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Cav1.2 L-type calcium channels regulate stress coping behavior via serotonin neurons

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Abstract

Human genetic variation in the gene *CACNA1C,* which codes for the alpha-1c subunit of Cav1.2 L-type calcium channels (LTCCs), has been broadly associated with enhanced risk for neuropsychiatric disorders including major depression, bipolar and schizophrenia. Little is known about the specific neural circuits through which *CACNA1C* and Cav1.2 LTCCs impact disease etiology. However, serotonin (5-HT) neurotransmission has been consistently implicated in these neuropsychiatric disorders and Cav1.2 LTCCs may influence 5-HT neuron activity during relevant behavioral states such as stress. We utilized a temporally controlled and 5-HT neuron specific Cacna1c knockout mouse model to assess stress-coping behavior using the forced swim test and dorsal raphe (DR) 5-HT neuron Fos activation. Furthermore, we assessed 5-HT1A receptor function and feedback inhibition of the DR following administration of the 5-HT1A antagonist WAY-100635. We find that 5-HT neuron Cacna1c knockout disrupts active-coping behavior in the forced swim test and that this behavioral effect is rescued by blocking 5-HT1A receptors. Moreover, Cacna1c knockout mice display enhanced Fos expression in caudal DR 5-HT neurons and an enhanced response to a 5-HT1A receptor antagonist in rostral DR 5-HT neurons, indicating that loss of Cacna1c disrupts both 5-HT neuron activation and 5-HT1A dependent feedback inhibition across the caudal to rostral DR. Collectively, these results reveal an important role for 5-HT neuron Cav1.2 LTCCs in stress-coping behavior and 5-HT1A receptor function. This suggests that alterations in *CACNA1C* function or expression could influence the development or treatment of neuropsychiatric disorder through serotonergic mechanisms.

Keywords

Serotonin; Dorsal Raphe; Cacna1c; L-type calcium channels; Cav1.2; psychiatric disorders

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1. Introduction

Polymorphisms in the gene *CACNA1C* are strongly and consistently associated with increased risk for the development of neuropsychiatric conditions including schizophrenia, bipolar disorder and major depression (Green et al. 2010; Wray et al. 2012; Ferreira et al. 2008; Cross-Disorder Group of the Psychiatric Genomics Consortium 2013)]. *CACNA1C* codes for the pore-forming alpha-1c subunit of Cav1.2 voltage-gated L-type calcium channels (LTCCs), which are expressed in the majority of CNS neurons (Sinnegger-Brauns 2009). Cav1.2 LTCCs open with fast kinetics following neuronal excitation above action potential threshold to dramatically increase calcium influx, yet have exceptionally slow inactivation kinetics, and therefore Cav1.2 LTCCs play a predominant role in the coupling neuronal excitation to long-term changes in calcium-dependent gene expression and functional activity (Wheeler et al. 2012). As the most consistently observed risk-associated polymorphism of *CACNA1C* is in a non-coding region, regionally specific increases or decreases in overall Cav1.2 LTCC expression are likely responsible for its behavioral impact (Heyes et al. 2015). The identification of specific neural circuits through which *CACNA1C* and Cav1.2 LTCCs impact behaviors that are relevant to the etiology and treatment of neuropsychiatric conditions would greatly inform the development of novel therapeutics.

Forebrain projecting serotonin (5-hydroxytryptamine; 5-HT) neurons have been consistently implicated in behaviors relevant to the etiology of neuropsychiatric conditions including the response to stress (Michelsen et al. 2007). The largest group of forebrain projecting 5-HT neurons arises from the dorsal raphe nucleus (DR) of the midbrain and prepontine hindbrain, and the role of *CACNA1C* and the Cav1.2 LTCCs expressed on these neurons has yet to be explored. Regionally specific innervation of the forebrain arises from distinct structural and functional subgroups of DR 5-HT neurons separated along the rostral (cell-group B7) to caudal (cell-group B6) axis of the DR (Commons 2016). Activation of these 5-HT neuron subgroups, as assessed by expression of the immediately early gene product Fos, changes under behavioral states such as acute stress. Since different subgroups of 5-HT neurons preferentially innervate different forebrain targets, this differential activation has the potential to generate region-specific increases and decreases in extracellular serotonin (Kirby and Lucki, 1997). Furthermore, there is evidence that feedback inhibitory mechanisms mediated by 5-HT1A receptors may help shape the selective activation of different subgroups of 5-HT neurons (Sperling and Commons, 2011; Bang et al., 2012). Due to these features of selective activation and their topographic patterns of projections, there is the emerging understanding that subgroups of serotonin neurons may be functionally distinct.

Cav1.2 LTCCs could potentially influence the regional pattern of DR 5-HT neuron activation, in part because they have been implicated in feedback inhibition. Specifically, somatodendritic release of 5-HT containing vesicles within the DR is dependent on calcium influx through LTCCs. This local release of serotonin has the capacity to engage 5-HT1A receptors and promote feedback inhibition (Chazal and Ralston 1987; Colgan et al. 2012). It is also possible that different groups of 5-HT neurons may be differentially sensitive to Cav1.2 LTCC function because of developmental differences between different groups of 5-HT neurons. The role of LTCC in regulating 5-HT neuron function may be particularly

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relevant during stress, since LTCC are prominent targets of corticosteroid hormones in the limbic forebrain (Joels and Karst, 2012). Taken together, these observations raise the possibility that Cav1.2 LTCCs could influence both overall activation and 5-HT1A dependent feedback inhibition of DR 5-HT neurons during behavioral states such as acute stress and these effects may be subregionally organized.

Importantly, 5-HT neurons express multiple subtypes of LTCCs and whether *CACNA1C,* encoding the Cav1.2 isoform, specifically contributes to DR 5-HT neuron function has not been directly assessed. We have recently studied aspects of 5-HT system structure and function in a mouse model of the neurodevelopmental disorder Timothy Syndrome (TS2-neo) that contains a brain-wide gain of function mutation in *Cacna1c* resulting in delayed Cav1.2 LTCC inactivation (Bader et al. 2011). Intriguingly, TS2-neo mice exhibit an enhanced active coping response during acute stress, increased Fos expression in 5-HT neurons of the caudal DR and enhanced 5-HT1A dependent feedback-inhibition of the rostral DR (Ehlinger and Commons 2017). These results raise the possibility that 5-HT neuron *Cacna1c* could directly regulate both stress coping behavior and the activation of ascending 5-HT neuron subgroups. However, *Cacna1c* might also act indirectly within efferent brain regions to modify both behavior and 5-HT neuron function (Dao et al 2010; Moosmang et al. 2005). Thus, whether *Cacna1c* acts directly on the DR 5-HT neuron system remains unknown.

In the present study, we assessed the direct impact of *Cacna1c* on stress coping behavior, DRN 5-HT neuron immediate early gene expression and 5-HT1A dependent feedback inhibition using a conditional and cell-type specific *Cacna1c* knockout mouse model. This study reveals for the first time that 5-HT neuron *Cacna1c* regulates the activation of DR 5-HT neuron subpopulations and the neurobehavioral response to acute stress.

2. Materials and Methods

2.1 Mice

All procedures involving mice were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee, and followed the National Institutes of Health guide for the care and use of laboratory animals and the additional institutional guidelines of Boston Children's Hospital and Harvard Medical School.

Original breeding pairs were purchased from Jackson Laboratory (Jax; Bar Harbor, ME). All transgenic and mutant strains were initially crossed to Jax #000664 C57BL/6J mice, including Jax #007914 homozygous B6;Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (*Ai14*^{tdTomato}), Jax #016584 hemizygous STOCK Tg(Tph2-icre/ERT2)6Gloss/J (*Tph2*^{iCre}), and Jax #024714 homozygous STOCK Cacna1c^{tm3Hfm}/J (*Cacna1c*$^{flox/flox}$). Initial crosses between these strains yielded breeders used for the generation of mice for subsequent experiments. Specifically, the breeding of 1) mice homozygous for a *Cacna1c*$^{flox/flox}$ transmutation and hemizygous for a tamoxifen (TAM) inducible Tph2-creERT2 transgene (*Tph2*^{icre}) with 2) mice homozygous for a *Cacna1c*$^{flox/flox}$ transmutation and heterozygous for the Cre-dependent tdTomato fluorescent reporter (*Cacna1c*$^{flox/flox}$*Ai14*^{tdTomato}) produced all experimental and control groups used in the following experiments. Previous

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characterization of *Cacna1c*$^{flox/flox}$ allele shows that with cre-recombination leads to about 80% loss of dihydropuridine sensitive LTCC currents in hippocampal neurons with residual currents likely dependent on Cav1.3 (Moosemang et al., 2005).

2.2 Tamoxifen administration protocol

In our mice, TAM administration induces a recombination event to disrupt Cacna1c expression within 5-HT neurons, allowing for specificity and temporal control in TAM treated *Tph2*^{iCre} mice. From postnatal day (P)35–40, mice received either 1mg of 10mg/ml TAM (Sigma-Aldrich #T5648) in sunflower oil (Sigma-Aldrich #S5007) or sunflower oil as vehicle (VEH) twice per day intraperitoneally (10 total injections), an administration protocol previously shown to exert efficient recombination in *Tph2*^{iCre} mice (Weber et al. 2008; Weber et al. 2015). The two main groups of experimental mice were 1) TAM treated *Tph2*^{iCre}*Cacna1c*–/– , in which 5-HT neuron Cacna1c expression is disrupted and 2) TAM treated *Cacna1c*+/+ , with unaltered Cacna1c expression. Initial behavioral characterization yielded no sex differences in the examined behaviors, therefore subsequent experiments were sex balanced but underpowered to determine additional sex differences. Two additional control groups were administered VEH rather than TAM: 1) VEH-*Tph2*^{icre}*Cacna1c+/+* mice, used to assess the impact of *Tph2*^{iCre} transgene expression alone and 2) VEH-*Cacna1c* +/+ mice, used to assess the impact of TAM treatment alone.

2.3 Behavioral testing

Experiments began approximately 4 weeks after the final TAM or VEH injection (P68±2) to avoid potential acute neurobehavioral effects of TAM (Vogt et al. 2008) (Figure 1A). Locomotor activity was assessed using an open-field apparatus (Photobeam Activity System, San Diego Instruments, San Diego, CA). Within clear plexiglass cages (10”×19”×8”h), accumulated beam breaks in *Tph2*^{iCre}*Cacna1c*–/– ($n = 13$) and *Cacna1c+/+* ($n = 15$), mice were counted, including ambulatory, fine and rearing movements over three 5-minute time bins for a total of 15 minutes. Movement measures were totaled within each time bin to acquire total locomotor activity scores. The percentage of time spent in the center rectangle (5” × 9.5”) of the open-field was calculated, with less time in the center compartment indicating avoidance of the more aversive portion of the open-field.

A forced swim test (FST) was used to assess stress-coping behavior and for subsequent analysis of DR 5-HT neuron Fos expression. Immediately following locomotor activity testing, experimental groups were injected subcutaneously with 0.9% NaCl (Saline) and returned to their home-cage for 10-minutes. As an additional behavioral control for the effect of *Tph2*^{iCre} transgene expression and TAM administration alone in the FST, a set of VEH-*Tph2*^{iCre}*Cacna1c+/+* ($n = 8$) and VEH-*Cacna1c*+/+ ($n = 13$) groups were subjected to the same procedure. Mice were then placed into a cylindrical glass tank (46cm high × 20cm diameter) filled 20cm deep with 25±1° C water for 15 minutes while being videotaped for behavioral analysis, a length of time that allows for a deeper characterization of the adaptation from active (swimming; movement through the tank with coordinated movement of both hindpaws) to passive (immobility; no movement through the tank and only minor movements of hindpaws to stay afloat) coping strategy during the forced swim stress and produces a robust elevation in DR 5-HT neuron Fos activation (Commons 2008; Ehlinger

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and Commons 2017). Coping strategy was scored as either active or passive using the 5-second time interval sampling method, in which mice are counted as active when swimming behavior was exhibited for the majority of each 5-second interval (Cryan et al. 2005). The number of swim counts was summed into three 5-minute time bins, making a total of 60 possible swim counts per 5-minute time bin. Thus active coping and passive coping counts sum to 60, and one measure can be derived from the other. To facilitate comparison with other work where passive coping is reported, a passive coping score totaling counts over the last 4-minutes of the first 6-minutes of the FST is also reported (Crawley et al. 2007). Immediately after the forced swim, mice were removed from the tank, quickly towel dried and returned to their home cage.

To examine the role of 5-HT1A receptors, a separate group of *Tph2^{iCre}Cacna1c*–/– (n = 11) and *Cacna1c*+/+ (n = 11) mice were administered the 5-HT1A antagonist WAY-100635 (WAY; 0.4 mg/kg; Sigma Aldrich #W108) 10-minutes prior to the FST and behavior was recorded as previously described. To determine whether FST behavioral effects observed following WAY administration reflected an altered state of locomotor activity, a final group of *Tph2^{iCre}Cacna1c*–/– (n = 9) and *Cacna1c*+/+ (n = 12) mice were administered WAY (0.4 mg/kg) 10-minutes prior to placement in the open-field, with locomotor activity and percentage center time recorded as previously described.

Statistical assesment of behavior was performed in R. For active coping behavior, mixed-ANOVA with time as a repeated measure and genotype (*Tph2^{iCre}* or *Tph2$^{+/+}$*), treatment (TAM or VEH) and drug (saline or WAY) as between-subjects factors was used. The Greenhouse-Geisser correction for degrees of freedom (df) was applied following a significant (p <.05) Mauchly's Test for Sphericity (corrected degrees of freedom denoted throughout the text by a subscript "G" prior to dfvalues). Following significant interaction with time (p < .05), ANOVA was performed within individual time bins followed by bonferroni corrected post-hoc t-tests to compare unique genotype, treatment and drug groups (significance at p < .016). For passive coping behavior, ANOVA with genotype, treatment and drug as between-subjects factors was used, followed by Bonferroni corrected post-hoc t-tests between unique groups (p < .016).

2.4 Immunofluorescence

To examine the role of *Cacna1c* on activation of DR 5-HT neuron subregions, a subset of the mice used in FST experiments were used for immunofluorescence experiments assessing the impact of *Cacna1c* knockout on expression of the immediately early gene product Fos within 5-HT neurons, as well as to assess recombination efficiency in *Tph2^{iCre}* mice. Fos was not measured in unstressed mice because Fos levels tend to be very low in 5-HT neurons in the absence of stress (Commons, 2008). 120 minutes following the FST, a time-point that allows for translation of the Fos protein a subset of *Tph2^{iCre}Cacna1c*–/– (n = 8), *Cacna1c*+/+ (n = 8), VEH-*Tph2^{iCre}Cacna1c*+/+ (n = 7), and VEH-*Cacna1c*+/+ (n = 9), as well as WAY exposed *Tph2^{iCre}Cacna1c*–/– (n = 9) and *Cacna1c*+/+ (n = 10) mice were perfused intracardially with 4.0% paraformaldahyde (PFA) in 0.1 M phosphate buffer. Brains were stored in the same 4.0% PFA solution overnight then equilibrated in a solution of 30.0% sucrose in 0.1 M phosphate buffer at 4.0° C. 40 μm thick coronal sections were

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made through the whole brain, storing every third section from the DR in separate containers. Immunofluorescence processing was performed on floating sections. 5-HT neuron and Fos co-labeling was detected by incubating sections in primary antisera for tryptophan hydroxlase 2 (TPH2; synthesizing enzyme for 5-HT) raised in rabbit (Novus Biologicals, #NB100–74555) diluted 1:1000 and Fos raised in goat (Santa Cruz, #SC-52) diluted 1:500 in PBS-BSA-TA at room temperature for 24 hours, followed by incubation in Alexa 488 anti-rabbit and Alexa 647 anti-goat raised in donkey (Invitrogen) diluted 1:500 at room temperature for 90 minutes. Sections were rinsed in 0.05 M phosphate buffer, mounted, dried, and cover-slipped with a glycerol-based mounting medium.

Every 3rd section throughout the rostral-caudal and dorsal-ventral extent of the DR (Paxinos and Franklin 2001) was imaged using an Olympus IX-81 spinning disk confocal fluorescence microscope with 20x objective, Hamamatsu Orca ER camera, Slidebook software (3i) and filter cubes selective for Alexa 488, tdtomato, and Alexa 647. For each channel of every 1344 × 1024 pixel (216 × 165μm) image, a 20 μm z-stack with 1 μm step-size was obtained while keeping imaging parameters identical for all samples. Max projection images were made for each channel.

Recombination efficiency was assessed in a subset of both TAM exposed $Tph2^{iCre}Cacna1c$ $-/-$ mice (n =6) and VEH exposed VEH-$Tph2^{iCre}Cacna1c+/+$ (n = 5) mice that were also heterozygous for a Cre-dependent fluorescent reporter ($Ai14^{tdTomato}$). After observing robust TdTomato expression in all rostral-caudal, dorsal-ventral, and lateral wings DRN subregions of $Tph2^{iCre}Cacna1c^{-/-}$ mice (Figure 1B), two 50μm × 50μm regions per mouse were selected from medial DRN sections with a high density of Tph2-positive neurons, and cells dually immunolabeled for Tph2 and tdTomato were visualized on individual and merged images of each channel in ImageJ and manually enumerated using the cell-counter plugin in FIJI (Figure 1C). The percentage of cells exhibiting recombination was calculated as the number of dually labeled cells divided by the total number of Tph2-positive cells and compared between groups using an independent samples t-test. As expected, TAM treatment induced an extremely robust Cre-mediated recombination exclusively in Tph2 neurons (Figure 1B, 1C). TdTomato expression was observed in a significantly greater percentage of Tph2 positive neurons from TAM treated $Tph2^{iCre}$ mice compared to VEH treated $Tph2^{iCre}$ mice (t[9] = 35.96, $p = 4.92 \times 10^{-11}$) (Figure 1C, 1D). Nonetheless, recombination was also observed in some 5-HT neurons (~28%) from VEH treated $Tph2^{iCre}$ mice, suggesting leakiness in this tamoxifen-inducible CreER driver line. While this observation represents a limitation of the current study, it underscores the importance of utilizing multiple control groups that either express or do no express the $Tph2^{iCre}$ transgene in our assessments of behavior and 5-HT neuron activation.

To quantitatively assess 5-HT neuron activation across DR subpopulations, cells dually labeled for Tph2 and Fos were quantified within 7 distinct subregions of the DR delineated across the rostral-caudal axis (3 regions: rostral, −4.16 to −4.24mm; middle, −4.36 to −4.84mm; caudal, −4.96 to −5.20), dorsal-ventral axis (2 regions: defined by characteristic separation of immunolabeled 5-HT neurons), and the lateral wings (Sperling and Commons 2011) using the cell-counter plugin in FIJI. This yielded two to three sections per region per animal, and counts were averaged to yield a single per section per region mean value for

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each animal. For statistical analysis, a mixed-ANOVA with within-subjects factor of DRN subregion and between-subjects factors of genotype (*Tph2^iCre^* or *Tph2^+/+^*), treatment (TAM or VEH) and drug (saline or WAY) was performed (with Greenhouse-Geisser correction for *df* as previously described). Following significant interaction with subregion, individual ANOVAs within DR subregion were performed and post-hoc comparisons between individual genotype, treatment and drug conditions via independent-samples *t*-tests using a false-discovery rate (FDR) of 5% based on the seven DR subregions examined ($p_{FDR} < .05$) (Benjamani and Hochberg 1995).

3. Results

First, we examined the neurobehavioral role of 5-HT neuron *Cacna1c* during acute stress by exposing *Tph2^iCre^Cacna1c*–/–, TAM-*Cacna1c+/+* and both VEH control groups to a 15-minute FST and measuring both active and passive coping behavior. A genotype by treatment interaction ($F_{(1,45)} = 4.503$, $p = .039$) and a trend towards a time by genotype interaction ($F_{G(1.66,\ 74.76)} = 3.235$, $p = .054$) was observed on active coping behavior, with the most pronounced behavioral effect observed during the first 5-minutes of the forced swim ($F_{(1,45)} = 7.219$, $p = .010$) (Figure 2A). Specifically, *Tph2^iCre^Cacna1c–/–* mice displayed lower active coping behavior compared to *Cacna1c+/+* mice during the first and second five minutes of the FST, which normalizes by the third five minutes of testing. Similarly, a genotype by treatment interaction was observed on passive coping behavior, in which *Tph2^iCre^Cacna1c*–/– mice exhibited increased immobility compared to *Cacna1c*+/+ mice during the initial portion of the FST. In contrast, coping behavior in VEH-*Tph2^iCre^Cacna1c+/+* and VEH-*Cacna1c+/+* was similar to that of *Cacna1c*+/+ mice, suggesting that the alterations in coping behavior were not simply a result of transgene expression or TAM exposure alone, and required the extensive recombination evident seen after TAM treatment and baseline recombination in VEH-*Tph2^iCre^Cacna1c*+/+ mice was insufficient to generate an altered behavioral response. No differences in general locomotor activity were observed between *Tph2^iCre^Cacna1c–/–* and *Cacna1c+/+* mice in the open-field test (Figure 2B), however *Tph2^iCre^Cacna1c–/–* mice spent less time in the open-field center compared to *Cacna1c+/+* mice (t[26] = 2.312, $p = .029$), suggesting that 5-HT neuron *Cacna1c* knockout also impacted behavior within this comparatively less stressful context.

The rostral and caudal DR are both anatomically and functionally distinct, as their activation level appears differentially regulated during acute stress (Commons 2008). We suspected that disrupting 5-HT neuron *Cacna1c* would influence the overall pattern of 5-HT neuron activation that occurs across DR 5-HT neuron subregions, and we examined this possibility by quantifying Fos expression within DR 5-HT neurons following acute stress to allow for the direct assessment of DR 5HT neuron activation across all unique rostral to caudal subregions within each subject. Through immunofluorescent labeling of Tph2 and Fos, we observed a subregion by genotype by treatment interaction ($F_{G(3.53,98.97)} = 5.863$, $p = .0003$) that reflected a dramatically elevated number of Fos expressing 5-HT neurons in the caudal DR subregions (caudal-dorsal, $F_{(1,28)} = 5.293$, $p = .029$; caudal-ventral, $F_{(1,28)} = 14.233$, $p = .0007$) of *Tph2^iCre^Cacna1c–/–* compared to *Cacna1c+/+* or either VEH control group (Figure 2D). These results reveal that 5-HT neuron *Cacna1c* has a regionally-specific influence on Fos expression within DR 5-HT neurons.

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The activation of DR 5-HT neurons is tightly regulated by 5-HT1A dependent feedback inhibition via local 5-HT neuron interconnections and local release of 5-HT. Given the regionally specific alteration in Fos expression and a known role for 5-HT neuron LTCCs in 5-HT1A dependent feedback-inhibition (Colgan et al. 2012), we then tested if altered coping behavior and the observed pattern of DR 5-HT neuron Fos expression persisted in *Tph2^iCre Cacna1c–/–* mice when 5-HT1A receptors were blocked. To explore this possibility, we administered the 5-HT1A antagonist WAY100635 (WAY; 0.4mg/kg) prior to the FST in a separate group of experimental mice. For active coping behavior in the FST, interactions between genotype and drug ($F_{(1, 45)} = 12.554$, $p = .0009$), genotype and time ($F_{G(1.72, 77.66)} = 9.685$, $p = .0004$), and drug and time ($F_{G(1.72, 77.66)} = 9.043$, $p = .0006$) were observed. During the first 5 minutes of the FST, WAY treatment rescued active coping behavior in *Tph2^iCre Cacna1c–/–* mice while it slightly decreased active coping behavior in *Cacna1c+/+* mice ($F_{(1, 45)} = 8.410$, $p = .006$). Similarly, WAY treatment completely normalized the increased passive-coping behavior previously observed in *Tph2^iCre Cacna1c–/–* mice yet had no effect on *Cacna1c+/+* mice (Figure 3A). Although active-coping behavior in *Cacna1c+/+* mice returned to normal for the remainder of the FST, *Tph2^iCre Cacna1c–/–* mice displayed a sustained elevation in active coping behavior throughout the second ($F_{(1, 45)} = 8.687$, $p = .005$) and third ($F_{(1, 45)} = 13.013$, $p = .0008$) five-minutes of the FST. These selective effects of WAY within *Tph2^iCre Cacna1c–/–* mice is specific to coping behaviors in the FST, as WAY administered to a separate group of experimental mice prior to open-field testing revealed no differences in either general locomotor activity nor center time (Figure 3B).

Given these unique neurobehavioral and pharmacological effects in *Tph2^iCre Cacna1c–/–* mice, we finally assessed whether the activation of DR 5-HT neuron subregions is also altered when 5HT1A receptors were systemically blocked. WAY increased Fos expression in 5-HT neurons across all DR subregions of both *Tph2^iCre Cacna1c–/–* and *Cacna1c+/+* ($F_{(1, 32)} = 83.436$, $p = 1.98 \times 10^{-10}$), suggesting that the 5-HT1A antagonist effectively disengaged feedback inhibition in both groups (Figure 3C). However, a subregion by genotype by drug interaction ($F_{G(4.32, 138.24)} = 4.044$, $p = .0008$) was revealed reflecting a larger increase of 5-HT neuron Fos expression in both the lateral wings ($F_{(1,}$ $32) = 7.244$, $p = .011$) and rostral-dorsal ($F_{(1, 32)} = 6.257$, $p = .018$) subregions of WAY exposed *Tph2^iCre Cacna1c–/–* mice compared to their *Cacna1c+/+* counterparts (Figure 3D, 3E). Collectively, these results suggest that loss of 5-HT neuron *Cacna1c* expression has region-dependent effects on both Fos expression and 5-HT1A-dependent feedback inhibition of DR 5-HT neurons.

4. Discussion

Polymorphism of *CACNA1C,* which encodes the pore-forming subunit of Cav1.2 LTCCs, is one of the most consistently identified genetic risk factors for schizophrenia, bipolar disorder and major depressive disorder, and it is critical to understand how this gene and its protein product impacts the neurobehavioral circuits most relevant for the etiology and treatment of these disorders. In the present study, we have identified a role for 5-HT neuron *Cacna1c* in regulating acute stress coping behavior, 5-HT neuron activation and 5-HT1A dependent feedback inhibition across the rostral to caudal extent of the DR. Given the roles of both stress and 5-HT system structure/function in neuropsychiatric disorder, the present

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results reveal a potential biochemical mechanism through which 5-HT neuron Cav1.2 LTCCs are poised to contribute to the neurobehaviorial stress response.

Acute stress is a primary risk factor for the development of a broad range of neuropsychiatric conditions (McLaughlin et al. 2010) and a disrupted neurobehavioral response to acute stress could either contribute to or be indicative of disease state progression (McEwen 2004). In animal models, disrupting the neural circuits that are active during acute stress can help elucidate the biochemical mechanisms that contribute to human conditions. We find that 5-HT neuron *Cacna1c* knockout reduces active coping behavior in the FST, with additional small but significant disruptions in the comparatively less stressful context of the open-field, indicating that 5-HT neuron Cav1.2 LTCCs provide critical control over normal stress-coping behavior. While this is the first direct neurobehavioral assessment of DR 5-HT neuron *Cacna1c*, previous research has characterized the behavioral impact of other regionally specific disruptions (Bhat et al. 2012). For example, conditional *Cacna1c* knockout in the hippocampus disrupts spatial memory (Moosmang et al. 2005; White et al. 2008) while conditional knockout in the prefrontal cortex causes avoidance of the aversive portion of the environment in the elevated plus maze (Lee et al. 2012). Most recently, it was shown that conditional knockout in the nucleus accumbens increases susceptibility to the negative impact of social defeat stress and increases anxiety-like behavior (Terrillion et al. 2017). Thus, the present results identify a new region of interest in the 5-HT system and a novel role for 5-HT neuron *Cacna1c* in the regulation of stress-coping behavior.

Known risk factors for neuropsychiatric disorder, such as stress, produce behavioral impairments in the FST while clinically effective treatments, such as anti-depressants, rescue behavioral impairments in the FST (Castagné et al. 2011; Lucki 1997). While the present results raise the possibility that disrupted *CACNA1C* function or expression within 5-HT neurons could contribute to the etiology of neuropsychiatric disorder, the most consistently identified risk-increasing polymorphism in *CACNA1C* is in a non-coding region of the gene and has often been shown to *increase* mRNA expression in both induced human neurons and in human brain tissue (Bigos et al. 2010; Yoshimizu et al. 2015). Interestingly, the effect of risk-associated *CACNA1C* polymorphisms on Cav1.2 expression may be regionally dependent, as *decreased* expression has been observed in the human cerebellum of risk-allele carriers (Gershon et al. 2014). How the human risk allele impacts Cav1.2 function or expression specifically within the DR, let alone in 5-HT neurons, is currently unknown. Importantly, previous results in mouse models would suggest that the brain-wide *pattern* of changes in Cav1.2 expression or function is most relevant when assessing altered neurobehavioral responses such as those revealed in the present study. That is, both whole brain Cav1.2 LTCC haploinsufficiency and alternatively a gain of function mutation in *Cacna1c* appear to *increase* active coping behavior in the FST (Ehlinger and Commons 2017; Dao et al. 2010), yet when *Cacna1c* knockout is isolated to 5-HT neurons we reveal *decreased* active coping-behavior. These results underscore the importance of considering circuit-specific versus brain-wide alterations in gene function or expression when assessing animal behavior models such as the FST and any potential relationship to neuropsychiatric disorder.

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Although the genetic approach used in this study would impact all 5-HT neurons, the largest group of forebrain-projecting 5-HT neurons arise from the DR and these are associated with regulating affective and emotive processes. Given the capacity of 5-HT neuron *Cacna1c* to influence stress-coping behavior we predicted that DR 5-HT neurons would display altered Fos-activation following *Cacna1c* knockout. Our results show increased Fos expression exclusively within the caudal DR, raising the possibility that caudal DR 5-HT neurons are particularly relevant for the observed changes in stress-coping behavioral responses in the FST. There is a consistent association of the caudal DR activity with stressful, aversive conditions as well as helplessness (Guo and Commons, 2017, Ehlinger and Commons, 2017; Hammack et al. 2002). However, Fos expression in the caudal DR 5-HT neurons doesn't correlate simply with coping behavior in the FST. Indeed, 5-HT neurotransmission plays a unique role in emotion and mood, which may not have a linear relationship to motor behavior or coping strategy. However, the selective changes seen in the caudal DR are intriguing given several lines of evidence implicating dysfunction of the caudal DR in the etiology of major depressive disorder. In humans, increased expression of Tph2 and loss of 5-HT1A receptors has been identified specifically in the caudal DR of depressed suicides (Bach-Mizrachi et al. 2008; Bodrini et al. 2008), while both afferents (orbitofrontal cortex, cingulate cortex) and an efferent target (hippocampus) of the caudal DR display reduced volume in patients with depression (Arnone et al. 2013; Wise et al. 2017). Taken together, the present results further identify the caudal DR (B6) as an integral component of the neural networks relevant to stress-coping behavior and indicate *Cacna1c* knockout disrupts both normal Fos activation of this area in conjunction with behavior.

Blocking 5-HT1A receptors completely reversed the behavioral deficit in the FST caused by disruption of 5-HT neuron *Cacna1c*. At the same time, the 5-HT1A antagonist WAY had no detectable effect in the relevant litter-mate control mice, although WAY sometimes has effects in other wild-type mouse lines depending on secondary experimental conditions including age and genetic background (O'Neill and Conway 2001; Ehlinger & Commons, 2017). Thus the present results suggest that increased 5-HT1A receptor activation following 5-HT neuron *Cacna1c* knockout is necessary for the behavioral deficit in stress-coping behavior. Like pharmacotherapies, in this study WAY was administered systemically such that the cellular or circuit mechanism is poorly resolved. However, this result may be particularly relevant for understanding previous reports on the combined treatment efficacy of 5-HT and LTCC acting pharmacotherapies, in which some clinical trials utilizing serotonin reuptake inhibitors in combination with LTCC antagonists have shown improved treatment outcomes (Taragano et al. 2001; Taragano et al. 2005). Furthermore, patient genotype with respect to *CACNA1C* may be highly relevant for the pharmacological treatment of psychiatric disorders, as a recent report suggests that mixed bipolar and major depression patients with risk-associated polymorphism in *CACNA1C* exhibit sustained cognitive impairments that would typically recover following 6-weeks of SSRI pharmacotherapy in patients without the risk allele (Lin et al. 2017). As genotype and cell-type specific targeting of neurotherapeutics becomes increasingly viable, it is possible that a combined targeting of Cav1.2 LTCCs in conjunction with traditional 5-HT system therapeutics would produce a sustained or more complete treatment response in certain populations.

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In addition to rescuing coping behavior in the FST, blocking 5-HT1A receptors revealed additional differences in the topography of 5-HT neuron Fos activation in *Cacna1c* knockout mice. Although the 5-HT1A antagonist elevated Fos expression throughout the rostral to caudal extent of the DR in both groups and activation of the caudal DR was similar between genotypes, substantially elevated Fos expression appeared in several rostral DR subregions of *Cacna1c* KO mice. This finding implicates a change in feedback regulation of rostral DR neurons in *Cacna1c KO* mice either by direct or indirect multisynaptic mechanisms. The rostral two-thirds (B7 cell-group) and caudal one third (B6 cell-group) of the nucleus are organized into two distinct structural network domains with distinct overall patterns of input and output connectivity (Commons 2016; Commons 2015). These areas may differentially function under different behavior conditions as we have previously found differences between the rostral and caudal DR following other behavioral and/or genetic manipulations [Ehlinger and Commons 2017; Sperling and Commons 2011). Furthermore, these areas may influence each other via 5-HT1A dependent feedback inhibition, as blocking 5-HT1A receptors reciprocally influences the rostral and caudal poles of the DR (Sperling and Commons 2011). The present results further support the notion that the rostral and caudal DR may also represent distinct functional network domains.

A parsimonious explanation of the Fos studies and the behavioral analysis together is that *Cacna1c* knockout in serotonin neurons alters the balance of excitation and feedback inhibition that exists between different groups of serotonin neurons and consequently disrupts behavior. Blocking 5HT1A receptors and 5-HT1A-receptor-dependent feedback inhibition restores behavior, possibly by compensating for altered topographic patterns of activity. It is tempting to speculate that serotonin neurons in the rostral and caudal poles of the DR reciprocally regulate each other, as the interconnectivity of 5-HT neurons within the DR has been previously proposed as an important mechanism for regionally dependent 5-HT1A dependent feedback inhibition (Bang et al. 2012). However, it's important to point out that there are many potential mechanisms for the differential response by neurons in different DR subregions. Neurons in these different areas are developmentally distinct (Alonso et al. 2013) and variation in expressed genes within individual 5-HT neurons of might make them respond differently to loss *Cacna1c* (Kast et al. 2017; Okaty et al. 2015).

In conclusion, we show that 5-HT neuron *Cacna1c* knockout disrupts the behavioral response to an acute stressor and that this behavioral deficit is rescued by blocking 5-HT1A receptors, revealing an important role for 5-HT neuron Cav1.2 LTCCs in the regulation of stress-coping behavior. This is consistent with literature associating LTCC as important mediators of the response to stress (Joels and Karst, 2012). Moreover, this manipulation regionally enhances 5-HT neuron Fos expression while altering 5-HT1A dependent feedback inhibition across the rostral to caudal extent of the DR, raising the possibility that Cav1.2 LTCCs directly impact the regional balance of DR 5-HT system activation to influence behavior. Understanding the mechanisms through which 5-HT neuron *Cacna1c,* and LTCCs more broadly, impact DR 5-HT system function will be important areas for future study, as well as the potential relationship between *CACNA1C* genotype and altered 5-HT system functional activity in clinical populations.

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Abbreviations

5-HT	Serotonin
5-HT1A	Serotonin type 1A receptor
DR	Dorsal raphe
LTCC	L-type calcium channel

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Highlights

- Loss of the L-type calcium channel subunit Cav1.2 selectively in serotonin neurons disrupts normal behavioral response to stress.
- Blocking 5-HT1A receptors restores normal behavior.
- There is evidence for altered balance of activation and feedback inhibition between different subgroups of serotonin neurons when Cav1.2 is lacking.

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Highlights

- Serotonin (5-HT) neuron Cacna1c knockout disrupts active coping behavior
- 5-HT neuron Cacna1c knockout enhances caudal dorsal raphe (DR) Fos expression
- Blocking 5-HT1A receptors rescues coping behavior and enhances rostral DR response

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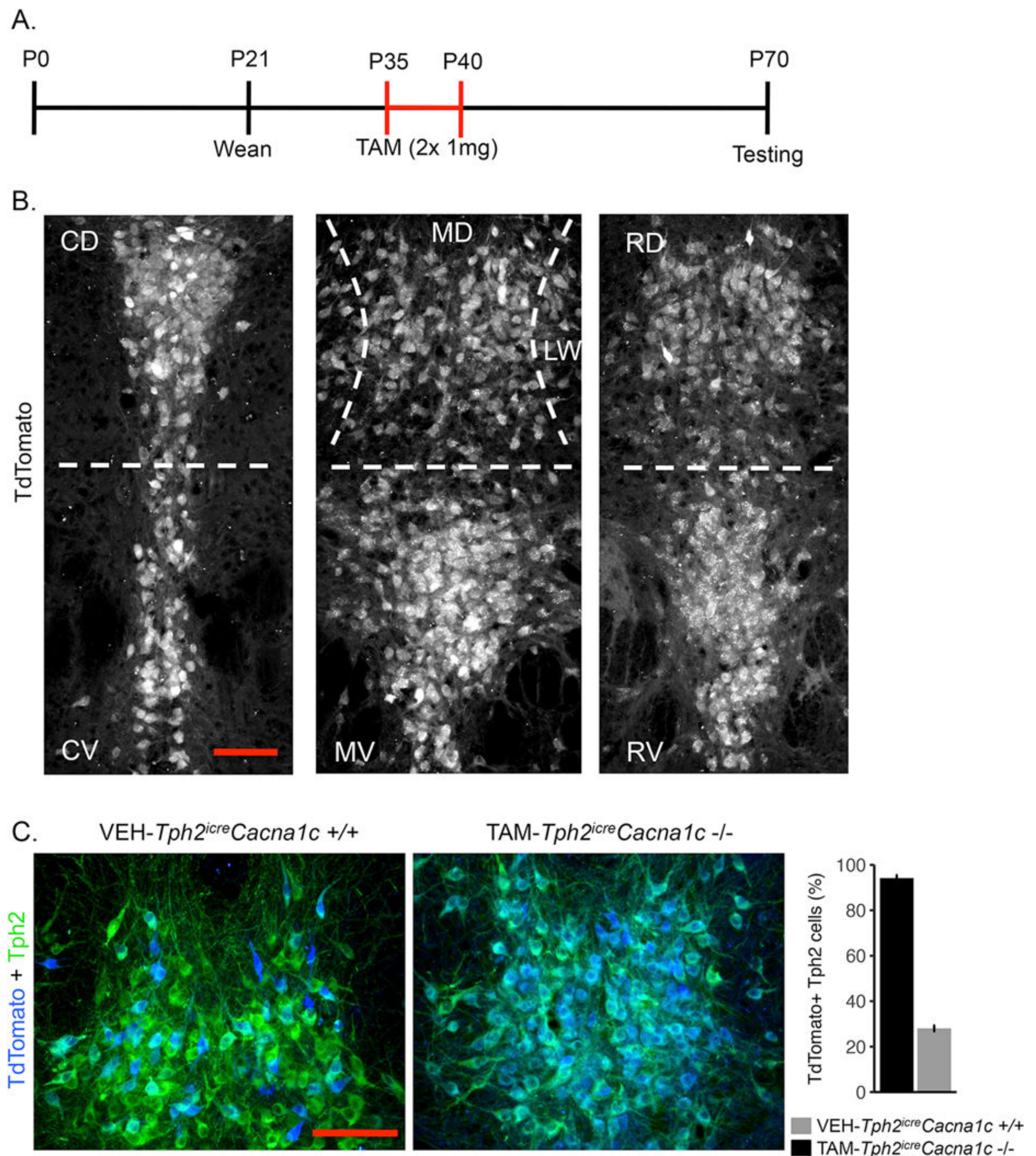

Figure 1.

Experimental timeline and TAM-induced recombination in 5-HT neuron Cacna1c knockout mice. *A*) Timeline (postnatal day; P) of TAM administration and behavioral/biological testing. B) TAMinduced recombination indicated by TdTomato expression in $Ai14^{TdTomato}Tph2^{icre}Cacna1c^{-/-}$ mice is exhibited throughout the caudal-rostral (*left* to *right*) and dorsal-ventral extent of the dorsal raphe nucleus. Dorsal raphe subregions abbreviated as *CD* = caudal-dorsal, *CV* = caudal-ventral, *MD* = medial-dorsal, *MV* = medial-ventral, *LW* = lateral wings, *RD* = rostral-dorsal, & *RV* = rostral-ventral. Scale bar

(red) = 100μm. C) *Left*, TdTomato expression (blue) within serotonergic neurons (Tph2 immunopositive, green) of VEH treated $Tph2^{icre}Cacna1c^{-/-}$ compared to TAM treated $Tph2^{icre}Cacna1c^{-/-}$ mice. Scale bar (red) = 100μm. *Right*, recombination rate (percentage of TdTomato+ serotonergic neurons) following TAM administration compared to VEH administration.

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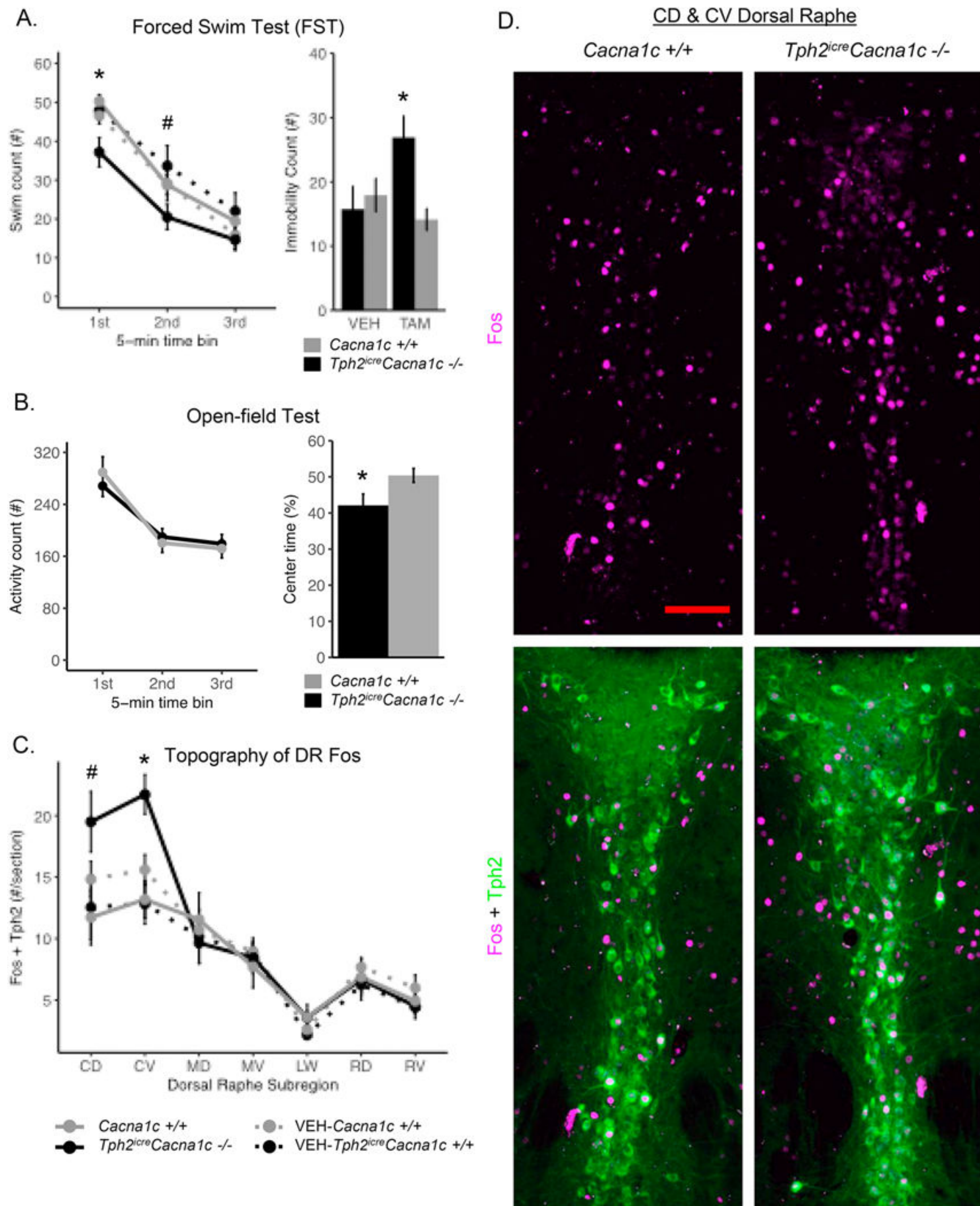

Figure 2.

Neurobehavioral effect of 5-HT neuron Cacna1c knockout. *A*) Forced-swim test swim count (active-coping behavior) measured across 5-minute time bins (right) or expressed as passive coping score over the last 4-minutes of the first 6-minutes (left), ±SEM. $^{*}p < .02$ and $^{\#}p < .05$ between TAM treated groups. *B*) *Left*, Open-field test beam-breaks (locomotor activity count) measured across 5minute time bins, ±SEM. *Right*, Percentage of time spent in the central rectangle of the open-field test, ±SEM. $^{*}p < .05$. *C*) Average number of serotonergic (Tph2-immunopositive) cells dually-labeled for Fos per tissue section examined, across

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distinct caudal-rostral, dorsal-ventral and lateral wings DR subregions, ±SEM. *$p_{FDR} < .05$ and #$p < .05$ between TAM treated groups. *D*) Representative images of the caudal DR from experimental mice. Immunofluorescent labeling of Fos (magenta; top) and cells dually labeled for Fos and Tph2 (green; bottom). Individual 216 × 165μm confocal images were merged using the pairwise stitching plugin of FIJI to display both CD and CV subregions on single representative sections. Scale bar (red) = 100μm.

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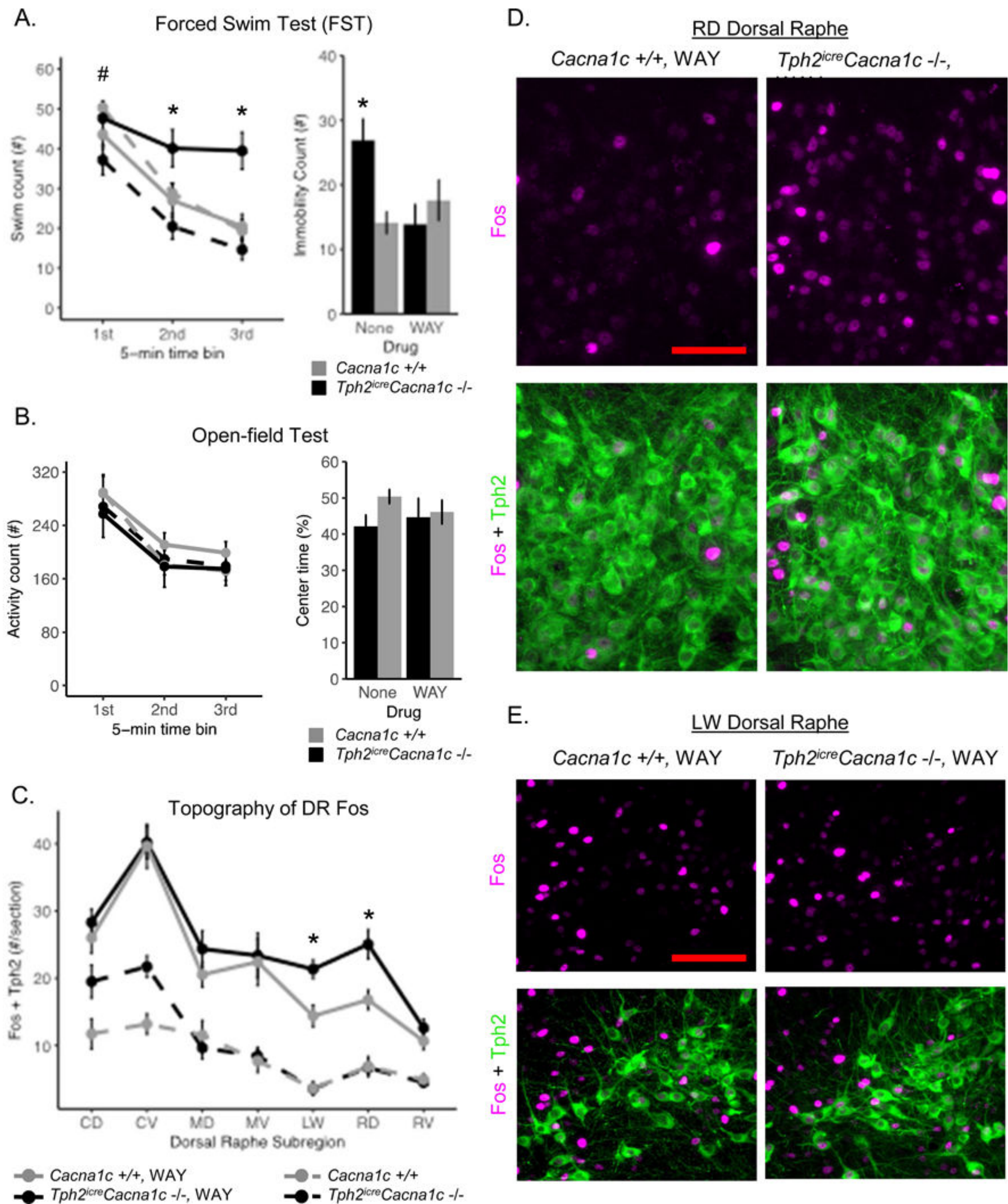

Figure 3.

Neurobehavioral effect of the 5-HT1A receptor antagonist WAY-100635 (WAY) following 5HT neuron Cacna1c knockout. *A*) Forced-swim test swim count (active-coping behavior) measured across 5-minute time bins (right) or expressed as passive coping score over the last 4-minutes of the first 6-minutes (left), ±SEM. $^{*}p < .02$ and $^{\#}p < .05$ for the effect of WAY in *Tph2*^{icre}*Cacna1c*$^{-/-}$ mice. *B*) *Left*, Open-field test beam-breaks (locomotor activity count) measured across 5-minute time bins, ±SEM. *Right*, Percentage of time spent in the central rectangle of the open-field test, ±SEM. *C*) Average number of serotonergic (Tph2-

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immunopositive) cells dually-labeled for Fos per tissue section examined, across distinct caudal-rostral, dorsal-ventral and lateral wings DR subregions, ±SEM. *$p_{FDR} < .05$ between WAY treated groups. *D*) Representative images of the rostral-dorsal (RD) DR from WAY-exposed experimental mice. Immunofluorescent labeling of Fos (magenta; top) and cells dually labeled for Fos and Tph2 (green; bottom). Scale bar (red) = 100μm. E) Representative images of the lateral wings (LW; one hemisphere only) of the DR from WAY-exposed experimental mice. Immunofluorescent labeling of Fos (magenta; top) and cells dually labeled for Fos and Tph2 (green; bottom). Scale bar (red) = 100μm.