

Supplemental Materials for

Large-scale mapping of the MCH network in ALS mice reveals the vulnerability of dopaminergic and GABAergic neurons in Zona Incerta

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Figure S1

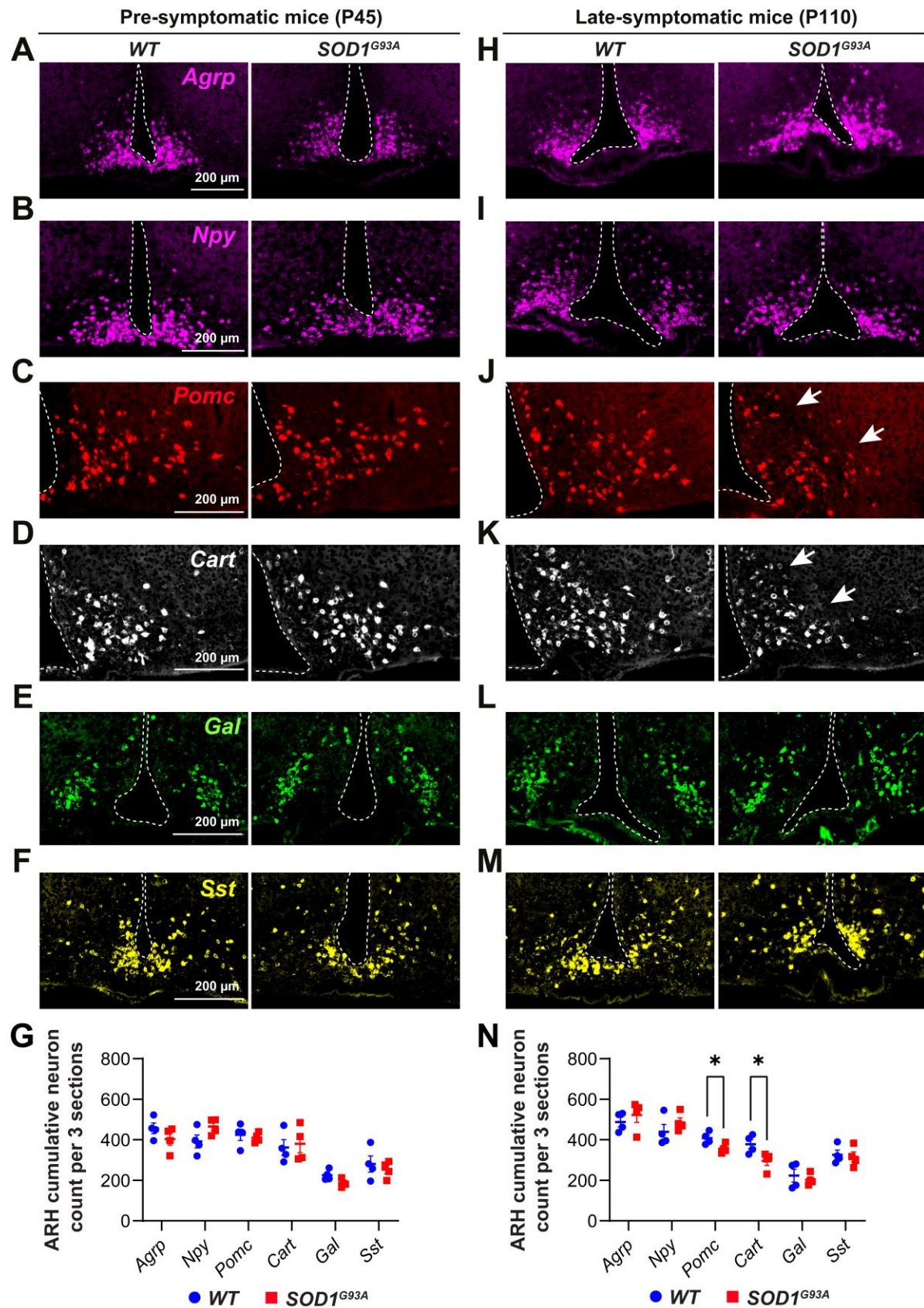


Fig. S1. Loss of POMC and CART populations in the ARH in late-symptomatic *SOD1*^{G93A} mice.

A-F, H-M. Representative images of the ARH of P45 (**A-F**), and P110 (**H-M**) *WT* (left panels) and *SOD1*^{G93A} (right panels) mice showing neuronal mRNA expression of specific neuropeptides: **A, H.** *Agrp* for agouti-related protein (in magenta), **B, I.** *Npy* for neuropeptide Y (in magenta), **C.** *Pomc* for pro-opiomelanocortin (in red), **D, K.** *Cart* (in grey), **E, L.** *Gal* (in green) and **F, M.** *Sst* for somatostatin (in yellow).

G, N. Quantification of neuropeptidergic populations located in the ARH of P45 (**G**), and P110 (**N**) *SOD1*^{G93A} (red) and *WT* (blue) mice. *Pomc* (p=0.03) and *Cart* (p=0.04) expressing neurons are significantly reduced in *SOD1*^{G93A} mice at P110 – late-symptomatic disease stage.

N=4 animals per genotype. (*) p-value<0.05, Multiple unpaired t-test per neuronal population. Data are displayed as the mean ± SEM, with each data point representing a single animal. Scale bars: 200 µm.

Figure S2

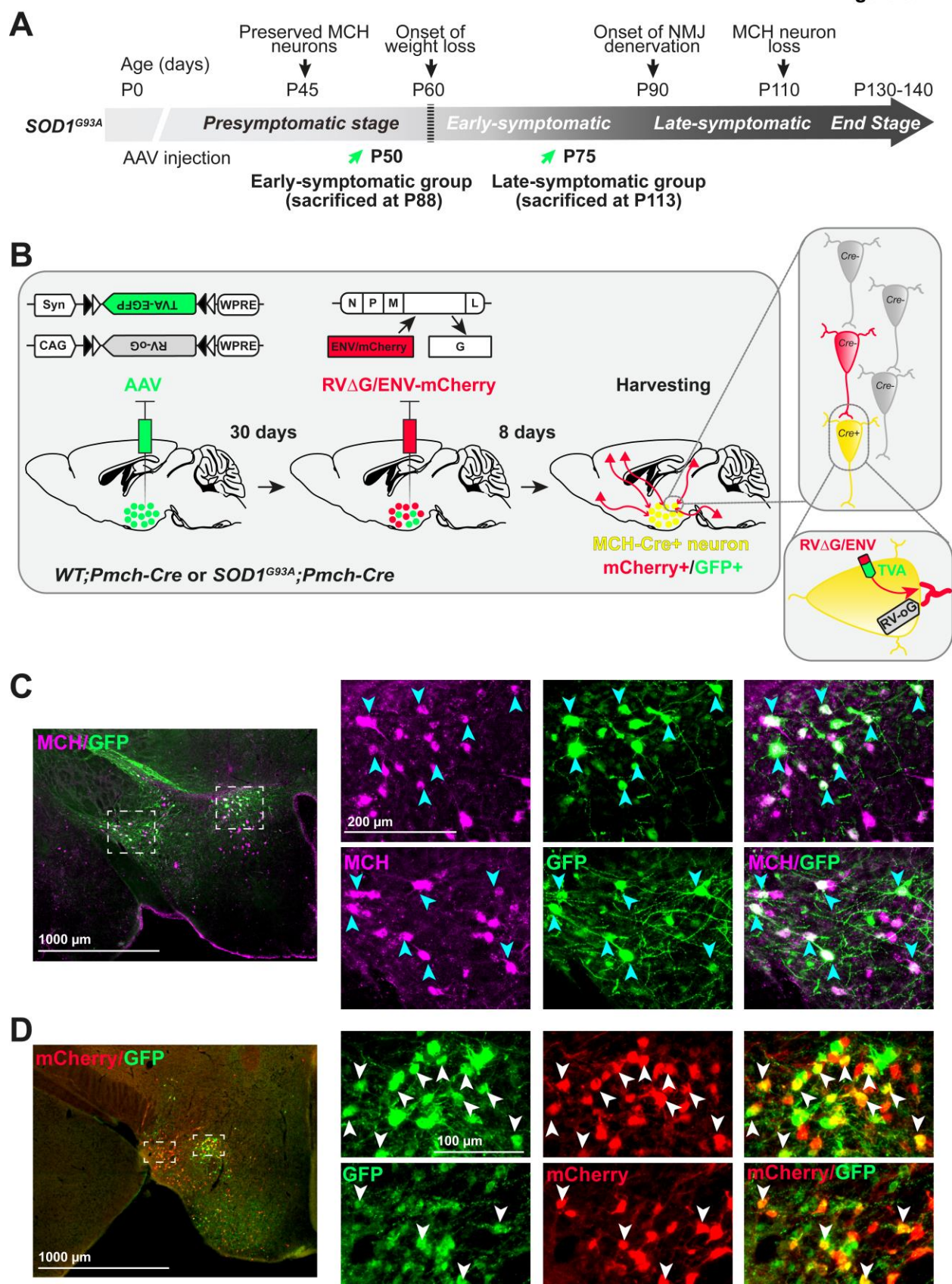


Fig. S2. Experimental strategy for the selective retrograde tracing of whole-brain monosynaptic inputs to MCH neurons.

A. Schematic of the disease stages in *SOD1^{G93A};Pmch-Cre* mice.

B. The scheme outlines the experimental design: AAV constructs expressing Cre-dependent TVA-EGFP and rabies oG protein (helper viruses) were injected at P50 (early-symptomatic mice) or P75 (late-symptomatic mice), followed by an injection of pseudotyped and modified rabies virus (RVΔG/ENV-mCherry) after 30 days and the mice being harvested 8 days later. The enlarged insets illustrate the mechanism of triple viral infection of Cre⁺ MCH neurons (starter neurons – yellow) and their monosynaptic inputs (red). The modified rabies virus can only cross synapses when it is completed by the oG protein inside the Cre⁺ MCH neuron.

C. Representative images of a coronal hypothalamic section (overview) and enlarged insets (white dashed line rectangle) from a *Pmch-Cre* mouse depict the selectively infected hypothalamic MCH neurons (magenta) co-expressing EGFP (green) delivered by AAV vectors, as demonstrated in **B** (cyan arrowheads).

D. An example of a hypothalamic section and enlarged insets showing MCH neurons (starter neurons – yellow), double positive for EGFP and mCherry (white arrowheads), and their direct, monosynaptic input neurons expressing only the mCherry reporter (red).

Scale bars: 1000 μm (for lower magnification images) and 100 μm (for higher magnification images).

Figure S3

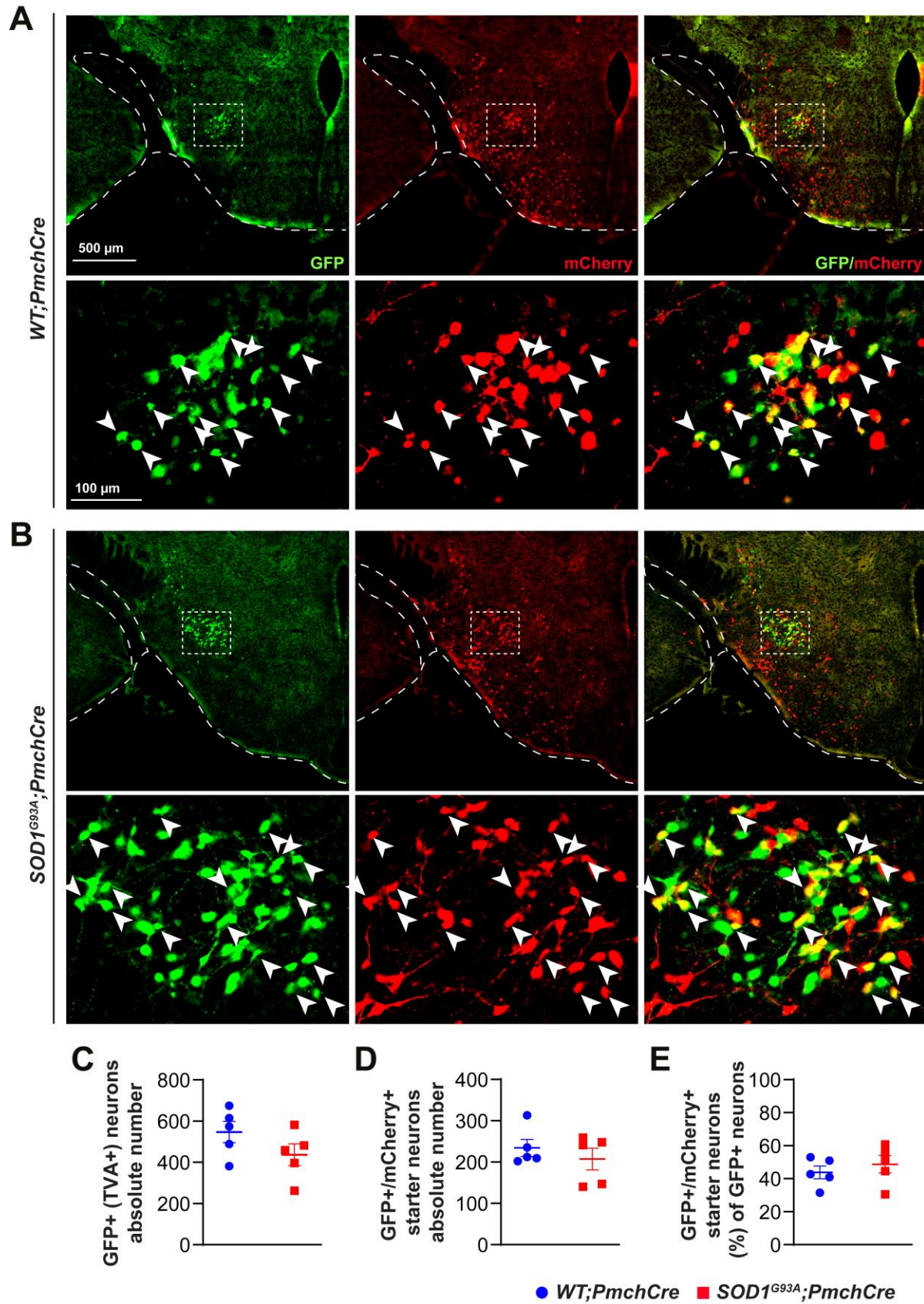


Fig. S3. Starter neurons engineered to enable the initial rabies infection and one-step viral spread to presynaptic partners.

A-B. Representative images of a coronal section of the hypothalamus (overview) and of the LHA (white dashed line square), depicting the starter neurons (yellow, white arrowheads) double positive for TVA-EGFP (green) and the mCherry-tagged modified rabies virus (red) in late-symptomatic *WT;Pmch-Cre* (**A**) and *SOD1^{G93A};Pmch-Cre* (**B**) mice.

C-E. The quantification plots show: a comparable number of neurons infected with TVA-EGFP (**C**), an unchanged number of neurons co-infected (starter neurons) with TVA-EGFP and modified rabies (**D**) and the same percentage of starter neurons (**E**) between genotypes.

N=5 animals per genotype. Two-tailed unpaired Student's t-test. Data are displayed as the mean $\pm$ SEM, with each data point representing a single animal. Scale bars: 500 μ m (for lower magnification images), 100 μ m (for higher magnification images).

Figure S4

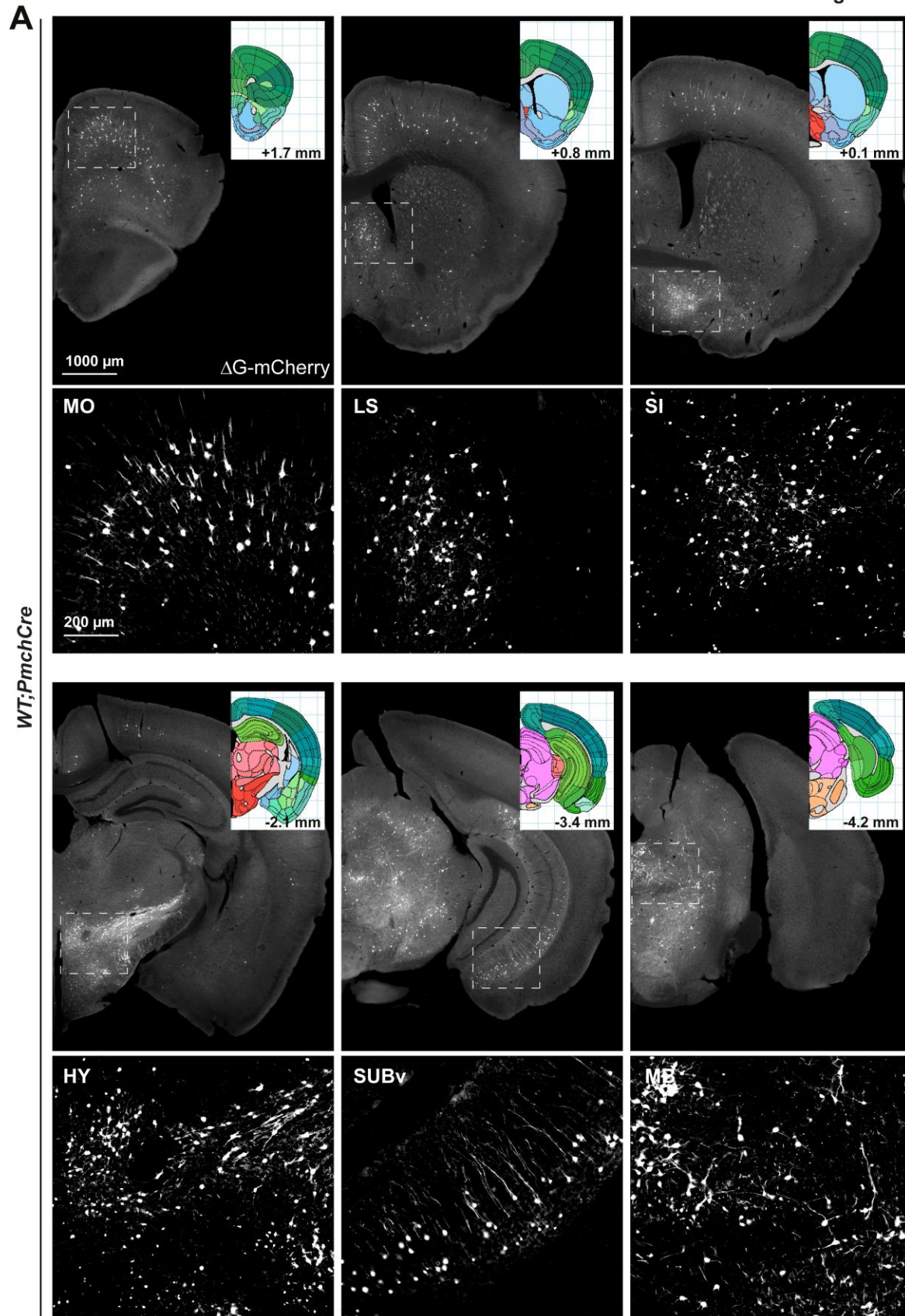
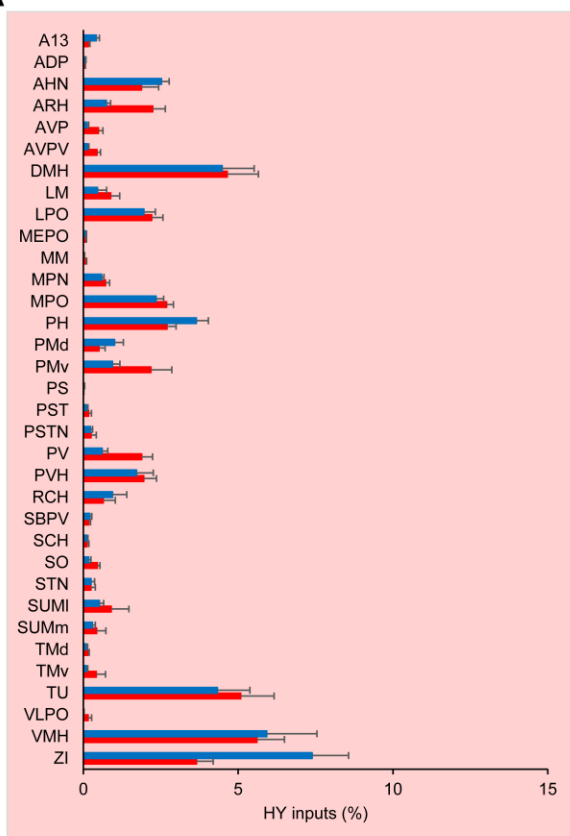
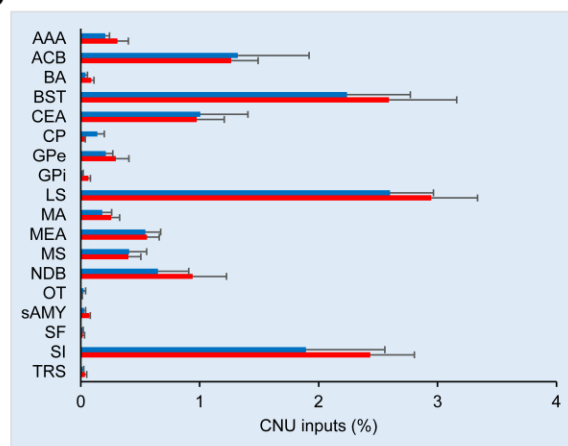
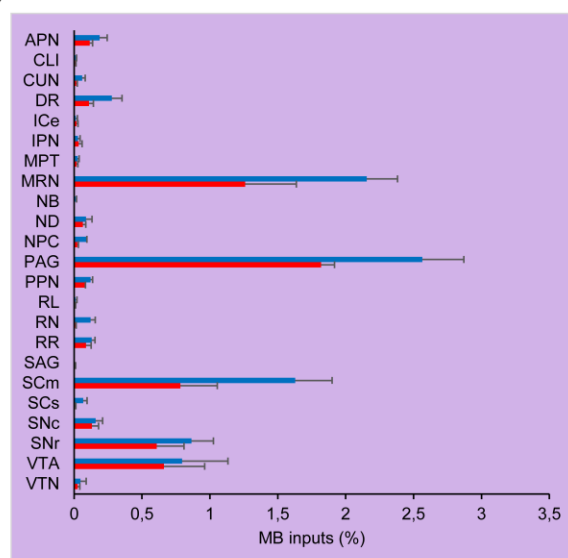


Fig. S4. Distribution of monosynaptic rabies-traced inputs to MCH neurons throughout the whole brain.

A. Representative native fluorescence images of coronal brain sections showing the distribution of mCherry-labelled input neurons to MCH neurons throughout the whole brain of an 88-day-old *WT;Pmch-Cre* mouse. Following modified rabies injection into the LHA, labelled neurons were consistently observed from Bregma +3 mm to Bregma –5 mm. The enlarged insets represent input neurons from the motor cortex (MO), lateral septum (LS), substantia innominata (SI), hypothalamus (HY), ventral subiculum (SUBv) and midbrain (MB). The slice schematics represent images from the Openbrainmap website corresponding to the Bregma positions of coronal brain sections along the anterior-posterior axis.

Scale bar: 1000 μm (for lower magnification images) and 200 μm (for higher magnification images).

Figure S5

A**B****D**

● WT;PmchCre ■ SOD1^{G93A};PmchCre

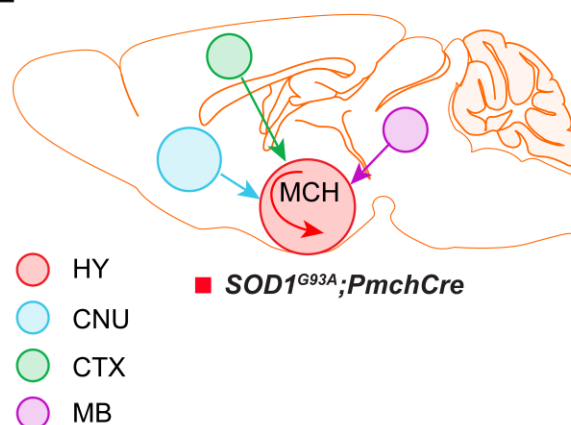
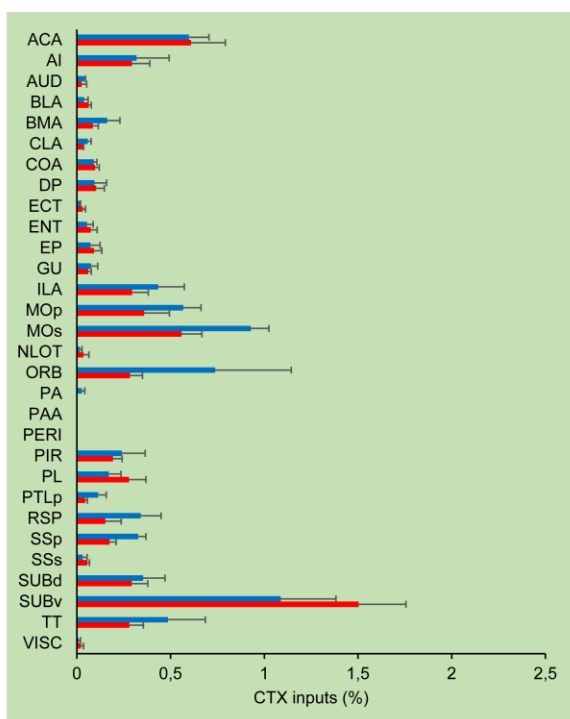
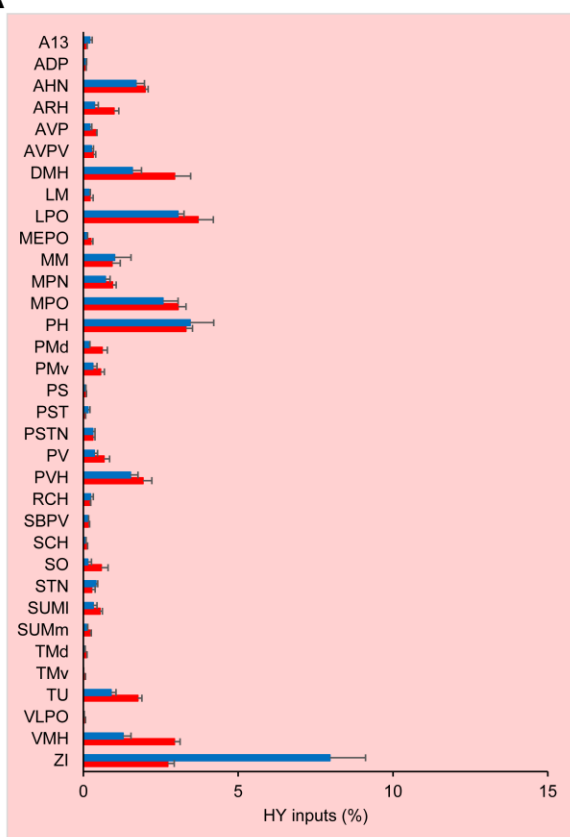
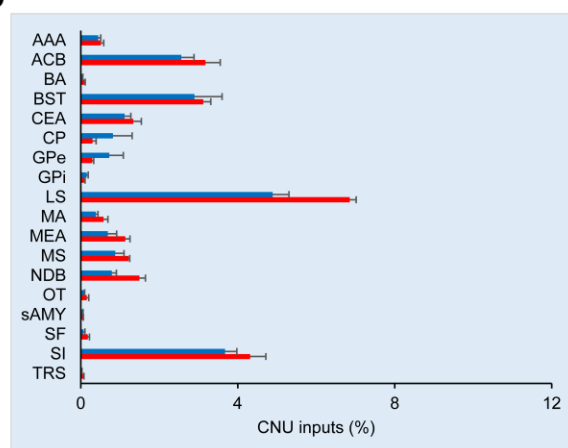
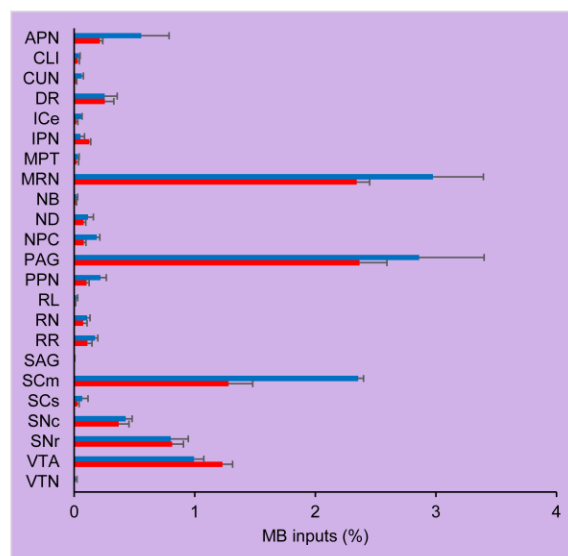
E**C**

Fig. S5. Meso-scale whole-brain mapping of neuronal inputs to MCH neurons traced using a low titer of ΔG rabies virus in early-symptomatic mice.

A-D. High-resolution quantification of monosynaptic inputs to MCH neurons across 130 brain areas of the hypothalamus (**A**), cerebral nuclei (**B**), cortex (**C**) and midbrain (**D**). Data are represented as the mean $\pm$ SEM of the proportion of inputs from each area/region in early-symptomatic *WT;Pmch-Cre* and *SOD1^{G93A};Pmch-Cre* mice (N=4 per genotype) injected with a low titer of modified rabies virus (LT). **e.** Schematic showing the relative contributions of four large-scale areas inputs to MCH neurons. The size of each circle represents the proportion of total whole-brain inputs and the arrows indicate the unchanged large-scale inputs in *SOD1^{G93A};Pmch-Cre* mice (thin line). Anatomical abbreviations are based on the Allen Brain Atlas (ABA), and are fully defined in **Table S3**.

Figure S6

A**B****D**

● WT;PmchCre ■ SOD1^{G93A};PmchCre

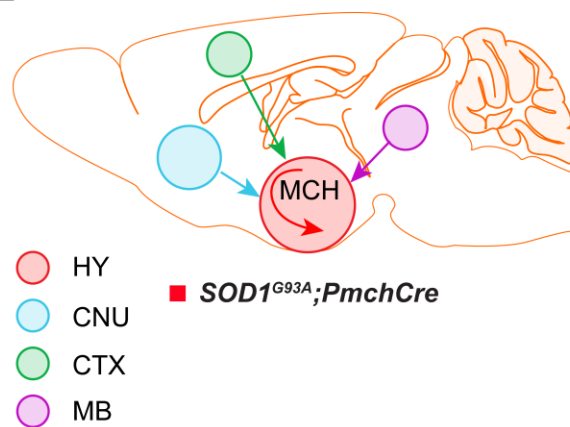
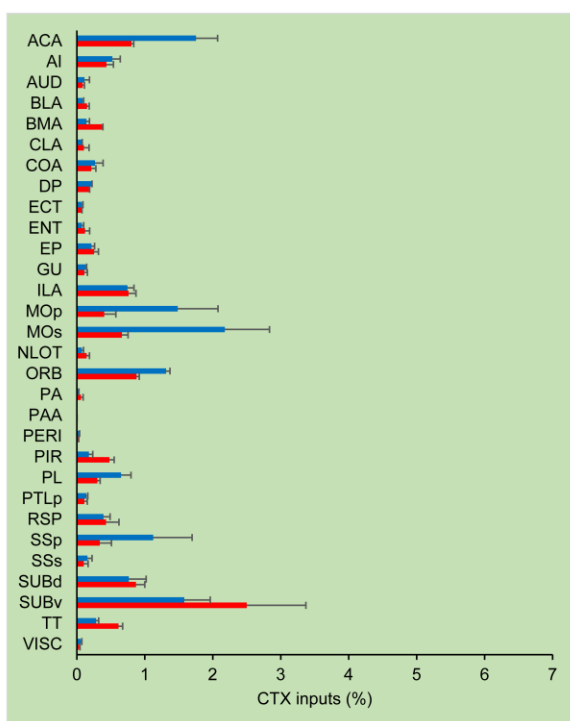
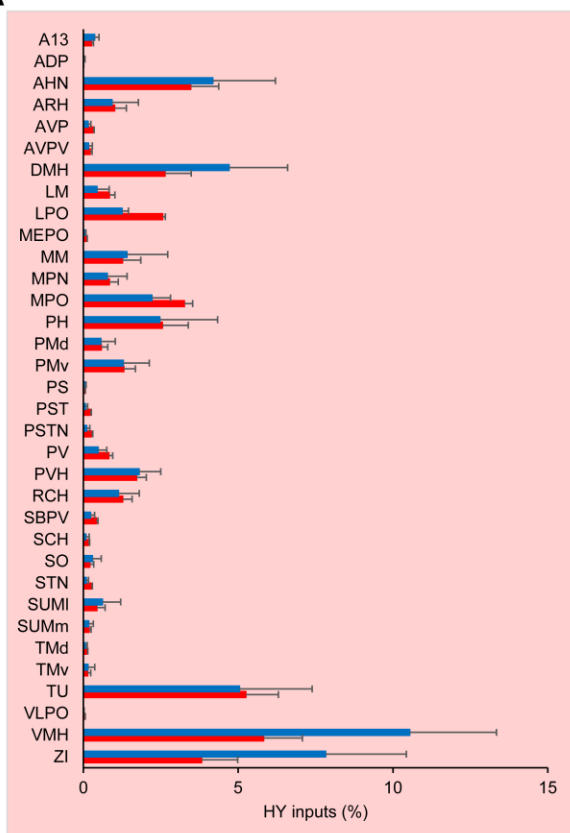
E**C**

Fig. S6. Meso-scale whole-brain mapping of neuronal inputs to MCH neurons traced using a high titer of ΔG rabies virus in early-symptomatic mice.

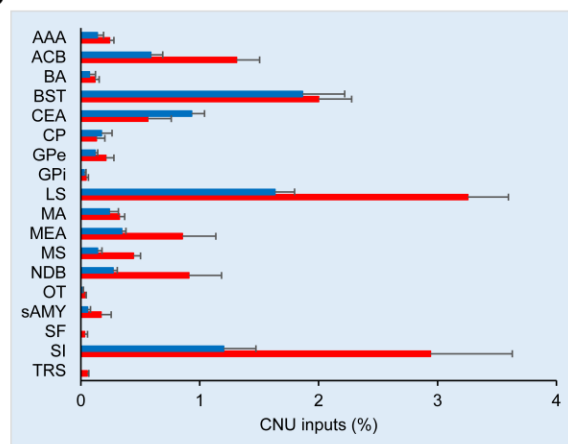
A-D. High-resolution quantification of monosynaptic inputs to MCH neurons across 130 brain areas of the hypothalamus (**A**), cerebral nuclei (**B**), cortex (**C**) and midbrain (**D**). Data are represented as the mean $\pm$ SEM of the proportion of inputs from each area/region in early-symptomatic *WT;Pmch-Cre* and *SOD1^{G93A};Pmch-Cre* mice (N=3 per genotype) injected with a high titer of modified rabies virus (HT). **e.** Schematic showing the relative contributions of four large-scale areas inputs to MCH neurons. The size of each circle represents the proportion of total whole-brain inputs and the arrows indicate the unchanged large-scale inputs in *SOD1^{G93A};Pmch-Cre* mice (thin line). Anatomical abbreviations are based on the Allen Brain Atlas (ABA), and are fully defined in **Table S3**.

Figure S7

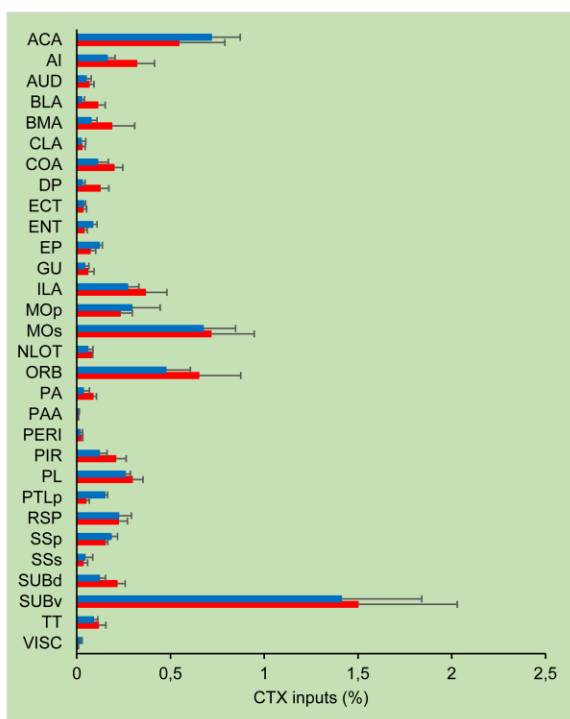
A



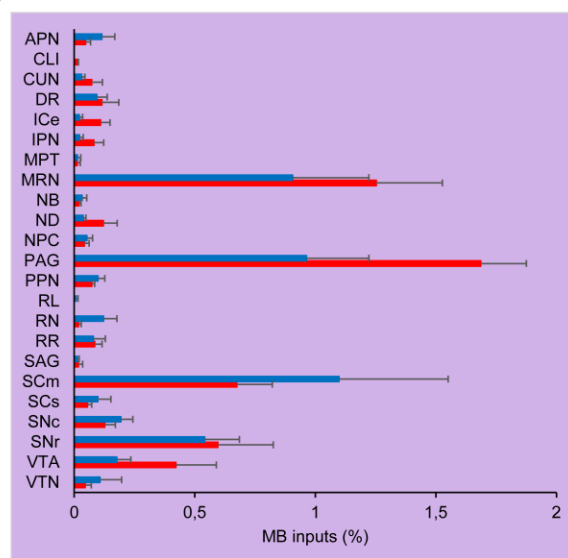
B



C



D



● WT;PmchCre ■ SOD1^{G93A};PmchCre

E

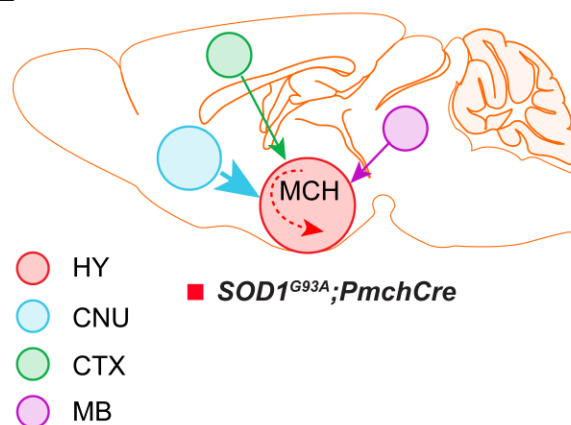


Fig. S7. Meso-scale whole-brain mapping of neuronal inputs to MCH neurons traced using a low titer of ΔG rabies virus in late-symptomatic mice.

A-D. High-resolution quantification of monosynaptic inputs to MCH neurons across 130 brain areas of the hypothalamus (**A**), cerebral nuclei (**B**), cortex (**C**) and midbrain (**D**). Data are represented as the mean $\pm$ SEM of the proportion of inputs from each area/region in early-symptomatic *WT;Pmch-Cre* and *SOD1^{G93A};Pmch-Cre* mice (N=5 per genotype) injected with a low titer of modified rabies virus (LT). **e.** Schematic showing the relative contributions of four large-scale areas inputs to MCH neurons. The size of each circle represents the proportion of total whole-brain inputs and the arrows indicate unchanged (thin line), decreased (dashed line), or increased (thick line) large-scale inputs in *SOD1^{G93A};Pmch-Cre* mice. Anatomical abbreviations are based on the Allen Brain Atlas (ABA), and are fully defined in **Table S3**.

Figure S8

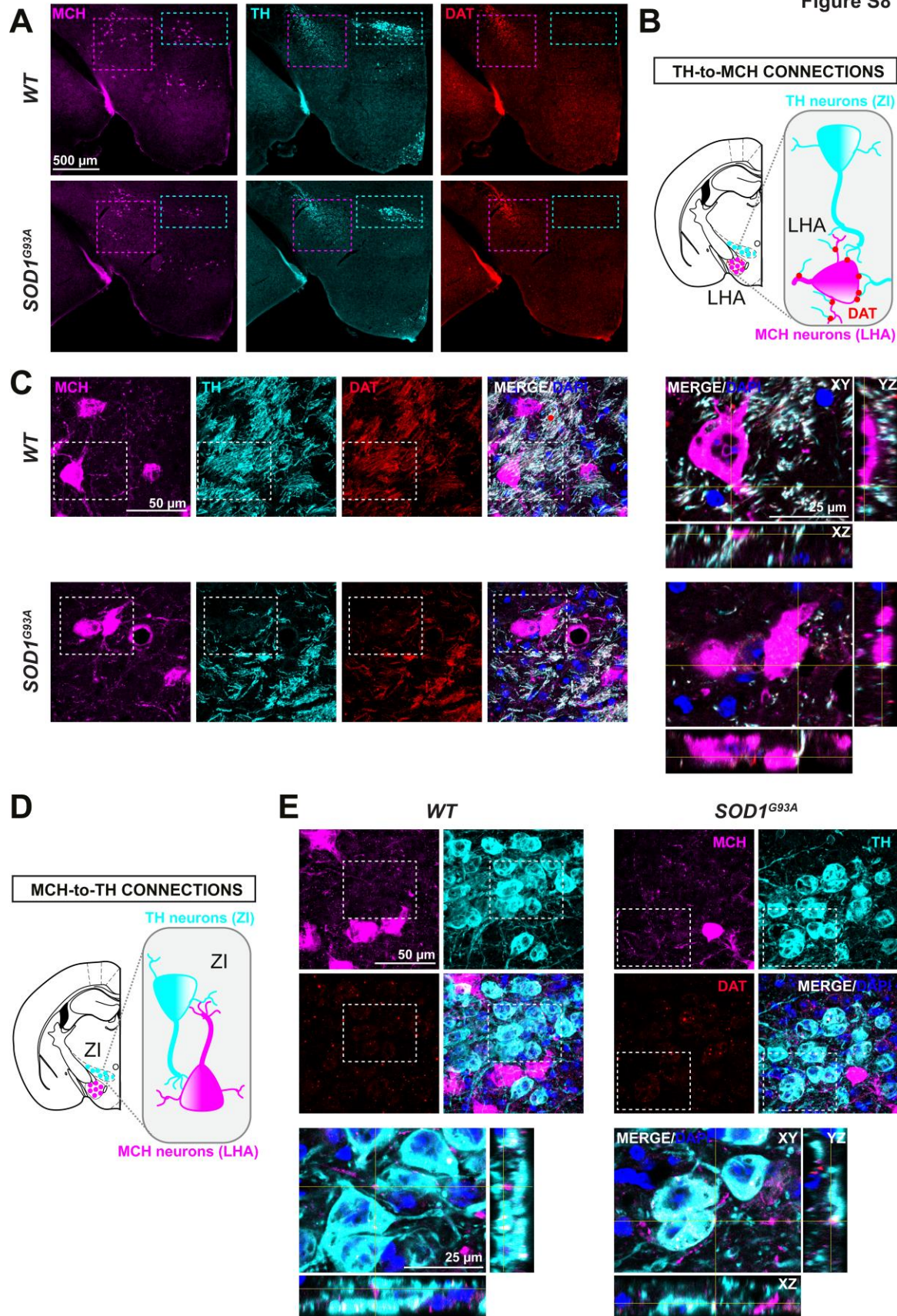


Fig. S8. Bidirectional connections between TH and MCH neurons.

A. Representative images of the coronal section of the hypothalamus (overview) and of regions of interest: LHA (magenta dashed line square) and ZI (cyan dashed line square).

B. A schematic showing projections from TH (cyan) to MCH (magenta) neurons and the expression of the dopamine transporter - DAT (red) in the LHA.

C. Representative confocal images of the LHA showing MCH⁺ neurons surrounded by dense TH⁺/DAT⁺ fibres/puncta. The enlarged inset (white dashed line square) and the orthogonal XZ and YZ views illustrate the co-localization of the TH⁺/DAT⁺ fibres/puncta with the cell body of an MCH⁺ neuron.

D. Schematic depicting projections from the MCH (magenta) to TH (cyan) neurons in the ZI.

E. Representative confocal images of the ZI depicting TH⁺ neurons surrounded by MCH⁺ fibres. The enlarged inset (white dashed line square) and the orthogonal XZ and YZ views illustrate the co-localization of MCH⁺ fibres with the cell body of a TH⁺ neuron.

Scale bar: Overview images 1000 μm . Confocal images 50 μm (for lower magnification images) and 25 μm (for higher magnification images).

Figure S9

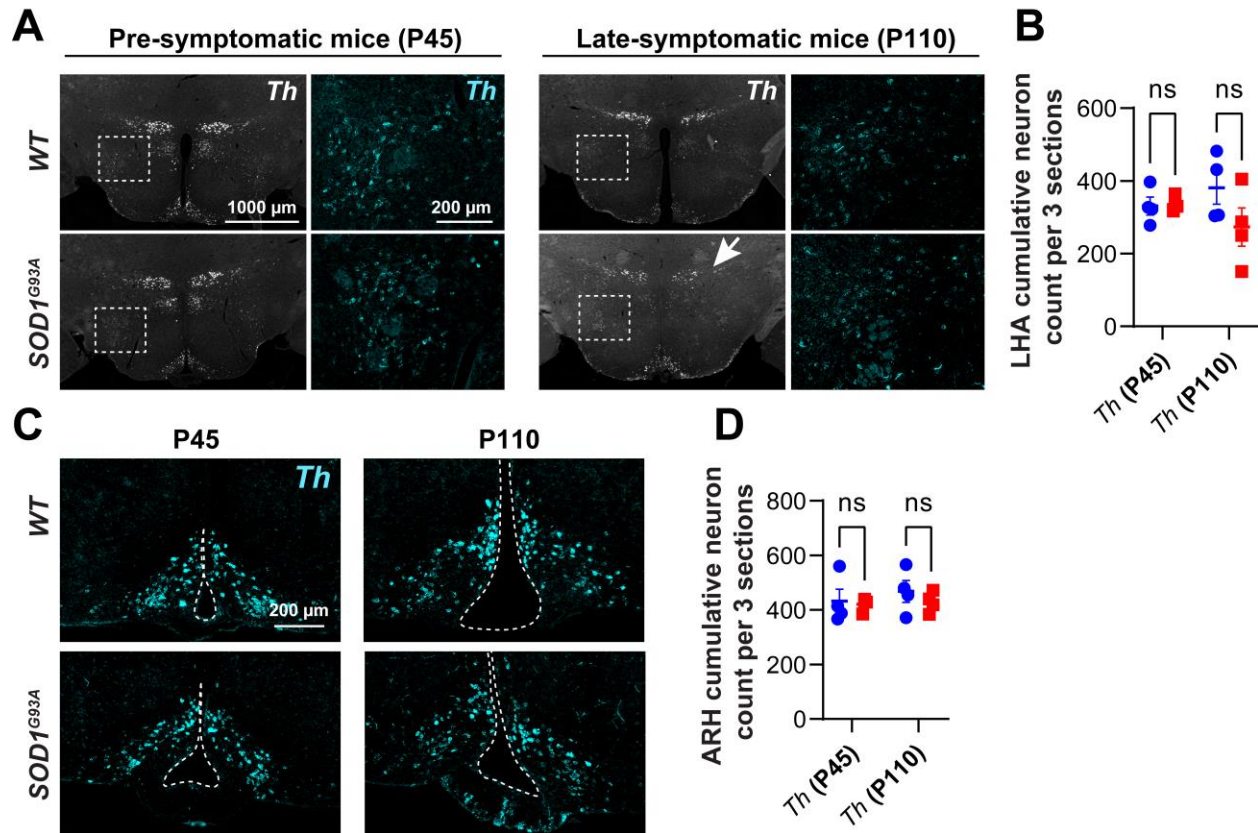


Fig. S9. DAergic neurons are preserved in the LHA and ARH.

A. Representative images show the distribution of *Th*⁺ neuronal populations (cyan) in the hypothalamus (overview) and in the LHA (white dashed line square) as detected by a RNAScope in WT (upper panels) and *SOD1*^{G93A} (lower panels) mice at P45 (left columns), and at P110 (right columns). The white arrow indicates the visible loss of *Th*⁺ neurons in the ZI of *SOD1*^{G93A} mice in the same section.

B. The graph shows the cumulative number of *Th*⁺ neurons in the LHA counted across three sections in WT (blue) and *SOD1*^{G93A} (red) mice.

C. Distribution of the *Th*⁺ neurons in the ARH around the third ventricle (white dashed line).

D. Quantification of *Th*⁺ neurons in the ARH.

N=4 animals per genotype. (ns) p-value>0.05. Multiple unpaired t-test per age. Data are presented as the mean ± SEM, with each data point representing a single animal. Scale bars: 1000 μm (for lower magnification images) and 200 μm (for higher magnification images).

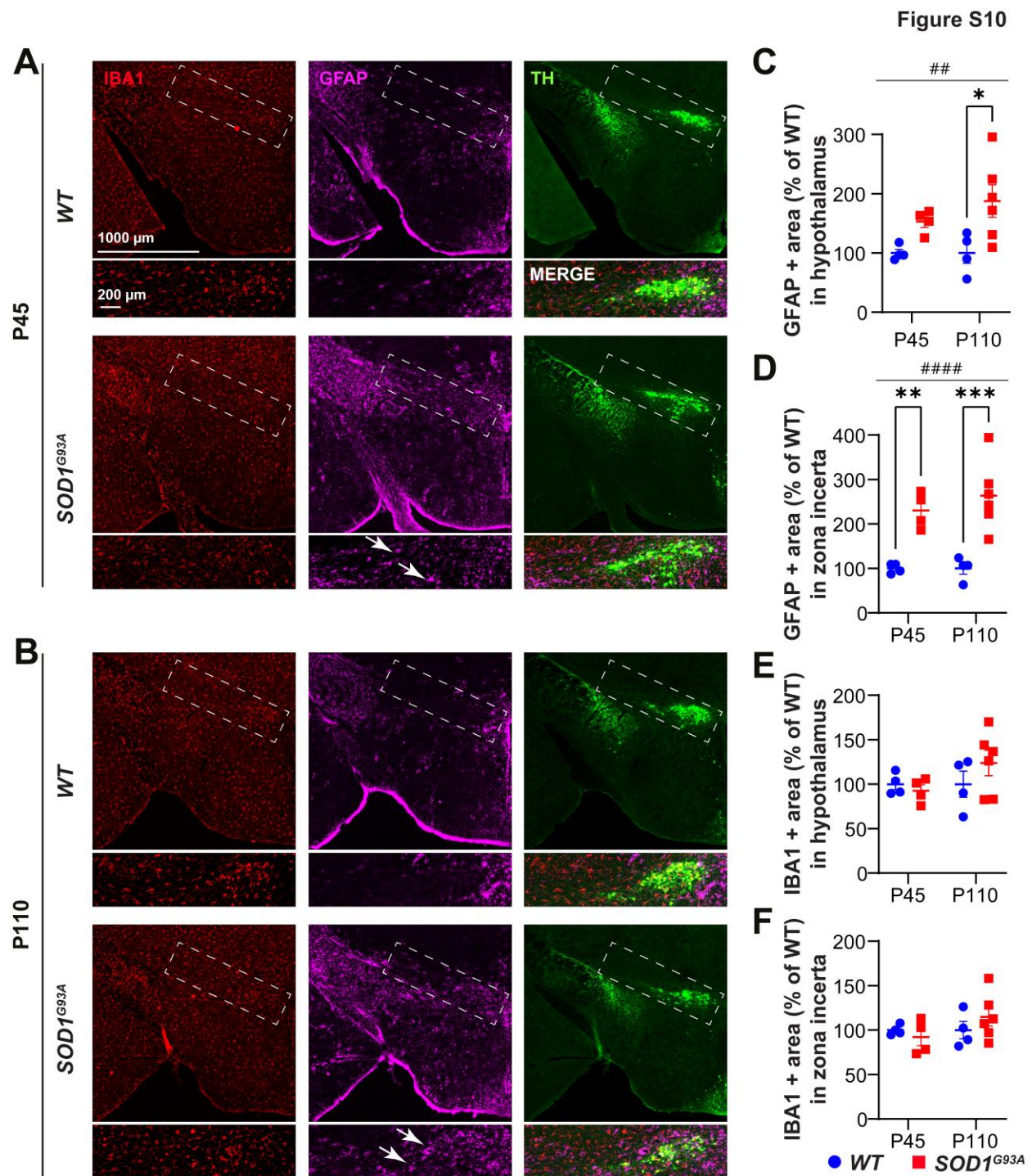


Fig. S10. Marked astrogliosis in the ZI occurs early, in pre-symptomatic *SOD1^{G93A}* mice.

A-B. Representative images of coronal hypothalamic sections (overview) and the ZI (enlarged insets – white dashed line square) showing immunolabelling for the microglia marker IBA1(red), astrocytes (GFAP, magenta) and DAergic TH⁺ neurons (green) in WT mice (upper panel) and high

GFAP expression in *SOD1^{G93A}* mice (lower panels) at P45 (**A**) and at P110 (**B**). The enlarged insets illustrate the increased density of GFAP+ cells in close proximity to TH+ neurons (white arrows). **C-D**. Quantification plots show a significant increase in the percentage of GFAP+ area throughout the HY (**C**) in *SOD1^{G93A}* mice compared to *WT* littermates ($p=0.02$ at P110, and genotype effect by 2-way ANOVA ($F_{1,14}=10.90$, $p=0.005$)), which is particularly pronounced in the ZI (**D**) at both ages ($p=0.006$ at P45; $p=0.0004$ at P110, and genotype effect by 2-way ANOVA ($F_{1,14}=35.77$, $p<0.0001$)).

E-F. Graphs show a comparable percentage of IBA1+ area in the HY (**E**) and ZI (**F**) between *SOD1^{G93A}* and *WT* mice at both ages.

N=3-6 animals per genotype. 2-way ANOVA followed by Bonferroni's multiple comparisons test, (##) $p\text{-value}<0.01$, (####) $p\text{-value}<0.0001$ for genotype effect and (*) $p\text{-value}<0.05$, (**) $p\text{-value}<0.01$, (***) $p\text{-value}<0.001$ for pairwise comparison. Data are presented as the mean $\pm$ SEM, with each data point representing a single animal. Scale bars: 1000 μm (for lower magnification images) and 200 μm (for higher magnification images)

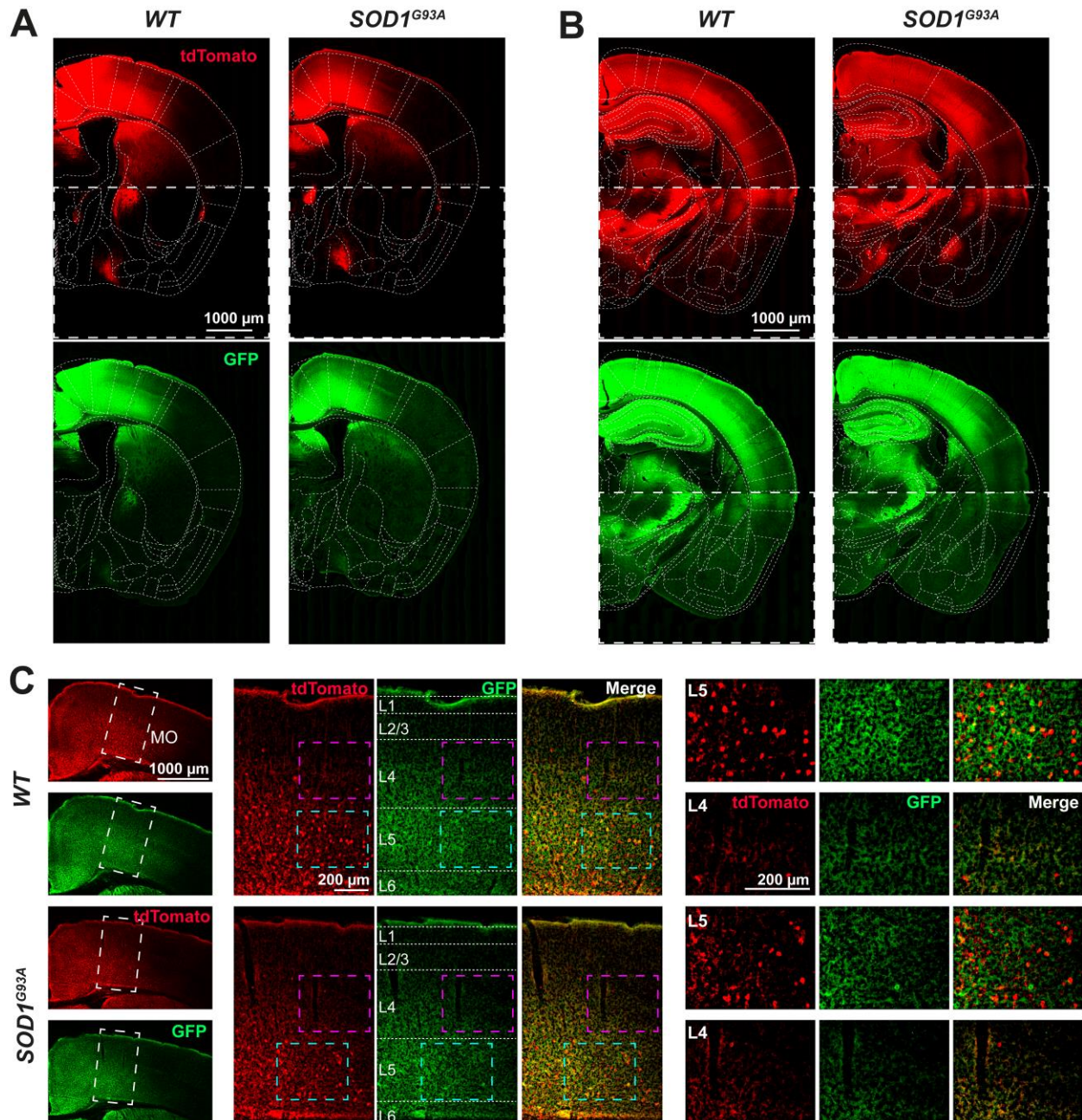


Fig. S11. Expression of anterograde viral tracer in the motor cortex.

A. Representative immunofluorescence (IF) images of coronal brain sections from WT and *SOD1^{G93A}* mice corresponding to the injection site (AP: -0.1 mm, ML: -0.7 mm, DV: -0.7 mm from Bregma), showing the expression of the fluorescent proteins tdTomato (red) and GFP (green), delivered by viral vectors, as indicated in Figure 8A. The white dashed line separates regions of the same section that were imaged using different exposure parameters.

B. Representative IF images of coronal brain sections used to investigate L5 PN connections to the hypothalamus corresponding to -2.0 mm and -1.6 mm caudal from Bregma.

C. Representative images of coronal sections of the mouse cortex (overview) and motor cortex (MO) (white dashed line square) showing tdTomato⁺ neurons and GFP⁺ puncta in layers 5 and 6 (cyan dashed line square), and the absence of these in layer 4 (magenta dashed line square) in both *WT* and *SOD1*^{G93A} mice.

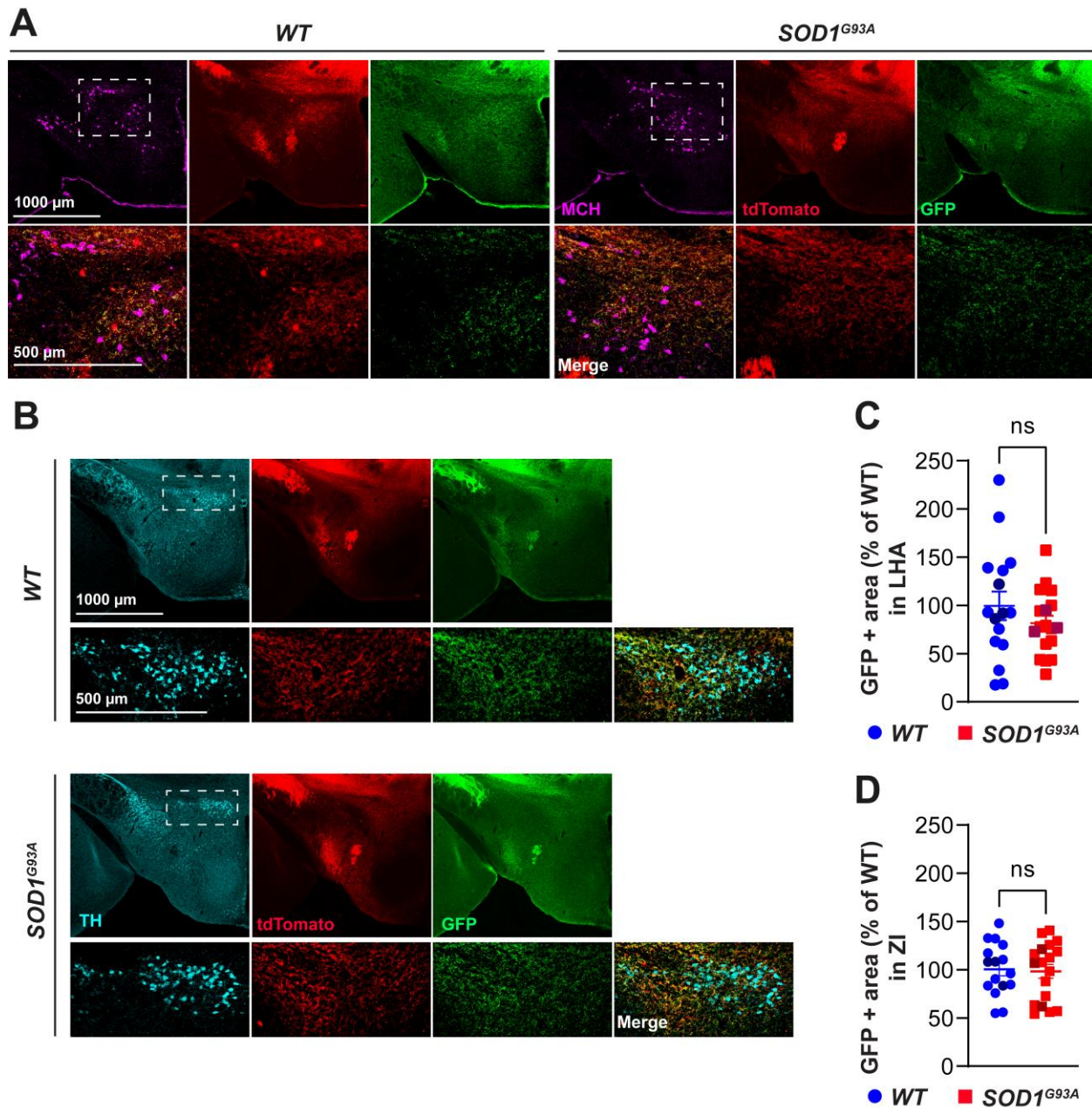


Fig. S12. Distribution of anterogradely traced cortical projections in the hypothalamus.

A-B. Representative IF images of coronal hypothalamic section (overview) and the LHA (**A**) or the ZI (**B**) (enlarged insets – white dashed line square) showing the distribution of tdTomato+ fibres (red) and GFP+ puncta (green) around MCH+ neurons (magenta) (**A**) and around TH+ neurons (cyan) (**B**) in *WT* and *SOD1^{G93A}* mice.

C-D. Quantification plots showing comparable percentage of the GFP⁺ area in the LHA (**C**) and in the ZI (**D**) between *SOD1*^{G93A} and *WT* mice.

N=3 animals per genotype. Two-tailed unpaired Student's t-test, (ns) p-value>0.05. Data are displayed as the mean $\pm$ SEM, with each data point representing a single animal. Scale bars: 1000 μ m (for lower magnification images), 100 μ m (for higher magnification images) and 50 μ m for confocal images.

Table S1. Catalogue numbers of the RNA probes and dilutions used for the RNAscope assay

Neuropetidergic population	Gene	Company	Channel	Catalog nr.	Dilution
Melanin-concentrating hormone (MCH)	<i>Pmch</i>	ACD	2	478721-C2	1:100
Orexin/hypocretin	<i>Hcrt</i>	ACD	2	490461-C2	1:100
Cocaine- and amphetamine-regulated transcript (CART)	<i>Cartpt</i>	ACD	1	42001-C1	1:50
Tyrosine hydroxylase	<i>Th</i>	ACD	3	317621-C3	1:50 or 1:100 (Fig.8C; Suppl.Fig.)
Galanin	<i>Gal</i>	ACD	1	400961-C1	1:50
Agouti-related peptide	<i>Agrt</i>	ACD	2	400711-C2	1:50
Neuropeptide Y	<i>Npy</i>	ACD	1	313321-C1	1:50
Pro-opiomelanocortin (POMC)	<i>Pomc</i>	ACD	2	314081-C2	1:50
Somatostatin	<i>Sst</i>	ACD	1	404631-C1	1:50
Tdtomato	<i>Tdtomato</i>	ACD	2	317041-C2	1:100
Gamma-aminobutyric acid (GABA) vesicular transporter (VGAT)	<i>Slc32a1</i>	ACD	3	319191-C3	1:100
Vesicular glutamate transporter 2 (VGlut2)	<i>Slc17a6</i>	ACD	3	319171-C3	1:100

Table S2. Catalogue numbers of the antibodies and dilutions used for immunolabelling

Primary antibodies	Host	Company	Catalog nr.	Dilution (application)
MCH	Rabbit	Phoenix Pharma	H-070-47	1:200 (IF)
GFP	Chicken	Abcam	ab13970	1:500 (IF)
RFP	Goat	Origene	AB8181-200	1:500 (IF)
Tyrosine hydroxylase (TH)	Guinea pig	SySy	213 104	1:200 (IF)
DOPA-decarboxylase (DDC)	Mouse	SySy	369 011	1:200 (IF)
DOPA-decarboxylase (DDC)	Rabbit	SySy	369003	1:200 (IF)
B8H10-SOD1 (human)	Mouse	Medimabs	MM-0070	1:500 (IF)
Iba1	Rabbit	SySy	234008	1:1000 (IF)
GFAP	Mouse	Sigma	G3893	1:400 (IF)
Matrin3	Rabbit	Abcam	84422	1:250 (IF)
Dopamine transporter (DAT)	Guinea pig	SySy	284 005	1:200 (IF)
Tyrosine hydroxylase (TH)	Chicken	SySy	213 106	1:200 (IF)

Secondary antibodies	Host	Company	Catalog nr.	Dilution (application)
Anti-chicken CF 488A	Donkey	Sigma	SAB4600031	1:200 (IF)
Anti-goat AF 568	Donkey	Invitrogen	A11057	1:500 (IF)
Anti-Rabbit AF 647	Donkey	Invitrogen	A31573	1:500 (IF)
Anti-guinea pig AF 488	Donkey	Jackson	706-545-148	1:500 (IF)
Anti-mouse AF 568	Donkey	Invitrogen	A10037	1:500 (IF)
Anti-Rabbit AF 568	Donkey	Invitrogen	A10042	1:500 (IF)
Anti-mouse AF Plus 488	Donkey	Invitrogen	A32766	1:500 (IF)
Anti-guinea pig AF 647	Donkey	Jackson	706-605-148	1:500 (IF)
Anti-mouse AF 647	Donkey	Invitrogen	A31571	1:500 (IF)
Anti-guinea pig CF 568	Donkey	Sigma	SAB4600469	1:500 (IF)
Anti-chicken CF 488A	Donkey	Sigma	SAB4600031	1:500 (IF)

Table S3. Anatomical abbreviations of meso-scale whole-brain structures (acronym based on the Allen Atlas)

Hypothalamus	
	acronym
Dopaminergic A13 group	A13
Anterodorsal preoptic nucleus	ADP
Anterior hypothalamic nucleus	AHN
Arcuate hypothalamic nucleus	ARH
Anteroventral preoptic nucleus	AVP
Anteroventral periventricular nucleus	AVPV
Dorsomedial nucleus of the hypothalamus	DMH
Lateral mammillary nucleus	LM
Lateral preoptic area	LPO
Median preoptic nucleus	MEPO
Medial mammillary nucleus	MM
Medial preoptic nucleus	MPN
Medial preoptic area	MPO
Posterior hypothalamic nucleus	PH
Dorsal premammillary nucleus	PMd
Ventral premammillary nucleus	PMv
Parastrial nucleus	PS
Preparasubthalamic nucleus	PST
Parasubthalamic nucleus	PSTN
Pariventricular hypothalamic nucleus	PV
Paraventricular hypothalamic nucleus	PVH
Retrochiasmatic area	RCH
Subparaventricular zone	SBPV
Suprachiasmatic nucleus	SCH
Supraoptic nucleus	SO
Subthalamic nucleus	STN
Supramammillary nucleus, lateral	SUMl
Supramammillary nucleus, medial	SUMm
Tuberomammillary nucleus, dorsal part	TMd
Tuberomammillary nucleus, ventral part	TMv
Tuberal nucleus	TU
Ventrolateral preoptic nucleus	VLPO
Ventromedial hypothalamic nucleus	VMH
Zona incerta	ZI

Cerebral nuclei	
Anterior amygdalar are	AAA
Nucleus accumbens	ACB
Bed nucleus of the accessory olfactory tract	BA
Bed nuclei of the stria terminalis	BST
Central amygdalar nucleus	CEA
Caudoputamen	CP
Globus pallidus, external segment	GPe
Globus pallidus, internal segment	GPI
Lateral septal nucleus	LS
Magnocellular nucleus	MA
Medial amygdalar nucleus	MEA
Medial septal nucleus	MS
Diagonal band nucleus	NDB
Olfactory tubercle	OT
Striatum-like amygdalar nuclei	sAMY
Septofimbrial nucleus	SF
Substantia innominata	SI
Triangular nucleus of septum	TRS
Cortex	
Anterior cingulate area	ACA
Anterior cingulate area	AI
Auditory areas	AUD
Basolateral amygdalar nucleus	BLA
Basomedial amygdalar nucleus	BMA
Clastrum	CLA
Cortical amygdalar area	COA
Dorsal peduncular area	DP
Ectorhinal area	ECT
Entorhinal area	ENT
Endopiriform nucleus	EP
Gustatory areas	GU
Infralimbic area	ILA
Primary motor area	MOp
Secondary motor area	MOs
Nucleus of the lateral olfactory tract	NLOT
Orbital areaaud	ORB
Posterior amygdalar nucleus	PA
Piriform-amygdalar area	PAA
Perirhinal area	PERI

Piriform area	PIR
Prelimbic area	PL
Posterior parietal association areas	PTLp
Retrosplenial area	RSP
Primary somatosensory area	SSp
Supplemental somatosensory area	SSs
Subiculum, dorsal part	SUBd
Subiculum, ventral part	SUBv
Taenia tecta	TT
Visceral area	VISC
Midbrain	
Anterior pretectal nucleus	APN
Central linear nucleus raphe	CLI
Cuneiform nucleus	CUN
Dorsal nucleus raphe	DR
Edinger-Westphal nucleus	EW
Interpeduncular nucleus	IPN
Medial pretectal area	MPT
Midbrain reticular nucleus	MRN
Nucleus of the brachium of the inferior colliculus	NB
Nucleus of Darkschewitsch	ND
Nucleus of the posterior commissure	NPC
Periaqueductal gray	PAG
Pedunculopontine nucleus	PPN
Rostral linear nucleus raphe	RL
Red nucleus	RN
Midbrain reticular nucleus, retrorubral area	RR
Nucleus sagulum	SAG
Superior colliculus, motor related	SCm
Superior colliculus, sensory related	SCs
Substantia nigra, compact part	SNc
Substantia nigra, reticular part	SNr
Ventral tegmental area	VTA
Ventral tegmental nucleus	VTN