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# Reduced vesicular monoamine transport disrupts serotonin signaling but does not cause serotonergic degeneration

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## Abstract

We previously demonstrated that mice with reduced expression of the vesicular monoamine transporter 2 (VMAT2 LO) undergo age-related degeneration of the catecholamine-producing neurons of the substantia nigra pars compacta and locus ceruleus and exhibit motor disturbances and depressive-like behavior. In this work, we investigated the effects of reduced vesicular transport on the function and viability of serotonin neurons in these mice. Adult (4–6 months of age), VMAT2 LO mice exhibit dramatically reduced (90%) serotonin release capacity, as measured by fast scan cyclic voltammetry. We observed changes in serotonin receptor responsivity in in vivo pharmacological assays. Aged (months) VMAT2 LO mice exhibited abolished 5-HT1A autoreceptor sensitivity, as determined by 8-OH-DPAT (0.1 mg/kg) induction of hypothermia. When challenged with the 5HT2 agonist, 2,5-dimethoxy-4-iodoamphetamine (1 mg/kg), VMAT2 LO mice exhibited a marked increase (50%) in head twitch responses. We observed sparing of serotonergic terminals in aged mice (18–24 months) throughout the forebrain by SERT immunohistochemistry and [$^3$H]-paroxetine binding in striatal homogenates of aged VMAT2 LO mice. In contrast to their loss of catecholamine neurons of the substantia nigra and locus ceruleus, aged VMAT2 LO mice do not exhibit a change in the number of serotonergic (TPH2 +) neurons within the dorsal raphe, as measured by unbiased stereology at 26–30 months. Collectively, these data indicate that reduced vesicular monoamine transport significantly disrupts serotonergic signaling, but does not drive degeneration of serotonin neurons.

### Keywords

Serotonin; VMAT2; Synaptic vesicle; Dorsal raphe; Parkinson's disease; 5-HT1A; 5-HT2

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## 1. Introduction

Parkinson's disease (PD) consistently features disruption and preferential degeneration of catecholamine neurotransmitter systems, including the destruction of the nigrostriatal dopamine (DA) system and the noradrenergic locus ceruleus (Manaye et al., 1992; Mann and Yates, 1983). Alterations in the serotonin (5-HT) system are less consistent. The serotonergic raphe nuclei also degenerate in PD (Halliday et al., 1990), though this pathology does not correlate with progression of motor symptoms (Politis et al., 2010), but rather is most pronounced in PD patients with depression (Jellinger and Paulus, 1992; Paulus and Jellinger, 1991). In contrast to striatal dopamine system, serotonergic innervation in the striatum is often preserved through later stages of PD (Bédard et al., 2011). Despite this resilience, reduced levels of 5-HT (Kish et al., 2008) and disruptions in serotonergic signaling likely contribute to the high incidence of comorbid depression and other psychiatric symptoms in PD (Fahn, 2003), and have been hypothesized to underlie the pathophysiology of L-DOPA induced dyskinesia (Cenci and Lundblad, 2006).

The basis for the shared vulnerability of monoaminergic neurons in PD may lie in their common molecular processes, which include the synthesis, storage, and metabolism of reactive monoamine neurotransmitters, all of which are influenced by the vesicular monoamine transporter 2 (VMAT2). Following synthesis or plasmalemmal uptake of monoamines, VMAT2 sequesters these transmitters from the cytosol into acidic synaptic vesicles for stable storage and exocytic release. Epidemiological studies have shown that *Slc18a2* promoter haplotypes that confer increased expression or function of VMAT2 are protective against PD (Brighina et al., 2013; Glatt et al., 2006), while reduced *Slc18a2* mRNA expression has been detected in the platelets of PD patients (Sala et al., 2010). Immunohistochemical analyses of postmortem brain tissue show that there is substantially less VMAT2 in caudate and putamen of PD cases than would be expected from degenerative loss (Miller et al., 1999a,b, 1997). This was recently confirmed to have a functional effect, when Pifl and colleagues demonstrated that vesicular transport of dopamine via VMAT2 is reduced in synaptic vesicles isolated from the striata of PD patients in comparison to control cases (Pifl et al., 2014).

While there is strong support for the role of reduced vesicular function in dopaminergic degeneration in PD, there has been less direct investigation into the relationship between vesicular function and the vulnerability of noradrenergic and serotonergic neurons in PD. However, these neurotransmitters are subject to similar cytosolic degradation pathways, which led us to hypothesize that reduced vesicular function contributes to their dysfunction and degeneration as well. In settings of reduced vesicular function, monoamines accumulate in the alkaline conditions of the cytosol, where they are subject to breakdown by spontaneous and enzymatic oxidative processes. Excessive cytosolic breakdown of DA and NE by either fate (enzymatic deamination or autoxidation) has been demonstrated to be neurotoxic (Caudle et al, 2007; Crino et al., 1989; Hastings et al., 1996; Mosharov et al., 2009; Taylor et al., 2014; Ulusoy et al., 2012). Less is known about the neurotoxic potential of cytosolic 5-HT, though mice with conditional knockout of VMAT2 in 5-HT neurons exhibit normal initial development of the raphe cytoarchitecture (Narboux-Neme et al., 2011).

Mice with globally reduced (~95%) expression of VMAT2 (VMAT2 LO) exhibit neurochemical depletion and increased cytosolic breakdown of DA (~95%),NE (90%), and 5-HT (80%). These mice develop normally, but undergo age-related degeneration of the SNpc and LC, and display motor and nonmotor symptoms (including depressive behavior) of PD (Caudle et al., 2007; Taylor et al., 2014, 2009). In this work, we used VMAT2 LO mice to investigate the effects of disrupted vesicular monoamine transport on the synaptic release of 5-HT. We then evaluated physiological consequences specific to the 5-HT system in tests of 5-HT1A and 5-HT2 receptor responsivity. Lastly, we evaluated the integrity of serotonergic innervation and the neuronal population of the dorsal raphe to determine if reduced vesicular 5-HT storage can cause neurodegeneration.

## 2. Materials and methods

### 2.1. Chemicals and reagents

(R)-(+)-8-OH-DPAT (#ab1210507) was purchased from Abcam (Cambridge, MA), DOI ((R)-(−)-2,5-dimethoxy-4-iodoamphetamine; #D153), levodopa (D150), benserazide (B7283), and fluoxetine (#F132) were purchased from Sigma-Aldrich (St. Louis, MO), and [$^{3}$H]-paroxetine (#Art177) was purchased from American Radiolabeled Chemical Inc. (St. Louis, MO). Antibodies and their sources are described below. All other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA).

### 2.2. Mice

Male and female VMAT2 LO mice (expressing 5% of wildtype VMAT2 levels) were generated as previously described (Caudle et al., 2007). Mice of both genders were used in all experiments. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Emory University.

### 2.3. Ages of mice used in experiments

From staging of catecholaminergic neurodegeneration in previous work (Caudle et al., 2007; Taylor et al., 2014), VMAT2 LO mice exhibit DAT and NET loss at 12 months, LC loss at 12 months, and nigral degeneration at 18–24 months. We have used aged mice as follows:

Young adult mice (between 4 and 6 months of age) were used in voltammetry experiments and in the DOI head-twitch response assay to determine the phenotypic, baseline effect of reduced VMAT2 expression on synaptic 5-HT release and postsynaptic signaling. Aged mice (between 18 and 24 months) for paroxetine-binding and SERT immunohistochemistry to capture a time point during which DAT and NET loss had already occurred (and LC and nigral degeneration had begun). 18–24 month old mice were also used in the 8-OH-DPAT assay. Advance-aged mice (26–30 months) were used the test for levodopa induced dyskinesia and for dorsal raphe stereology to capture a most neurodegenerative state.

### 2.4. Immunohistochemistry

Mice were transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. Their brains were then removed and stored in 4% paraformaldehyde for

24 h, and cryopreserved in 30% sucrose for 48 h. Brains were then sliced on a freezing microtome at 40 μm and collected into cryopreservative. Immunohistochemistry was performed as previously described (Caudle et al., 2007; Taylor et al., 2014). Following tissue slicing, free-floating sections were incubated with a polyclonal rabbit antibody against either TPH2 (1,5:000; Novus NB100-74555, Littleton, CO) overnight at 4°, or SERT for 48 h at room temperature (1:10,000; Immunostar 24,330, Hudson, WI). Secondary incubations were performed with biotinylated polyclonal goat anti-rabbit antibody (1:200; Jackson 111-065-003, Hudson, WI).

### 2.5. Stereology of the dorsal raphe nucleus

Stereology was performed to determine the number of serotonergic (TPH2 +) neurons in the dorsal raphe nucleus of aged (26–30 months) mice. Following immunohistochemistry against TPH2, tissue slices were mounted on microscope slides, processed, and counterstained with cresyl violet to identify cell nuclei as previously described (Taylor et al., 2014). Stereological sampling (West et al., 1991) was performed using Stereo Investigator software (MicroBrightField, Colchester, VT). Dorsal raphe contours (comprising the dorsal, ventral, and intrafascicular regions of the nucleus) were outlined as defined by the atlas of Paxinos and Franklin (2001), using a 4× objective. Final tissue thickness was 24 μm; guard zones of 2 μm were used to exclude lost profiles on the top and bottom of tissue sections. Cells were counted with a 40× objective (1.3× numerical aperture), using a counting frame of 50 × 50 μm, and sampling grid of 80 × 100 μm, at an evaluation interval of three sections (yielding 6–7 sections per animal). A serotonergic neuron was defined as an in-focus TPH2-immunoreactive cell body with a TPH2-negative nucleus. Nissl + cells were defined as having a cresyl-violet positive nucleus or TPH2 + cell body. The Gundersen (m = 1) coefficient of error was less than 0.1 for each animal.

### 2.6. Fast scan cyclic voltammetry in substantia nigra pars reticulata

Fast scan cyclic voltammetry was performed as previously described (Lohr et al., 2014), with modification to measure 5-HT release in the substantia nigra pars reticulata (SNr). Adult wildtype and VMAT2 LO mice (aged 4–6 months, n = 3) were decapitated and their brains quickly transferred to ice cold oxygenated (95% $O_2$/5% $CO_2$) artificial cerebrospinal fluid (aCSF). Brains were mounted in aCSF onto the stage of a vibratome (Leica VT100S) and sliced at 300 μm. Midbrain slices were collected and maintained in oxygenated aCSF at room temperature. After 30 min, slices were placed in a slice perfusion chamber. Stimulating and carbon-fiber recording electrodes were placed in the lateral region of the SNr, as defined by Paxinos. A four-recording survey of three different sites within the SNr was taken for each animal with a 5-min rest interval between each stimulation (20 pulses, 100 Hz, 350 μA, 4 ms, monophasic). Neurochemical identity of 5-HT was confirmed with a 5-HT-specific waveform and by observing increased signal in the presence of 10 μM fluoxetine. Synaptic clearance of 5-HT was assessed by measuring the rate constant *tau*, which is a measure of the time required for the 5-HT signal to decay to 1/3 of its peak height.

Carbon-fiber microelectrodes were calibrated with 5-HT standards using a flow-cell injection system. Kinetic constants were extracted using nonlinear regression analysis of

release and uptake of 5-HT. Application of wave-form, stimulus, and current monitoring was controlled by TarHeel CV [University of North Carolina (UNC)] using a custom potentiostat (UEI, UNC Electronics Shop). The waveform for 5-HT detection consisted of a −0.2 V holding potential versus an Ag/AgCl (In Vivo Metric) reference electrode. The applied voltage ramp ranged from −0.2 V to 1.0 V and back to −0.2 V, applied at a rate of 1000 V/s at 10 Hz.

### 2.7. [$^3$H]-paroxetine binding to quantify SERT expression in striatal homogenates

Paroxetine binding was performed as previously described (Caudle et al., 2006). Aged (18–24 months) wildtype and VMAT2 LO mice were decapitated and their brains were subsequently removed and dissected. Whole, bilateral striata were collected into a glass Wheaton homogenizer tube containing 5 ml ice-cold assay buffer (50 mM Tris, 120 mM NaCl, 5 mN KCl, pH 7.4), homogenized with a Teflon homogenizer at 1000 rpm, and centrifuged at 48,000 ×*g*. The resulting pellet was resuspended, homogenized, and centrifuged again. The final pellet was resuspended in 500 μl assay buffer. 100–200 μg (post-hoc determination) of sample was incubated in a 400 μl reaction mixture of 2 nM paroxetine. Nonspecific binding was determined in the presence of 10 μM fluoxetine.

### 2.8. Test for expression of L-DOPA induced dyskinesia

The evaluation of L-DOPA induced dyskinesia was modeled after Ding and colleagues (Ding et al., 2007). Aged wildtype and VMAT2 Lo mice (26–30 months, n = 5–7/group) were injected with saline or a mixture of L-DOPA (25 mg/kg) and the AADC inhibitor benserazide (12.5 mg/kg), i.p., every 12 h. Mice were placed in glass cylinder for video-recorded observation and evaluated for the presence of dyskinetic limb movement, as defined in the original study.

### 2.9. DOI-induced head twitch response

Methods were modeled after Canal and Morgan (2012). DOI was prepared fresh in 0.9% bacteriostatic sodium chloride. Adult mice (4 months of age) of either gender were injected with saline or DOI (1 mg/kg, i.p.), and individually placed into a clean cage for video-recorded observation. 12 days later, a crossover study was performed. Behavior was recorded from 10 to 20 min post injection. A blinded observer counted the number of head twitches over a 10-min interval. Video was played at 0.6× speed to distinguish head twitches from other rapid furtive movements. Head twitches were defined as rapid rotational head movements as previously described (Canal and Morgan, 2012).

### 2.10. 8-OH-DPAT-induced hypothermia

The 8-OH-DPAT-induced hypothermia assay was adapted from Martin and colleagues (Martin et al., 1992). Mice of either gender were individually placed into clean cages. Baseline core body temperatures ($t_0$) were measured with a rectal thermometer. Mice were then injected with saline or 8-OH-DPAT (0.1 mg/kg; s.c.). Core body temperatures were again measured 20 min following injection ($t_{20}$), and the differences between $t_0$ and $t_{20}$ were calculated to yield the change in core body temperature.

### 2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA). Experiments with two factors were analyzed by two-way ANOVA, followed by Bonferroni posttests for pairwise comparisons. One-factor experiments were analyzed with t-tests for independent means. Results were reported as mean ± SEM, and statistical significance set at $\alpha = 0.05$. In figures, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

## 3. Results

### 3.1. VMAT2 LO mice have drastically reduced synaptic release of 5-HT

To determine the extent to which reduced vesicular function affected the ability of serotonergic neurons to release 5-HT, we performed fast scan cyclic voltammetry in the brain slices of adult (2 months) wildtype and VMAT2 LO brain slices (Fig. 1). We recorded the stimulated synaptic release of 5-HT in the SNr, which is the most densely serotonergically innervated structure in the brain and is a major output nucleus of the basal ganglia. In response to electrical stimulation, wildtype mice had a peak release of 1.0±0.3 μM5-HT (Fig. 1B),whereas VMAT2 LO littermates had a peak release of 0.1 ± 0.0 μM 5-HT constituting a 90% decrease from control animals, barely exceeding the threshold of detection. We observed no difference in the synaptic clearance of 5-HT between genotypes, as measured by the rate constant *tau* (Fig. 1C).

### 3.2. Reduced vesicular function disrupts serotonergic signaling

We examined potential changes to serotonergic signaling in vivo. 5-HT1A receptors are expressed on somatodendrites and function as inhibitory autoreceptors. Physiologically, 5-HT1A agonism elicits a hypothermic effect in rodents and humans, though this effect is lost in depressive states (Jacobsen et al., 2012a; Lesch, 1991). We tested the thermic response of aged (18–24 months) VMAT2 LO mice to the 5-HT1A agonist, 8-OH-DPAT (Fig. 2). There were significant effects of genotype ($F_{1,27} = 41.2$, $p < 0.0001$), and treatment ($F_{1,27} = 77.1$, $p < 0.0001$) on thermic response with an interaction between these factors ($F_{1,27} = 17.7$, $p = 0.0003$). Wildtype and VMAT2 LO mice had similar baseline core body temperatures (WT: 37.6 ± 0.2; LO: 37.4 ± 0.2; $p = 0.37$). After administration of 8-OH-DPAT (0.1 mg/kg; IP), wildtype mice exhibited a −4.0 ± 0.4° change in core body temperature 20 min following treatment; this response was attenuated in VMAT2 LO mice, which exhibited a −1.0 ± 0.3° change in core temperature ($p < 0.0001$).

5-HT2 receptors are expressed postsynaptically and mediate excitatory ($G_{q/11}$) signaling. Behaviorally, selective 5-HT2 agonism causes hallucinations in humans and a head-twitch response in rodents (Canal and Morgan, 2012; Corne et al., 1963). To investigate the effect of reduced VMAT2 expression on the responsiveness of 5-HT2 receptors, we examined the DOI-induced head-twitch response in wildtype and VMAT2 LO mice (Fig. 3). There were significant effects of genotype ($F_{1,30}$=16.7; $p = 0.0003$) and treatment ($F_{1,30} = 189.4$; $p < 0.0001$) in the DOI-induced head-twitch response, with an interaction between these factors ($F_{1,30} = 15.9$; $p = 0.0004$). After administration of DOI (1 mg/kg, IP), wildtype animals elicited 29 ± 3 head twitches over 10 min of recorded observation. VMAT2 LO mice were

more sensitive to DOI, and exhibited a 79% increase in head-twitches (52 ± 5 HTRs; WT vs. LO, p = 0.0009).

We performed a study to test if VMAT2 LO mice develop L-DOPA induced dyskinesia in advanced age. Aged (26–30 months) wildtype and VMAT2 Lo mice were injected (i.p.) with a mixture of L-DOPA (25 mg/kg) and benserazide (12.5 mg/kg) every 12 h for 1 week. We observed no dyskinetic behavior (abnormal limb or orofacial movement) on Days 1, 4, or 7 (data not shown).

### 3.3. Preservation of SERT expression

We next sought to determine if serotonergic innervation of rostral brain regions was disrupted in aged (18–24 months) VMAT2 LO mice. Reduced expression of SERT may indicate 5-HT terminal dysfunction or loss preceding neurodegeneration. Using immunohistochemistry against SERT, we evaluated serotonergic innervation of the SNr, somatomotor cortex, and striatum (Fig. 4A–C). In each region surveyed, we observed no overt changes to serotonergic morphology or expression. We quantified striatal SERT expression in striatal homogenates with [$^3$H]-paroxetine binding (Fig. 4D), and observed no change in the number of specific paroxetine-binding sites (WT: 426 ± 41 fmol/mg protein; LO: 420 ± 25 fmol/mg; p = 0.9).

### 3.4. Reduced vesicular function does not cause age-related neurodegeneration of the dorsal raphe nucleus

As VMAT2 deficiency drives age-related neurodegeneration of the SNpc and LC (Caudle et al., 2007; Taylor et al., 2009), we examined the effects of this genetic disruption on the integrity of the dorsal raphe nucleus. Using immunohistochemistry against tryptophan hydroxylase 2, we observed no apparent changes to the serotonergic cytoarchitecture of the dorsal raphe (Fig. 5). We counted serotonergic (TPH2 +) neurons within the nucleus using unbiased stereology. At 26–30 months (following the age at which LC and SNpc degeneration were severe), we observed no significant change in the serotonergic neuronal population in the dorsal raphe nucleus between genotypes (WT: 3027 ± 210 TPH2 + neurons; LO: 2646 ± 253 neurons; p = 0.73), and no difference between the numbers of Nissl + neurons in the dorsal raphe (p = 0.84).

## 4. Discussion

Significant attention has been directed toward exploring the connection between vesicular function and neuronal vulnerability in PD (Alter et al., 2013; Sonsalla, 2003). Reduced vesicular function occurs in monoaminergic neurons in the striata of PD patients (Pifl et al., 2014), and has been demonstrated to drive neurodegeneration in the SNpc and LC of mouse models (Taylor et al., 2014; Ulusoy et al., 2012). In this work, we demonstrated that disrupting the vesicular storage of 5-HT causes dysfunction of serotonergic signaling, despite the structural preservation of 5-HT neurons.

### 4.1. Reduced vesicular function diminishes 5-HT storage and release capacity

While prior studies revealed an 80% reductions in tissue 5-HT content by HPLC (Taylor et al., 2009), we wanted to determine to what extent synaptic release of 5-HT would be affected. Despite the dramatic (95%) suppression of VMAT2, there was still detectable synaptic release of 5-HT. As measured by fast scan cyclic voltammetry, VMAT2 LO mice exhibited a 90% decrease in synaptic release of 5-HT in the SNr.

### 4.2. Depressive-like thermic and behavioral responses

We explored the possibility that reduced vesicular function in 5-HT neurons could contribute to a depressive state. While clinical and animal data have shown that drastically reduced VMAT2 expression causes abnormal psychiatric phenotypes in humans and mice (Rilstone et al., 2013; Taylor et al., 2009), these can be the result of disruptions in any of the neuronal monoamine systems. Studies evaluating catecholaminergic and serotonergic innervation in patients have found that comorbid depression in PD correlates most closely with neuronal loss in the dorsal raphe (Paulus and Jellinger, 1991). Because catecholaminergic degeneration can also contribute to depression in PD (Remy et al., 2005), we examined the effects of genetic VMAT2 suppression, specifically on 5-HT signaling.

In this study, we evaluated the sensitivity of the 5-HT1A/2 receptors in tests that correspond with human biomarkers of depression (Jacobsen et al., 2012b). VMAT2 LO mice had an abolished hypothermic response to the 5-HT1A autoreceptor agonist 8-OH-DPAT, which is a selective 5-HT1A agonist that suppresses 5-HT release from raphe neurons, eliciting a hypothermic response (Martin et al., 1992). While it could be expected that 5-HT1A sensitivity would increase in a setting of reduced extracellular 5-HT (the inverse has been observed in SERT KO mice (Li et al., 1999), raphe neurons may adaptively blunt the 5-HT1A autoinhibition. Our results are consistent with observations in other hyposerotonergic mouse models (Jacobsen et al., 2012b; Mosienko et al., 2014). This bears translational relevance, as unmedicated depressed patients also exhibit a blunted hypothermic response to 5-HT1A agonists (Cowen et al., 1994; Lesch, 1991).

VMAT2 LO mice exhibited a markedly increased head-twitch response to the 5-HT2 agonist, DOI. DOI is a substituted amphetamine selective for 5-HT2A/C postsynaptic receptors that elicits hallucinations in humans and head-twitches in rodents (Canal and Morgan, 2012). 5-HT2 receptor expression and function have been shown to increase in experimental models of hyposerotonergia and serotonergic denervation (Jacobsen et al., 2012a), while increased 5-HT2A binding has been observed in depression and suicide (Shelton et al., 2009). Observations of the altered responsiveness of 5-HT1A and 5-HT2A have led to their consideration as biomarkers of chronic hyposerotonergia and depression (Jacobsen et al., 2012b). In VMAT2 LO mice, we have demonstrated that these phenotypes are capitulated by disrupting vesicular storage of 5-HT (though we cannot rule out DA and NE deficits as contributing factors). These findings indicate that reduced vesicular function, which exacerbates or drives Parkinsonian pathology, can also cause depressive features in mice, providing a single molecular mechanism that could plausibly drive the comorbidity of PD and depression. We have further supported this argument by demonstrating that

overexpression of VMAT2 protects against MPTP-derived neurotoxicity and confers a resilient, euthymic phenotype in depressive behavior tests (Lohr et al., 2014).

### 4.3. Reduced vesicular storage of 5-HT does not cause loss of serotonergic innervation

In light of the drastic changes in 5-HT content, release, and signaling, we explored the possibility that VMAT2 LO mice may undergo loss of serotonergic terminals in brain regions relevant to Parkinsonian pathophysiology. We observed no qualitative loss of SERT expression in the cortex, SNr, or striatum by immunohistochemistry (Fig. 4). We also observed no change in SERT expression in striatal tissue by [$^3$H]-paroxetine binding. This is in stark contrast to what we observed of dopaminergic innervation of the striatum, which featured progressive dopamine transporter loss beginning at 12 months of age, with near-complete terminal loss at 24 months (Caudle et al., 2007).

The preservation of striatal serotonergic innervation is of particular interest in the context of levodopa-induced dyskinesia. In a well-supported hypothesis of levodopa-induced dyskinesia, 5-HT neurons transport L-DOPA, convert it to DA via AADC, and release it in a dysregulated manner (Carta et al., 2007; Cenci and Lundblad, 2006). In patients, an increased ratio of SERT to DAT expression in the striatum is predictive of the severity of levodopa-induced dyskinesia following fetal midbrain graft implantation (Politis et al., 2011), while a recent study in primates has demonstrated that serotonergic lesioning with MDMA suppresses dyskinesia in MPTP-treated monkeys (Beaudoin-Gobert et al., 2015). Upon observing the resilience of 5-HT striatal innervation in VMAT2 LO mice, we performed a study modeled after that performed in Pitx3-deficientmice, a mouse model which exhibits DA-specific striatal denervation (Ding et al., 2007), but observed no dyskinetic movement in treated animals; it is possible that the 5-HT neurons of VMAT2 LO mice do not have the storage capacity to release sufficient levodopa-derived DA to express levodopa-induced dyskinesia. An important limitation to this experiment is that it was performed without a positive control, as the aphakia mouse model (Ding et al., 2007) is the only known murine model that exhibits L-DOPA-induced dyskinesia in the absence of a chemical lesion.

### 4.4. Reduced vesicular storage of 5-HT does not cause degeneration of the dorsal raphe in VMAT2 LO mice

As we previously reported severe, age-related degeneration of the SNpc and LC of VMAT2 LO mice (Caudle et al., 2007; Taylor et al., 2014), we asked if suppressed VMAT2 expression would also drive 5-HT neuron loss in the dorsal raphe. Others have demonstrated that conditional knockout of VMAT2 or TPH2 in serotonergic neurons does not affect the initial development of the raphe cytoarchitecture (Narboux-Neme et al., 2011). In this work, we demonstrate that VMAT2 LO mice of advanced age (26–30 months) do not exhibit change to the quantity of TPH2 + cell populations within the dorsal raphe by unbiased stereological counting. The preservation of serotonergic innervation and the cytoarchitecture of the raphe nuclei in aged VMAT2 LO mice suggest that the vesicular storage of 5-HT is less critical to serotonergic neuronal health than vesicular catecholamine storage is to the viability of the LC or SNpc.

### 4.5. Comparative pathology between VMAT2 LO mice and PD

Patterns of neuronal loss in that we observe in VMAT2 LO mice are similar to those in human PD. Just as in PD, VMAT2 LO mice lose both the LC and SNpc, but not the VTA (Caudle et al., 2007; Taylor et al., 2014). In this work we report preservation of the dorsal raphe and serotonergic innervation throughout the brain despite dramatic neurochemical 5-HT depletion. A recent investigation specifically examined the number of serotonergic neurons in the dorsal of raphe of post mortem PD cases, as well as anterior striatal SERT abundance and 5-HT content (Cheshire et al., 2015). Cheshire and colleagues reported that PD patients exhibited a significant reduction in striatal 5-HT, with no loss of SERT expression in the anterior striatum or quantity of serotonergic neurons in the dorsal raphe.

It is important to note several limitations to this comparison. Degeneration of the serotonergic median raphe and raphe obscurus occur in PD (Halliday et al., 1990). Qualitatively, we did not observe any overt changes to the large but sparsely populated serotonergic neurons in the ventrolateral periaqueductal gray or the cytoarchitecture of the median raphe (given its narrow dimensions, this region is less amenable to stereological counting). Moreover, serotonergic innervation is preferentially lost in the caudate, while putamenal innervation is preserved (Kish et al., 2008). In the present study, we did not differentiate between the striatal subregions, and thus cannot comment on their differential SERT abundance.

## 5. Conclusions

VMAT2 LO mice exhibit reduced synaptic release of 5-HT and altered serotonergic signaling, which drive physiological and behavioral correlates of depression. Disrupting the vesicular storage of 5-HT did not cause degeneration of serotonergic neurons in the dorsal raphe, indicating that cytosolic 5-HT is not sufficiently neurotoxic to drive neurodegeneration in this model. These data, along with our previous reports, demonstrate that disruption of a single neuronal function—vesicular transport—can exacerbate or drive dysfunction in each monoamine system, contributing to the motor and nonmotor symptoms of PD.

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## Abbreviations

| | |
|---|---|
| **5-HT** | 5-hydroxytryptamine serotonin |
| **DA** | dopamine |
| **NE** | norepinephrine |
| **SERT** | serotonin transporter |
| **TPH2** | tryptophan hydroxylase 2 |

**VMAT2** vesicular monoamine transporter 2

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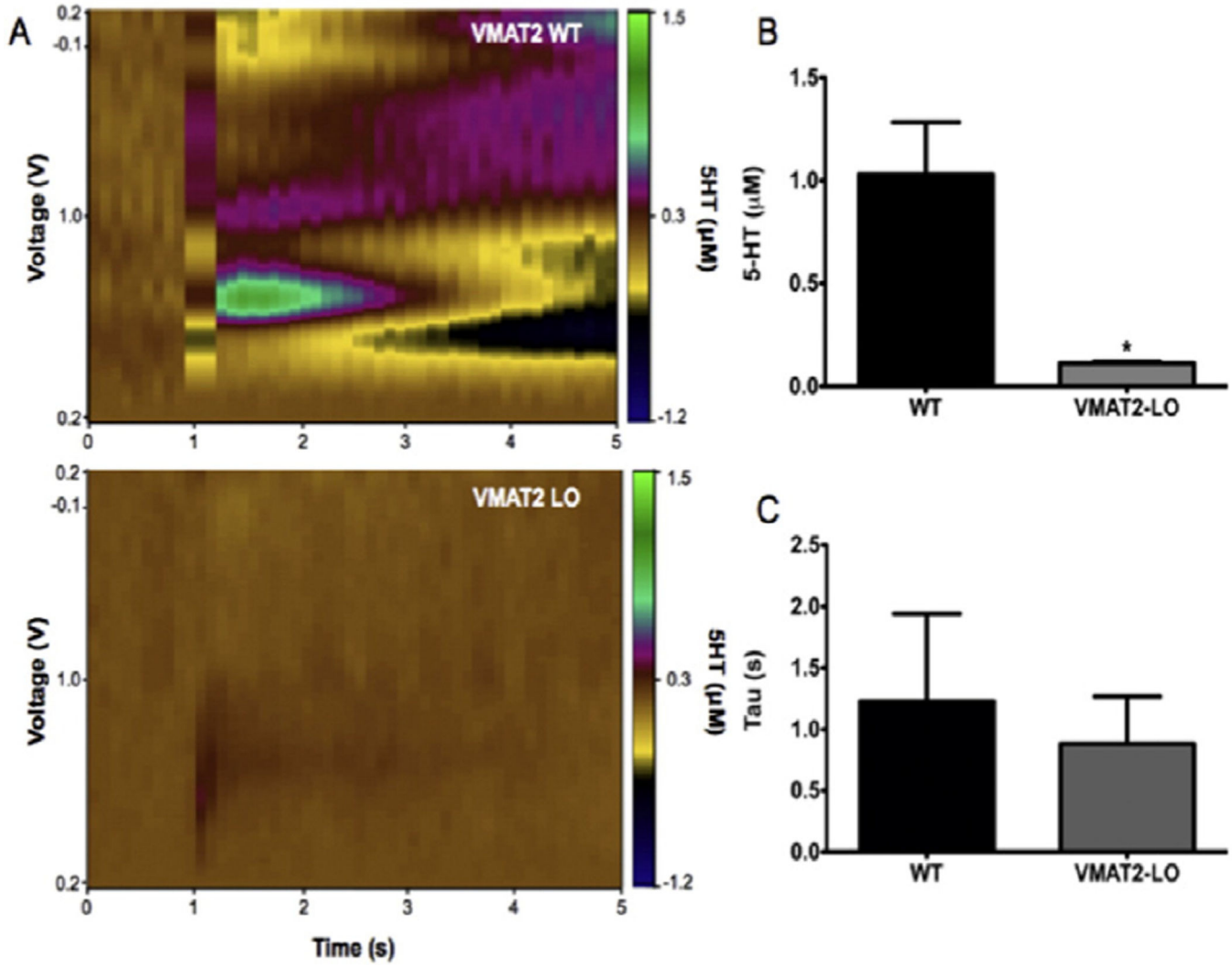


**Fig. 1.**

Genetic VMAT2 suppression causes reduced synaptic release of 5-HT in the SNr. Synaptic release of 5-HT in the SNr was measured with fast scan cyclic voltammetry A. Top color plot, adult (4–6 months) VMAT2 WT animals exhibited a peak release of 1 μM 5-HT in the SNr in response to electric stimulation (20 pulses, 100 Hz, 350 μA, 4 ms, monophasic). Bottom color plot. VMAT2 LO littermates had drastically reduced peak release. Representative color plots for single recordings are shown. B. VMAT2 LO mice exhibited a 90% reduction in mean peak release (*p = 0.02). C. There was no significant difference in the synaptic clearance rate of 5-HT, as reflected by *tau*. Four sample recordings were taken at 3 sites within the lateral SNr of each mouse; n = 3 mice per genotype.

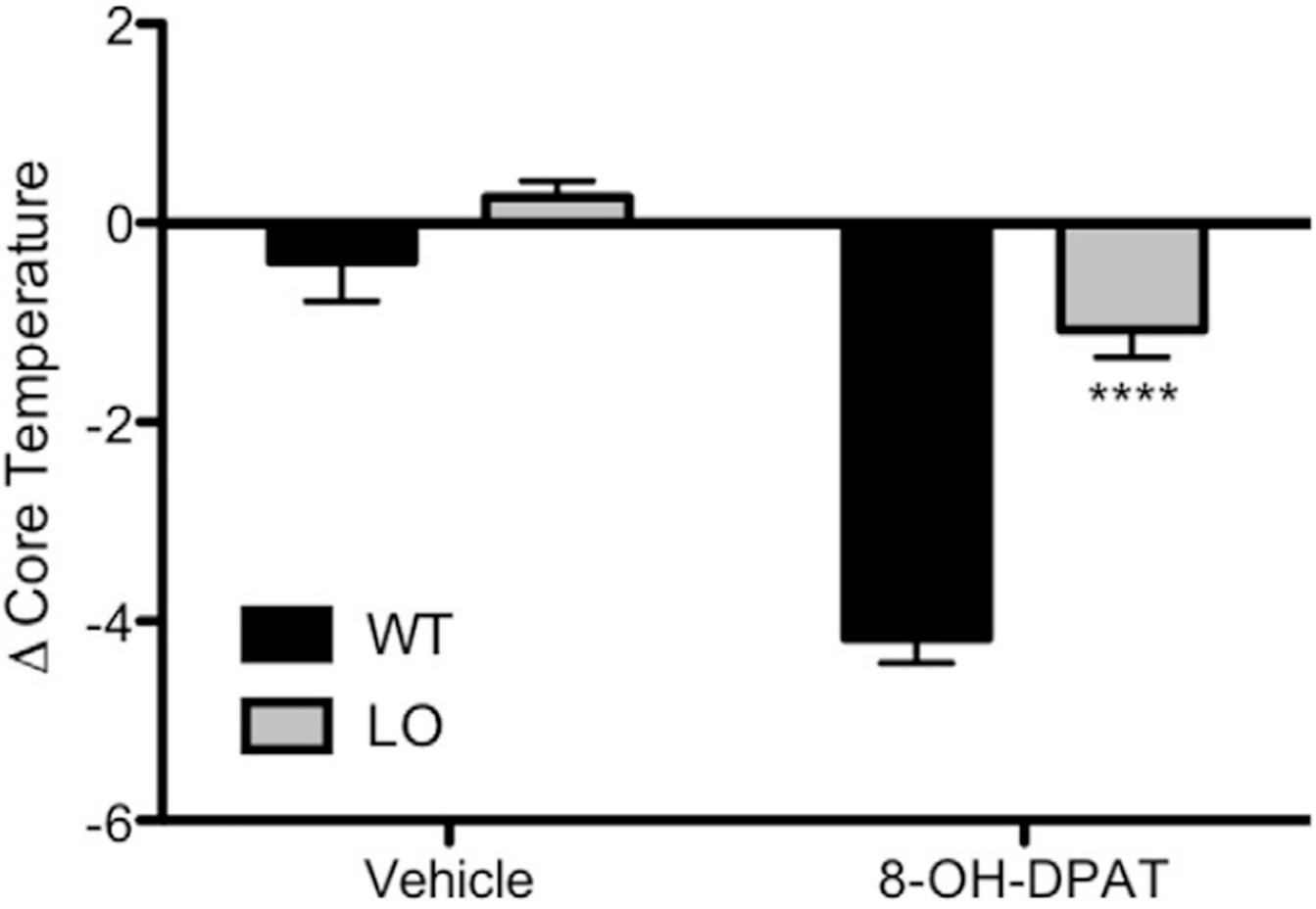


**Fig. 2.**

VMAT2 LO mice have an ablated hypothermic response to the 5-HT1A agonist 8-OH-DPAT. Aged (18–24 months) wildtype and VMAT2 LO mice were injected with saline or 8-OHDPAT (0.1 mg/kg; i.p.) to assess thermic response to 5-HT1A activation. Graph represents the mean of differences between core body temperature taken at baseline and 20 min following injection for each animal. Wildtype animals exhibited a −4° change in core body temperature, while VMAT2 LO mice had an ablated response of −1° (****$p < 0.0001$, n = 7–8 mice per group).

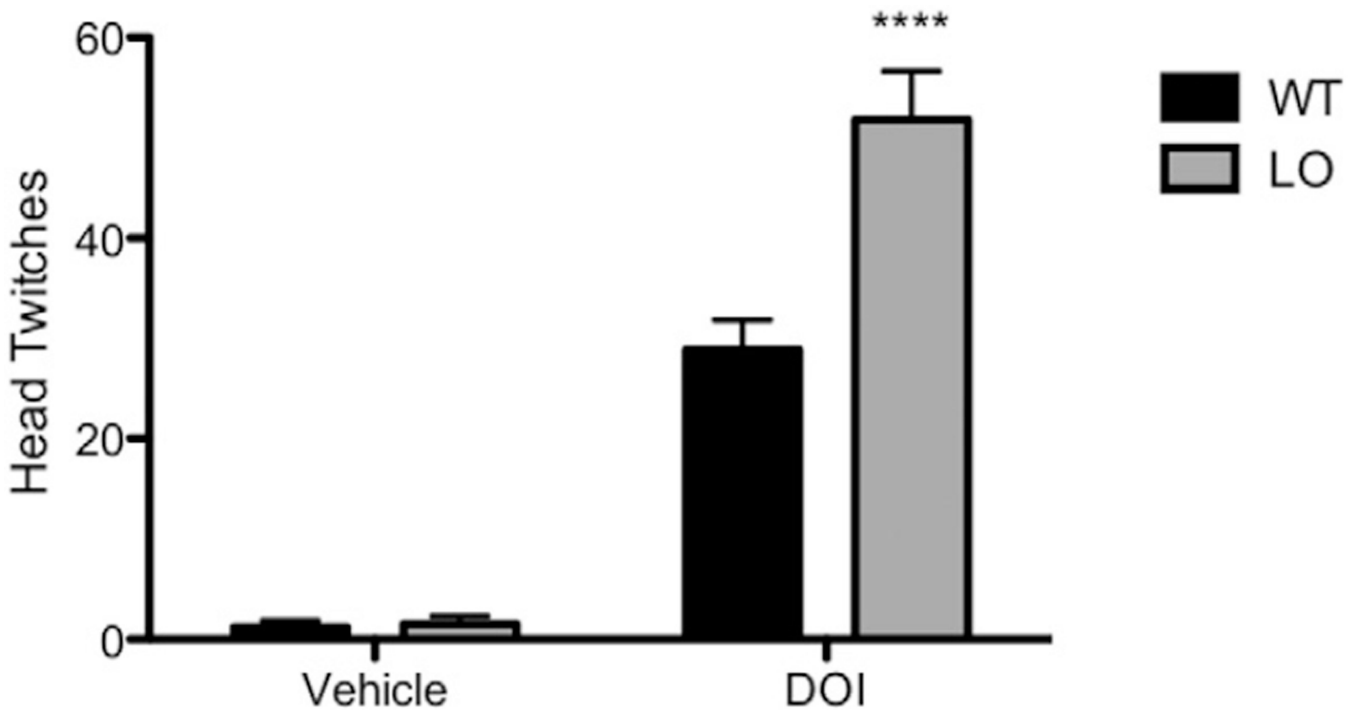


**Fig. 3.**

VMAT2 LO mice have increased sensitivity to the 5-HT2 agonist DOI. Wildtype and LO mice (4–6 months of age) were injected with saline or DOI (1 mg/kg) and placed in a cage for 10 min video-recorded observation of head twitches. When challenged with DOI, VMAT2 LO mice exhibited a 79% increase in the number of head twitches over wildtype mice (****$p < 0.0001$, $n = 8–9$ mice per group,).

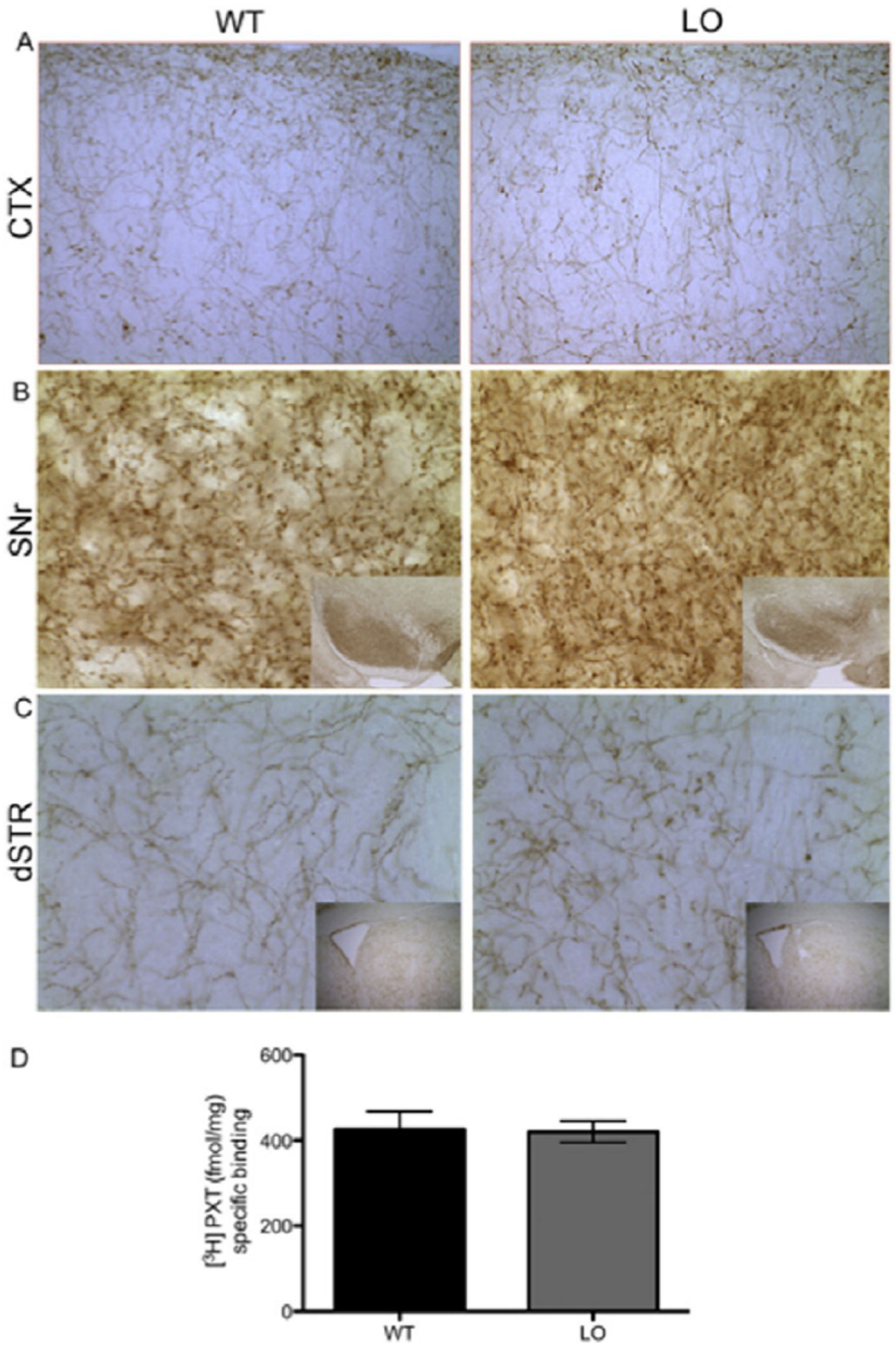


**Fig. 4.**

Serotonergic innervation is preserved in aged VMAT2 LO mice. Immunohistochemical staining against SERT reveals no substantial change in serotonergic innervation between aged (24 months) wildtype and VMAT2 LO mice. Regions depicted are the somatomotor cortex (A, 200× magnification), SNr (B, 400× magnification, inset at 25×), and dorsal striatum(C, 400× magnification, inset at 25×) D. [3 H]-paroxetine binding in striatal homogenates reveals no change in binding sites between aged (22–24 months) wildtype and VMAT2 LO mice (n = 6, p = 0.9).

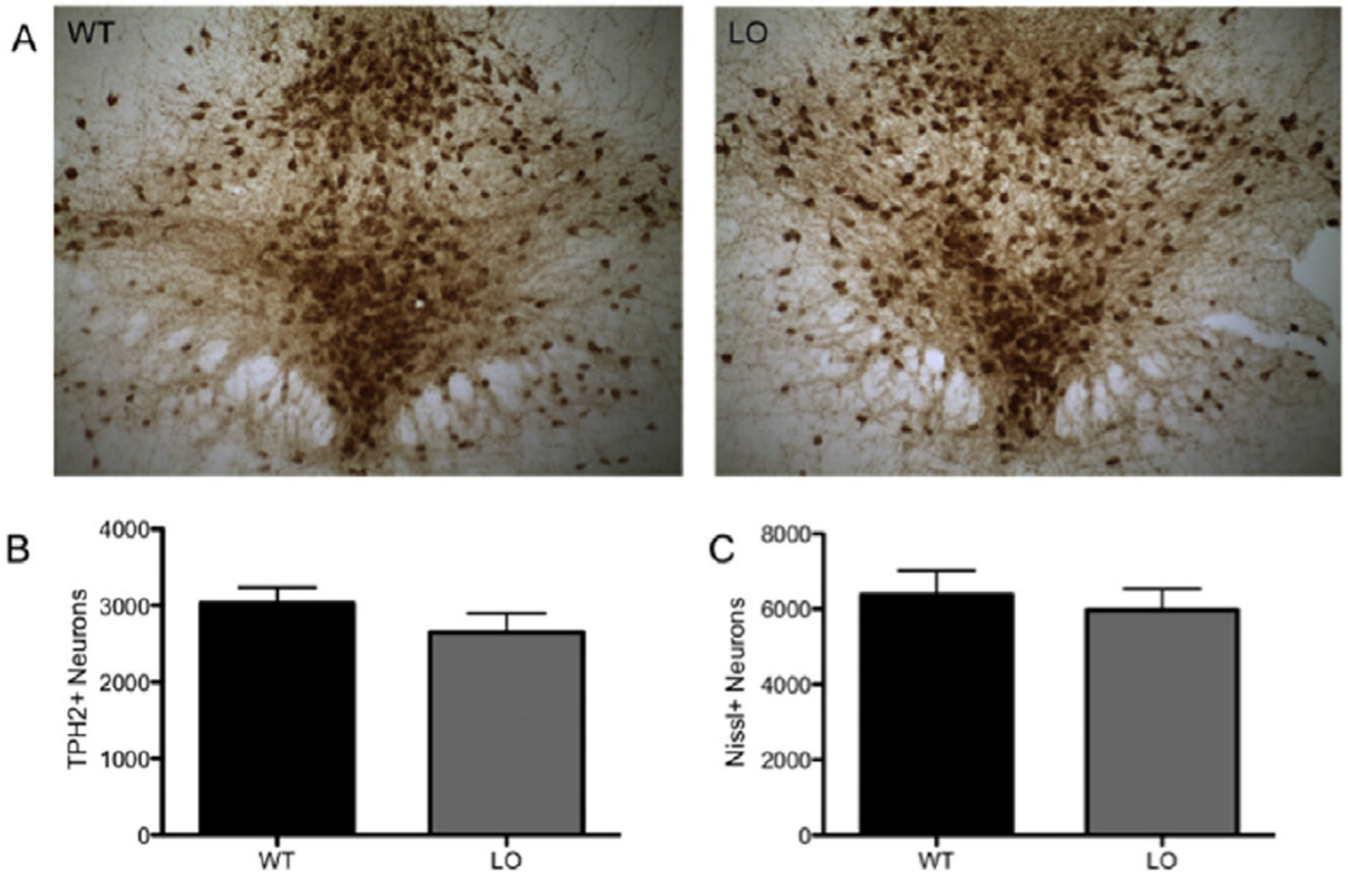


**Fig. 5.**

Preservation of the dorsal raphe nucleus in aged VMAT2 LO mice. A. Immunohistochemical staining against TPH2 in the dorsal raphe shows normal cytoarchitecture in VMAT2 LO mice at 24 months of age (images taken at 50× magnification). B. Unbiased stereological sampling revealed no significant change in the quantity of TPH2 + neurons in the dorsal raphe between aged WT and VMAT2 LO mice; the quantity of Nissl + neurons (inclusive of TPH2 + neurons) was also unchanged. Analysis was performed at section intervals of 3, with a total of 6–7 dorsal raphe sections per animal. The Gundersen coefficient of error was <0.1 for all animals (n = 5 mice, p = 0.73).