Increased serotonin transporter expression reduces fear and recruitment of parvalbumin interneurons of the amygdala

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Supplementary Materials and methods

Fear conditioning

Mice underwent cued fear conditioning training and testing over two days (day 1: training, day 2: test). At the start of the training session, mice were placed into an operant chamber and allowed to explore the environment for 180 s. Mice were then presented with 5 auditory cues (2900 Hz pure tone; 30 s duration; 80 dB), with a mean inter-stimulus interval of 120 s (range: 90-150 s; standard deviation: 24.5 s). For mice in the paired condition, the tone always co-terminated with a footshock (0.3 mA, 0.5 s); in the unpaired condition, tone and footshock were never paired together. Mice in the tone-alone condition received presentations of the auditory cues but no footshocks at any point. The total session duration was 840 s. After the training session, mice were taken in individual cages to a darkened room and left for 2 h to habituate them to the procedure that would follow after the testing session on the following day.

For the testing session (24 h later), mice were placed into a novel context: an operant chamber with the same dimensions as the training context but with distinct flooring, visual pattern on the walls, and odor. At the start of the testing session mice were allowed to explore the environment for 180 s before stimulus presentation. Then, mice from all training groups were presented with a single tone (2900 Hz, 60 s duration, 80 dB) and they remained in the chamber for 180 s after tone offset. No shocks were delivered to any of the mice during the testing session and the total session duration was 420 s. After the testing session, mice were taken in individual cages to a darkened room and left for 2 h before perfusion.

Fear conditioning apparatus

Fear conditioning was conducted in one of two operant chambers (ENV-307A, Med Associates Inc.), each with distinct visual and olfactory cues. The floor was made of stainless steel metal bars through which the shocks were delivered (Med Associates shock generator). Fear testing was conducted in a novel context (i.e. a mouse trained in context A would be tested in context B). A plastic insert was placed on top of the bars on the testing day to provide an additional cue to signal the shift in context compared to the training session. Training and testing contexts were counterbalanced across conditions and genotypes. Stimulus delivery was controlled by software written in the Med Associates programming language (MED-PC). All sessions were recorded via a video camera mounted in the ceiling of the chamber and stored on a digital video recorder.

Electrophysiological protocols and data analysis

Inhibitory postsynaptic currents (IPSCs) were recorded by voltage clamping the neurons at -50 mV. Membrane potential during 5-HT application was monitored while holding PNs at -60±1 mV and INs at -70±1 mV. Hyperpolarizing (-50 pA) and depolarizing (+10 - +180 pA)

current steps were injected every 10 s to monitor input resistance (R_{in}) and firing, respectively. Sinusoidal current injections (6 Hz frequency, 1.7 s duration) were injected every 10 s in PNs recorded in current clamp at -60±1 mV. Current amplitude was set to evoke firing in ~50% of the oscillation peaks in baseline conditions (baseline firing rates: 3.3 ± 0.6 Hz in WT and 3.6 ± 0.9 Hz in 5-HTTOE, $p>0.5$).

Analysis of synaptic currents and intrinsic membrane properties were performed using IGOR Pro 6 (Wavemetrics Inc.). IPSCs were detected using TaroTools toolbox for Igor Pro (https://sites.google.com/site/tarotoolsregister/). Frequency of sIPSCs and membrane potential were normalized by subtracting the average baseline values (respectively 3 and 4 min preceding drug application) for each neuron. Electrophysiological parameters of single neurons were analyzed as previously described (Manko *et al*, 2012). Specifically, the input resistance (R_{in}, MΩ) was calculated from the slope of steady-state voltage responses to a series of 8-10 subthreshold current injections (-30 - +60 pA) lasting 400 ms. The fast afterhyperpolarization (fAHP, mV) was determined from the first spike in response to current injection just above threshold. The duration of the action potential (AP, ms) was measured as the width at half amplitude between the threshold potential and the peak of the action potential, which was evoked by a strong (800-1000 pA) and short (2-5 ms) depolarizing current pulse. The membrane time constant (τ, ms) was estimated from the monoexponential curve fitting of voltage responses to a -30 pA hyperpolarizing pulse. The rheobase (pA) was determined as a 50 ms current injection able to generate a spike in 50% of the cases out of 10 trials. The instantaneous frequency (Hz) was defined as the number of action potentials evoked during a 1 s depolarizing current pulse of twice the amplitude of the rheobase current. The membrane capacitance (nF) was calculated as the ratio between τ and R_{in}. The adaptation index (range: 0-1) was defined as the ratio between the first and last interspike intervals (ms) elicited by the same pulse used to measure the instantaneous frequency. PNs were

distinguished from INs by their larger somata (≥20 μm diameter), lower (≤150 MΩ) R_{in}, adapting firing patterns in response to prolonged current injections, broad spikes (~1 ms half-width) and higher τ (see Table S2 for comparison of electrophysiological properties differing between PNs and PVINs), in line with previous reports (Sosulina *et al*, 2006).

Immunohistochemistry

Sections from fear conditioned mice were blocked with 10% normal donkey serum (NDS) for 1 h at RT, following overnight incubation at 4°C. with guinea pig anti-PV (1:2000, Synaptic Systems) and rabbit anti-c-Fos primary antibodies (1:500, Abcam), with 1% NDS. Following washing with PBS, sections were incubated overnight at 4°C in AMCA-conjugated anti-guinea pig (1:250) and DyLight®Cy3-conjugated anti-rabbit (1:500) secondary antibodies (both raised in donkey, from Jackson Immunoresearch).

Sections containing recorded and biocytin-filled neurons were incubated overnight at 4°C in 1:1000-3000 Cy3-conjugated (Life Technologies) or Alexa488-conjugated Streptavidin (Invitrogen). Sections containing the soma of the recorded neuron were blocked with 10% NDS for 1 h at RT, following overnight incubation at 4°C. with either guinea pig anti-PV (1:2000, Synaptic Systems) or goat anti-PV (1:500, Abcam) primary antibodies, conjugated with DyLight® 649 secondary antibody (1:250; Jackson Immunoresearch). All reagents were diluted in PBS containing 0.3% Triton X-100. Immunoreactivity of the recorded neurons was visualized using an epifluorescence microscope (AxioImager M2, Zeiss) or a laser-scanning confocal microscope (LSM 710, Zeiss).

Quantification of c-Fos and PV-positive neurons

Sections containing the BA from fear conditioned mice were imaged with the epifluorescence microscope mentioned above and StereoInvestigator software (MBF Bioscience). A region of

interest delineating the BA was defined using a 5x 0.16 NA objective lens. Stereological sampling was carried out in both hemispheres from 1 out of 4 sections in the range -1.4 to 2 mm from bregma. Series of tiled stacked images were acquired using a 40x 1.3 NA oil-immersion objective and 1 µm steps at depths of 2 to 22 µm ('optical sections') from the upper surface of each section. In order to minimize artifacts arising from surface irregularities, the first 2 µm from the upper surface were defined as 'guard zone' and not scanned. Counting frames (240x180 µm, consisting of two perpendicular exclusion lines and two inclusion lines) were generated for unbiased two-dimensional counting using an 'optical dissector'. Counting was performed offline in StereoInvestigator. A neuron was only counted once if its immunopositive nucleus came into focus with the dissector. Nuclei already in focus in the top optical section were not counted (West, 1999). The experimenter was blind to genotype and behavioral testing conditions. Data were exported to Excel (Microsoft) and pooled for further analysis.

Supplementary references

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West M (1999). Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends Neurosci* 22: 51–61.

Figure S1

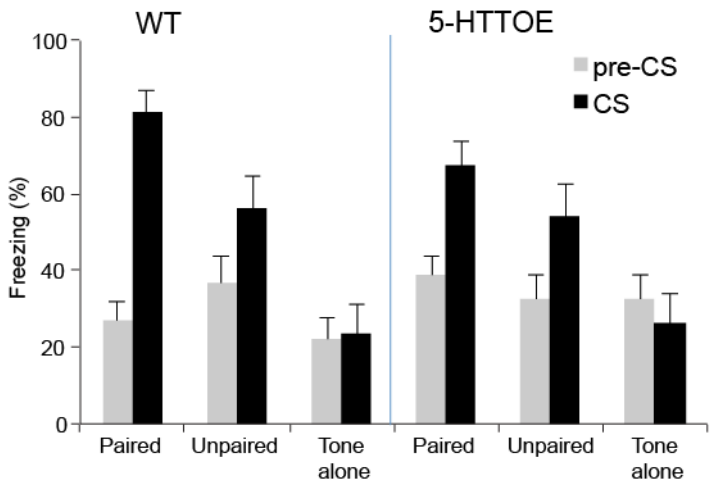

Figure S1

Freezing levels during pre-CS and CS periods in WT and 5-HTTOE mice.

Percentage of time spent freezing before and during CS presentation in the test session. The time spent freezing during CS presentation in the paired condition does not differ significantly between WT and 5-HTTOE. However, WT mice display a larger CS – pre-CS freezing difference compared to 5-HTTOE (see Fig. 1A).

Figure S2

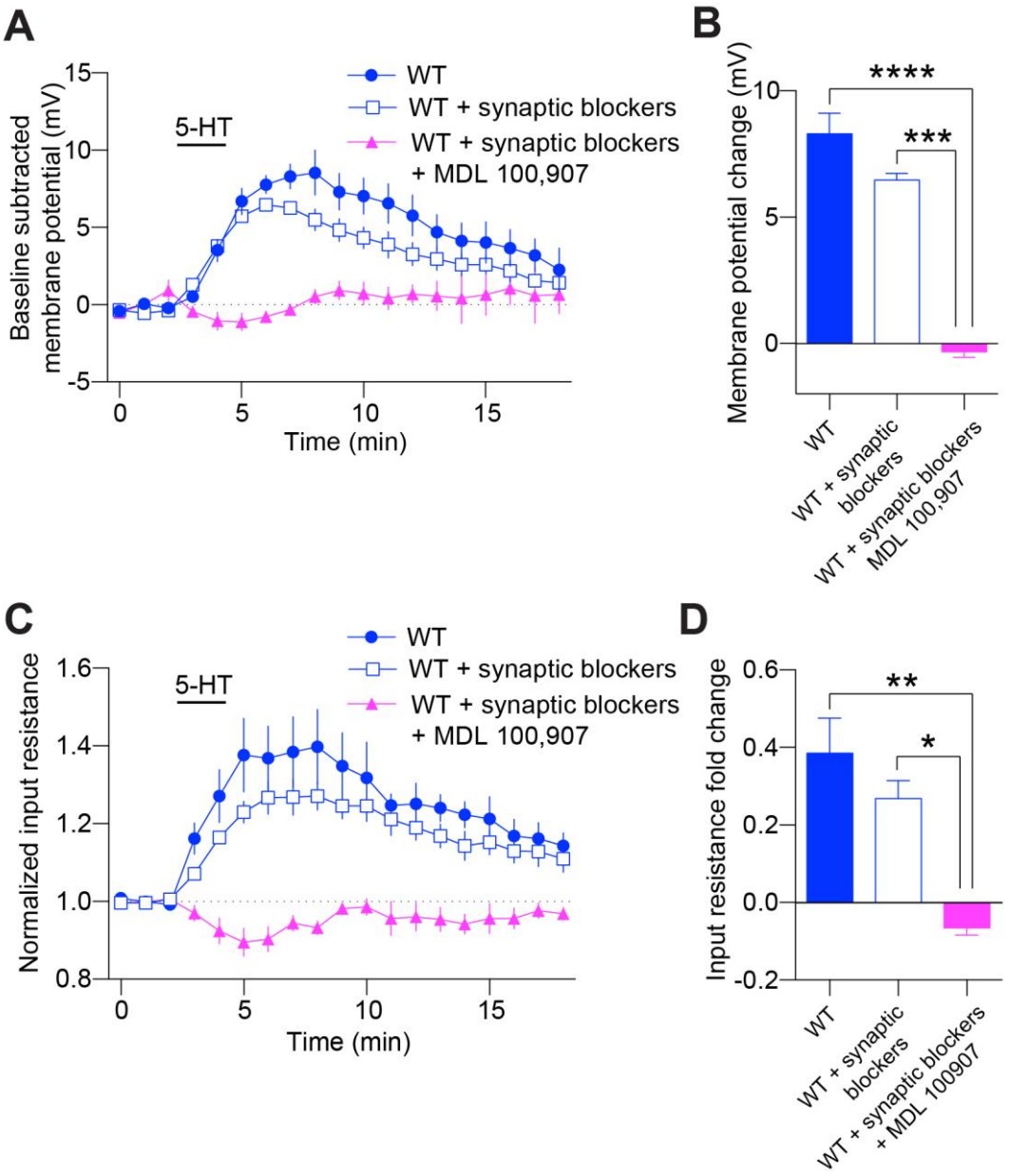

Figure S2

Direct 5-HT depolarization of PVINs via 5-HT$_{2A}$ receptors

A) Time course of the effect of bath application of 5-HT (50 µM) on BA PVINs membrane potential with and without pre-incubation with MDL 100,907 (150 nM) and/or kynurenic acid (3 mM), SR95531 (10 µM) and CGP 54626 (5 µM). B) Membrane depolarization occurs when glutamatergic and GABAergic transmissions are blocked (n=5 from 3 animals),

demonstrating direct effect. Further blockade of 5-HT_{2A} receptor with MDL 100,907 abolishes membrane depolarization (n=5 from 4 animals). C) Time course of the effect of bath application of 5-HT on BA PVINs input resistance with and without pre-incubation with MDL 100,907 (150 nM) and/or kynurenic acid (3 mM), SR95531 (10 μM) and CGP 54626 (5 μM). D) 5-HT increases the input resistance when glutamatergic and GABAergic transmissions are blocked (n=5 from 3 animals) suggesting a direct effect. Further blockade of 5-HT_{2A} receptor with MDL 100,907 abolishes the increase in input resistance (n=5 from 4 animals). **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

Table S1

	WT PN (n=11)	5-HTTOE PN (n=10)
R_{in} (MΩ)	145 ± 11.3	171.9 ± 18.3
Membrane τ (ms)	32 ± 4.8	34.7 ± 4.6
C_m (pF)	0.224 ± 0.031	0.226 ± 0.04
Rheobase (pA)	121 ± 13	128 ± 18
Instantaneous frequency (Hz)	11.6 ± 1.4	15.5 ± 0.8
Adaptation index	0.148 ± 0.03	0.183 ± 0.047
fAHP (mV)	14.8 ± 3.1	21.2 ± 2.4
AP half width (ms)	0.99 ± 0.07	0.98 ± 0.09
RMP (mV)	-67.6 ± 1.7	-65.9 ± 1.3
	WT PVIN (n=7)	**5-HTTOE PVIN (n=5)**
R_{in} (MΩ)	486.9 ± 34.4	458.1 ± 139.9
Membrane τ (ms)	20.8 ± 2.5	14.8 ± 1.9
C_m (pF)	0.044 ± 0.005	0.045 ± 0.011
Rheobase (pA)	58 ± 14	74 ± 22
Instantaneous frequency (Hz)	39.3 ± 7.2	38.7 ± 5.6
Adaptation index	0.576 ± 0.123	0.381 ± 0.077
fAHP (mV)	22.1 ± 2.7	16.7 ± 1.7
AP half width (ms)	0.46 ± 0.03	0.46 ± 0.09
RMP (mV)	-68.7 ± 2.3	-68.9 ± 2

Table S1

Overexpression of the 5-HTT does not alter electrophysiological membrane responses of BA PNs and PVINs.

Inter-genotypes comparisons of intrinsic membrane responses of PNs and PVINs. No significant genotypic difference in intrinsic membrane responses was detected from recorded PNs and PVINs. WT PNs n=11 from 6 animals; 5-HTTOE PNs n=10 from 7 animals; WT PVINs n=7 from 6 animals; 5-HTTOE PVINs n=5 from 5 animals. R_{in}: input resistance; C_m: membrane capacitance; fAHP: fast after-hyperpolarization; AP: action potential; RMP: resting membrane potential.

Table S2

	PN (n=11)	PVIN (n=7)
R_{in} (MΩ)	145 ± 11.3	486.9 ± 34.4 ***
Membrane τ (ms)	32 ± 4.8	20.8 ± 2.5
C_m (pF)	0.224 ± 0.031	0.044 ± 0.005 ***
Rheobase (pA)	121 ± 13	58 ± 14 **
Instantaneous frequency (Hz)	11.6 ± 1.4	39.3 ± 7.2 ***
Adaptation index	0.148 ± 0.03	0.576 ± 0.123 ***
fAHP (mV)	14.8 ± 3.1	22.1 ± 2.7
AP half width (ms)	0.99 ± 0.07	0.46 ± 0.03 ****
RMP (mV)	-67.6 ± 1.7	-68.7 ± 2.3

Table S2

Electrophysiological differences between BA PNs and PVINs.

Comparison of intrinsic membrane responses between BA PNs and PVINs of WT mice. WT PNs n=11 from 6 animals; WT PVINs n=7 from 6 animals; R_{in}: input resistance; C_m: membrane capacitance; fAHP: fast after-hyperpolarization; AP: action potential; RMP: resting membrane potential. ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

Figure S3

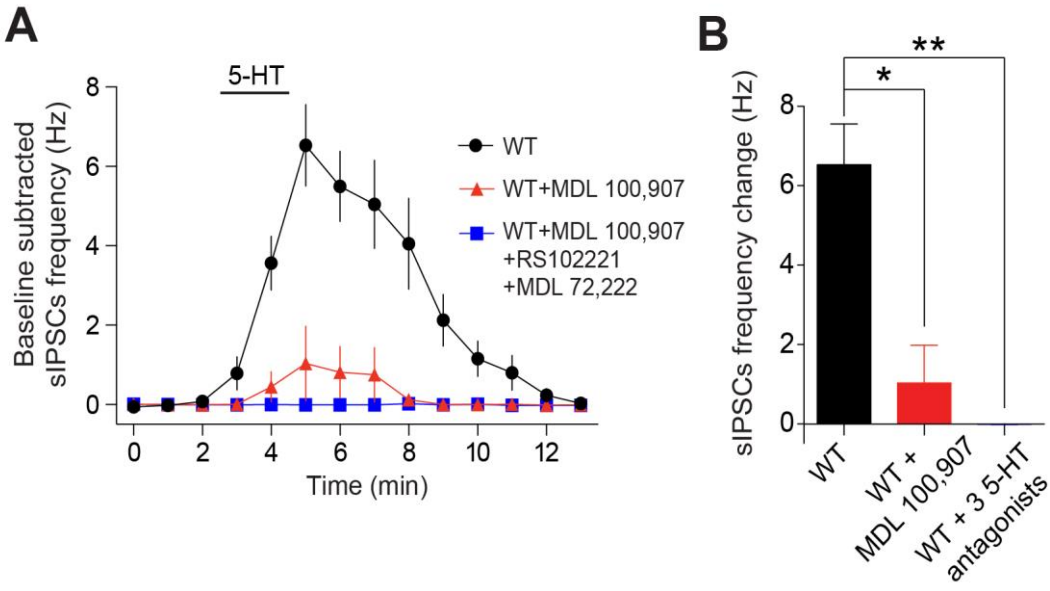

Figure S3

5-HT promotes indirect inhibition of BA PNs primarily through 5-HT$_{2A}$ receptors.

A) Time course of the effect of bath application of 5-HT (50 µM) on sIPSCs recorded from PNs of the BA in control conditions and in presence of MDL 100,907 (150 nM), RS 102221 (5-HT$_{2C}$ antagonist, 1 µM) and MDL 72,222 (5-HT$_3$ antagonist, 20 µm). B) Blocking 5-HT$_{2A}$ receptors with MDL 100,907 blocks 79.2±17.3 % of the 5-HT-mediated sIPSCs frequency increase (n=5 from 4 animals). Additional antagonism of 5-HT$_{2C}$ and 5-HT$_3$ receptors with RS 102221 and MDL 72,222 completely blocks the effect of 5-HT (n=4 from 4 animals). * $p<0.05$; ** $p<0.01$.

Figure S4

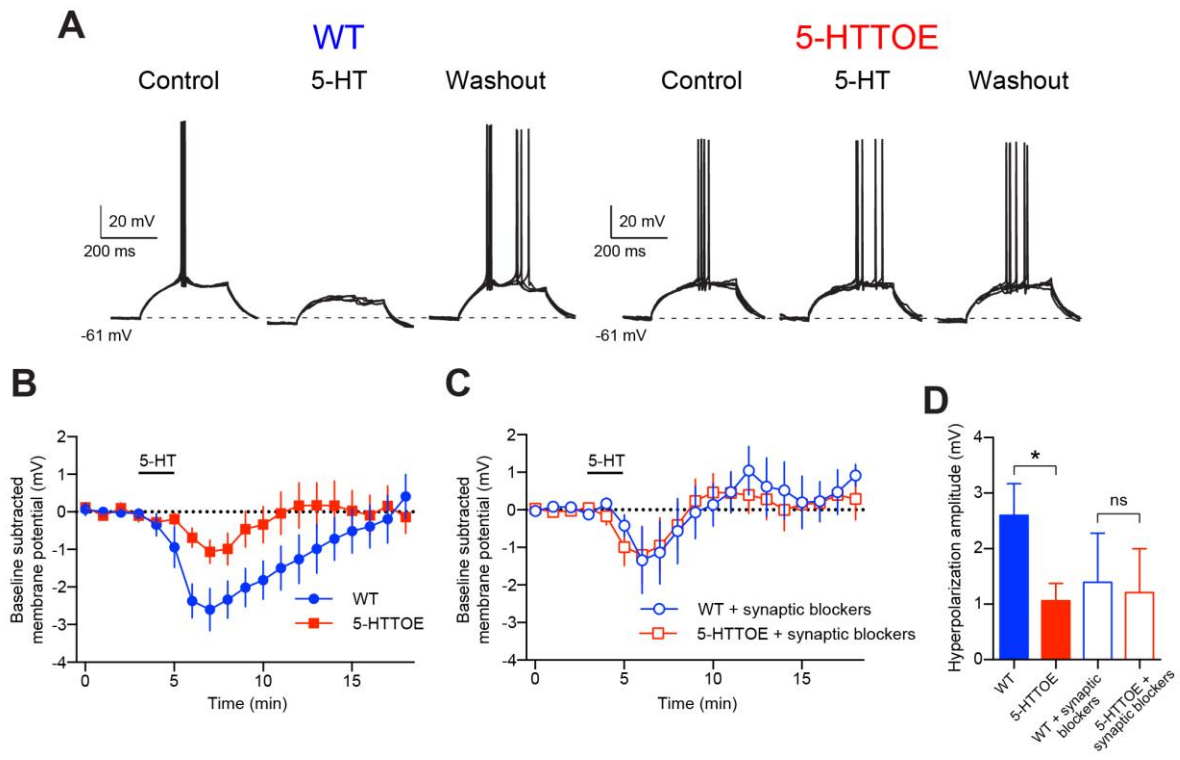

Figure S4

Impairment of serotonergic facilitation of GABA release leads to reduced hyperpolarization of PNs in 5-HTTOE mice.

A) Five superimposed voltage responses to depolarizing current injections of representative PNs from a WT (100 pA, 300 ms , left) and 5-HTTOE mouse (70 pA, 300 ms, right) during control, bath application of 5-HT (50 µM) and washout. In control, the current intensities used were set just above threshold to evoke an action potential. B) Time course of the effect of 5-HT on the membrane potential of WT and 5-HTTOE PNs. 5-HT evokes stronger hyperpolarization in WT compared to 5-HTTOE PNs. C) Time course of the effect of 5-HT on the membrane potential of WT and 5-HTTOE PNs in presence of kynurenic acid (3 mM), SR95531 (10 µM) and CGP 54626 (5 µM). In these conditions, 4/9 WT and 3/7 5-HTTOE PNs hyperpolarized following 5-HT application, suggesting a direct response. Direct inhibitory responses are similar in WT and 5-HTTOE. D) 5-HT evoked hyperpolarization

amplitude compared to control. 5-HT membrane hyperpolarization observed in WT PNs (n=8 from 5 animals) is significantly reduced in PNs of 5-HTTOE (n=10 from 7 animals). In contrast, direct hyperpolarization by 5-HT observed when glutamatergic and GABAergic transmissions are blocked is not significantly different in PNs of WT and 5-HTTOE mice (n=9 from 6 animals and n=7 from 2 animals, respectively). * $p<0.05$.