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Vesicular monoamine transporter 2 mediates fear behavior in mice

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Abstract

A subset of people exposed to a traumatic event develop post-traumatic stress disorder (PTSD), which is associated with dysregulated fear behavior. Genetic variation in *SLC18A2*, the gene that encodes vesicular monoamine transporter 2 (VMAT2), has been reported to affect risk for the development of PTSD in humans. Here, we use transgenic mice that express either 5% (VMAT2-LO mice) or 200% (VMAT2-HI mice) of wild-type levels of VMAT2 protein. We report that VMAT2-LO mice have reduced VMAT2 protein in the hippocampus and amygdala, impaired monoaminergic vesicular storage capacity in both the striatum and frontal cortex, decreased monoamine metabolite abundance, and a greatly reduced capacity to release dopamine upon stimulation. Furthermore, VMAT2-LO mice showed exaggerated cued and contextual fear expression, altered fear habituation, inability to discriminate threat from safety cues, altered startle response compared to wild-type mice, and an anxiogenic-like phenotype, but displayed no deficits in social function. By contrast, VMAT2-HI mice exhibited increased VMAT2 protein throughout the brain, higher vesicular storage capacity, and greater dopamine release upon stimulation compared to wild-type controls. Behaviorally, VMAT2-HI mice were similar to wild-type mice in most assays, with some evidence of a reduced anxiety-like responses. Together, these data demonstrate that presynaptic monoamine function mediates PTSD-like outcomes in our mouse model, and suggest a causal link between reduced VMAT2 expression and fear behavior, consistent with the correlational relationship between VMAT2 genotype and PTSD risk in

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humans. Targeting this system is a potential strategy for the development of pharmacotherapies for disorders like PTSD.

Keywords

posttraumatic stress disorder; PTSD; vesicular monoamine transporter; VMAT2; monoamine; fear; mouse; transgenic; behavior; fast scan cyclic voltammetry; FSCV

Introduction

Posttraumatic stress disorder (PTSD) is characterized by aberrant fear behaviors including intrusive memories, avoidance symptoms, and negative changes in cognition and mood following a traumatic event (American Psychiatric Association 2013). Although exposure to severe trauma is relatively common, only a small proportion of people go on to develop PTSD (Kessler *et al.* 1995; Breslau *et al.* 1999; Shiromani *et al.* 2009), a risk mediated by genetic and social factors (Ozer *et al.* 2003; Charney 2004; Binder *et al.* 2008; Li *et al.* 2016). Prevention and treatment of PTSD would be greatly advanced by the identification of biomarkers that predict and/or mediate PTSD risk.

One avenue of research is to examine the genetic differences between trauma-exposed individuals who develop PTSD and those that do not (Norrholm & Ressler 2009). Transgenerational and twin studies indicate that genetics, particularly variation in monoamine systems, play a role in the etiology of PTSD (Naß & Efferth 2017). In a recent candidate gene analysis of more than 3,000 single nucleotide polymorphisms across more than 300 genes, the gene *SLC18A2*, which encodes the vesicular monoamine transporter 2 (VMAT2), was identified as a risk haplotype for PTSD diagnosis in a cohort of 2,538 European women and replicated in an independent cohort of 748 male and female African Americans (Solovieff *et al.* 2014). VMAT2 protein abundance is reduced in post-mortem brains of PTSD patients compared to the post-mortem brains of people without a PTSD diagnosis (Bharadwaj *et al.* 2016). Other large genetic studies implicate presynaptic monoamine regulation as an important risk factor for PTSD (Nievergelt *et al.* 2019). Furthermore, the psychiatric disorders that often present concurrently with PTSD have been independently associated with VMAT2 dysfunction (Zubieta *et al.* 2001; Zucker *et al.* 2002; Schwartz *et al.* 2003; Eiden & Weihe 2011). Taken together, these findings indicate that VMAT2 dysfunction may be an underlying cause for a variety of associated psychiatric disorders, including PTSD. For these reasons, the function of VMAT2 merits further analysis as it relates to PTSD and psychiatric risk.

VMAT2 transports monoamine neurotransmitters (dopamine, serotonin, norepinephrine, epinephrine, and histamine) into presynaptic neuronal vesicles in preparation for release into the synaptic space (Peter *et al.* 1995). The monoaminergic neurons release neurotransmitter broadly across the brain, particularly in the dorsal and ventral striatum and frontal cortex. Multiple monoaminergic systems also converge on the amygdala (Goldstein *et al.* 1996), a key structure for the expression of fear and anxiety responses in both rodents and humans (Davis 1992). Neurotransmitter-specific manipulations do not capture the state of globally altered monoaminergic transmission suggested by the human genetic literature. As such,

experimental data are needed to establish directionality and causality in the relationship between global VMAT2 expression and PTSD outcomes.

One approach to understand this relationship is to measure fear behavior after pharmacological modulation of VMAT2. A historical experiment showed that administration of reserpine, a potent and specific VMAT2 inhibitor, increases the behavioral response to a conditioned fear stimulus (Brady 1956), first suggesting some connection between VMAT2 and the behavioral fear response. Further experiments have continued to show a link between pharmacological VMAT2 inhibition and fear behavior (Savage 1962; Fernandes *et al.* 2008). These results have been complicated by the sedative effects of VMAT2 inhibitors (Wilkins & Judson 1953; Beleslin *et al.* 1981) and by the lack of a positive modulator for VMAT2. Furthermore, pharmacological manipulation does not best reflect the genetic nature of VMAT2 and PTSD risk. To overcome these limitations, we turned to genetic models of VMAT2 function.

Behavioral studies in VMAT2 knock-out mice are limited by the fact that completely ablating VMAT2 is neonatal lethal (Wang *et al.* 1997). To circumvent this issue, our laboratory has developed and characterized strains of transgenic mice that provide a continuum of VMAT2 gene dose. Specifically, these mice express differing amounts of VMAT2: 95% reduced (VMAT2-LO) (Cliburn *et al.* 2016), normal (VMAT2-WT), or 200% elevated levels (VMAT2-HI) (Lohr *et al.* 2014, 2016). Our laboratory and others have previously demonstrated that VMAT2-LO mice show decreased extracellular dopamine and norepinephrine concentrations compared to those measured in wild-type mice (Wang *et al.* 1997; Taylor *et al.* 2009, 2011). VMAT2-LO mice also display decreased serotonergic transmission (Alter *et al.* 2016). Conversely, VMAT2-HI mice show an increased capacity to store and release monoamines, particularly dopamine (Lohr *et al.* 2015). Thus, VMAT2 is a critical bidirectional modulator of monoaminergic neurotransmission throughout the brain, and our continuum of VMAT2 transgenic mice reflect reduced or increased capacity to transmit monoamines. This continuum of VMAT2 expression is an ideal tool for testing whether the functional dysregulation of VMAT2 is a causal factor in producing an aberrant fear response.

We have previously characterized the impact of VMAT2 gene dose on VMAT2 protein abundance in regions containing monoaminergic cell bodies, including the midbrain, paraventricular nuclei, locus coeruleus, raphe nucleus, and tuberomammilary nucleus (Cliburn *et al.* 2016). Here, we tested how VMAT2 gene dose affects VMAT2 protein abundance in the amygdala and hippocampus, brain areas critical for the expression and maintenance of fear. We also investigated the consequences of VMAT2 transgenic alterations on monoamine function, as measured by total brain monoamine metabolite content, radioactive vesicular monoamine uptake in the frontal cortex and striatum tissue, and ex vivo neurotransmitter release. Next, we measured PTSD-like fear phenotypes using several behavioral assays that reflect different aspects of the human condition, including learning of cued and contextual fear (Orr *et al.* 2000; Norrholm *et al.* 2015a), habituation (Guthrie & Bryant 2006), startle responses (Glover *et al.* 2011), and fear generalization (Levy-Gigi *et al.* 2015; Morey *et al.* 2015). Because PTSD also commonly co-occurs with depression, social anxiety, and social withdrawal (Kessler *et al.* 1995; Pietrzak *et al.* 2011;

Spinhoven *et al.* 2014), we also tested whether VMAT2 gene-dose impacts affective and social behavior. We hypothesized that decreased VMAT2 protein expression would result in reduced presynaptic monoaminergic function and lead to increased PTSD-like outcomes, while increased VMAT2 protein expression would increase presynaptic monoaminergic function and be associated with increased trauma resilience.

Materials and Methods

Mice.

The original VMAT2-deficient mouse strain was created by insertion of a hypomorphic allele of the gene *Vmat2* using gene targeting techniques as fully described in Mooslehner *et al.* 2001. Following receipt of this mouse line, we were informed by researchers at the Babraham Institute that the VMAT2-deficent mouse strain was carrying a null mutation for alpha synuclein sourced to a subpopulation of Harlan C57BL/6OlaHSD used in its creation. This issue has been documented in the literature and fully described with the nomenclature for this subpopulation of Harlan C5BL/6OlaHsd being renamed C57BL/6S (Specht & Schoepfer 2001). In our laboratory, we confirmed the presence of this alpha-synuclein null mutation and began a within-colony mating scheme to remove it from this VMAT2-deficient mouse strain (Caudle *et al.* 2007; Guillot *et al.* 2008; Taylor *et al.* 2009, 2014). We then backcrossed these VMAT2-deficient mice to Charles River C57BL/6NCrl mice for 4 generations using a marker assisted selection (ie, “speed congenic”) approach. Mouse genomes were assessed at the DartMouseTM Speed Congenic Core Facility at Dartmouth Medical School, which uses the Illumina, Inc (San Diego, California) GoldenGate Genotyping Assay to examine 1449 single nucleotide polymorphisms (SNPs) throughout the genome. Raw SNP data were analyzed using DartMouse’s SNaP-MapTM and Map-SynthTM software, determining the genetic background at each SNP location for each mouse. These back-crossed VMAT2-deficient mice on the C57BL/6NCrl background are referred to as VMAT2-LO in publications from our laboratory (Cliburn *et al.* 2016; Lohr *et al.* 2016).

VMAT2-HI mice were generated by using a bacterial artificial chromosome (BAC RP23–292H20) mediated transgene to insert three additional copies of the murine *SLC18A2* (VMAT2) gene, including its endogenous promotor and regulatory elements. BAC DNA was introduced into Charles River C57BL/6NCrl embryos via pronuclear injection. Positive founders were identified by PCR genotyping using primers against BAC sequences and confirmed by Southern blotting. These founders were then maintained on a Charles River C57BL/6NCrl background at Emory University (Lohr *et al.* 2014). Notably, both the VMAT2-LO and VMAT2-HI mouse line are currently on a C57BL/6NCrl genetic background.

In our studies of these mice, the wild-type littermates of VMAT2-LO mice and the wild-type littermates of VMAT2-HI mice show no difference in neurochemical or behavioral outcomes so graphs show collapsed data from VMAT2-HI and -LO wild-type littermate controls. Male and female mice were used for all experiments. Mice were singly-housed and received food and water *ad libitum* on a 12:12 light cycle. Experiments were conducted during the light phase of the light cycle. Adult male and female mice (age 6–10 months) were used all

behavioral experiments. Notably, using mice of this age avoids the neurodegenerative effects seen in aged VMAT2-LO mice (Taylor *et al.* 2011). All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University.

Immunohistochemistry.

Tissue was incubated at 70°C in Citra (BioGenix) antigen retrieval solution for one hour. Non-specific antibody binding was blocked with a 10% normal goat serum block for one hour at room temperature. Tissue was incubated overnight at 4°C in polyclonal rabbit anti-VMAT2 serum (1:50,000, developed in our laboratory, see Cliburn et al, 2016). Tissue was then incubated at room temperature in biotinylated goat anti-rabbit (1:200, Jackson ImmunoResearch) secondary antibody and visualized using a 60-second 3.3'-diaminobenzidine (DAB) reaction. The reaction was terminated with a PBS rinse. All images were acquired with NeuroLucida (MicroBright-Field, Williston, VT). We did not perform densitometry on IHC data because IHC is dependent on a horseradish peroxidase diaminobenzidine reaction, in which color density does not linearly correlate with protein amount. Furthermore, we did not quantify the IHC via stereology because while it would show the number of VMAT2-positive neurons, it would not quantify the total amount of VMAT2, since we know that the amount of VMAT2 changes on the vesicle itself (Lohr *et al.* 2014).

Immunoblot.

Immunoblot was used to quantify the relative amount of VMAT2 protein in VMAT2 transgenic animals. To produce the crude synaptosomal preparation, brains were homogenized in ice-cold homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4) and protease inhibitors (1:1000) using an immersion homogenizer (Tissue Tearor) for approximately 15 seconds. This homogenate was spun at 1000 x *g* for 10 minutes and the resultant supernatant was centrifuged at 20,000 x *g* for 20 minutes. To generate the membrane-associated fraction, the crude synaptosomes were osmotically lysed in pure water, then neutralized by addition of HEPES and potassium tartrate (final concentration: 25 mM and 100 mM, respectively). The lysed synaptosomes were centrifuged at 20,000 x *g* for 20 minutes. The pellet was suspended in assay buffer (25 mM HEPES, 100 mM potassium tartrate, 100 μM EDTA, 50 μM EGA, pH 7.4). Samples were *not* boiled. We used 400 mM dithriothrietol (DTT, Sigma) in NuPage LDS Sample Buffer 4X (Invitrogen) to make 4X loading buffer. We specify these parameters because boiling samples and using non-DTT containing loading buffers appears to destroy the VMAT2-specific epitope. Samples were run on a NuPage 10% bis tris gel (Life Technologies) and transferred to a PVDF membrane. Nonspecific antibody binding was blocked with a 7.5% milk solution and the membrane was then incubated in primary antibody overnight at 4°C. Primary antibodies used were polyclonal rabbit anti-VMAT2 serum (1:10,000). The following day, the membrane was incubated with the appropriate HRP-linked secondary antibody (1:5,000, Jackson ImmunoResearch) for one hour. Densitometric analysis was performed and calibrated to coblotted dilutional standards of pooled striata from all control samples. Actin blots were used to ensure equal protein loading across all samples. Differences between groups were

determined via one-way ANOVA with genotype (LO, WT, HI) as factor and with post-hoc Dunnett's test to determine differences between groups.

Vesicular radioactive monoamine uptake.

Three to five striatal or frontal cortical dissections from each genotype were homogenized in homogenization buffer. Uptake was performed as described previously (Caudle et al. 2007). ANOVA with post-hoc Dunnett's tests were performed to determine differences between genotypes.

High performance liquid chromatography (HPLC).

HPLC was performed as described previously (Lohr *et al.* 2014), with a few modifications. Briefly, half brains cut sagittally with the cerebellum removed were sonicated in 10x their weight of 0.1 M perchloric acid and were pelleted at 10,000 x *g* for 10 min. Supernatants were filtered at 0.2 μm. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were detected using an MD-150 × 3.2 mm C18 column (ESA). The mobile phase consisted of a 1.5 mM 1-octanesulfonic acid sodium, 75 mM NaH_2PO_4, 0.025% trimethylamine, and 8% (vol/vol) acetonitrile at pH 2.9. A 20 μL sample was injected. The detected concentrations of three groups of analytes (dopamine group: DA and DOPAC; norepinephrine group: NE; serotonin group: HVA and 5-HIAA) were analyzed using separate two-factor MANOVAs, with genotype (LO, WT, HI) as between-subjects factor and the metabolites as dependent variables.

Fast scan cyclic voltammetry (FSCV).

In order to measure peak stimulated dopamine release, slice FSCV was performed in the lateral dorsal striatum of male and female adult VMAT2-LO, -WT, or -HI mice. Mice were decapitated and the brain rapidly removed and placed in ice-cold, oxygenated (95% O_2/5% CO_2) sucrose aCSF (193 mM sucrose, 11 mM d-glucose, 1.2 mM dihydrous $CaCl_2$, 4.5 mM KCl, 25 mM $NaHCO_3$, 20.5 mM NaCl, 1.2 mM NaH_2PO_4 monobasic, 2.6 mM $MgCl_2$, adjusted to pH 7.4) then sliced coronally at 300 μM using a vibratome (Leica VT1000 S, Buffalo Grove, IL). Striatal slices were selected and bathed in oxygenated HEPES aCSF (19.7 mM HEPES, 11 mM d-glucose, 2.4 mM dihydrous $CaCl_2$, 25 mM $NaHCO_3$, 126.4 mM NaCl, 2.5 mM KCl, 1.2 mM monobasic NaH_2PO_4, 2.6 mM $MgCl_2$, adjusted to pH 7.4) at 20°C for 30 mins prior to recording. For recording, a slice was transferred to a slice dish where it was superfused in 30 °C HEPES aCSF for the course of the experiment. The recording electrodes, cylindrical carbon-fiber (6 um diameter, Thornel, Greenville, SC) microelectrodes, were fabricated in-house by sealing the carbon fiber in a glass capillary such that the length of the protruding carbon-fiber was approximately 65 μM. A bipolar tungsten stimulating electrode (MicroProbes, Gaithersburg, MD) and a recording electrode were placed on the surface of the slice in the nucleus accumbens core. For each recording, dopamine release was effected via a single computer-generated stimulation (monophasic, 60 Hz, 2.31 V to produce a 700 μA stimulation). Stimulations were separated by five minutes. Application of waveform, stimulus, and current monitoring was controlled by TarHeel CV (University of North Carolina) using a custom potentiostat (UEI, UNC Electronics Shop). The waveform for dopamine detection consisted of a −0.4 holding potential versus an Ag/

AgCl (World Precision Instruments, Inc., Sarasota, FL) reference electrode. The applied triangular voltage ramp went from −0.4 V to 1.0 V and back to −0.4 V at a rate of 600 V/s at 60 Hz. The current at the peak oxidation potential for dopamine (0.6 vs Ag/AgCl) was used to evaluate dopamine concentration changes with time. Primary outcome measure was peak dopamine release, which was evaluating by calculating the maximum current and dividing it by the calibration constant for that electrode. Measuring peak dopamine release is a common practice to determine stimulated neurotransmitter release (Lohr *et al.* 2014; Alter *et al.* 2016; Stout *et al.* 2016). ANOVA with post-hoc Dunnett's tests were performed to determine differences in peak stimulated dopamine release between genotypes.

Fear acquisition, contextual fear, cued fear, and fear habituation.

On day 1, mice were placed in the fear conditioning apparatus (7" W, 7" D, 12" H, Coulbourn Instruments, Whitehall, PA) composed of Plexiglass with a metal shock grid floor and were allowed to explore the enclosure for 3 min. Following this habituation period, 3 presentations of a conditioned stimulus (CS) (tone, 20 s, 10 kHz, 85 dB, 1 min inter-trial interval) co-terminated with 3 presentations of an unconditioned stimulus (US) (foot shock, 2 s, 0.5 mA). Shock was delivered via a Precision Animal Shocker (Coulbourn Instruments, Whitehall, PA) connected to each fear-conditioning chamber. One min following the last CS-US presentation, animals were returned to their home cage. On day 2, mice were presented with a context test, during which they were placed in the same conditioning chamber used on day 1 for 7 min and the amount of freezing was recorded via FreezeFrame 3 (Coulbourn Instruments, Holliston, MA). No shocks were administered during the context test. On day 3, a tone test was presented, during which mice were placed in a novel compartment with a texturally distinct metal floor. Initially, animals were allowed to explore the novel context for 2 min. Following this habituation period, the CS was presented for 6 continuous min without shocks, and the amount of freezing behavior was recorded. On day 4 the tone test was repeated, and freezing during the continuous tone over time was taken as a measure of habituation. Freezing threshold was determined individually for each mouse using FreezeFrame 3 (Coulbourn Instruments, Whitehall, PA). For each assay, we performed a two-way ANOVA with genotype (LO, WT, HI) as a between-subjects factor and time as a within-subjects factor. Post-hoc Dunnett's tests were used to analyze differences between genotype (GraphPad Prism, San Diego, CA).

Acoustic startle and prepulse inhibition.

Startle assays were performed as described in Flandreau et al. (2015). Briefly, mice underwent multiple sessions in order to assess startle magnitude and prepulse inhibition. Because mice that are physically heavier produce a more robust startle response and VMAT2-LO mice tend to be physically smaller than VMAT2-WT and -HI mice, each startle response was normalized to that mouse's average startle response to a 120 dB tone. For each assay, a separate two-way ANOVA with genotype (LO, WT, HI) as a between-subjects factor and tone intensity (80, 90, 100, 110 dB for startle test, 67, 70, 74, 80 dB for prepulse test) as a within-subjects factor was performed. Post-hoc Dunnett's tests were used to analyze differences between genotypes (GraphPad Prism, San Diego, CA).

Fear discrimination.

VMAT2-LO and -WT mice were trained using the protocol described in McHugh et al. (2015). Briefly, on the first day, the mice habituated to the fear chambers and were exposed to the auditory tones. Briefly, on the first day, the mice habituated to the fear chambers and the auditory tones (termed the “pre-test” day). Over the next three days, mice training in which one tone (CS+) was always co-terminated with a mild foot shock (0.5 mA, 0.5 s), another tone (CS-) was never co-terminated with shock, and a third tone (CS20) was co-terminated with mild foot shock for 20% of its presentations. In this way, the CS20 represents a ‘probabilistic’ fear cue. Each auditory tone (tone 1, 10 kHz, 85 dB, 30 s; tone 2, 7 kHz, 85 dB, 30 s; tone 3, 2.9 kHz, 85 dB, 30 s) was randomly assigned to be CS+, CS-, or CS20 for each mouse. On the fifth and final day of this protocol, mice were moved to a new fear testing apparatus in order to reduce contextual fear, and were exposed to multiple presentations of the three auditory cues. Each mouse’s freezing behavior was recorded for each day. Freezing threshold was determined individually for each mouse using FreezeFrame 3 (Coulbourn Instruments, Holliston, MA). The change in freezing in response to each CS was determined by subtracting the percentage freezing during the CS by the percentage freezing during the thirty seconds of silence immediately preceding the CS. Note that the frequency of the tones is the only difference between this protocol and that described in McHugh et al (2015). Data were analyzed using a 2-way ANOVA with genotype (LO, WT) as a within-subjects factor and stimulus (CS+, CS-) as a repeated measure. We display the data from the CS+ and CS- in order to show the most informative differential of responses to potential fear cues in Figure 5. However, a figure which contains the probabilistic fear cue data is included as supplementary material (Fig S1). Post-hoc Sidak’s multiple comparisons test were used to analyze differences between stimulus types within each genotype.

Open field test.

Mice were individually placed in a circular chamber (96.5 cm diameter, 28 cm height) and allowed ten min to freely explore. Time spent in the center of the apparatus, time spent in the sides of the apparatus, and total movement was recorded by TopScan 2.0. CleverSysm Inc (Reston, VA). ANOVA with post-hoc Dunnett’s test was performed to determine differences between groups.

Sucrose preference.

For a period of 5 d, a bottle of 2% sucrose and a bottle of plain water were placed in the hoppers of individually housed mice. Each day, the bottles were weighed to determine the amount of liquid consumed. The relative positions of the bottles were switched each day to control for side preferences. Percent sucrose preference was determined by dividing the amount of sucrose water consumed divided by the total amount of liquid consumed. Differences among genotype were determined using ANOVA with post-hoc Dunnett’s tests (Graphpad Software, La Jolla, CA).

Forced swim test.

Forced swim test assays were administered as previously described (Lohr et al. 2014). Briefly, mice were placed in glass cylinders (24 × 16 cm) with 15 cm of water maintained at 25°C and were video recorded for 6 min. After the first 2 min, videos were scored in 5 s bins, and the primary behavior (either immobile, passive swimming, or active struggle) was scored for each bin. Behavior as measured by a rater blind to the experimental groups had over 95% concordance between independent scoring sessions. Differences among genotypes were determined using ANOVA with post-hoc Dunnett's test. Because experimental observers noticed different patterns of behavior during the forced swim test that were not captured simply by measuring immobility time. Videos were re-analyzed and proportion of time spent immobile, passively swimming, and actively struggling were compared between VMAT2-LO and VMAT2-WT mice using a chi-square test (Graphpad Software, La Jolla, CA).

Marble burying.

Mice were placed in their home cages with the nestlet, food, and water removed from the cage for the duration of the test. Extra bedding was added to each cage such that there was ~6" of loose bedding throughout the cage. In each cage, 20 marbles were arranged evenly in 5 rows of 4 marbles. The mouse was placed in the cage for 30 min, and the number of marbles at least 2/3 covered was recorded. ANOVA with post-hoc Dunnett's test was performed to determine differences between groups.

Repetitive behaviors.

Mice were individually placed in a clean, new cage and allowed to habituate for 20 minutes. After habituation, mice were recorded for ten minutes using a GoPro Hero 5 Session. Videos were played via QuickTime movie player and duration of repetitive behaviors was timed via hand-held stopwatch. Amount of time spent self-grooming was recorded. ANOVA with post-hoc Dunnett's test was performed to determine differences between groups.

Social Approach.

A social approach assay was used to quantify social approach behavior and social memory in mice. Mice were individually placed in the center chamber of a 3-chambered apparatus and allowed 10 min to habituate. The sides were then opened and the mouse was allowed 10 min to habituate to the whole apparatus. Time spent in each chamber was later analyzed through video recordings to test for side biases. A stimulus mouse was then placed under a mesh wire cup (3.8-cm bottom diameter, rust-proof/rust-resistant, noncorrosive, steel wire with space between bars to allow for interaction) on one side of the chamber, and an identical empty upside-down cup was placed on the opposite side. To prevent the subject mouse from climbing on top of the cup, a right-side up Solo cup was placed on top of the inverted cup and a weight was placed in the Solo cup. The subject mouse was given 10 min to freely explore the apparatus. Time spent near the stimulus mouse under the cup (novel mouse) and time spent near the inverted cup without the mouse (novel object) was later analyzed through video recordings. The relative placement of the novel mouse was counterbalanced across all subjects. In the final stage, the original stimulus mouse was

moved to the opposite side of the chamber, and a new subject mouse was placed under a cup on the original side. The subject mouse was given 10 min to explore the apparatus, and time near the novel and familiar stimulus mouse was observed through video recordings. The apparatus was cleaned between subjects. All videos were scored by an experimenter blind to the experimental groups. ANOVA with post-hoc Dunnett's test was performed to determine differences between groups.

Results

VMAT2 gene dose mediates VMAT2 expression and function.

Using immunohistochemistry, we showed that VMAT2 was sparsely present in both the anterior basolateral nucleus of the amygdala and in the dentate gyrus of the hippocampus in VMAT2-WT and -HI mouse tissue, and that VMAT2 staining was negligible in VMAT2-LO tissue (Fig 1A). VMAT2-LO mice have greatly reduced VMAT2 protein amount wheras VMAT2-HI mice have increased VMAT2 protein amount ($F(2,9) = 730.4$, $p < 0.0001$, Dunnett's test LO<WT $p < 0.0001$ and HI>WT $p<0.0001$) (Fig 1B), consistent with previous descriptive findings that VMAT2 protein expression varies across dopaminergic, noradrenergic, and serotonergic circuits in VMAT2 transgenic mice (Cliburn *et al.* 2016). However, the presence of VMAT2 in anatomical regions critical to expression and maintenance of fear behavior (amygdala and hippocampus) has not been verified in adult mice.

VMAT2 genotype is associated with functional consequences.

We first performed radioactive monoamine uptake in isolated vesicles from the striatum and the frontal cortex. Compared to the wild-type, vesicles isolated from VMAT2-LO animals transported less radioactive tracer than vesicles isolated from wild-type animals, while VMAT2-HI animals were better able to take up radioactive tracer in both the cortex ($F(2, 33) = 56.09$, $p < 0.05$, Dunnett's test LO<WT $p < 0.0001$ and HI>WT $p < 0.05$) and in the striatum ($F(2,30) = 115.5$, $p<0.0001$, Dunnett's test LO<WT $p < 0.0001$ and HI>WT $p<0.001$) (Fig 2A). We performed HPLC to test whether VMAT2 genotype resulted in changes in monoamine and monoamine metabolite content in the mouse brain. We performed analysis on three groups of monoamines and their metabolites (dopamine group: DA and DOPAC; norepinephrine group: NE; serotonin group: HVA and 5-HIAA). We found that VMAT2 gene dose had an effect on metabolite content in the dopamine group (main effect of genotype, $F(4,28) = 4.712$, $p < 0.01$; LO<WT $p < 0.001$) and norepinephrine group (main effect of genotype, $F(2,14)=4.801$, $p < 0.05$) content, but not on the serotonin group, between VMAT2-LO, -WT, and -HI mice, (Fig 2B). Lastly, we performed FSCV to measure stimulated dopamine release in the dorsal striatum. Compared to the wild-type, VMAT2-LO tissue exhibited reduced peak stimulated dopamine release while VMAT2-HI exhibited enhanced peak stimulated dopamine release ($F(2,19) = 33.85$, $p < 0.0001$, Dunnett's test LO<WT $p < 0.001$ and HI>WT $p<0.01$) (Fig 2C).

VMAT2-LO mice display increased fear acquisition, exaggerated cued and contextual fear responses, and altered habituation.

VMAT2-LO mice displayed increased fear acquisition, as shown by increased freezing during CS-US pairings on the training day (Fig 3A, main effect of genotype $F(2,107)=16.16$, $p<0.0001$; Dunnett's test LO<WT, $p<0.0001$). Furthermore, compared to wild-type controls, VMAT2-LO mice froze more in response to a context (Fig 3B, main effect of genotype $F(2, 632)=17.87$, $p<0.0001$; Dunnett's test LO<WT, $p<0.0001$) and tone (Fig 3C, main effect of genotype $F(2,576)=19.62$, $p<0.0001$; Dunnett's test LO<WT, $p<0.0001$) previously paired with foot shocks. Lastly, VMAT2-LO mice show an altered time course of fear habituation (Fig 3D, genotype × time interaction effect, $F(10,35) = 1.997$, $p<0.05$; Dunnett's test LO<WT at minute 2, $p<0.01$). VMAT2-LO mice exhibit a small but significant increase in 'baseline' freezing, as measured by amount of freezing before the tone presentation of the first day in the fear-conditioning chamber. By contrast, the performance of VMAT2-HI mice was indistinguishable from wild-type in all assays.

VMAT2-LO mice displayed altered startle reactivity.

We tested two different aspects of startle reactivity. The first test measured startle response to tones of varying loudness. VMAT2-LO mice startled more in response to these auditory tones overall, particularly the loudest tone (Fig 4A, main effect of genotype $F(2, 252)=5.888$, $p<0.01$; Dunnett's test LO>WT at 110 dB, $p<0.01$). In a separate assay, we tested for the ability of an auditory tone (called a 'prepulse') to inhibit a startle response to a very loud tone (120 dB) that immediately followed the prepulse. While the 70 dB prepulse had no effect on startle in WT and VMAT2-HI mice, it potentiated startle in VMAT2-LO animals (genotype × intensity interaction $F(6,756)=2.173$, $p<0.05$; Dunnett's test LO<WT at 70 dB, $p<0.001$). Prepulse inhibition at the higher decibel prepulses were equivalent in the three genotypes.

VMAT2-LO showed inability to discriminate threat and safety cues.

As expected, VMAT2-WT mice responded with higher freezing to a danger cue and lower freezing to a safety cue, indicating that they can distinguish between a danger signal and a safety signal. By contrast, VMAT2-LO mice froze at the same rate for both the safety and threat cues (Fig 5A, main effect of genotype $F(1,43)=10.31$ $p<0.01$, Sidak's test WT-CS +>WT-CS-, $p<0.01$; LO CS+ vs LO CS- is not significant), and froze more in response to all cues (Fig 5A, 2-way repeated measures ANOVA, main effect of genotype, $p < 0.05$). This VMAT2 generalization effect was not due to an increased tendency to freeze to all stimuli, as VMAT2-LO mice showed less freezing to novel tone presentations (Fig 5B, main effect of genotype $F(1,44) = 7.287$, $p<0.01$).

VMAT2 genotype mediates some affective behaviors in mice.

In a series of assays that measure the effect of VMAT2 gene dose on anxiety- and depressive- like behavior, there was variation due to genotype in some measures. There was no difference due to genotype in the amount of time spent close to the edges of an open field enclosure compared to time spent in the middle (main effect of genotype, $F(2,24) = 0.0689$, $p = 0.9336$) (Fig 6A). VMAT2-HI mice buried fewer marbles in a marble burying assay

(F(2,13) = 53.18, p < 0.0001, HI>WT p < 0.0001) (Fig 6B). Furthermore, VMAT2-LO mice exhibited a decreased preference for a 2% sucrose solution (F(2,26) = 8.372, p < 0.01, LO<WT, p<0.001) (Fig 6C) and there was a trend towards increased self-grooming in the VMAT2-LO mice (F(2,55) = 3.134, p = 0.0514) (Fig 6D). VMAT2-LO mice spent less time immobile during a forced swim test (F(2,15) = 4.983, p < 0.05, LO<WT, p < 0.05) (Fig 6E) due to an increase in active climbing compared to wild-type mice, which paddled but did not climb ($\chi^2(1) = 79.5$, p < 0.0001) (Fig 6F). We also performed power analysis for each of these assays, since the number of mice used for each group varied among each test (See Supplementary Resources 1).

VMAT2 transgenic animals show similar patterns of social memory.

There were no differences among VMAT2-LO, -WT, and –HI mice in regards to amount of time spent exploring a novel mouse versus a novel object (F(2,36) = 1.518, p = 0.2328) (Fig S2A) or in the amount of time spent exploring a novel mouse versus a familiar mouse (F(2,36) = 0.2158, p = 0.8069) (Fig S2B).

Discussion

Summary.

Together, these data point to VMAT2 as a regulator of monoamine content and function, whose downregulation results in potentially maladaptive behavioral changes which may be important for understanding PTSD neurobiology. We show that drastic reduction of the VMAT2 protein in mouse brain reduces monoamine content, packaging, and release, and results in aberrant fear behavior. While genetically increasing global VMAT2 protein amount produces increased monoamine content, packaging, and release, it does not result in dramatic behavioral phenotypes. Together, these data point to VMAT2 as a regulator of monoamine content and function that results in potentially maladaptive behavioral changes in the case of reduced VMAT2, which may be important in the understanding of PTSD etiology and treatment.

VMAT2 regulates functional monoamine dynamics.

Here, we showed for the first time that VMAT2 protein is present in wild-type mouse brain in areas critical to expression of learned fear: the hippocampus and the amygdala, and that this expression varies based on VMAT2 genotype. We have previously shown variation in VMAT2 expression in canonical monoaminergic circuits in the continuum of VMAT2 mice (Cliburn *et al.* 2016). VMAT2 genotype also mediates the ability of the vesicle to store monoamine neurotransmitter. The striatum primarily contains dopamine terminal projections from the midbrain, while noradrenergic and serotonergic neurons, projecting from the locus coeruleus and raphe nucleus, respectively, are the primary drivers of vesicular monoamine uptake in the frontal cortex. Testing and comparing vesicular monoamine uptake in these two regions enabled us to characterize how VMAT2 genotype affects the vesicular storage capabilities of various monoamine systems. Furthermore, we show that VMAT2 gene dose results in brain-wide alterations in monoamine metabolite content and dramatic alterations to the capacity of terminals to release neurotransmitter in response to a stimulation.

VMAT2 mediates behavioral fear phenotypes in mice.

Because the architecture of fear biology is highly conserved in mammals, mice serve as a useful tool for studying fear behavior in humans (Flandreau & Toth 2017). Fear conditioning paradigms capitalize on this shared circuitry and can be used in rodents and humans to model fear-related phenotypes (Jovanovic *et al.* 2005; Jovanovic & Ressler 2010). We show that global reduction of VMAT2 leads to a broadly aberrant fear phenotype, including increased fear acquisition, increased contextual fear, increased cued fear, altered rate of fear habituation, altered startle reactivity, and inability to discriminate between threat and safety cues. These phenotypes correspond to clinical observations of patients with PTSD. Dysregulated fear responses have been observed in PTSD, including poor discrimination between threat and safety cues, impaired inhibition of fear to safety signals, deficits in fear habituation, heightened fear expression during habituation, and exaggerated startle responses (Grillon *et al.* 1996; Jovanovic *et al.* 2012; Morey *et al.* 2015; Norrholm *et al.* 2015b). These fear phenotypes may be behavioral indices of amygdala hyperactivity, which has been frequently reported in PTSD neuroimaging studies (Rauch *et al.* 2000; Stevens *et al.* 2017).

Evidence for VMAT2 regulation of affective behavior.

VMAT2-LO mice displayed an anxiety-like phenotype across multiple assays. The reduced preference for sucrose solution demonstrated by VMAT2-LO mice could be interpreted as an anhedonic-like response, or neophobia associated with an anxiety-like phenotype. The increased escape-like behavior observed in our VMAT2-LO mice in the forced swim test is consistent with the increase in escape behavior observed in mice with 95% depletion of VMAT2 in SERT-expressing neurons (Narboux-Nême *et al.* 2011). Previous researchers have suggested that this pattern of behavior is indicative of a high-anxiety panic-like phenotype. We observed a trend towards increased repetitive self-grooming in the VMAT2-LO mice, which can be an indicator of a coping mechanism in response to acute stress or discomfort in the environment (Kalueff & Tuohimaa 2004; Denmark *et al.* 2010; Kalueff *et al.* 2016).

No evidence for VMAT2 effect on social behavior.

We found that VMAT2 genotype did not impact social behavior. These social tests involved interactions with a novel mouse, however, which does not encapsulate the range of social deficits or social anxiety phenotypes exhibited in other disorders (PTSD, bipolar disorder, schizophrenia, depression) in which VMAT2 is implicated (Zubieta *et al.* 2001; Schwartz *et al.* 2005). These disorders often include aspects of social anxiety, social withdrawal, social dysfunction, and altered empathic responses. In humans, the social issues associated with these disorders are not limited to interactions with novel individuals. In order to better address the role of VMAT2 in the social components of these disorders, future studies could use familiar conspecifics, thus more closely mirroring the social phenotype seen in humans.

Overexpressing VMAT2 changes monoamine function but does not impact behavioral phenotypes.

The VMAT2-LO behavioral phenotypes, in conjunction with our previously published literature showing that VMAT2-LO mice show altered sleep patterns (Taylor *et al.* 2011)

indicate that the VMAT2-LO mice model a variety of human PTSD symptomology. We also show that while overexpression of VMAT2 results in biochemical changes, there is no evidence that this leads to changes in fear behavior, social behavior, or preference for natural rewards (i.e. sucrose), but rather appears to be mildly anxiolytic. Indeed, in our laboratory, we have yet to observe any dramatic behavioral phenotypes in the VMAT2-HI mice as measured in motor, sensory, affective, appetitive, or social assays (Lohr *et al.* 2014). It is possible that a period of chronic stress is necessary to 'unmask' any protective effect of overexpression of VMAT2. The possibility of a VMAT2-HI 'resiliency' phenotype could be tested under more intense fear paradigms that produce fear generalization and delayed extinction in WT mice.

Understanding monoaminergic contributions to behavioral outcomes.

VMAT2 acts in all monoaminergic brain regions, and thus variation in its expression has wide-ranging effects on neurochemical transmission (Caudle *et al.* 2007; Lohr *et al.* 2014; Alter *et al.* 2016). It is difficult to attribute any one of the behavioral phenotypes described in this study to a particular monoamine neurotransmitter system or brain region; indeed, genetic variation in multiple monoaminergic systems, including in monoaminergic vesicular packaging itself, has been implicated in risk for PTSD (Solovieff *et al.* 2014; Naß & Efferth 2017). All three major central monoamine systems (dopamine, norepinephrine, and serotonin) interact with each other, and individually to contribute to PTSD outcomes. Further investigation of specific circuits via optogenetics could provide more specific therapeutic treatments or better tease out the role of specific circuits in these behaviors. The Giros laboratory at McGill University has bred VMAT2(lox/lox) mice with DBHcre, SERTcre, and DATcre mice to create conditional VMAT2 knockout in norepinephrine, serotonin, or dopamine circuits, respectively, but reported difference in assays of motor or affective behavioral assaysnin heterozygous animals (Isingrini *et al.* 2016). Conditional knock-out mice could be ideal for initial testing of which neurotransmitter system is critical for the VMAT2-depletion effects on fear behavior.

The periphery and monoamines.

Another consideration of reduced VMAT2 function that was not explored in this study is the idea that monoaminergic tone can significantly alter basic growth and health of the body. VMAT2-LO mice show an increased baseline core body temperature and show a consistently smaller body size. In our VMAT2 hypomorph line of mice, we assume that the primarily non-neuronal isoform VMAT1 is compensating in the absence of robust VMAT2 expression in non-neuronal cells. However, the paucity of VMAT2 in these peripheral cell types may be influencing the health and growth of our VMAT2-LO line of transgenic mice. Alternately, reduced monoaminergic signaling in the central nervous system could influence peripheral function such that VMAT2-LO animals are typically smaller than their wild-type counterparts.

PTSD heterogeneity.

PTSD is a heterogeneous disorder with multiple subtypes (Norrholm & Jovanovic 2010). Previous work shows that PTSD symptoms tend to cluster into four classes (re-experiencing, avoidance/numbing, negative cognitions, and hyperarousal), and that accurate identification

of the patient's particular symptom cluster is paramount to effective treatment (Norrholm & Jovanovic 2010). The phenotypes observed in VMAT2-LO mice are most consistent with the "avoidance/numbing" and "hyperarousal" symptom clusters, as shown by the anhedonic sucrose response, increase in contextual fear response, blunted fear response to an ambiguous fear cue, and increased escape behavior. It is possible that humans with PTSD who have lower VMAT2 expression tend to disproportionately experience these symptom clusters. This is a testable hypothesis, given that VMAT2 function is readily assessed in humans using PET ligands (Okamura *et al.* 2010).

Future directions.

These results point to VMAT2 as a potential therapeutic target for the treatment of fear disorders. The canonical pharmacological tools for PSTD and other fear disorders are selective serotonin reuptake inhibitors, which are not universally effective and produce unwanted side effects (Kelmendi *et al.* 2016). Augmenting vesicular function would lead to an action potential-dependent increase in synaptic transmission, an approach which could reduce side effects common in drugs that affect synaptic machinery or increase extracellular monoamine concentration regardless of neuronal activity. Future drug discovery and development aimed at positive allosteric modulators of VMAT2 may produce candidate pharmacological therapeutics for the treatment of fear symptoms associated with PTSD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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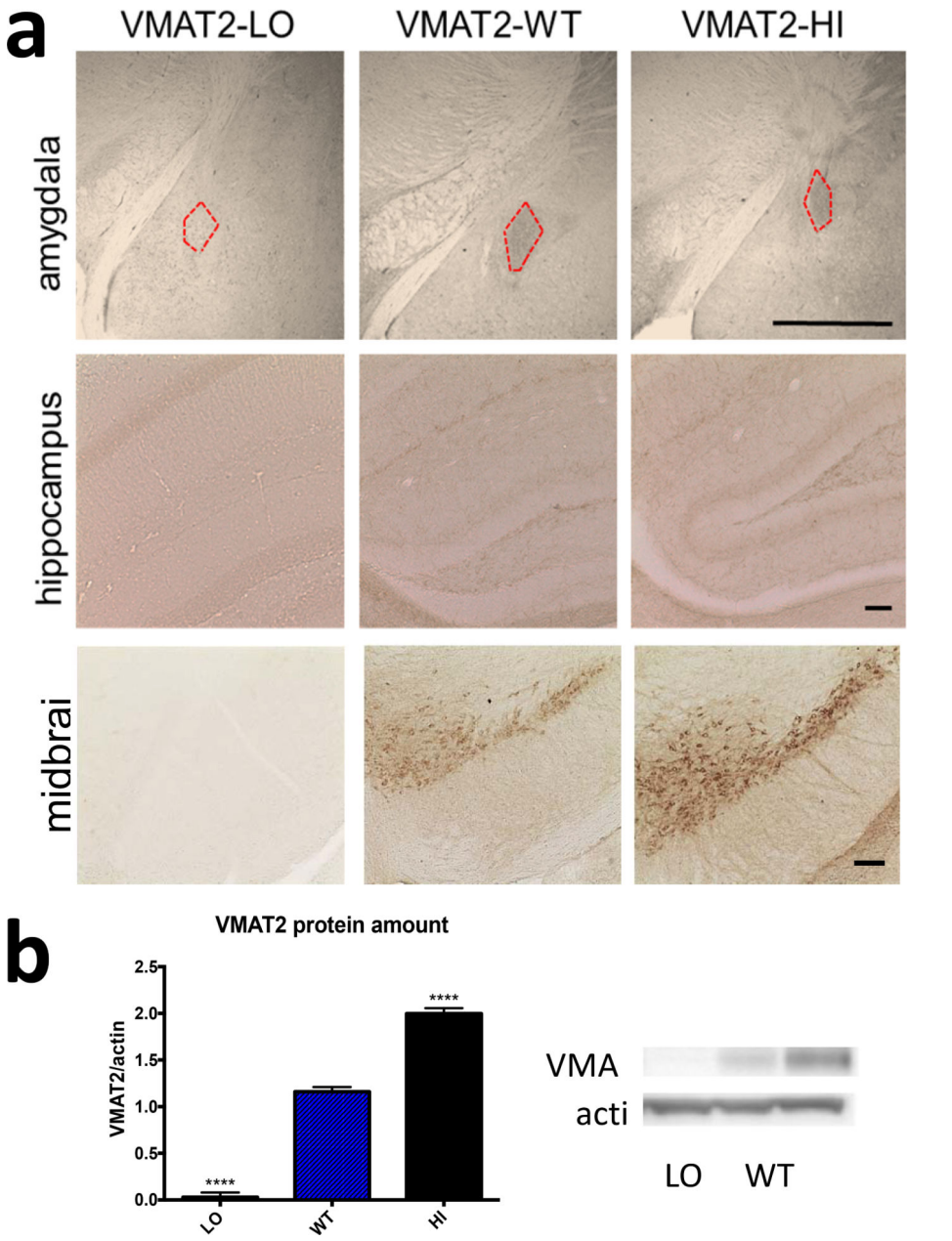

Fig 1. VMAT2 immunohistochemistry in mouse amygdala, hippocampus, and midbrain.

A.) VMAT2-LO mice show negligible VMAT2 immunohistochemical staining in the amygdala (outlined in red), hippocampus and midbrain, while VMAT2-WT and –HI mice show VMAT2 protein expression in these areas. Midbrain staining is included to show visible under- and over-expression of VMAT2 in monoaminergic areas. Polyclonal rabbit anti-VMAT2 was used at 1:50 000. Scale bar at 1 mm for amygdala, 100 μm for hippocampus and midbrain. B.) Variation in VMAT2 expression due to VMAT2 genotype is quantified via immunoblot.

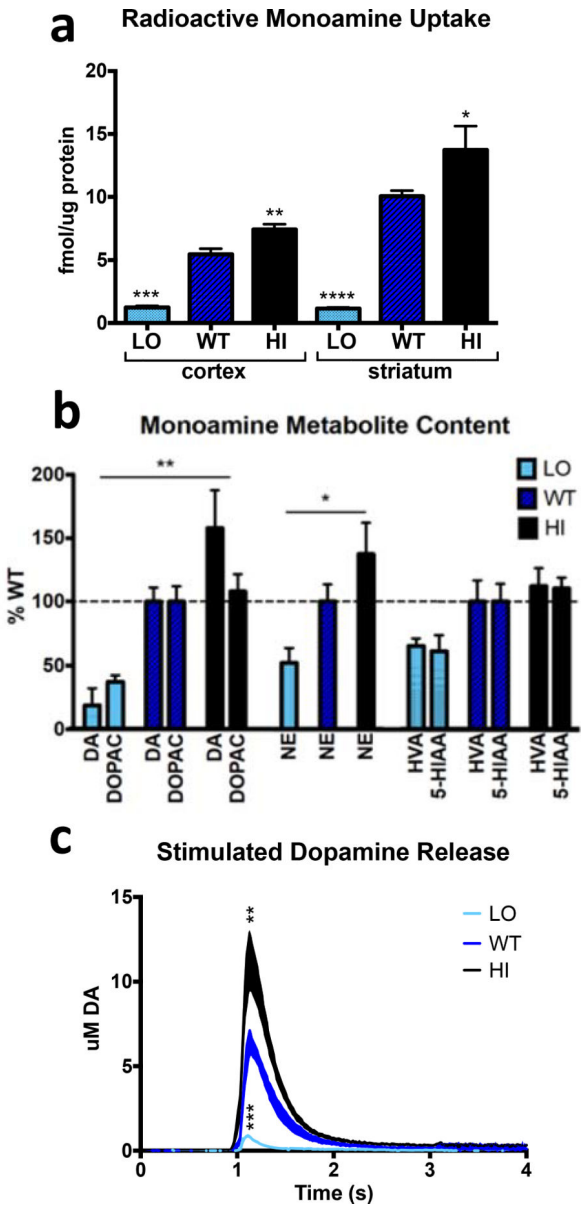

Fig 2. VMAT2 genotype determines monoamine function and content.

A.) VMAT2-LO mice show a reduced capacity to take up radioactive monoamine in vesicles isolated from both the cortex and striatum, while VMAT2-HI mice show an increased capacity to take up radioactive monoamine. N = 3–5 for each group. B.) Monoamine and monoamine metabolite content varies across VMAT2 genotype. HPLC analysis from half brain homogenate shows a reliable trend towards increased monoamine or monoamine content as gene dose of VMAT2 increases. N = 4–8 for each group. C.) VMAT2 genotype mediates stimulated dopamine release in the dorsal striatum. *Ex vivo* slice preparation of VMAT2-HI striata exhibited enhanced stimulated dopamine release while VMAT2-LO striata displayed a reduction in stimulated dopamine release. Trace shows the standard error of the mean of an average dopamine release time course for that genotype. n = 6–10; * $p<0.05$; ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

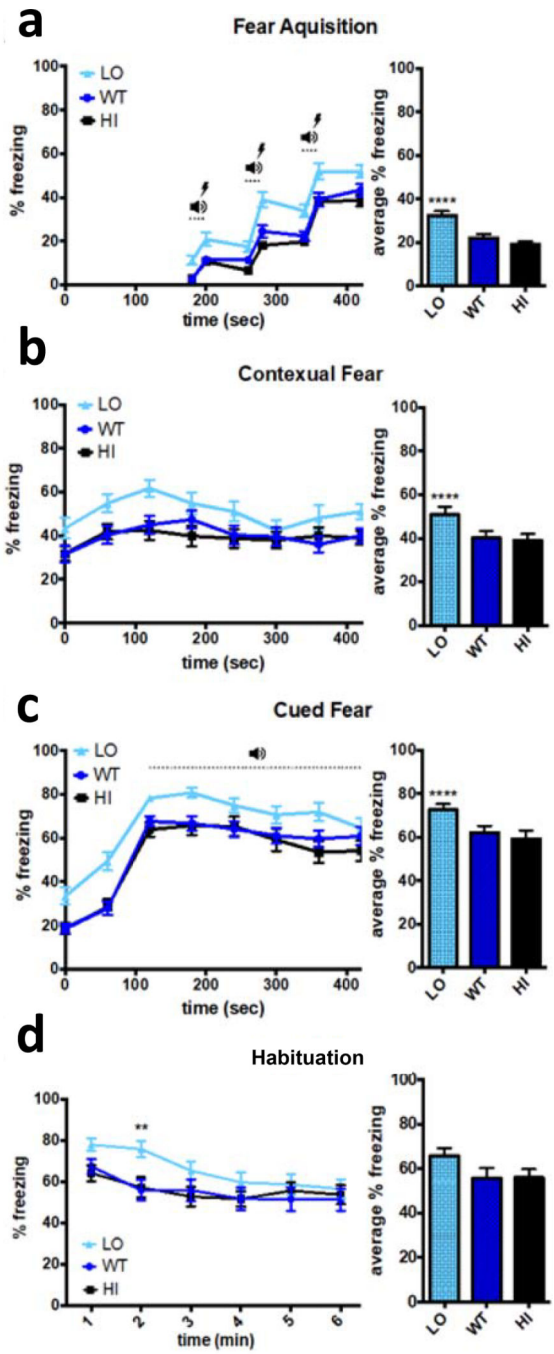

Fig 3. VMAT2-LO mice display increased fear behavior.

A.) VMAT2-LO mice show increased fear learning during CS-US pairings B.) VMAT2-LO mice show increased freezing in response to context previously associated with footshocks C.) VMAT2-LO mice show increased freezing response to an auditory tone previously associated with a footshock D.) VMAT2-LO mice show an altered rate of fear habituation. n = 22–40 animals per group, ** $p<0.01$, **** $p<0.0001$

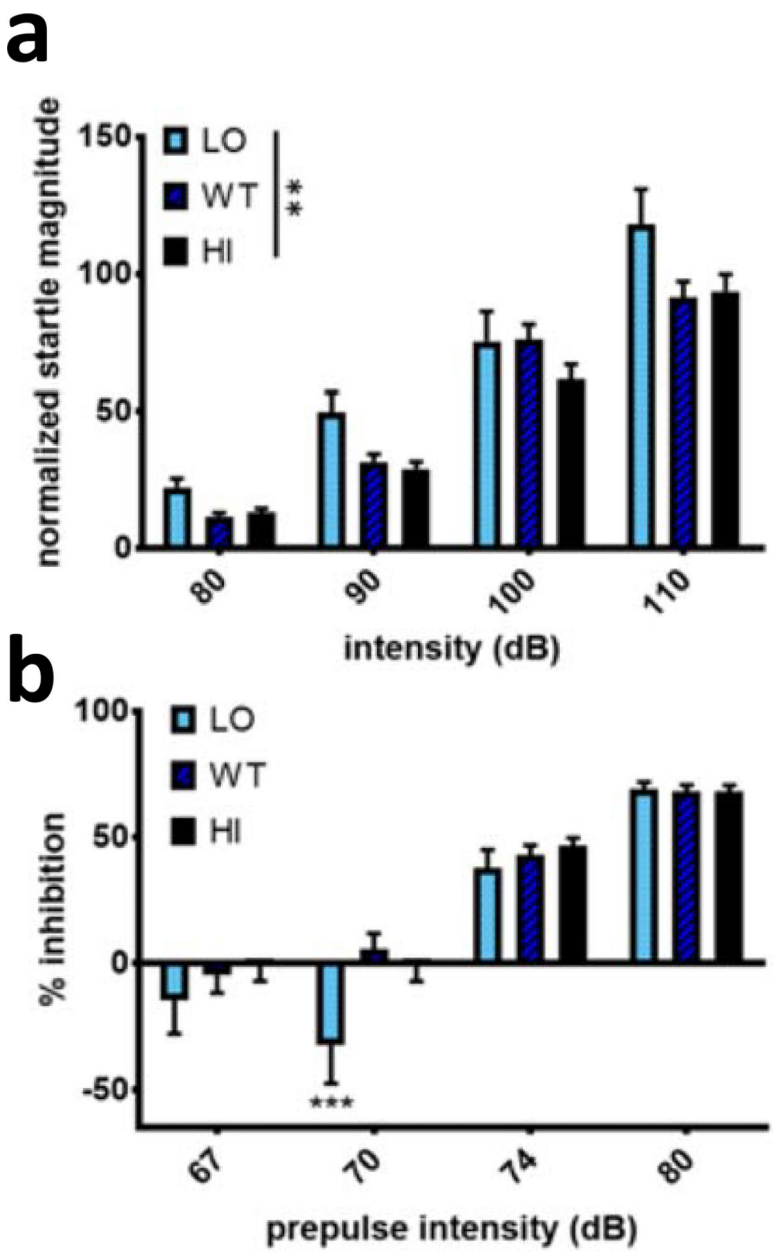

Fig 4. VMAT2-LO mice show altered startle responses.

A.) VMAT2-LO mice displayed increased corrected acoustic startle magnitude in response to a range of acoustic stimulus intensities (main effect of genotype, $p < 0.01$) B.) VMAT2-LO mice were less able to inhibit a startle response with lower-intensity prepulse tones. n = 11–20 animals per group, ** $p<0.01$; *** $p<0.001$

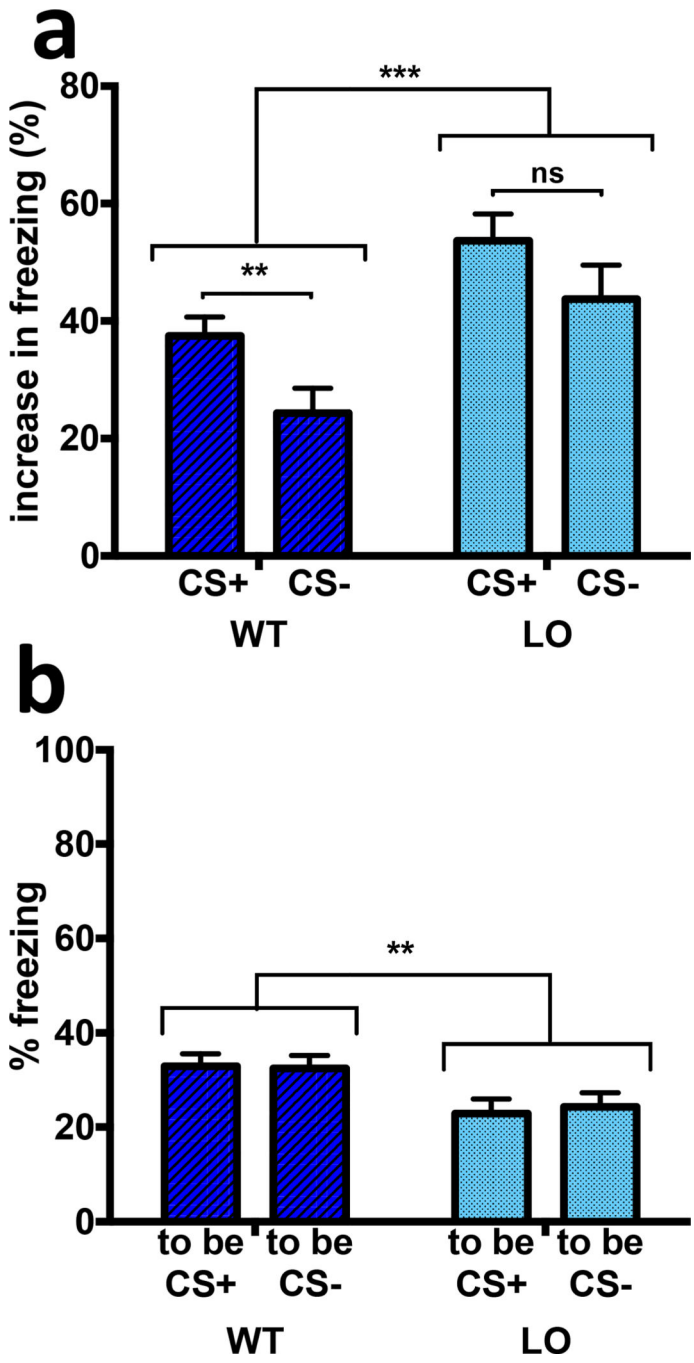

Fig 5. VMAT2-LO show increased fear generalization and increased fear response to multiple cues.

A.) Following training to associate one auditory cue with a foot shock (CS+) and another auditory cue with safety (CS-), VMAT2-LO mice have an increased freezing response across both tone types and fail to discriminate between a tone which reliably predicts a footshock (CS+) compared to a tone which does not predict footshock (CS-). B.) This increase in freezing is not due to an increased tendency for VMAT2-LO to freeze to novel tones. VMAT2-LO mice freeze less in response to novel tones compared to VMAT2-WT. n = 22–25 mice per group, * $p<0.05$, ** $p<0.01$, ns=not significant

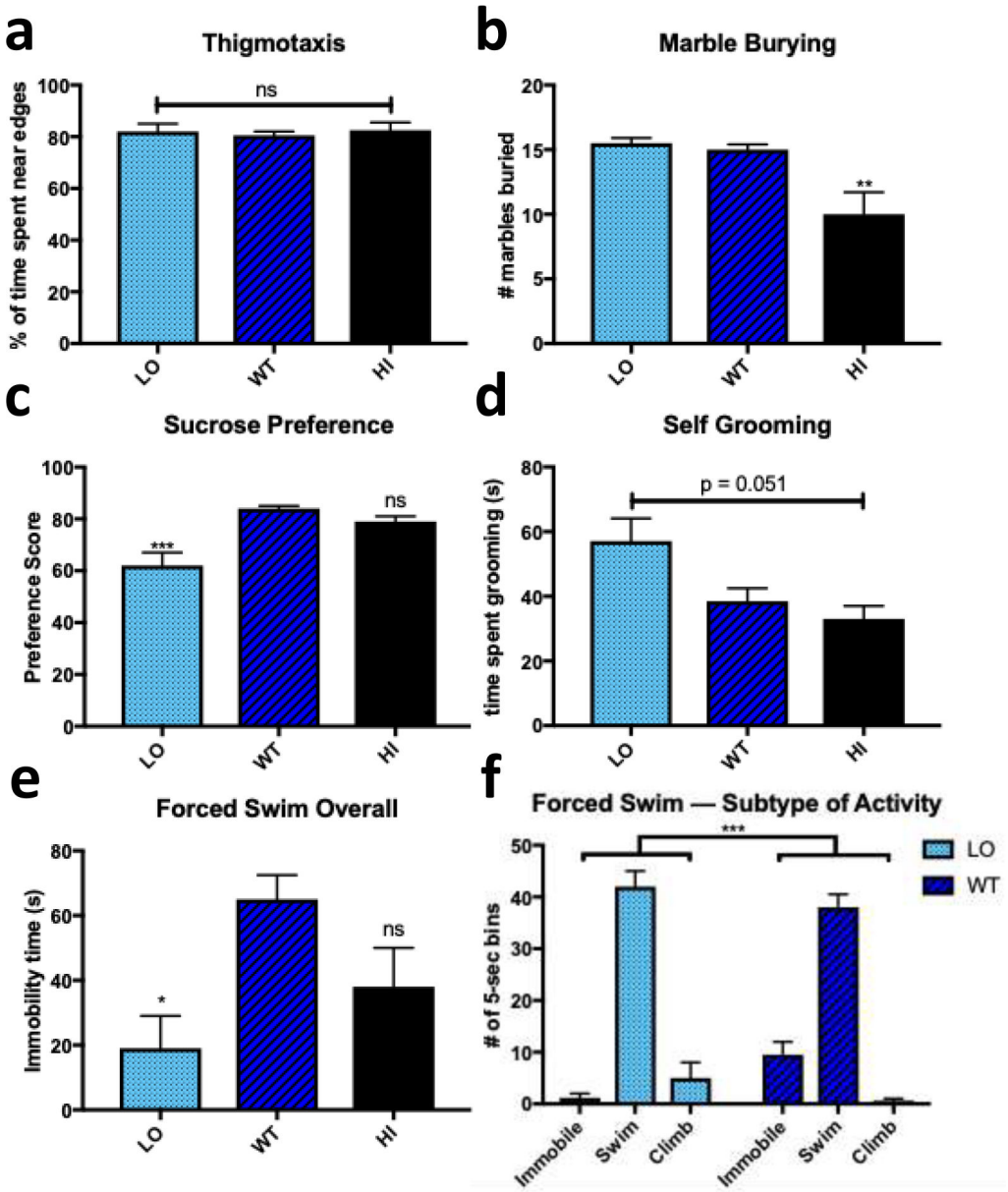

Fig 6. VMAT2-LO animals show alterations in some tests of depressive- and anxiety- like behavior.

VMAT2 transgenic animals underwent a number of assays to determine affective behavioral phenotypes. A.) VMAT2 transgenic animals did not vary in the amount of time spent near the edges of an open enclosure. B.) VMAT2-HI mice bury fewer marbles in a test a marble-burying assay. C.) VMAT2-LO mice have a reduced preference for sucrose-sweetened drinking water. D.) VMAT2 may mediate time spent self-grooming, as variation in this behavior due to VMAT2 genotype was on the cusp of significance. E.) VMAT2-LO spend less time immobile in a forced swim test. F.) The reduction in VMAT2-LO immobility time is due to an increase in climbing (i.e. actively struggling to climb up the walls of the forced

swim apparatus), not due to an increase in amount of time treading water. n = 5–24 animals per group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant