG or biotinylated horse anti-mouse IgG (using Sigma B7389 and B0529 at 1:250 for mouse tissue, and Vector labs, Burlingame, CA BA2000 and BA1000 at 1:200 for human tissue). Immunoreactivity was visualized by incubating for two hours in either ExtrAvidin peroxidase (Sigma E2886 at 1:1000) for mouse tissue or avidin-biotin-complex (Vector Labs PK-6100 at 1:100) for human tissue, followed by incubation in 0.05% diaminobenzidine tetrahydrochloride (Sigma

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D5637 DAB), with either 0.04% nickel ammonium sulphate (for mouse tissue) or 2% nickel ammonium sulphate and 2.5% cobalt chloride (for human tissue) in PBS for 3 minutes, followed by the addition of 0.1% H_2O_2. The addition of nickel and cobalt result in the peroxidase complex forming a dark black stain that could be easily distinguished from the lighter brown neuromelanin pigment present in human dopamine nigral neurons. After rinsing in PBS, all sections were mounted and dried overnight, dehydrated, delipidated, and coverslipped. Bright field histological preparations were photographed using an Aperio Scanscope.

To avoid experimental variability in the double-immunofluorescent labeling, the series of serial sections from all 5 human cases were processed in the one immunorun for each antibody pair, as were the series of serial sections from all 6 mouse brains. Sections were blocked for one hour in 5% normal serum and 0.05% TX-100, then incubated for two nights at 4ºC in the following primary antibodies; anti-TH/ anti-GIRK2 (goat/rabbit both at 1:200 for human; mouse/rabbit 1:2000/1:200 for mice), anti-TH/ anti-calbindin (rabbit/mouse both at 1:1000 for mice) and anti-calbindin/anti-GIRK2 (mouse/rabbit 1:1000/1:200 for both human and mice). Immunofluorescent labeling was visible after incubation for three hours in Alexa fluor 594 anti-rabbit, Alexa fluor 488 anti-mouse, and Alexa fluor 488 anti-goat (Invitrogen A21207, A11001, A11055, each at 1:250). The mouse tissue sections were washed and counterstained with 4´,6-diamidino-2-phenylindole, dilactate (Invitrogen D3571). All sections were washed, mounted, and coverslipped with fluorescent mounting medium (Dako S3023). Fluorescent images were captured using a Nikon Microscope ECLIPSE 90i confocal microscope and Nikon D-ECLIPSE C1 high-resolution camera. Double-labeled images were converted from red-green to magenta-green using Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) as required for the Journal of Comparative Neurology.

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Analysis

Initial comparative analyses of the GIRK2-immunoperoxidase reaction in spaced serial sections was performed for all human upper brainstems (n=5) and whole mouse brains (n=6) in order to identify the regional distribution of GIRK2-immunopositive neurons and fibers compared with the atlases of the human brainstem {Paxinos, 1995 #22} and the mouse brain {Franklin, 2008 #25}. The number of sections containing GIRK2-immunopositive structures in the upper brainstem of the mice was three of the 40 µm think sections in the spaced series (one in three series spaced 120 µm apart, only these three levels contained the SNC), and for direct comparison three similar levels containing the greatest extent of the same subregions the human material (original one in sixteen series spaced every 800 µm apart, average of 11-12 sections per upper brainstem, final three sections spaced approximately 3 mm apart) were selected from each human case for further analysis.

For the identification of the SNC and VTA subregions of interest in the selected sections, the atlases of the human brainstem (Paxinos and Huang, 1995), the mouse brain (Franklin and Paxinos, 2008), the published comparative cytoarchitectural study between the rat and human (McRitchie et al., 1996), and recent observations in the mouse brain (Franklin and Paxinos, personal communication) were used to confirm the borders distinguishing the upper brainstem dopamine cell groups. The simplified nomenclature for the anatomical divisions of the human SNC (the dorsal and ventral tiers, the pars lateralis and the pars medialis) and the medial and dorsal VTA subregions was used (McRitchie et al., 1996) and the boundaries were traced using Stereoinvestigator software (MicroBrightField, Williston, VT) installed on an Olympus BX51 microscope at X1.25 prior to subsequent analysis at the three comparable rostrocaudal levels (see **Figure 1**).

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To establish the pattern of GIRK2 immunoreactivity, all upper brainstem pigmented cell groups in humans and all upper brainstem TH-immunoreactive cell groups in mice were initially identified

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To determine the proportion of pigmented (human) or TH-immunoreactive (mouse) neurons containing GIRK2 immunoreactivity, neurons exhibiting any immunoreactivity clearly within the cell soma using a X20 objective (human) or a X60 objective (mouse) were categorized as immunopositive and the proportion determined at each of the three levels in one representative control human and one mouse (rostrocaudal variability within regions is given as SEM). For comparison, co-localization of calbindin-immunoreactivity in the pigmented (human) or TH-immunoreactive (mouse) neurons in the same regions was also determined. The proportion of double-labeled neurons was assessed by two raters in 5% of the tissue sections and their numbers were highly correlated (Pearson R=0.87, p=0.025) and not significantly different using paired t test (p=0.5).

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GIRK2 immunoreactivity was noted to be variable within the cell bodies, particularly in humans, so further assessment of the neuronal intensity of GIRK2 compared with TH (both human and mouse) or calbindin (mouse only) immunoreactivity was performed in the subregions identified in the immunoperoxidase series. This analysis was performed on the fluorescent images captured using constant settings on a Nikon Microscope ECLIPSE 90i confocal microscope with a Nikon D-ECLIPSE C1 high-resolution camera to ensure adequate image processing for evaluation. Images were captured separately in grayscale for each channel at x40 (human) or x60 (mice) and the series of images within each of the selected sections sewn together using Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA). The grayscale intensity of the GIRK2 immunoreaction was directly compared with the grayscale intensity of either the TH or calbindin immunoreactivity in the double-labeled neurons using Image J software and a scale of 0-255 (v1.42q Java, National Institute of Health, USA). Neurons with GIRK2 immunoreactivity at >50% of either the TH or

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calbindin immunoreactivity were identified as strongly labeled (**Figure 2**), and the proportion of double-labeled neurons with strong GIRK2-immunoreactivity determined. The cut-off value of 50% intensity was determined in separate workup experiments to identify the majority of neurons in the ventral SNC, as this region was previously identified as having strongly double-labeled neurons (Mendez et al., 2005; Thompson et al., 2005). The proportion of double-labeled neurons with strong GIRK2 immunoreactivity was assessed by two raters in 50% of the tissue sections and their numbers were highly correlated (Pearson R=0.98, $p<0.0001$) and not significantly different using paired t test ($p=0.4$).

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***** Figures 1 & 2 about here *****

RESULTS

Cell clusters of the SNC

Dopamine neurons are present in each of the dorsal and ventral tiers and the pars lateralis and pars medialis of the SNC in both the human and mouse brain, but the proportional sizes of the tiers are species-dependent (**Figure 1**). As previously shown (Hardman et al., 2002), there is an enlarged ventral tier in humans compared with mice (**Figure 1**). The comparable ventral SNC cell clusters concentrate at mid transverse levels of the mouse SN, whereas they populate a greater rostrocaudal extent in humans as well as expanding significantly laterally. By contrast, the mouse dorsal tier is a continuous compact cell group, whereas in humans a more cell sparse area occurs in the midregion of the dorsal tier (**Figure 1**). As previously identified in humans (McRitchie et al., 1996), the pars medialis is located caudally and contains smaller cells than those of the dorsal and ventral tiers, and a similar cell cluster was observed in the medial aspects of the mouse SNC (**Figure 1**). The pars lateralis is positioned dorsolaterally in the SNC (**Figure 1**) and is identifiable by the orientation and shape of its neurons in the mouse and a notably decreased cellular density in the human brain.

GIRK2-immunoreactive neurons and fibers in SNC neurons and cell clusters

Immunohistochemical localization of GIRK2 revealed that the protein is expressed in the cell bodies and fibers (but not nuclei) of SNC neurons (**Figure 3**). Neuronal GIRK2 immunoreactivity within the cytoplasm appeared diffuse and granular (**Figure 4B,D**). In pigmented human SNC neurons, GIRK2 immunoreactivity was strongest in cell processes compared with somata (**Figure 3M-W**), although variation in the intensity of cell body staining was apparent (**Figure 2C,D).** In contrast, cell body staining was stronger in the mouse SNC compared with those in humans (**Figure 3A-L**) with stronger and weaker immunoreactive neurons also apparent (**Figure 2A,B)**. In both species strong fiber staining had a punctate or varicose appearance (**Figure 4**). Neurons in nearby regions, including the SN pars reticulata (**Figure 5A,B**) and the striatum (data not shown) did not contain GIRK2 immunoreactivity, although a proportion of non-pigmented and TH-negative neurons in the dorsal raphe nucleus were immunoreactive for GIRK2 (not shown). In mice but not humans, GIRK2-immunopositive but TH-negative neurons were observed in the red nuclei and strong GIRK2-immunopositive fibers were located in the interpedunclar nucleus (**Figure 5A).**

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***** Figures 2-5 about here *****

GIRK2-immunoreactive fibers appeared particularly prominent in the human compared with the mouse SN, with very long trajectories extending through the transverse sections (**Figure 4A,C**). In humans there was a dense network of GIRK2-immunoreactive fibers in the caudal, ventral and medial SN, within the ventral tier and pars medialis SNC cell clusters as well as in the pars reticulata ventral to the SNC cell clusters (**Figure 6G,F**). Many pigmented or TH- and GIRK2-immunoreactive neurons and dendrites in these more caudal regions appeared to be in close association with GIRK2-immunoreactive but TH-negative thin varicose fibers (**Figure 4D**). Similar to humans, many TH- and GIRK2-immunoreactive neurons and

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dendrites in the caudal SN of the mice appeared to be in close association with GIRK2-immunoreactive but TH-negative thin varicose fibers (**Figure 5E**). More rostrally, colocalization of GIRK2 and TH immunoreactivity was observed in fibers projected laterally from the dorsal SNC tier (**Figure 5C**).

***** Figure 6 about here *****

A very similar pattern of SNC cell clusters containing strong and weak GIRK2 immunreactivity was observed in both the human and mouse (**Figure 6**). All SNC subdivisions throughout their entire rostrocaudal extent had a high proportion of neurons that expressed strong GIRK2 immunoreactivity in TH neurons (**Figure 6**). In contrast, only a small proportion of SNC TH (mouse) or pigmented (human) neurons contained calbindin immunoreactivity and these were confined to the dorsal, lateral and medial regions of the SNC, being absent from the ventral tier (**Figure 7B-D, Table 4**). Quantitation of TH and/or pigmented neurons in the SNC subdivisions revealed that the majority of human neuromelanin-pigmented and mouse TH-immunoreactive neurons contained GIRK2 immunoreactivity with a strong intensity (**Table 3**). In both species, an average of 88% of ventral tier neurons were strongly double-labeled, with the remainder of TH-immunoreactive neurons displaying weak GIRK2 immunoreactivity in mice (**Table 3**). This pattern of GIRK2 colocalization in dopamine neurons was consistent throughout the mouse SNC regions, while in humans slightly less double labeled neurons were observed in the dorsal, lateral and medial SNC regions (**Table 3**). A very small proportion of SNC neurons contained only GIRK2 immunoreactivity in both species (**Table 3**), while more SNC neurons contained only neuromelanin pigment in humans (14±3%) or only TH immunoreactivity in mice (3±5%) (**Table 3**). The similar pattern and proportion of GIRK2-immunoreactive neurons in the mice and human material suggests that post-mortem delay or time in fixative have had minimal effect.

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Quantitation of calbindin neurons in the SNC subdivisions of mice revealed only occasional GIRK2 double-labeled neurons in the dorsal tier with the majority of dorsal tier neurons containing GIRK2 immunoreactivity alone (**Figure 7A,B, Table 4**). While a proportion of neurons in the pars medialis were double labeled for calbindin and GIRK2, most were not strongly immmunoreactive for GIRK2 (**Figure 7A,D, Table 4**) and this region had a relatively low proportion of TH/calbindin-immunoreactive neurons (**Table 4**). In contrast, all calbindin-immunoreactive neurons in the pars lateralis of mice were double labeled for GIRK2 with the majority strongly immmunoreactive (**Figure 7A,C, Table 4**). This region of the SNC had a relatively high proportion of TH/calbindin-immunoreactive neurons (**Table 4**).

***** Figure 7 about here *****

Assessment of GIRK2 immmunoreactive neurons in VTA neurons located dorsomedially

GIRK2 immunoreactivity in the dorsomedial VTA neurons was similar in intracellular location to that observed in the SNC, with both cell bodies and fibers (but not nuclei) containing GIRK2 immunoreactivity (**Figures 5, 7-8**). In both human and mouse VTA, all subregions contained GIRK2-immunoreactive neurons (**Figures 5, 7-8**). Unlike human SNC neurons, a large proportion of the human VTA dopamine neurons do not contain neuromelanin pigment (Hirsch et al., 1989), with a slightly lower proportion of pigmented VTA neurons also containing GIRK2 immunoreactivity (**Figure 8G-J**) and a larger number of non-pigmented GIRK2-immunoreactive neurons observed (**Table 3**). Double labeling showed that a very small proportion of GIRK2-immunoreactive neurons in the VTA regions were not TH-immunoreactive (**Table 3**). In contrast to the low proportion of calbindin- and TH-immunoreactive SNC neurons, over 30% of pigmented or TH-immunoreactive neurons in the dorsomedial VTA on average contained calbindin immunoreactivity (**Table 4**). In humans

the paranigral nucleus (PN) had about as many neurons containing calbindin immunoreactivity alone as those colocalizing with neuromelanin pigment, although this was not a major feature of the other VTA subregions (**Figure 7I**). In mice nearly all PN TH-immunoreactive neurons also contained calbindin immunoreactivity, while 43% on average of the pigmented neurons in this region were calbindin-immunoreactive in humans (**Table 4**). This could suggest that most non-pigmented calbindin-immunoreactive neurons in this region in humans would also be TH-immunoreactive. Lower proportions of pigmented or TH-positive neurons in the other VTA regions abutting the SNC also contained calbindin immunoreactivity (**Table 4**).

***** Figure 8 about here *****

In contrast to the SNC, a smaller proportion of pigmented or TH-immunoreactive VTA neurons also had strong GIRK2 immunoreactivity with more variability between subregions and species (compare **Figures 8 with 6, Table 3**). In humans the parabrachial pigmented nucleus (PBP) directly abutting the red nucleus had the highest proportion of strongly GIRK2-immunoreactive dopamine neurons (**Figure 8G, Table 3**). In mice the rostral VTA contained a relatively high proportion of strong GIRK2 intensity dopamine neurons (**Figure 8E, Table 3**), while this region had weak GIRK2 immunoreactivity in humans (**Figure 8J, Table 3**). The reverse was observed in the PN where a high proportion of dopamine neurons contained GIRK2 immunoreactivity in humans but not mice (**Figure 8D,I, Table 3**). Taking both strong and weak GIRK2 immunoreactivity into account, the majority of dopamine neurons in the VTA regions contained some GIRK2 immunoreactivity in both species (**Table 3**).

Because few SNC neurons were calbindin-immunoreactive, the colocalization of GIRK2 with calbindin occurred mainly in VTA neurons (**Figure 7**). In the VTA a relatively small

proportion of calbindin-immunoreactive neurons did not contain at least some GIRK2 immunoreactivity (**Table 4)**. As noted above, PN contained a high proportion of calbindin-immunoreactive neurons and many also had GIRK2 immunoreactivity, although strong expression was only observed in 25% (**Figure 7A,F**, **Table 4)**. A similar proportion of strong GIRK2/calbindin-immunoreactive neurons were observed in the other major VTA subregions, although few were found in the VTAR (**Table 4)**.

DISCUSSION

This study details the upper brainstem localization of GIRK2 protein in humans and mice using double labeling with pigment, TH and calbindin to determine the dopamine cell groups involved. While the cellular localization of calbindin and TH immunoreactivity found in the SN and VTA regions in both species confirmed previously published studies (Damier et al., 1999; Liang et al., 1996a; McRitchie et al., 1996), the localization of GIRK2 immunoreactivity in the same tissue did not yield expected results. As detailed in the introduction, the expression of GIRK2 protein is thought to differentiate SN neurons from those in the VTA. More particularly, GIRK2 expression is thought to differentiate those SN neurons in the ventral tier which are most vulnerable to Parkinson's disease, and GIRK2 immunohistochemistry has been widely used for identifying such cells in studies assessing dopamine neural grafts (Stromberg et al., 2010). There has been only one previous human tissue study assessing GIRK2 expression in the upper brainstem (Mendez et al., 2005). This study found that GIRK2-expressing dopamine neurons were located predominantly in the ventral tier of the SNC. In contrast, our results using the same antibody suggest no significant differentiation between ventral and dorsal tier SNC neurons in the amount of their GIRK2 immunoreactivity. Further, we found that a significant proportion of dopamine neurons in the

human VTA also had strong GIRK2 immunoreactivity, particularly in the paranigral and parabrachial pigmented regions immediately adjacent to the SN. As most VTA regions do not have any significant neuronal loss even in end-stage Parkinson's disease (McRitchie et al., 1997), these data show that GIRK2 is expressed in human dopamine neurons that are both vulnerable and resistant to Parkinson's disease.

In order to determine whether our findings in the human brainstem were not due to a species difference, we have used the same techniques to directly compare GIRK2 immunoreactivity in the upper brainstem of mice. Our results in mice are similar to those we observed in humans and are also similar to those described by Lammel and coworkers (2008) showing that the entire SNC and lateral VTA regions express GIRK2 protein. This contrasts with some studies in mice describing that SNC but not VTA dopamine neurons express GIRK2 protein (Aron et al., 2010; Chung et al., 2005; Thompson et al., 2005). Consistent with our mice and human data, previous localization studies in rats also show that the entire SNC and lateral but not midline VTA regions contain GIRK2-immunoreactive dopamine neurons (Eulitz et al., 2007). All of these studies show that GIRK2 protein is localized in dopamine neurons in the entire SNC, and does not differentiate the ventral from the dorsal tier.

Another reason for the concept that GIRK2 expression is important for the survival of ventral tier SN neurons is the pattern of cell loss observed in mutant GIRK2 Weaver mice (Graybiel et al., 1990; Slesinger et al., 1996). The loss of brainstem dopamine neurons in Weaver mice has been described to be selective for the ventral tier of the SN, largely sparing the VTA and dorsal tier (Graybiel et al., 1990; Slesinger et al., 1996). However, more careful analysis of these studies shows that the pattern of strong GIRK2 immunoreactivity we observed in mice dopamine neurons is very similar to the pattern of cell loss observed in Weaver mice, with the

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following Weaver mice description noted. “Neuronal loss in the VTA was not evident on qualitative analysis, but at the border between the lateral VTA cell group and the medial SN cell group there was obvious loss of TH-immunostained neurons” (Graybiel et al., 1990). The discrepancy between these previous and more recent work is the anatomical identification of the dopamine neuronal clusters of the upper brainstem. Again the data are consistent with no differentiation between the true ventral and dorsal tiers of the SN with respect to GIRK2 protein expression.

Differential GIRK2 intensity in SNC and VTA neurons

GIRK2 expression in VTA dopamine neurons has not been reported in most previous immunohistochemical studies, although studies assessing both GIRK2 mRNA and protein have identified considerable variation in expression levels (Eulitz et al., 2007; Saenz del Burgo et al., 2008). Our study is the first to quantify this variability and determine the relative levels of GIRK2 immunoreactivity in different dopamine cell groups. Strong GIRK2 immunoreactivity was found in most SNC cells (Mice: 82%, Humans: 77%) but also in a relatively large proportion of VTA cells (Mice: 58%, Humans: 55%). We (and others) also observed weak GIRK2 protein expression in nearly all remaining upper brainstem dopamine neurons. It should be noted that dopamine neurons with low GIRK2 mRNA but high protein levels have also been observed (Eulitz et al., 2007), suggesting that modulation of turnover can contribute to the variability in the number of GIRK2 channels on dopamine neurons. While this may contribute to the differences observed in GIRK2 localization between studies, we believe the erroneous impression that GIRK2 expression is largely confined to SN neurons is due to the difference in the proportion of strong GIRK2-immunoreactive neurons between the SN and VTA, in association with cell size differences (smaller VTA compared with SN dopamine neurons (Halliday et al., 2005; Poirier et al., 1983)).

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GIRK2 controls the neuronal membrane excitability of dopamine neurons through its activation of D2 or GABAb receptors (Wickman and Clapham, 1995) and interactions with other ion channels, such as the K-ATP channel (Liss et al., 1999). It is of interest that the K-ATP channel is also considered to promote the differential cell death between SNC and VTA neurons (Liss et al., 2005).

GIRK2 and calbindin colocalization

Previous studies have reported several genes as being differentially expressed in specific subsets of midbrain dopamine neurons (Chung et al., 2005; Duke et al., 2007; Greene et al., 2005; Grimm et al., 2004), supporting the concept of functionally diverse populations of brainstem dopamine neurons. In this context, the protein calbindin is mainly restricted to VTA regions (Damier et al., 1999; Liang et al., 1996b; McRitchie and Halliday, 1995; Yamada et al., 1990), a finding corroborated in the present study. In the context of GIRK2 localization studies, calbindin immunoreactivity has been used to separate dopamine neurons in the VTA (which contain this calcium binding protein) from those in the SNC (Mendez et al., 2005; Thompson et al., 2005). These studies suggest that GIRK2 and calbindin are not expressed together in dopamine neurons. Our observations show that around 25% of calbindin-immunoreactive VTA neurons also had strong GIRK2-immunoreactivity. This supports the concept that these proteins are largely confined to different populations of dopamine neurons, although we observed a greater overlap than expected in the strong colocalized expression of these proteins.

Speculation on the non-dopaminergic GIRK2-immunoreactive fibers in the SNC

A proportion of GIRK2-immunopositive fibers did not contain TH immunoreactivity. This

was found both within the ventral SNC cell clusters as well as ventrally in the pars reticulata of both species. Similar strong GIRK2 immunoreactive fiber staining has also been identified in the rat SN (Liao et al., 1996; Murer et al., 1997; Saenz del Burgo et al., 2008). As no GIRK2 mRNA occurs in neurons of the pars reticulata (Karschin et al., 1996; Liao et al., 1996), it is unlikely that the fiber staining is from neurons in this region. As the fibers do not colocalize TH immunoreactivity and have a restricted localization in the SN, it is unlikely they are from the dopamine neurons of the SNC or VTA. The restricted regional localization and highly varicosed nature of the GIRK2 positive/TH negative fibers impinging on GIRK2/TH-immunoreactive dendrites in the caudal and ventral regions of the SN suggests they could be afferent fibers to the SN.

The striatum is one of the main regions with projections to the SN. From mRNA and localization studies, there is no evidence for GIRK2 protein expression in striatal neurons (Mouse:(Schein et al., 1998); Rat; (Saenz del Burgo et al., 2008)), consistent with our preliminary experiments in this region showing no GIRK2-immunopositive striatal neurons (data not shown). The SN also receives a dense 5-HT innervation from the dorsal raphe nucleus (DR) (Fahn et al., 1971; Mackay et al., 1978), with electron microscopy showing that these terminals form synaptic junctions with both the SNC dopamine neurons and the non-dopamine neurons in the pars reticulata (Herve et al., 1987; Kalivas, 1993; Moukhles et al., 1997). This 5-HT innervation matches the regional localization of the GIRK2 positive/TH negative fibers within the SN (Corvaja et al., 1993; van der Kooy and Hattori, 1980; Wirtshafter et al., 1987). GIRK2 mRNA is found in the majority of DR neurons, although fewer DR neurons contain GIRK2 immunoreactivity (Saenz del Burgo et al., 2008). We can confirm the presence of GIRK2 positive/TH negative neurons in the human DR (data not shown). GIRK2 channels are coexpressed with 5-HT autoreceptors in the DR as well as with

5-HT heteroreceptors in the SN (Hopwood and Stamford, 2001; Penington et al., 1993; Saenz del Burgo et al., 2008) and we suspect that the GIRK2 positive/TH negative fibers observed in the SN are on 5-HT fibers projecting from the DR.

Summary

Our study has found that GIRK2 protein is expressed in the vast majority of human and mouse SN neurons and that its expression does not differentiate ventral from dorsal tier neurons. We also found that the majority of nearby VTA regions contain a high proportion of intensely stained GIRK2-immunoreactive dopamine neurons. It therefore seems unlikely that the differential expression of the GIRK2 channel is responsible for the selective neuronal vulnerability observed within the SNC in Parkinson's disease. Further work is required to identify the molecular mechanism and proteins involved in such selective dopamine neuron vulnerability.

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ABBREVIATIONS

3n	third nerve fascicles
5-HT	serotonin
ABBN	Australian Brain Donors Programmes
ACEC	Animal Care and Ethics Committee
Aq	aqueduct
cp	cerebral peduncle
DAB	diaminobenzidine tetrahydrochloride
DR	dorsal raphe nucleus
fr	fasciculus retroflexus
GIRK2	G protein-activated inward rectifier potassium channel 2
H_2O_2	hydrogen peroxide
ml	medial lemniscus
MT	medial terminal nucleus of the optic tract
PAG	periaqueductal gray
PaP	parapeduncular nucleus
PBS	0.1M phosphate-buffered saline
PBP	parabrachial pigmented nucleus
PIF	parainterfascicular nucleus
PN	paranigral nucleus
R	red nucleus
RLi	rostral linear nucleus
RRF	retrorubral fields
SN	substantia nigra
SND	dorsal tier of the substantia nigra pars compacta

SNL	substantia nigra pars lateralis
SNM	substantia nigra pars medialis
SNV	ventral tier of the substantia nigra pars compacta
SNC	substantia nigra pars compacta
SNR	substantia nigra pars reticulata
TH	tyrosine hydroxylase
VTA	ventral tegmental area
VTAR	rostral ventral tegmental area
xscp	decussation of the superior cerebellar peduncle

FIGURE LEGENDS

Figure 1: Representative transverse sections through three upper levels (indicated at left) of the mouse (**A,C,E**) and human (**B,D,F**) brainstem immunohistochemically stained with tyrosine hydroxylase (TH) (counterstained with cresyl violet in the mouse) showing the comparative location of the cytoarchitecturally different dopamine cell clusters. Scale in **E** equivalent for **A** and **C**. Scale in **F** equivalent for **B** and **D**. The cell clusters in the substantia nigra (SN) are more extensive in humans compared with mice occupying more of the lateral and caudal regions of this structure. In particular, there is an enlarged ventral tier (SNV) in humans compared with mice. The mouse dorsal tier (SND) is a continuous compact cell group, whereas in humans a more cell sparse area occurs at mid levels of the SN. The ventral tegmental area (VTA) cell groups extend more dorsally in humans compared with mice.

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Figure 2: Representative 1µm confocal images of double immunohistochemically labeled neurons in the VTA of the mouse (**A,B**) showing the variable intensity and location of GIRK2 immunoreactivity in TH immunoreactive neurons. Arrows at right indicate the rating of the intensity from grayscale images which were compared to determine strong (50-100% of the intensity of the TH immunoreactivity) and weak (<50% of the intensity of the TH immunoreactivity) GIRK2 immunoreactivity in the TH-immunoreactive neurons. TH only immunoreactive neurons are also shown. Similar representative images in the human SN taken under normal fluorescence microscopy (**C,D**) also show the variable intensity of GIRK2 immunoreactivity in TH immunoreactive neurons. Note that confocal images were used to determine the relative intensity of the GIRK2 immunoreaction in human sections, but the dark pigment shown is not seen using confocal microscopy.

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Figure 3: Photomicrographs of GIRK2 immunohistochemically stained sections through different levels (at left) of the SN in mice (**A-L**) and humans (**M-W**) showing immunoreactive neurons in all SNC cell clusters. Scale bar in L is equivalent for all figures. In humans neuromelanin pigment (found in dopamine neurons) is brown, in contrast to the nickel-cobolt enhanced GIRK2 immunoreactivity (black), indicating that the GIRK2 immunoreactivity occurs in these human dopamine neurons. The majority of SNC neurons in these regions contained enhanced diffuse and granular GIRK2 immunoreactivity. GIRK2 immunoreactivity was strongest in SNC cell processes compared with somata in humans (**M-W**) with cell body staining stronger in the mouse SNC (**A-L**).

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Figure 4: Photomicrographs of mouse (**A,B**) and human (**C,D**) sections through the SN immunofluorescently stained for both TH (green) and GIRK2 (magenta converted from red). High magnification shows diffuse and granular cytoplasmic colocalization (white in the merged images) of TH and GIRK2 immunoreactivity in SNC neurons (**B**,**D**) and double-labeled varicose fibers dominating the pars reticulata (**A,C**). Double labeled TH/GIRK2 immunoreactive neurons in the caudal SNC appeared to be in close association with GIRK2-immunoreactive but TH-negative fibers (**B,D**).

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Figure 5: Photomicrographs of TH (green)/GIRK2 (magenta converted from red) double immunoflourescence labeled transverse brainstem sections from mice (**A,C-E)** and a GIRK2 nickel cobolt enhanced immunoperoxidase labeled human transverse brainstem section (**B**). Scale in **E** is equivalent for **C** and **D**. GIRK2 immunoreactivity colocalized (white) with TH-immunoreactive dopamine neurons and varicose fibers in mice (**A,C-E)** but was more restricted to only the dopamine cell clusters in humans (**B**). GIRK2-immunoreactive but TH-negative neurons were located in the red nuclei with GIRK2-immunoreactive fibers in the

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interpeduncular in mice (**A**) but not humans (even with enhanced immunoperoxidase staining as shown in **B**). The degree of colocalization of TH and GIRK2 immunoreactivity in SN fibers differed depending on the rostrocaudal location of the transverse brainstem section. Rostrally (**C**) many TH/GIRK2-immunopositive varicose fibers occurred in the pars reticulata, with less overlap and some GIRK2 only-immunoreactive fibers observed at mid levels (**D**), while caudally (**E**) GIRK2 only immunoreactive varicose fibers predominated.

Figure 6: Photomicrographs of TH (green)/GIRK2 (magenta converted from red) double immunofluorescence labeled SN sections from mice (**A-D)** and humans (**E-H**). Scale in **D** is equivalent for **A-C**. Scale in **H** is equivalent for **E-G**. The majority of neurons in all the SN subregions were strongly double labeled with both TH and GIRK2 (white in the merged images) with only occasional TH only-immunoreactive neurons observed in any of the cell clusters.

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Figure 7: Photomicrograph of calbindin (green)/GIRK2 (magenta converted from red) double immunofluorescence labeled transverse brainstem sections from mice (**A-H**) and a calbindin immunoperoxidase labeled human transverse brainstem section (**I**). Scale in **H** equivalent for **B-F**. Note that calbindin immunoreactivity was observed in nuclei as well as the cytoplasm of positively stained neurons (**B-H**). There is more limited overlap (white) of calbindin and GIRK2 immunoreactivity in SN and VTA neurons (**A-H**, compare with **Figures 5, 6**). As previously described, few SN neurons contained calbindin immunoreactivity **(A-**D) with only some SNM neurons (**A,D**) but more SNL neurons (**A,C**) also strongly immunoreactive for GIRK2. There was variable overlap (white) of calbindin and GIRK2 immunoreactivity in VTA subregions, with very few high GIRK2 intensity, double-labeled neurons in the VTAR (**E**) and up to 25% of calbindin-immunoreactive neurons in other VTA subregions also

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containing GIRK2 immunoreactivity (**F-H**). In contrast to other VTA subregions, most PN neurons were at least weakly immunreactive for GIRK2 (**F**). A large proportion of the human VTA dopamine neurons do not contain neuromelanin pigment (Hirsch et al., 1989), and many calbindin-immunoreactive non-pigmented paranigral (PN) neurons (arrows in **I**) were observed. In other human VTA subregions, calbindin immunoreactivity occurred mainly in pigmented neurons (**I**).

Figure 8: Photomicrographs of TH (green)/GIRK2 (magenta converted from red) double immunoflourescence labeled transverse brainstem sections from mice (**A-E)** and GIRK2 nickel cobolt enhanced immunoperoxidase labeled human transverse brainstem sections (**F-J**). Scale in **E** is equivalent for **B-D**. Scale in **J** is equivalent for **G-I**. In all VTA subregions, GIRK2 immunoreactivity was found in most TH-immunoreactive (**A-E**) and/or pigmented dopamine neurons (**G-J**, arrows indicate GIRK2-immunoreactive pigmented neurons), although occasional neurons expressing only TH immunoreactivity and/or pigment were observed (asterisks in **C-D,J**). The intensity of GIRK2 was highly variable in the VTA subregions, with the majority of subregions close to the SN having many high GIRK2 intensity, double-labeled neurons (**B,E**) while those closer to the midline contained mostly low GIRK2 intensity, double-labeled neurons (**C,D**).