BRIEF REPORT

The Small GTP-Binding Protein Rhes Influences Nigrostriatal-Dependent Motor Behavior During Aging

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ABSTRACT

Background: Here we aimed to evaluate: (1) *Rhes* mRNA expression in mouse midbrain, (2) the effect of *Rhes* deletion on the number of dopamine neurons, (3) nigrostriatal-sensitive behavior during aging in knockout mice.

Methods: Radioactive in situ hybridization was assessed in adult mice. The beam-walking test was executed in 3-, 6- and 12-month-old mice. Immunohistochemistry of midbrain tyrosine hydroxylase (TH)–positive neurons was performed in 6- and 12-month-old mice.

Results: *Rhes* mRNA is expressed in TH-positive neurons of SNpc and the ventral tegmental area. Moreover, lack of *Rhes* leads to roughly a 20% loss of nigral TH-positive neurons in both 6- and 12-month-old mutants, when compared with their age-matched controls. Finally, lack of *Rhes* triggers subtle alterations in motor performance and coordination during aging.

Conclusions: Our findings indicate a fine-tuning role of Rhes in regulating the number of TH-positive neurons of the substantia nigra and nigrostriatal-sensitive motor behavior during aging.

Key Words: Rhes; Rasd2; dopamine neurons; midbrain; TH-positive neurons

Rhes is a small GTP-binding protein highly enriched in the striatum,[1] developmentally regulated by thyroid hormone[2,3] and by dopamine (DA) innervation in adult rat brain.[4] Within the striatum, *Rhes* mRNA is localized in GABAergic medium-sized projection neurons of mice and humans,[5-7] as well as in large aspiny cholinergic interneurons,[8] where it modulates DA-dependent transmission. Recent findings showed that Rhes binds to and activates mTORC1, a key modulator of several biological processes,[9] including levodopa (L-dopa)–induced dyskinesia.[10] Accordingly, lack of *Rhes* attenuates L-dopa-induced motor disturbances in hemiparkinsonian mutants. In addition, Snyder and coworkers demonstrated that Rhes influences Huntington's disease physiopathology through its ability to act as a selective E3 ligase of mutant huntingtin.[11] Furthermore, they also reported the ability of this striatal GTPase to bind to Beclin-1 and, in turn, activate autophagy-related processes.[12]

In addition to its enriched striatal localization, *Rhes* mRNA is also expressed in the hippocampus, olfactory tubercle, anterior nuclei of the thalamus, inferior

colliculus, and granular layer of cerebellum.[1] Here, we evaluated the *Rhes* mRNA expression pattern in midbrain regions and investigated its potential involvement in regulating the number and density of DA neurons, as well as nigrostriatal-sensitive behavior during aging.

Materials and Methods

Animals

Male $Rhes^{+/+}$ and $Rhes^{-/-}$ mice without PGK-neo cassette,[8] backcrossed to F11 generation to the C57BL/6J strain, were used in the present study. Experiments were performed in conformity with protocols approved by the veterinary department of the Italian Ministry of Health and in accordance with the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian laws and European Union's directives (no. 86/609/EC). All efforts were made to minimize the animals' suffering.

Neuroanatomical Studies

Single and double in situ hybridization (ISH) analyses were performed according to protocols previously described.[7,13] Digoxigenin-(*Rhes, TH, Gad67*) and fluorescein-conjugated (*TH*) or ^{35}S-labeled (*Rhes*) antisense riboprobes were used. Nitro blue tetrazolium (NBT)/ 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate in combination with 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate (HNPP)/Fast Red Fluorescent Detection Set was used in double ISH. For radioactive ISH, slides were exposed to Biomax MR x-ray films.

For combined ISH and immunohistochemistry (IHC), animals were perfused transcardially with 4% paraformaldehyde (PFA) and brain sections 50 μm thick were obtained with a vibratome (Leica Microsystems). Free-floating sections were incubated over night at 4 °C with a primary mouse anti-TH antibody (1:400, Millipore), followed by a 2-hour incubation at room temperature with an Alexa Fluor 488 goat anti-mouse (1:500, Molecular Probes). Following IHC, ISH analysis was performed as described above using a fluorescein-labeled *Rhes* antisense riboprobe. HNPP/Fast Red was used as labeling method taking care to avoid quenching the Alexa Fluor 488 fluorescence masking the TH signal in dopaminergic neurons. Images were taken with a SP8 confocal microscope (Leica).

Behavioral Tests

Motor performance and coordination of mice was evaluated with a beam-walking test.[14,15] Mice were trained to traverse the length of a Plexiglas beam. Mice received 2 days of training before testing that ended when all animals complete 5 unassisted runs across the length of a Plexiglas beam (4 sections of 25 cm each, 1 m total length, with a different width: 1, 2, 3, and 4 cm). On test day, a mesh grid (1 cm^2) of corresponding width was placed over the beam. Mice were videotaped for 5 trials. An error was counted when, during a movement, a limb slipped through the grid. An individual animal could make a maximum of 4 slips (errors) per step. Time to traverse, number of steps, and errors per step scores were calculated across 5 trials and averaged for each group.[15]

Immunohistochemistry

After behavioral tests, mice were anesthetized and perfused for immunohistochemistry. Three coronal sections (40 μm thick) from striatum (A: 1.10, 0.74, 0.38 mm from bregma) and 6 sections from the SNpc/ventral tegmental area (VTA; A: -2.92 to -.64 mm from bregma) were cut on a vibratome, according to Paxinos and Franklin.[16] SNpc/VTA sections were immunoreacted with an anti-TH antibody, whereas sections from the striatum were immunoreacted with an anti–glutamic acid decarboxylase 67 (GAD67) antibody. Reaction was amplified as described.[17]

Stereological analysis of total number and density of TH-positive neurons in the SNpc were counted on both hemispheres, using software (Stereologer) linked to a motorized stage on a light microscope.[18] The SNpc region was outlined at low magnification (2×), and sampling of cells was achieved using automatically randomized sampling and an optical dissector ($50 \times 50 \times 15$ μm). Cells were sampled with a 40 × objective through a defined depth with a guard zone of 2 μm. Coefficient of error ranged from 0.05 to 0.1.[18] GAD67 and TH-positive neurons of the VTA were counted in both hemispheres. Images were captured and evaluated with Pixelink software. The absolute number of GAD67 and TH-positive neurons obtained for each VTA and striatal level showed no differences; therefore, the data obtained were pooled together.

Statistics

Data are presented as mean ± SEM. For all parameters, differences were evaluated by 1-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. Significance was set at $P < 0.05$.

Results

Rhes Is Coexpressed With TH-Positive Neurons in SNpc and VTA

To characterize *Rhes* expression in the midbrain, we performed in situ hybridization on coronal sections of adult mice labeled with a specific ^{35}S-labeled riboprobe for *Rhes* (Fig. 1A-D, A′-D′). Results indicated expression of *Rhes* in a domain along the anteroposterior

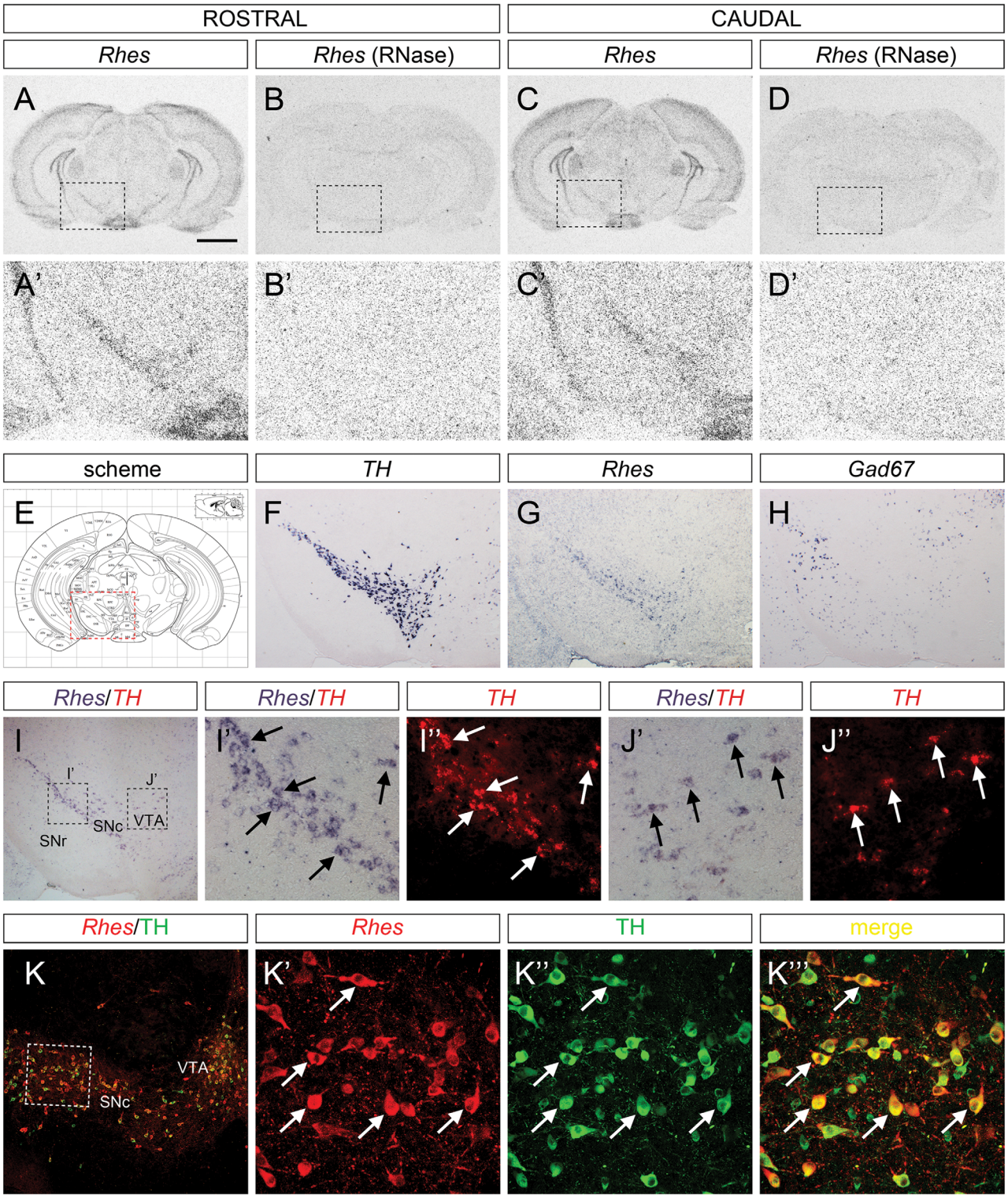

FIG. 1. Rhes expression in midbrain DA neurons of the SNpc and VTA. (**A-D′**) Radioactive ISH on coronal sections of adult mice hybridized with a specific ^{35}S-labeled Rhes riboprobe show expression of *Rhes* mRNA in midbrain territories corresponding to SNpc and VTA (**A-A′, C-C′**). Black-boxed regions in A-D are shown at higher magnification in A′-D′. (**B, B′, D, D′**) Control RNase-treated sections showing the specificity of the Rhes riboprobe. (**E-H**) Nonradioactive ISH on parallel coronal sections of adult mouse brains labeled with specific probes for TH (**F**), Rhes (**G**), and Gad67 (**H**). Images show the area highlighted by the red-boxed region in the schematic view (**E**). TH-expressing cells are localized in the SNpc territory, where it is also evident Rhes expression (**F-G**). Conversely, Rhes is not expressed at detectable levels in the SNpr, where Gad67-positive cells are present (**G-H**). (**I-J″**) Double ISH on coronal sections of adult brains labeled with specific probes for Rhes (blue precipitate in **I, I′, J′**) and TH (red fluorescence in **I″, J″**), analyzed in SNpc and VTA (black-boxed panels in I). Rhes expression is detectable in the SNpc and VTA regions, where it colocalizes with TH (arrows in **I-J″**). (**K-K‴**) Representative confocal image of brain coronal section processed for combined in situ hybridization and immunohistochemical labeling. Boxed region in **K** is shown at higher magnification in **K′-K‴**. Arrows highlight neurons coexpressing *Rhes* mRNA and TH protein. Scale bar: 1.2 mm (**A, B, C, D**); 300 μm (**A′, B′, C′, D′, F-I**); 75 μm (**I′-J″**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

extent of the ventral midbrain, corresponding to where SN and VTA are localized (Fig. 1A, A′, C, C′). The specificity of the riboprobe labeling was confirmed by treating parallel adjacent sections with RNase A before the hybridization step (Fig. 1B-B′, D-D′). DA- and GABA-expressing cells collectively constitute the 2 main neuronal populations of the ventral midbrain, having partially overlapping localization domains in both the SN and VTA, but showing very little, if any, colocalization.[19] To assess whether *Rhes*-expressing neurons could belong to 1 of these 2 populations, we compared, by means of ISH on adjacent sections, the expression profile of *Rhes* with that of *TH* and *Gad67* mRNA, which encode DA- and GABA-synthetic enzymes, respectively. Results showed that *Rhes* is expressed in the same territory as *TH*-expressing cells (ie, the SNpc and VTA; Fig. 1E-G). In contrast, ISH data did not show any obvious expression of *Rhes* in the SN pars reticulata (SNpr), where *Gad67*-positive cells are mainly present (Fig. 1G-H). These results led us to hypothesize that Rhes might be expressed in midbrain DA neurons. Hence, we performed double ISH combining *Rhes*- and *TH*-specific riboprobes, focusing our analysis on SNpc and VTA (Fig. 1I-J″). To further confirm such a hypothesis, we combined fluorescent immunohistochemistry and HNPP/Fast Red in situ hybridization protocol to examine the colocalization of TH protein and *Rhes* mRNA (Fig. 1K, K′-K‴). Results demonstrated that *Rhes* mRNA expression could be detected in a large extent of TH-positive neurons, both in SNpc (Fig. 1I, I′-I″, K, K′-K‴) and VTA (Fig. 1I, J′-J″, K, K′-K‴).

Evaluation of Midbrain TH- and Striatal GAD67-Positive Neurons in *Rhes* Mutants

A mild decrease in the total number of TH-positive nigral neurons was found in $Rhes^{-/-}$ mutants, compared with age-matched $Rhes^{+/+}$ (6 months, $F_{1,23} = 6.9$, $P < 0.05$; 12 months, $F_{1,18} = 18.14$, $P < 0.001$; Fig. 2A-B). Also, density of TH-positive neurons in SNpc of 6- and 12-month-old mutants demonstrated a significant decrease (6 months, $F_{1,23} = 10.07$, $P < 0.005$; 12 months, $F_{1,18} = 4.42$, $P < 0.05$; not shown). Conversely, the number of TH-positive neurons in VTA and the number of GAD67-positive neurons in striatum at 12-month of age were indistinguishable between genotypes (VTA: $F_{1,18} = 0.99$, $P = 0.33$), Fig. 2D; striatum: $F_{1,18} = 0.66$, $P = 0.43$, Fig. 2C).

To explore whether lack of *Rhes* influences coordination and motor behavior during aging, we performed the beam-walking test, a well-established behavioral paradigm to evaluate the integrity of the nigrostriatal system.[14] $Rhes^{-/-}$ and $Rhes^{+/+}$ of 3, 6, and 12 months of age were tested (Fig. 2E).

The 3-, 6- and 12- month-old mutants made a significantly higher number of steps to traverse the beam, compared with controls (3 months, $F_{1,39} = 4.82$, $P < 0.05$; 6 months, $F_{1,42} = 4.25$, $P < 0.05$; 12 months, $F_{1,45} = 13.44$, $P < 0.005$).

Three-month old mutants spent similar time to traverse the beam ($F_{1,39} = 0.58$, $P = 0.45$). In contrast, 6- and 12-month-old $Rhes^{-/-}$ mice spent a significantly longer time to traverse the beam (6 months, $F_{1,42} = 4.18$, $P < 0.05$; 12 months, $F_{1,45} = 4.06$, $P < 0.05$).

Moreover, 3- and 6-month-old $Rhes^{-/-}$ mice made similar errors per step to traverse the beam (3 months, $F_{1,39} = 0.37$, $P = 0.55$; 6 months, $F_{1,42} = 1.96$, $P = 0.169$). In contrast, 12-month-old mutants made significantly more errors per step in traversing the beam ($F_{1,45} = 13.18$, $P < 0.005$; Fig. 2E).

Discussion

In the present study we documented that *Rhes* mRNA, beyond its main striatal localization in GABAergic medium spiny neurons,[5-7] cholinergic large aspiny interneurons,[8] and the hippocampus,[6] is also expressed, although to a lesser extent, in TH-positive neurons of SNpc and VTA of the mouse midbrain. In addition, we showed that lack of *Rhes* leads to a mild, although significant, 20% of nigral TH-positive neuronal loss in both 6- and 12-month-old mutant animals, when compared with their age-matched controls. Moreover, in *Rhes* knockouts we found subtle motor coordination deficits, as measured by the beam-walking test, during aging.[14,15]

Although it is still unclear whether such motor defect is selectively associated with a SNpc dysfunction or rather is linked to striatal abnormalities,[8,11] our data point to an unexpected influence of Rhes in selectively regulating the number of nigral DA midbrain neurons, but not those of VTA or striatal projecting neurons. This observation fits with previous data evidencing a role for Rhes in modulating signaling pathways implicated in neuronal survival, such as mTOR[20] and Beclin-1.[12] On the other hand, based on previous in vitro findings indicating that Rhes, by influencing Gαi-coupled GPCR signaling, affects the voltage-dependent inhibition of Cav2.2 channels,[21] we cannot exclude involvement of Rhes in regulating local calcium homeostasis and thereby neurosecretion,[22] which, in turn, could explain the progressive detrimental effect on the nigrostriatal survival pathway seen in mutants.

Present data showing that the thyroid target gene *Rhes* is expressed in both SNpc and VTA midbrain regions significantly enlarges the conceptual meaning of this gene, previously thought to be expressed only in dopaminoceptive neurons.[5,6]

In conclusion, even though we argue that Rhes does not have a causative role in Parkinson's disease, however, considering its peculiar expression throughout DA system, we suggest that this protein could regulate such neurotransmission at both at pre- and postsynaptic sites.

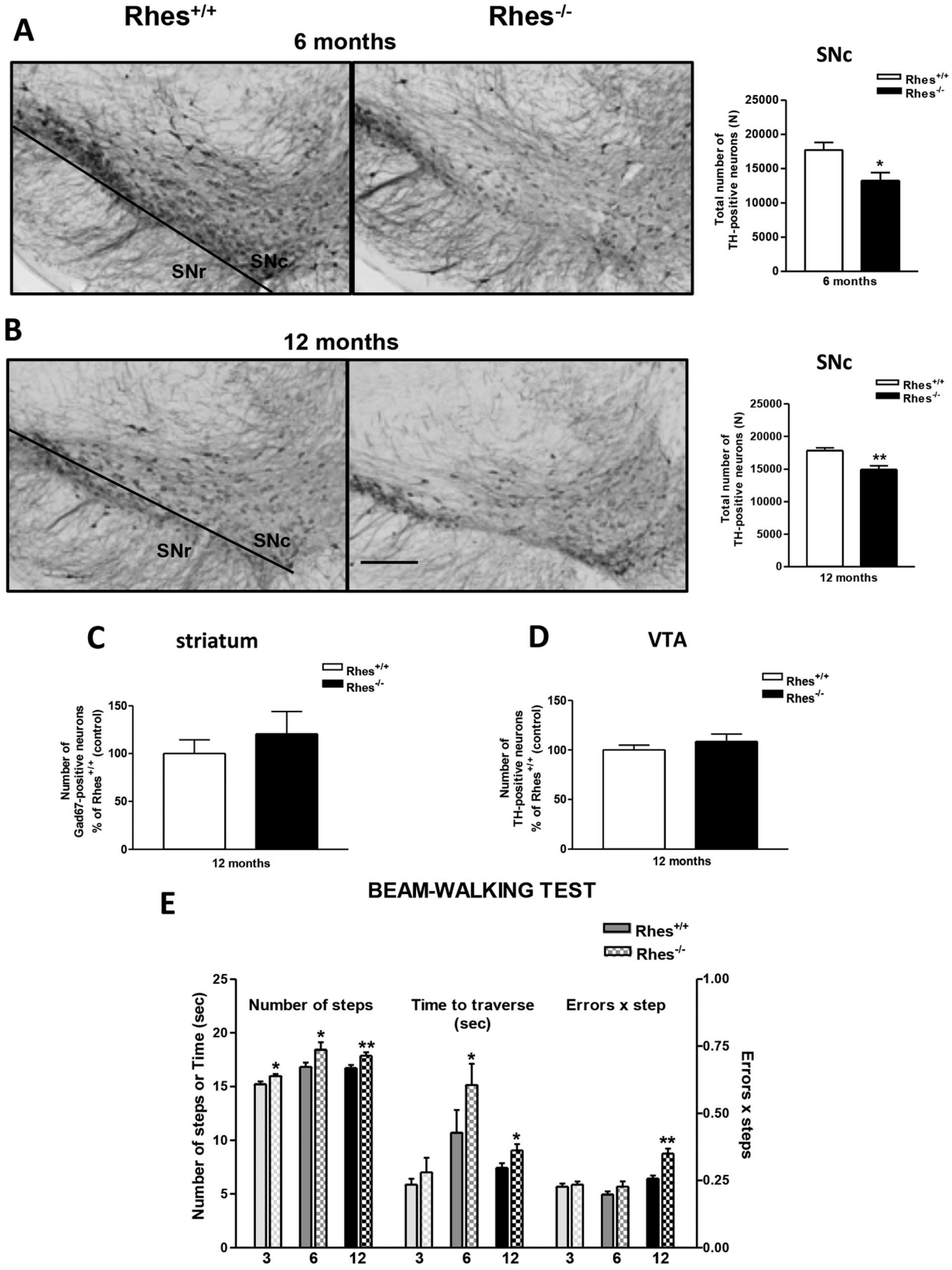

FIG. 2. DA neurons in the SNpc and VTA and analysis of motor performance and coordination in *Rhes*$^{-/-}$ and *Rhes*$^{+/+}$ mice. Representative photomicrographs of brain coronal sections showing TH-positive cells in the SNpc of *Rhes*$^{-/-}$ (center) and *Rhes*$^{+/+}$ (left) mice of 6 (**A**) and 12 (**B**) months (scale bar = 50 μm). Histograms (**A-B**, right) showing stereological quantification of TH-positive neurons in the SNpc of *Rhes*$^{-/-}$ (black column) and *Rhes*$^{+/+}$ (white column) mice of 6 (**A**, right) and 12 (**B**, right) months. Results are mean ± SEM of total number of TH-positive neurons (6 months: *Rhes*$^{+/+}$ n = 15, *Rhes*$^{-/-}$ n = 10; 12 months: *Rhes*$^{+/+}$ n = 12, *Rhes*$^{-/-}$ n = 8). Histogram (**C**) showing quantification of *Gad67*-positive neurons in the striatum of *Rhes*$^{-/-}$ (black column) and *Rhes*$^{+/+}$ (white column) mice of 12 months. Results are mean ± SEM of total number of *Gad67*-positive neurons expressed as a percentage of *Rhes*$^{+/+}$ (control) mice (12 months: *Rhes*$^{+/+}$ n = 12, *Rhes*$^{-/-}$ n = 8). Histogram (**D**) shows quantification of TH-positive neurons in the VTA of *Rhes*$^{-/-}$ (black column) and *Rhes*$^{+/+}$ (white column) mice of 12 months. Results are mean ± SEM of total number of TH-positive neurons expressed as a percentage of *Rhes*$^{+/+}$ (control) mice (12 months: *Rhes*$^{+/+}$ n = 12, *Rhes*$^{-/-}$ n = 8). Three separate groups of *Rhes*$^{+/+}$ and *Rhes*$^{-/-}$ mice of 3, 6, and 12 months old were tested in the beam-walking test (**E**). Results are mean ± SEM of the number of steps, time to traverse the beam in seconds (left axis), and errors × step (right axis) (**E**) (3 months: *Rhes*$^{+/+}$ n = 20, *Rhes*$^{-/-}$ n = 21; 6 months: *Rhes*$^{+/+}$ n = 25, *Rhes*$^{-/-}$ n = 19; 12 months: *Rhes*$^{+/+}$ n = 22, *Rhes*$^{-/-}$ n = 25). Statistical significance was determined by 1-way ANOVA followed by the Newman–Keuls post hoc test. **P* < 0.05, ***P* < 0.005, versus Rhes$^{+/+}$ (control) mice at the same age.

Moreover, we can also hypothesize that putative downregulation of Rhes might affect the vulnerability of nigral cells to death triggered by aging processes and/or environmental insults. ■

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