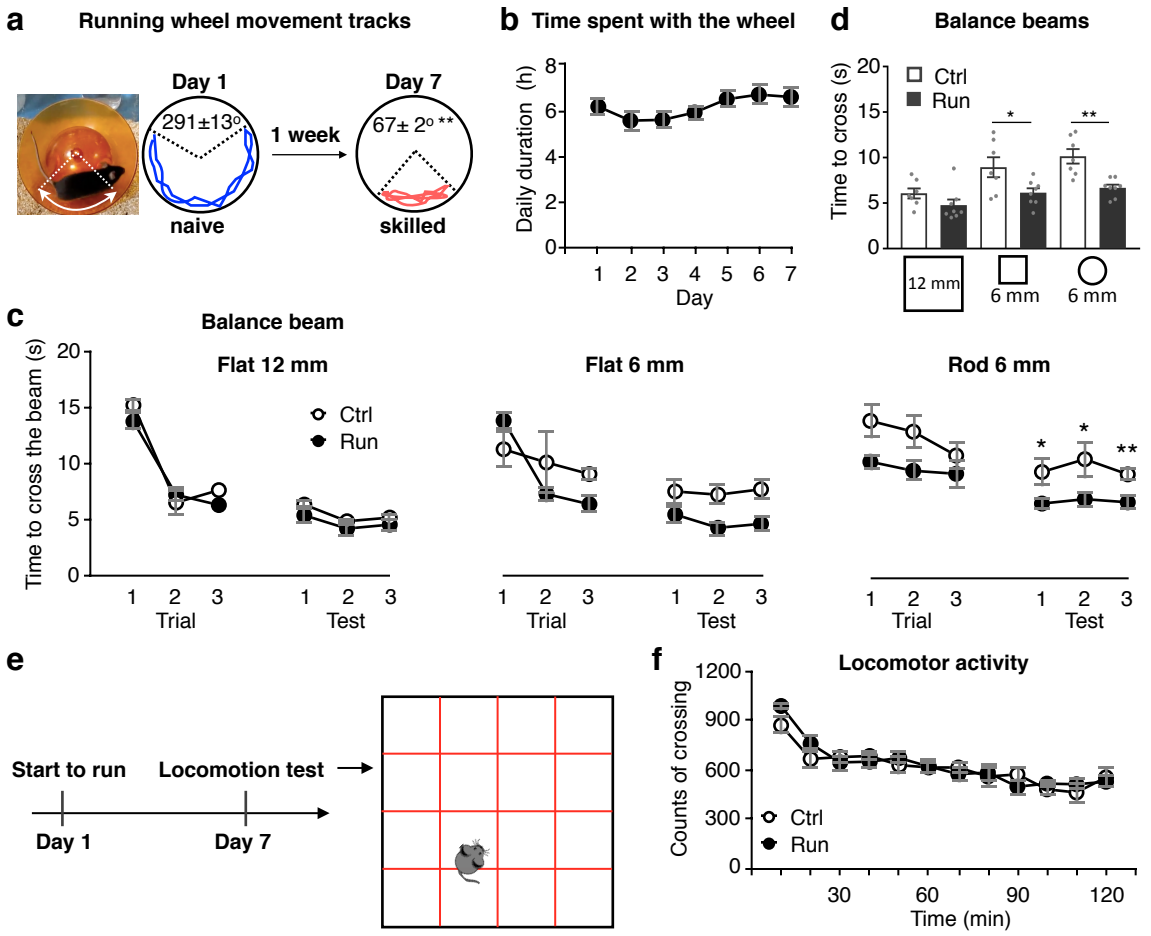
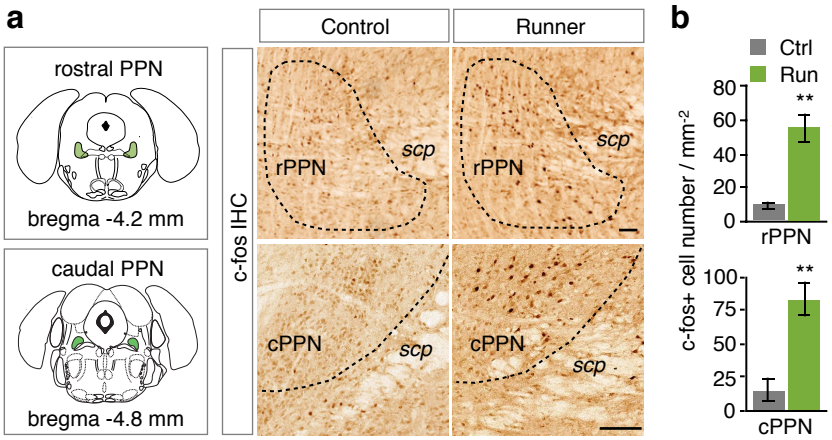


Supplementary Figure 1

Running enhances motor skill learning but does not affect basic locomotor activity.

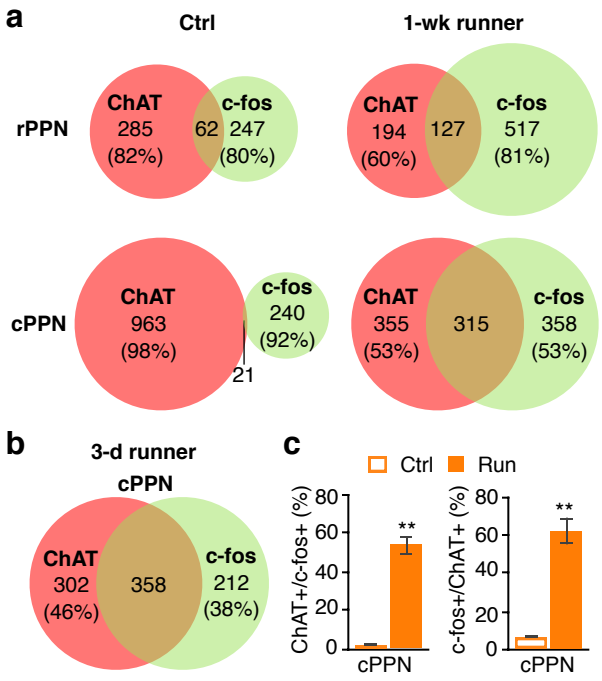
a, Image of a runner mouse and examples of mouse movement tracks on the running wheel. Angles are mean mouse movements subtended while running. n=6 tracks from 3 animals/group. **b**, Daily duration that mice spent with running wheels during one week of running. n=8 animals/group. **c,** Time to cross a 1 meter long, 0.75 meter high balance beam during each trial of training or each test on the day after training. Beam shape (flat or rod) and size (diameter) are indicated. **d,** Mean time to cross the balance beams in three tests on the day after training. For (**c**,**d**), n=7 animals for Ctrl and 8 animals for Run. **e,** Experimental design for locomotion test. **f**, Average counts of mice crossing laser beams in the novel locomotion chamber (**e**) in a 2-hour test. n=6 animals/group. Statistical significance *p<0.05, **p<0.01 was assessed by Welch's t-test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 2

Running activates both rostral and caudal PPN.

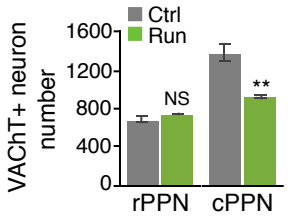
a, Left panels show the PPN (green) in coronal brain sections. Middle and right panels illustrate 3,3'-diaminobenzidine (DAB) immunohistochemistry of c-fos in rostral PPNs (rPPN, upper) or caudal PPNs (cPPN, lower) of controls and 1-week runners. Dotted lines outline the PPN. Dark brown stain indicates c-fos+ neurons. Scale bar, 100 µm. **b,** Counts of c-fos+ neurons in the rostral and caudal PPN. n=6 animals/group. Statistical significance $^{**}p<0.01$ was assessed by Mann–Whitney U test. Data shown are mean± SEM.



Supplementary Figure 3

A substantial fraction of PPN c-fos+ neurons are cholinergic and cPPN cholinergic activation is more relevant to running.

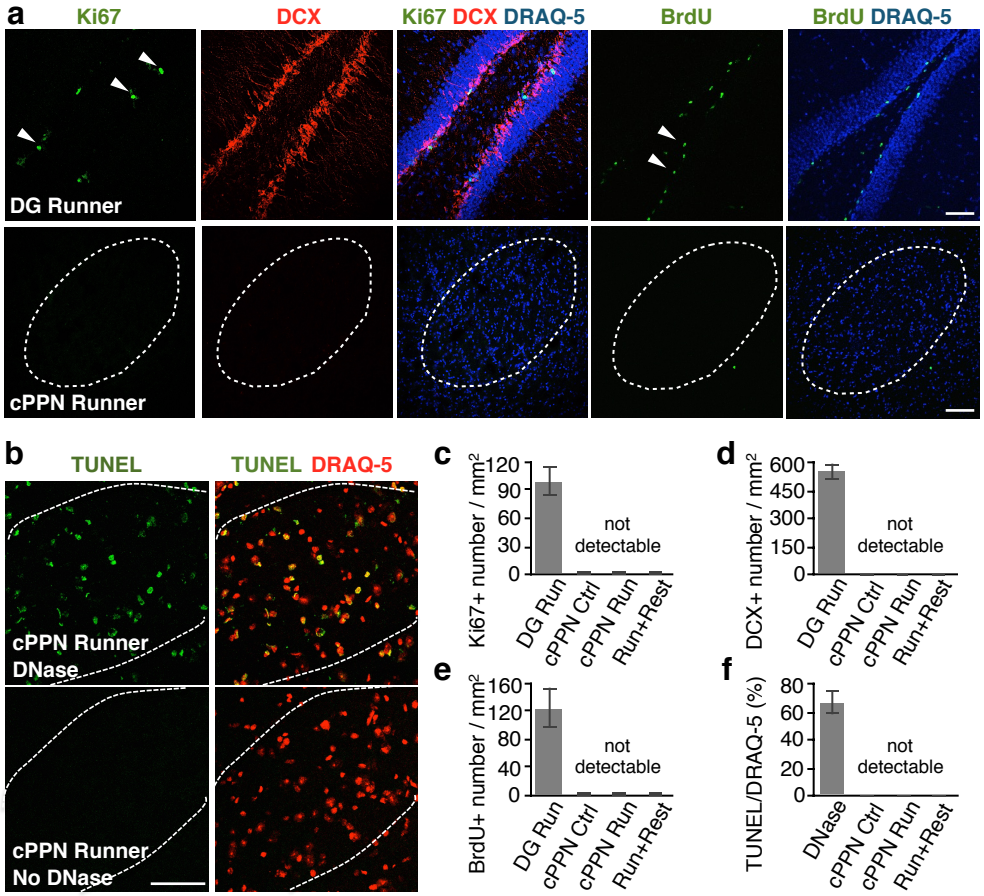
a, Co-localization of ChAT and c-fos in the PPN of controls and 1-wk runners. Cell numbers and percentages of cholinergic (red) and non-cholinergic (green) neurons are shown. n=3 animals/ group. **b**, Co-localization of ChAT and c-fos in the caudal PPN of mice that had run for 3 days. Cell numbers and percentages are shown. n=3 animals. **c**, The percentage of the ChAT+, c-fos+ neurons in the ChAT+ neuron population (left) or in the c-fos+ neuron population (right) in the cPPN of 3-day runners and controls. n=3 animals/group. Statistical significance $^{**}p<0.01$ was assessed by Mann–Whitney U test. Data shown are mean± SEM.



Supplementary Figure 4

Running reduces the number of VAChT+ neurons in the cPPN but not in the rPPN.

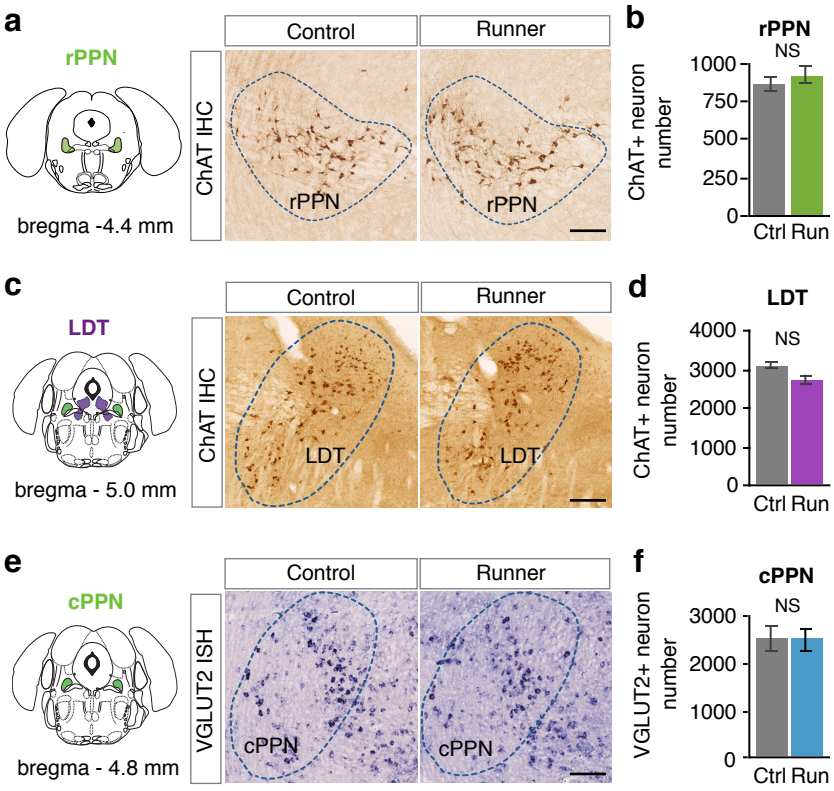
Stereological counts of VAChT+ neurons. n=6 animals/group. Statistical significance $^{**}p<0.01$ was assessed by Mann–Whitney U test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 5

Apoptosis and neurogenesis are not apparent in the caudal PPN of runner mice.

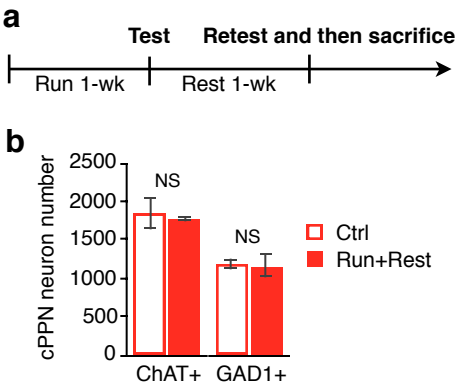
a, Sections of dentate gyrus (DG, upper panels) and cPPN (lower panels) from 1-week runners triple-stained for Ki67, DCX, and DRAQ-5 (nuclear marker) (Columns 1-3) or double-stained for BrdU and DRAQ-5 (columns 4-5). For BrdU labeling, mice were i.p. injected with BrdU (50 mg/kg) once every 12 hr for 1 week. Dotted lines outline the cPPN. Arrowheads point to Ki67+ (upper panel in column 1) or BrdU+ cells (upper panel in column 4). Scale bar, 100 µm. **b,** Sections of the cPPN of a 1-wk runner are double-stained for TUNEL and DRAQ-5. Dotted lines outline the cPPN. Upper panels: DNase-treated tissue as positive control. Lower panels: no DNase treatment. Scale bar, 100 µm. **c-e,** Statistical summary of (**a**) combined with littermate non-runner controls and mice that ran for 1 week followed by 1 week of rest. DG Run, dentate gyrus of a runner mouse. The region of interest that was quantified includes only the granule layer and not the hilus of the dentate gyrus. n=3 animals, 12 sections per group. **f,** Statistical summary of (**b**) combined with littermate non-runner controls and mice that ran 1-week followed by 1 week of rest. n=3 animals, 12 sections per group. Data shown are mean± SEM.



Supplementary Figure 6

Running does not change the number of ChAT+ neurons in the rostral PPN (rPPN) or the laterodorsal tegmental nucleus (LDT) or the number of VGLUT2+ neurons in the caudal PPN (cPPN).

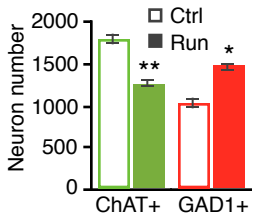
a, The left panel shows the rPPN (green) in a coronal brain section. Middle and right panels illustrate DAB staining of ChAT in rPPN of a control and 1-week runner. Dotted lines outline the rPPN. Dark brown stain indicates ChAT+ neurons. Scale bar, 100 µm. **b,** Stereological counts of ChAT+ neurons in the rostral PPN. n=6 animals/group. **c,** The left panel shows the LDT (purple) and the caudal PPN (green) in coronal brain sections. Middle and right panels illustrate DAB staining of ChAT in LDT of a control and 1-week runner. Dotted lines outline the LDT. Dark brown stain indicates ChAT+ neurons. Scale bar, 100 µm. **d,** Stereological counts of ChAT+ neurons in the LDT. n=4 animals/group. Scale bar, 100 µm. **e,** The left panel shows the cPPN (green) in a coronal brain section. Middle and right panels illustrate *in situ* hybridization staining of VGLUT2 of a control and a 1-week runner. Dotted lines outline the cPPN. Dark blue-purple stain indicates VGLUT2+ neurons. Scale bar, 100 µm. **f,** Stereological counts of VGLUT2+ neurons in the cPPN. n= 4 animals for Ctrl and 5 animals for Run. Statistical significance was assessed by Mann–Whitney U test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 7

Running-induced transmitter switching reverses after 1 week of rest, even though mice experienced behavioral tests immediately after running and retests one week later.

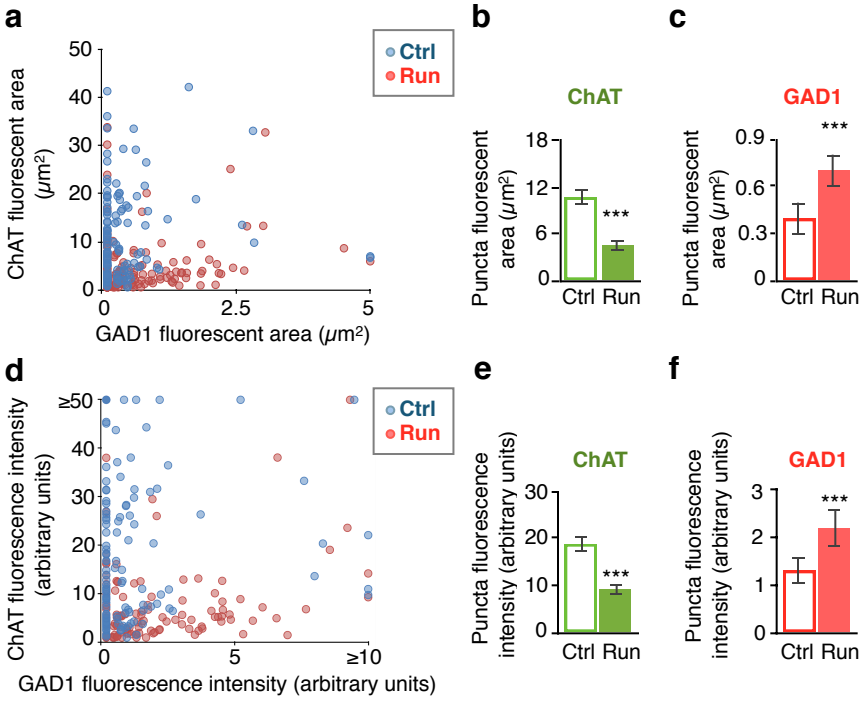
a, Experimental design. **b,** cPPNs from Ctrl or "Run+Rest" mice of the "1-wk rest group" (**Fig. 1g,h**) were stained for ChAT or for GAD1. n=5 animals/group. Statistical significance was assessed by Mann–Whitney U test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 8

Running for 1 week decreases the number of ChAT+ neurons and increases the number of GAD1+ neurons in the caudal PPN of a ChAT-IRES-Cre mouse line.

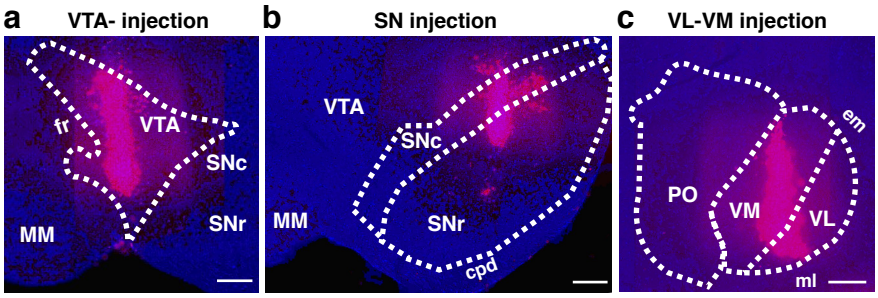
ChAT was detected by DAB staining. GAD1 was detected by *in situ* hybridization. n=5 animals/group. Statistical significance *p<0.05, **p<0.01 was assessed by Mann–Whitney U test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 9

Changes of ChAT and GAD1 transcript area and intensity in nNOS neurons.

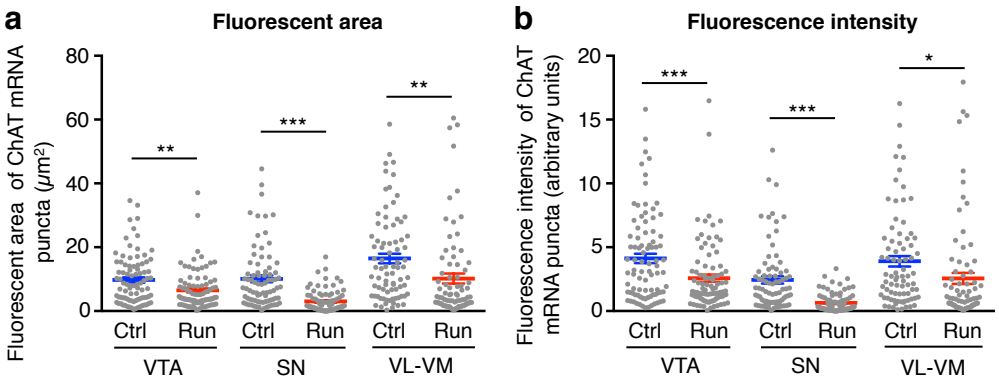
a, Scatterplot of fluorescent area of ChAT transcripts (y-axis) against fluorescent area of GAD1 transcripts (x-axis) in nNOS neurons. Each dot represents one neuron. **b,c**, Mean fluorescent area of ChAT (**b**) or GAD1 (**c**) transcripts in single nNOS+ cells. **d**, Scatterplot of fluorescence intensity of ChAT transcripts (y-axis) against fluorescence intensity of GAD1 transcripts (x-axis) in nNOS cells. Each dot represents one neuron. **e,f**, Mean fluorescence intensity of ChAT (**e**) or GAD1 (**f**) transcripts in single nNOS+ neurons. For (**a-f**), n=4 animals/group; n=123 cells for Ctrl and 137 cells for Run. Statistical significance ***p<0.001 was assessed by Welch's t-test. Data shown are mean± SEM.



Supplementary Figure 10

Anatomical evidence of retrobead injection.

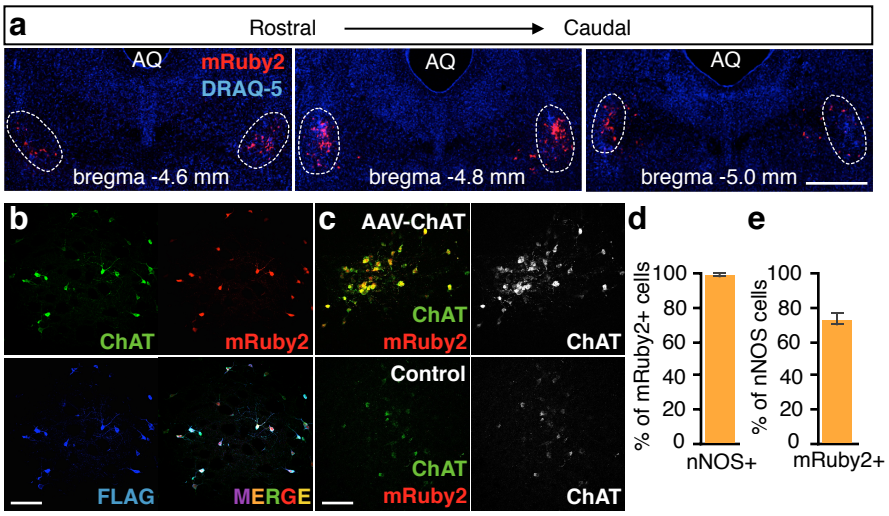
Representative coronal sections show retrobeads (magenta) injected into the VTA (**a**), SN (**b**) and VL-VM (**c**). MM, medial mammillary nucleus; fr, fasciculus retroflexus; SNc, substantia nigra, compact part; SNr, substantia nigra, reticular part; cpd, cerebral peduncle; PO, posterior complex of the thalamus; ml, medial lemniscus; em, external medullary lamina of the thalamus. The boundaries of the nuclei are drawn based on the comparison with the Allen mouse brain atlas. Scale bar, 200 μm.



Supplementary Figure 11

Fluorescent area and fluorescence intensity of ChAT transcripts in nNOS neurons that project to each brain region.

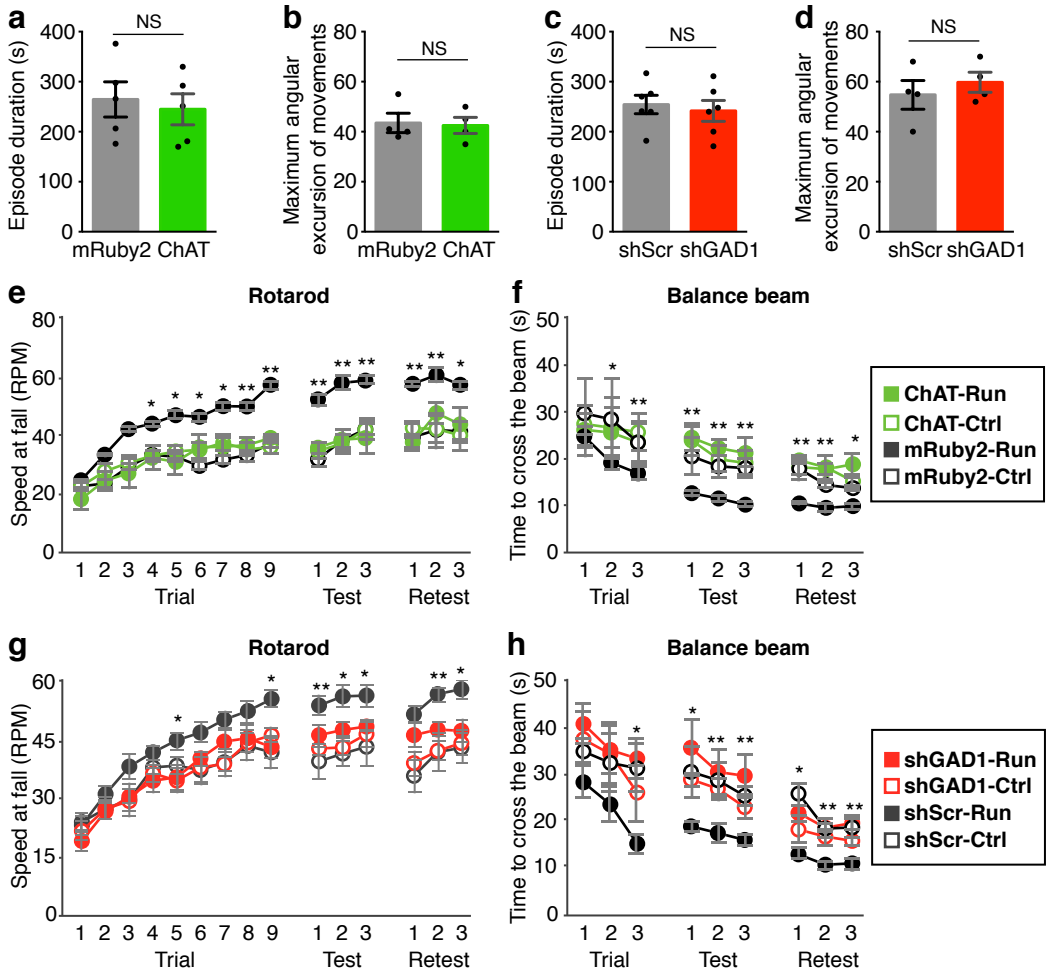
Mean fluorescent area (**a**) or fluorescence intensity (**b**) of ChAT transcripts in nNOS neurons that project to corresponding brain regions (x-axis). Each dot represents one cell. n=4 animals/group. n=89 cells for VTA-Ctrl, 91 for VTA-Run, 81 for SN-Ctrl, 87 for SN-Run, 82 for VL-VM-Ctrl, 80 for VL-VM-Run. Statistical significance *p<0.05,**p<0.01, ***p<0.001 was assessed by Welch's t-test. Data shown are mean± SEM.



Supplementary Figure 12

Validation of the use of the AAV-DIO-ChAT construct.

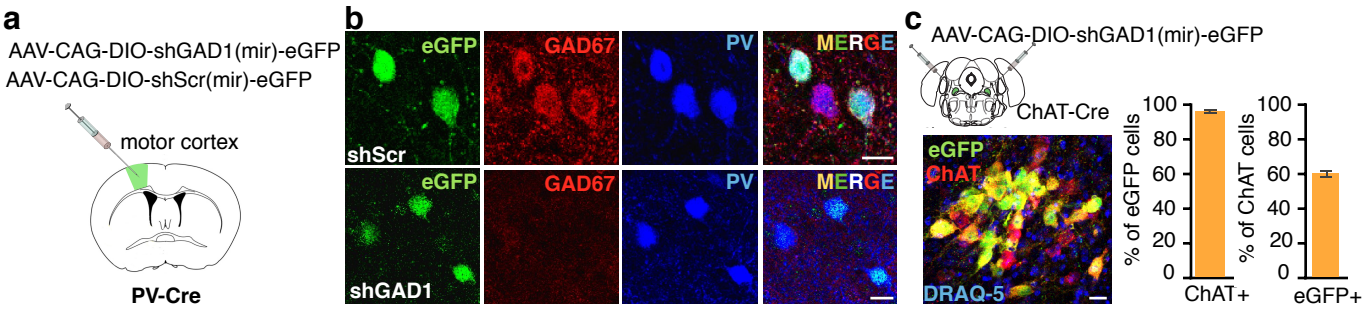
a, mRuby2 expression in rostral-to-caudal sections of the cPPNs (dotted lines) of a single animal bilaterally-injected with AAV-DIO-ChAT. Coordinates adapted to Allen Brain Atlas. AQ, aqueduct. Scale bar, 1 mm. **b,** Triple-labeled images of ChAT, mRuby2, FLAG and merged image in a cPPN of an animal injected with AAV-DIO-ChAT. Scale bar, 100 µm. **c,** Double-stained images of ChAT and FLAG in both AAV-DIO-ChAT-injected (upper panels) or contralateral uninjected control (lower panels). Statistical analysis is in (**Fig. 5c**). Scale bar, 100 µm. **d**, The percentage of nNOS+mRuby2+ neurons in the mRuby2+ population (cell type specificity). n=4 animals/group. **e,** The percentage of nNOS+mRuby2+ population in the nNOS+ neurons (transfection efficiency). n=4 animals/group. Data shown are mean± SEM.



Supplementary Figure 13

Running is not affected but running-induced improvements in motor skill learning are impaired when transmitter switching is overridden.

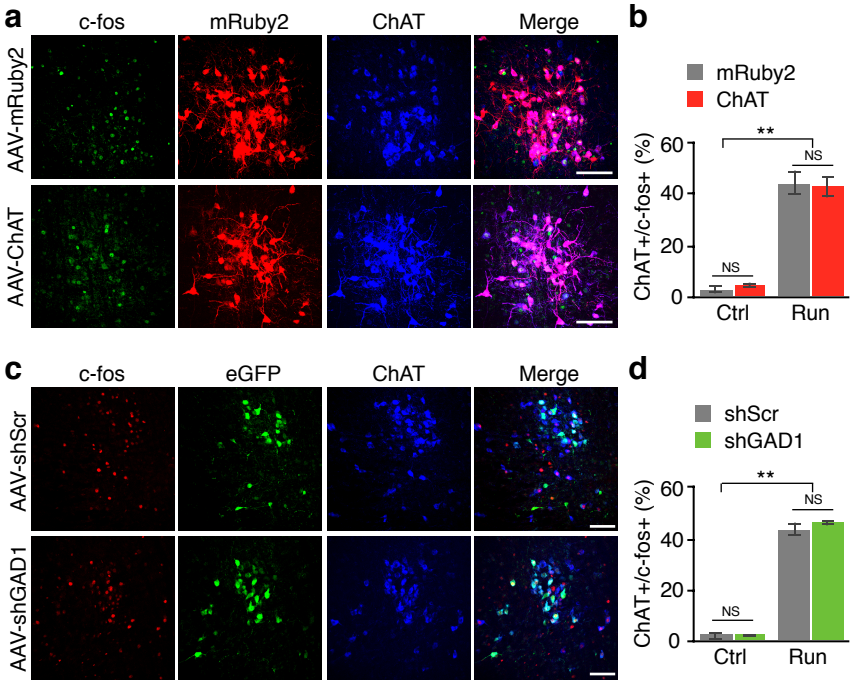
a-d, Mean duration of running episodes and maximum angular excursion of mouse movements on the running wheels for 1-week trained ChAT-Cre runner mice that were injected with AAV-DIO-mRuby2, AAV-DIO-ChAT, AAV-DIO-shScr, or AAV-DIO-shGAD1 at the cPPN. For (**a,c**), n=5 animals for mRuby2 and ChAT, and 6 for shScr and shGAD1. For (**b,d**), n=4 animals for each group. **e,f**, Mean speed at fall on a rotarod and mean time to cross a 1 meter long, 0.75 meter high, 4 mm rod balance beam at each trial during training, at each test on the day after training, and at each retest 1 week after test. n=9 animals for mRuby2-Run and 18 animals for ChAT-Run. **g,h,** Same as (**e,f**). n=8 animals for shScr-Run and 9 for shGAD1-Run. Statistical significance **p*<0.05, ***p*<0.01 was assessed by Welch's t-test. NS, not significant. Data shown are mean± SEM.



Supplementary Figure 14

Validation of the use of the AAV-DIO-shGAD1 construct.

a, Knockdown efficiency of GAD67 was verified *in vivo* by expressing either AAV-DIO-GAD1-shRNAmir-eGFP or AAV-DIO-scramble-shRNAmir-eGFP constructs in the cortex of a PV-Cre mouse. **b,** Brain sections from (**a**) were immunostained for GAD67 and PV**.** Scale bar, 20 µm. **c**, Triple-labeled images of eGFP, ChAT, and DRAQ-5 in the cPPN of a non-runner ChAT-IRES-Cre mouse injected with AAV-DIO-shGAD1. The specificity of transduction was measured as the percentage of ChAT+eGFP+ neurons in the eGFP population (>95%, 197/208) and the efficiency was measured as the percentage of ChAT+eGFP+ neurons in the ChAT population (~60%, 197/330). Similar specificity (>95%) and efficiency (~60%) were also observed for the control shScr construct. Scale bar, 20 µm. n=3 animals/group. Data shown are mean± SEM.



Supplementary Figure 15

Overriding transmitter switching does not affect c-fos expression in cholinergic cPPN neurons.

a, Triple labeling of c-fos, mRuby2 and ChAT in 1-week runners that were injected with AAV-DIO-mRuby2 or AAV-DIO-mRuby2-P2A-ChAT. Scale bar, 100 µm. **b,** Summary of (**a**) and non-runner controls. n=214 cells for mRuby2-Ctrl, 204 for ChAT-Ctrl, 223 for mRuby2-Run and 206 for ChAT-Run. n=3 animals/group. **c,** Triple labeling of c-fos, eGFP and ChAT in 1-week runners that were injected with AAV-DIO-shScr-eGFP or AAV-DIO-shGAD1-eGFP. Scale bar, 100 µm. **d,** Summary of (**c**) and non-runner controls. n=213 cells for shScr-Ctrl, 360 for shGAD1-Ctrl, 277 for shScr-Run and 315 for shGAD1-Run. n=3 animals/group. Statistical significance $^{**}p<0.01$ was assessed by ANOVA followed by Tukey's test. NS, not significant. Data shown are mean± SEM.