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Ca_v3.1 isoform of T-type calcium channels supports excitability of rat and mouse ventral tegmental area neurons

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Abstract

Recent data have implicated voltage-gated calcium channels in the regulation of the excitability of neurons within the mesolimbic reward system. While the attention of most research has centered on high voltage L-type calcium channel activity, the presence and role of the low voltage-gated T-type calcium channel (T-channels) has not been well explored. Hence, we investigated T-channel properties in the neurons of the ventral tegmental area (VTA) utilizing wild-type (WT) rats and mice, Ca_v3.1 knock-out (KO) mice, and TH-eGFP knock-in (KI) rats in acute horizontal brain slices of adolescent animals. In voltage-clamp experiments, we first assessed T-channel activity in WT rats with characteristic properties of voltage-dependent activation and inactivation, as well as characteristic crisscrossing patterns of macroscopic current kinetics. T-current kinetics were similar in WT mice and WT rats but T-currents were abolished in Ca_v3.1 KO mice. In ensuing current-clamp experiments, we observed the presence of hyperpolarization-induced rebound burst firing in a subset of neurons in WT rats, as well as dopaminergic and non-dopaminergic neurons in TH-eGFP KI rats. Following the application of a pan-selective T-channel blocker TTA-P2, rebound bursting was significantly inhibited in all tested cells. In a behavioral assessment, the acute locomotor increase induced by a MK-801 (Dizocilpine) injection in WT mice was abolished in Ca_v3.1 KO mice, suggesting a tangible role for 3.1 T-type channels in drug response. We conclude that pharmacological targeting of Ca_v3.1 isoform of T-channels may be a novel approach for the treatment of disorders of mesolimbic reward system.

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Conflict of interest

The authors received no compensation, nor do they have any conflicting financial interests in regards to the work described in this manuscript.

Keywords

ventral tegmental area; dopamine; T-type calcium channel; TTA-P2; rebound spiking; burst firing; low-voltage-activated

1. Introduction

Voltage-gated calcium channels (VGCC) play a role in neuronal excitability and have been implicated in a number of disease processes involving hyperexcitability, including epilepsy (Cain and Snutch, 2010) and addiction (Lin et al., 1999; Uslaner et al., 2010). There are multiple classes of VGCC including the high-voltage-activated L, P/Q, N, R and low-voltage-activated T-type, each with unique structural and electrophysiological properties. Regions in which specific VGCC have been shown to have a significant contribution include the ventral tegmental area (VTA).

The VTA is a heterogeneous region of the midbrain that is highly involved in motivated actions, including aversive and reinforcement-related behaviors. Although different classes of VGCC may be involved in the regulation of neuronal excitability, most attention involving the VTA has focused on L-type calcium channels (Licata et al., 2000; Liu et al., 2014, 2007; Liu and Chen, 2008). However, the T-type VGCC is ideally suited to regulate neuronal excitability since it has the unique property of activating at low voltages to produce an influx of calcium ions, which in turn supports the excitability of the neuron allowing for more frequent action potentials. It has been demonstrated that both dopaminergic neurons and inhibitory interneurons in the midbrain may fire rebound spikes following periods of membrane hyperpolarization (Grace, 1991; Tateno and Robinson, 2011). However, ionic conductances underlying rebound spiking in the VTA region remain poorly characterized.

Unlike any other subtypes of VGCCs, T-type channels are deinactivated following a brief period of membrane hyperpolarization. After a subsequent return to resting membrane potential, T-type channels open and allow calcium influx into the cell, raising the membrane potential and triggering an action potential, a process defined as a low-threshold spike (LTS). Previous *in-situ* hybridization studies have shown that the $\text{Ca}_v3.1$ isoform of T-channels is highly expressed in VTA neurons (Talley et al., 1999). However, kinetic properties of T-currents and their effect on neuronal excitability in the VTA are not well studied. Here, we tested hypothesis that T-channels may be a significant contributor to neuronal excitability in the VTA and that this, in turn, may play a role in the behavioral effects of drugs of abuse. Therefore, understanding how T-type channels contribute to the excitability of VTA neurons may provide a novel insight into the mechanisms that govern neuronal excitability in this brain region that is heavily implicated in motivational behavior, drug abuse and disorders like schizophrenia (Belardetti et al., 2009).

2. Materials and Methods

2.1. Animals

A total of 23 transgenic TH-eGFP (tyrosine-hydroxylase-eGFP) rats on Sprague-Dawley background (Taconic Biosciences/Michael J. Fox Foundation, New York, NY), 24 wild-type

Sprague-Dawley rats (Harlan, Madison, WI), 4 $\text{Ca}_v3.1$ knock-out mice, and 5 C57BL6/J mice (Jackson Laboratory, Bar Harbor, ME) were used for the electrophysiology experiments. For the behavioral experiment, 6 $\text{Ca}_v3.1$ knock-out mice, and 6 C57BL6/J wild-type mice (Jackson Laboratory, Bar Harbor, ME) were used. $\text{Ca}_v3.1$ KO mice were obtained from Riken BRC through the National Bio-Resource Project of the MEXT (Japan) and were generated as described previously (Petrenko et al., 2007). Animals were housed with their mother until postnatal day 21 and thereafter in groups of three. All animals were maintained under a 12 h light/dark cycle and experiments were performed during the light phase of the cycle. Laboratory chow (Harlan, Madison, WI) and water were available *ad libitum* prior to electrophysiology experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado-Denver and were in accordance with NIH guidelines (National Research Council, 2011).

2.2. Brain slice preparation for patch-clamp experiments and immunohistochemistry

The electrophysiological properties of VTA neurons were assessed in acute midbrain horizontal slices containing the VTA from post-natal P17-36 adolescent rats. Patch-clamp recordings focused on T-type VGCCs. Animals were anesthetized briefly with isoflurane and decapitated, and brains were rapidly removed as described previously (Joksimovic et al., 2017). Fresh horizontal or sagittal brain slices were sectioned 250–300 μm thick at 4 °C in pre-chilled solution containing (in mM): sucrose 260, D-glucose 10, NaHCO_3 26, NaH_2PO_4 1.25, KCl 3, CaCl_2 2, MgCl_2 2, using a vibrating micro slicer (Leica VT 1200S). Brain slices were then immediately incubated for 45 minutes in solution containing (in mM): NaCl 124, D-glucose 10, NaHCO_3 26, NaH_2PO_4 1.25, KCl 4, CaCl_2 2, MgCl_2 2 at 37 °C prior to use in electrophysiology recordings; all recordings were conducted at room temperature. During incubation, slices were constantly perfused with a gas mixture of 95 vol% O_2 and 5 vol% CO_2 .

The procedure for labeling recorded neurons was based on our previously published method (Joksovic et al., 2006). The internal solution of the recording pipette contained 0.5% biocytin. After recording T-type currents from biocytin-filled neurons, slices were fixed overnight in 4% paraformaldehyde and then washed with 0.1% phosphate buffer (PB). Neurons were revealed using the avidin–biotin–peroxidase complex method (ABC elite kit; Vector Laboratories, Burlingame, CA, USA). After blocking endogenous peroxidase with 0.3% H_2O_2 in 0.4% Triton X-100 and PB, slices were incubated in the ABC reagent for 2 h. Incubated slices were washed several times in PB before immersion in a solution containing 0.05% of 3,3'-diaminobenzidine tetrahydrochloride (DAB reagent) in 0.1% Tris buffer and 0.02% H_2O_2 . After several washes in PB, slices were mounted on gelatin-coated slides and mounted with an aqueous mounting medium and then imaged immediately following immersion. The position of labelled neurons within the VTA was confirmed by using the brain atlas of Paxinos and Franklin, 2001.

2.3. Electrophysiological Studies

Slices were placed in a recording chamber (1-ml bath volume, RC-24N, Warner Instruments, Hamden, CT) that was steadily superfused at a rate of 2 ml/min with carbogenated recording solution of appropriate composition. The external solution for current-clamp

electrophysiology experiments consisted of the following (in mM): NaCl 125, D-glucose 25, NaHCO₃ 25, NaH₂PO₄ 1.25, KCl 2.5, MgCl₂ 1, CaCl₂ 2. This external solution was supplemented with picrotoxin (20 μ M), D-2-amino-5-phosphonovalerate (D-AP5; 50 μ M), and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 5 μ M) to block inhibitory and excitatory synaptic currents, respectively. For current-clamp experiments, the internal solution consisted of the following (in mM): potassium-D-gluconate 130, EGTA 5, NaCl 4, CaCl₂ 0.5, HEPES 10, Mg ATP 2, Tris GTP 0.5, pH 7.2. The external solution for voltage-clamp experiments with T-currents was identical to one for current-clamp experiments with addition of 1 μ M of TTX to block voltage-gated sodium currents. This solution was equilibrated with a mixture of 95 vol% O₂ and 5 vol% CO₂ for at least 30 min with a resulting pH of 7.4. The internal solution for voltage-clamp experiments with T-currents consisted of the following (in mM): tetramethyl ammonium (TMA)-OH 135, EGTA 10, MgCl₂ 2, HEPES 40, titrated to pH 7.2 with hydrofluoric acid (HF) (Todorovic and Lingle, 1998). VTA neurons were visually identified by video microscopy (high performance CCD camera, Cohu, San Diego, CA) combined with a BX51WI microscope with IR-DIC illumination (Olympus, Hamburg, Germany). Glass microelectrodes (Sutter Instruments, OD 1.2 mm) were pulled using a Sutter Instruments model P1000 and fabricated to maintain an initial resistance of 4–6 M Ω . Neuronal membrane responses were recorded using an Axopatch 200 B amplifier (Molecular Devices). Voltage current commands and digitization of the resulting voltages and currents were performed with Clampex 10.7 software (Molecular Devices) running on a PC-compatible computer. Resulting current and voltage traces were analyzed using Clampfit 10.7 (Molecular Devices). Data aggregation, statistical tests, and graphical analyses were performed using Rstudio (RStudio Team), GraphPad Prism 7.04 software (GraphPad Software), and Origin 7.0 (OriginLab), respectively. Results typically are presented as means from different neurons $\pm$ SEM unless stated otherwise.

2.4. Current-Clamp Studies

Both tonic and hyperpolarization induced rebound action potential properties of VTA neurons were examined by using multistep protocols with synaptic blockers (20 μ M picrotoxin, 50 μ M d-APV, and 5 μ M NBQX) in the external solution. For hyperpolarizing-induced rebound spiking, VTA neurons were patched in the whole-cell configuration, and resting membrane potentials were recorded for a 100 ms period. To investigate hyperpolarization induced rebound action potential patterns, the neurons were injected with 500 msec long hyperpolarizing currents in 10–75 pA intervals across 11 interval steps. Input resistance was determined from series of 500 msec long negative current injections delivered *via* recording pipette. The resulting amplitudes of membrane hyperpolarizations induced by given current injections were measured at the end of the hyperpolarizing step.

2.5. Drug applications and data analysis

A racemic mixture of TTA-P2 (3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide) (Alomone Labs, Israel) was prepared as a 3 mM stock solution in dimethyl sulfoxide (DMSO); aliquots were stored at –20 °C and diluted for use at final concentrations of 5 μ M, which is known to selectively block T-type calcium channels (Choe et al., 2011; Dreyfus et al., 2010; Shipe et al., 2008). Data from the

pre-drug baseline and following five and ten minutes after application of TTA-P2 were compared by 2-way analysis of variance (ANOVA) using within-subject mixed-design measures (Negus and Miller, 2014). Similarly, we used 2-way mixed measures ANOVA to analyze the phenotype of neurons from TH-eGFP KI rats. For the voltage clamp experiments comparing the peak current magnitude in wild-type and Cav 3.1 knock-out mice, an unpaired t-test and Mann-Whitney U test was performed to determine statistical significance.

2.6. T-current properties

T-channel activation was measured by stepping the membrane potential from an initial holding potential (V_h) of -90 mV to test potentials (V_t) of -80 mV to -20 mV in 2.5 mV increments over a period of 200 ms. Current-voltage (I-V) curves were generated, and peak current amplitudes and inactivation properties of current waveforms were established. Steady-state inactivation curves were generated by using a standard double-pulse protocol with 3.6 s-long pre-pulses to variable voltages (from -120 to -50 mV in 5 mV increments) and test potentials to -50 mV. The voltage dependencies of activation and steady-state inactivation were described with single Boltzmann distributions of the following forms:

$$\text{Activation: } I(V) = I_{\max}/(1+\exp [-(V-V_{50})/k])$$

$$\text{Inactivation: } I(V) = I_{\max}/(1+\exp [(V-V_{50})/k])$$

In these forms, $I_{\max}$ is the maximal amplitude of current; V_{50} is the voltage at which half of the current is activated or inactivated; and k represents the voltage dependence (slope) of the distribution. The time constant of T-current inactivation in VTA neurons was assessed by fitting the decaying portions of the current waveforms at the peak potential of the I-V relationships with a single exponential function $f(t) = A_1 \exp(-t/\tau_1)$, yielding one time constant (τ_1) and its amplitude (A_1).

Recordings from intact brain slices offer great advantages for studying neurons in an intact setting *ex vivo*. However, the presence of extensive neuron processes compromises voltage control in whole-cell recordings from slices so that all biophysical measurements must be interpreted with caution. Accordingly, we paid close attention to signs of good voltage control. Specifically, we analyzed cells in which there was no extensive delay in the onset of current; also, the onset and offset kinetics depended on voltage, not on the amplitude of current. In whole-cell experiments, because intact VTA neurons have long processes, rapid components of recorded current, such as tail currents, are unlikely to reflect the true amplitude and time course of calcium current behavior. The use of internal solution with TMA and HF allowed us to isolate T-currents from high-voltage-activated (HVA) calcium currents (Todorovic and Lingle, 1998). The amplitude of T-currents was measured from the peak, which was subtracted from the current at the end of a depolarizing test potential to avoid contamination with a small HVA current component.

Similarly, application of drugs has some limitations in intact slices. TTA-P2 was applied until an apparent steady-state effect was achieved. The quantitative assessment of drug effects is limited since delivery of drug-containing solutions *in vitro* may be compromised due to diffusion through the sliced tissue. Therefore, the actual concentrations of all

compounds at their sites of action are likely to be lower than those reported. To perform the study of neuronal excitability under similar conditions, fluctuations of resting membrane potential during drug applications were corrected with direct injections of currents *via* the recording electrode. Hence, for the data presented in Figure 2, membrane potential was kept constant by correcting any TTA-P2-induced hyperpolarization in order to perform comparisons of neuronal excitability before and after drug application that was not confounded by the changes of membrane potential.

2.7. Confocal laser scanning microscopy

Transgenic TH-eGFP knock-in rats were deeply anesthetized with 5% isoflurane and transcardially perfused with phosphate buffered saline (PBS pH 7.4, Life Technologies). Brains were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours. Brains were rinsed in PBS, embedded in 3% agarose and brain sections (40 μ m) were prepared on a microtome (Leica VT1200). Slices were rinsed three times in PBS and a heat antigen retrieval process was performed in boiling citric buffer (0.1M, pH 6.0). Sections were blocked with 5% normal donkey serum in PBST (0.05% Triton $\times$ 100 in PBS) for 30 minutes, and incubated with primary antibody (rabbit anti-NeuN, 1:100, Millipore) diluted in 1% normal donkey serum in PBST overnight at 4°C. Slices were rinsed 3 $\times$ 5 minutes in PBST followed by 5 min rinsing in PBS and then incubated for 2 h with secondary anti-rabbit antibody (1:100, Alexa 555, Invitrogen) at room temperature and washed 3 $\times$ 5 minutes in PBS. The sections were mounted in fluorescent mounting medium (Vector laboratories) and the fluorescent signals were examined using an Olympus FluoView FV1000 confocal laser scanning microscope. Specificity of the antibody was confirmed by omitting the primary antibody in control slices.

2.8. MK-801-induced hyperlocomotion

Locomotor activity of wild-type (WT; n=6) and Cav3.1 KO mice (n=6) was recorded in an open field chamber (44 $\times$ 44 $\times$ 30 cm³) under white light for a total of 90 min using EthoVision XT videotracking software (Noldus). The test was performed in three sessions: first - baseline locomotor activity (15 min); second - locomotor activity after intra-peritoneal (i.p.) injection of saline vehicle (15 min); and third - locomotor activity after i.p. injection of MK-801 (0.1 mg/kg; 60 min).

3. Results

3.1. T-type Channel Activity Utilizing Current and Voltage Clamp in Wild-type Rats

Many neurons in the VTA receive a strong inhibitory synaptic input that generates hyperpolarizing synaptic potentials (Johnson and North, 1992). Importantly, T-type calcium channels are deinactivated during periods of membrane hyperpolarization and support characteristic rebound after-depolarizing potentials (ADP) that often crown with one or multiple rebound action potentials (APs) upon return to the baseline membrane potentials. Hence, we used current-clamp recordings from WT rats to assess whether VTA neurons exhibit ADP and rebound firing of APs. Current-clamp stimulus waveform for the dual step protocol (brief depolarizing stimulus followed by a hyperpolarizing stimulus) is shown in Figure 1A. Injections of 50 pA (red traces) and 100 pA (black traces) induced regular

spiking firing pattern upon depolarization in both neurons (Fig. 1B and 1C). However, the dual step protocol resulted in hyperpolarization-induced rebound spiking in some neurons in the VTA, as shown in Figure 1B, while other neurons did not produce hyperpolarization-induced ADP and rebound spiking (Figure 1C). Note that the representative VTA neuron in panel B fired nine APs upon depolarization with 100 pA, while the neuron depicted in panel C fired only three APs. Consistent with this pattern, subsequent experiments using similar dual step protocols demonstrated that neurons with ADP had significantly higher depolarization-induced AP firing frequency (45.14 ± 3.93 Hz, $n = 37$ neurons from 23 rats) than neurons without ADP (33.83 ± 2.76 Hz, $n = 30$ neurons from 16 rats; $p = 0.028$, two-tailed t-test). In addition, we found that baseline membrane potentials in our recording conditions within the same cohorts were more depolarized in neurons with ADP (-50.9 ± 0.5 mV) than in neurons without ADP (-54.0 ± 0.7 mV; $p = 0.001$, two-tailed t-test). Neurons patched in Figures 1B and 1C were also stained with biocytin and are shown in Figures 1b and 1c, respectively. We examined 14 VTA neurons from eight WT rats using the dual step protocol in conjunction with biocytin in the recording pipette; 9/14 neurons (64%) showed hyperpolarization-induced ADP and rebound spiking.

Next, we set out to determine if rebound spike firing and ADP could be abolished in the presence of the pan-selective T-channel blocker, TTA-P2. Figure 2A (top panel) shows the stimulus waveform for the dual step protocol overlapped with baseline control traces (middle panel) and five minutes after application of $5 \mu\text{M}$ TTA-P2 (bottom panel). Baseline traces show multiple depolarization-induced APs and hyperpolarization-induced rebound spiking following a return to baseline. Although a 10-min exposure of neurons to TTA-P2 did not affect depolarization-induced firing, rebound spikes were completely ablated (Figure 2A, bottom panel). Aggregation of eleven observations from brain slices exposed to TTA-P2 from seven WT rats were averaged and compared for statistical significance (Figure 2B-D). We found that TTA-P2 produced a significant effect on hyperpolarization-induced rebound spiking [$F(10, 100) = 8.875$ $p < 0.0001$] resulting in decreased spiking from 30–100% of maximal current intensity (Figure 2B). In contrast, TTA-P2 failed to produce a significant change in the depolarization-induced firing frequency in the same neurons (Figure 2C). Interestingly, TTA-P2 produced a significant interaction [$F(20, 180) = 3.008$, $P < 0.0001$] resulting in an increase in input resistance at 10–100% of maximal current at the 5 and 10 min time points after application of TTA-P2 (Figure 2D). Importantly, VTA neurons ($n=3$) that were not exposed to TTA-P2, hyperpolarization-induced rebound spiking and input resistance remained stable up to ten minutes of recording (data not shown). Interestingly, TTA-P2 caused a small but significant membrane hyperpolarization of 3.04 ± 0.76 mV in VTA neurons ($n=5$; $p = 0.016$, two-tailed paired t-test). Hence, for the data presented in Figure 2, the membrane potential was kept constant by injecting current *via* the recording pipette to correct TTA-P2-induced hyperpolarization. This allowed comparison of neuronal excitability before and after drug application that was not confounded by the changes of membrane potential.

Next, we performed voltage-clamp recording from VTA neurons of WT rats in order to investigate biophysical properties of T-currents. We focused on the properties of voltage-dependent activation and voltage-dependent inactivation kinetics, specifically, because they can drastically influence neuronal excitability. Voltage-clamp stimulus waveform and

representative family of current traces for the activation current-voltage protocol are shown in Figure 3A demonstrating activation and inactivation kinetics of macroscopic currents becomes progressively faster with stronger depolarizing steps, which produces a characteristic crisscrossing pattern. Analysis of peak current for each trace across nine WT rats was aggregated and normalized to determine a V_{50} of -59.60 mV for the voltage-dependent activation of T-currents in VTA neurons (Figure 3B). Similarly, we also plotted current density (current amplitudes divided by cell's capacitance) and inactivation time constant (τ) against test potential for the current-voltage protocol (Figure 3C, 3D respectively). Inactivation τ became progressively faster with stronger depolarizing steps and largely voltage-independent at more positive potentials, reaching a steady-state value of approximately 40 msec.

Voltage-clamp stimulus waveform and representative current traces for the inactivation protocol are shown in Figure 4A. Analysis of peak current for each trace across neurons from brain slices of WT rats was aggregated and normalized to determine a V_{50} of -94.35 mV for the voltage-dependent inactivation of T-currents in VTA neurons (Figure 4B). Next, normalized current and conductance were plotted against test potential for both the inactivation (black) and activation (red) protocol respectively, to show the small overlapping region termed “window” current (Figure 4C). These “window” currents result from the fact that a small fraction of T-channels is persistently available near the resting membrane potential, which in turn allows a basal inward flux of calcium ions and membrane depolarization at rest (Perez-Reyes, 2003). It is well documented that T-type “window” currents play an important role in regulating functional states of thalamic neurons (Cope et al., 2005; Dreyfus et al., 2010). Furthermore, it is likely that inhibition of T-type “window” currents by TTA-P2 in VTA neurons underlies the hyperpolarization and increased input resistance that we observed in our current-clamp experiments (Figure 2D).

3.2. T-type Channel Activity Utilizing Voltage Clamp in Wildtype and $\text{Ca}_v3.1$ Knock-out Mice

There are three known isoforms of T-type calcium channels based on the cloned pore-forming $\alpha 1$ subunit: $\text{Ca}_v3.1$ ($\alpha 1G$), $\text{Ca}_v3.2$ ($\alpha 1H$) and $\text{Ca}_v3.3$ ($\alpha 1I$) that are encoded by *CACNA1G*, *CACNA1H* and *CACNA1I* genes, respectively (Perez-Reyes, 2003). Furthermore, TTA-P2 possesses a comparable affinity for all three T-channel isoforms (Shipe et al., 2008). *In-situ* hybridization has demonstrated rat VTA neurons are enriched in mRNA for the $\text{Ca}_v3.1$ isoform; however, $\text{Ca}_v3.2$ and $\text{Ca}_v3.3$ were below threshold for detection (Talley et al., 1999). Hence, we recorded well-isolated T-currents from brain slices of WT mice and global $\text{Ca}_v3.1$ knock-out (KO) mice to determine the molecular entity of T-currents in VTA neurons. The voltage-clamp stimulus waveform for the inactivation protocol is shown in top panel of Figure 5A. Example peak sweeps are shown from activation traces ($V_h -90$ mV, $V_t -40$ mV) of a neuron in the VTA for a wild-type (black trace) and $\text{Ca}_v3.1$ KO (red trace) mouse (bottom panel of Figure 5A). The amplitude of the T-current in KO mice was less than 5% of the amplitude obtained in WT mice. Average time constant (τ) for macroscopic current inactivation in WT mice was 42.3 ± 1.9 msec, which was similar to the corresponding values of 43.7 ± 4.9 msec obtained from WT rats (Figure 3D). Bar graph on Figure 5B shows the peaks of T-currents ($n=10$ neurons from 4 WT mice) compared to

Cav3.1 KO mice (n=10 neurons from 3 KO mice), suggesting that VTA T-currents were significantly diminished in KO mice ($p<0.001$, Mann-Whitney U Statistic).

3.3. Horizontal Slice Imaging of VTA from TH-eGFP Rat Neurons

Our patch-clamp recordings and results with TTA-P2 in WT rats clearly demonstrated expression of classic T-type currents that underlie ADP and rebound burst firing in a subset of VTA neurons. However, it is well known that the VTA contains heterogeneous populations of dopaminergic neurons as well as both inhibitory and excitatory interneurons, all of which play critical roles in regulating motivated behaviors (Morales and Margolis, 2017). Hence, we wished to determine if there was a functional significance to our findings and if T-channels might support excitability in one or both cell populations in the VTA. To address this issue and identify dopaminergic and non-dopaminergic neurons in the VTA, we took advantage of transgenic rats with the dopamine-producing enzyme tyrosine-hydroxylase (TH) labeled with eGFP (Iacovitti et al., 2014). To label all neuronal nuclei (both dopaminergic and non-dopaminergic neurons) in the VTA and adjacent areas in this region, brain slices from the TH-eGFP rats were labeled with the general neuronal marker NeuN and imaged with confocal microscopy (Figure 6A and 6B). A representative brain slice of the VTA region examined by dual imaging (Figure 6A) shows all NeuN positive neuronal nuclei (red), and dopaminergic neurons, which express TH-eGFP (green). The white square in Figure 6A is enlarged in Figure 6B, which shows TH-eGFP (top), NeuN (bottom), and the merged image (middle). Similar images were obtained from a total of three TH-eGFP rats, suggesting the VTA contains both dopaminergic (TH-eGFP positive/NeuN positive) and non-dopaminergic (TH-eGFP negative/NeuN positive) neurons.

3.4. Electrophysiological properties of VTA Positive and Negative TH-eGFP Rat Neurons

We next performed current-clamp recordings from identified dopaminergic (TH-eGFP positive, TH-POS) and non-dopaminergic (TH-eGFP negative, TH-NEG) neurons in the VTA of slices from TH-eGFP transgenic rats. Figure 7A (top) depicts family of current injections protocol used to study depolarization-induced and rebound firing properties of TH-POS and TH-NEG neurons in the VTA. The membrane was first depolarized for 500 msec, then hyperpolarized for 500 msec with 10 incremental steps of current injections *via* the recording pipette. Consequently, we studied resulting rebound excitability upon return to the baseline membrane potential for 300 msec following termination of current injection. Traces from representative TH-POS (green traces) and TH-NEG (red traces) neurons are depicted in the middle and lower panels, respectively, of Figure 7A. Both cell types fired multiple APs when depolarized with escalating current injections. Note that in both types of neurons hyperpolarization-activated current, indicative of I_h current, is noticeable during negative current injections. Upon return of membrane potential to the baseline levels, both cell types displayed bursts of APs. Figure 7B shows input-output curves representing the number of rebound APs as a function of the percentage of maximal current injection between TH-POS neurons (n = 13 neurons from 12 TH-eGFP rats) and TH-NEG neurons (n = 17 neurons from 11 TH-eGFP rats). Although both cell types had similar input-output curves, there was a statistically significant increase in the ability for TH-NEG neurons to produce more rebound APs at 70%, 80% and 90% of maximal current injections. In contrast, there was no statistical difference between the depolarization-induced firing frequency of

TH-POS and TH-NEG neurons (Figure 7C). While all tested neurons displayed depolarization-induced firing mode, rebound spike firing was observed in 13 out of 31 studied TH-POS neurons (42%) and 17 out of 31 TH-NEG neurons (55%). Finally, we tested the ability of TTA-P2 to inhibit rebound APs in TH-POS and TH-NEG neurons. Figure 7D shows representative traces of rebound APs before application of TTA-P2 (green trace) and after 10 minutes of application of TTA-P2 (gray trace) in the same TH-POS neuron. TTA-P2 completely inhibited rebound spiking of an AP, which was repeated in a total of three neurons from three rats. Figure 7E shows representative traces of rebound APs before application (red trace) and after 10 minutes of application of TTA-P2 (gray trace) in the same TH-NEG neuron. TTA-P2 completely and reproducibly inhibited both APs during rebound spiking in a tested TH-NEG neurons (n=5 neurons from 5 rats).

We also compared passive and active membrane properties between TH-POS and TH-NEG neurons by measuring the following: baseline membrane potential, input resistance (R_{in}), threshold hyperpolarizing current injection to evoke rebound spike (rheobase), latency to the first rebound spike measured from the end of hyperpolarizing current injection step, and threshold membrane potentials to evoke first rebound spike. Table 1 summarizes these data and indicates that with the exception of a lower rheobase in TH-NEG neurons, there was no statistical significance in any of these parameters between the TH-POS and TH-NEG neurons (two-tailed t-test and Mann-Whitney U test). It is noteworthy that in our recording conditions, we did not observe spontaneous electrical activity in any of the studied VTA neurons.

3.5. MK-801-induced hyperlocomotion

An important role of the VTA in basal dopamine output in mesolimbic and mesocortical dopamine terminals is well established (Mathé et al., 1999). MK-801, a potent and selective NMDA receptor antagonist, induces stereotyped behaviors and a marked increase in locomotor activity in rodents resembling motor symptoms of schizophrenia in humans (Tricklebank et al., 1989). Many of these locomotor effects of MK-801 appear to be mediated by VTA neurons (Mathé et al., 1998; Narayanan et al., 1996). Based on our patch-clamp results with VTA neurons lacking $Ca_v3.1$ channels, we hypothesized that $Ca_v3.1$ KO mice could display reduced locomotor responses when injected with MK-801.

The effects of $Ca_v3.1$ T-channels on hyperlocomotion induced by 0.1 mg/kg MK-801 are summarized in Figure 8. The baseline locomotor activity and activity after saline injection were not significantly different between WT and $Ca_v3.1$ KO mice (two-way mixed-design ANOVA). When monitoring the mice for an hour immediately after MK-801 injection (5 min time-bins), WT, but not $Ca_v3.1$ KO mice, exhibited a dramatic increase in locomotor activity (factor genotype $F(1,10) = 7.43$, $p = 0.02$, Figure 8A). Similar lack of MK-801 effect in $Ca_v3.1$ KO mice was observed when the total distance was analyzed (two-tailed unpaired t-test, $p = 0.02$, Figure 8B). Only the $Ca_v3.1$ KO mice failed to respond to the stimulatory properties of MK-801. In fact, when comparing the periods of 15 minutes before and immediately after the MK-801 injection, $Ca_v3.1$ KO mice displayed a significant decrease in locomotion (paired t-test, KO: $p=0.015$; WT: $p=0.222$), consistent with continuing habituation to the open field arena. Taken together, these results strongly suggest

that the $\text{Ca}_v3.1$ isoform of T-channels is necessary to relay the excitatory input from the VTA that mediates MK-801-induced hyperlocomotion.

4. Discussion

The major goal in this paper was to identify and categorize the presence of T-channel activity in the VTA neurons in rats and mice. To accomplish the goal of isolating VTA T-type currents and to determine pertinent patterns in its distribution we used a combination of genetic, pharmacological and electrophysiological techniques. Here, we showed that for a subpopulation of neurons within the VTA, in both TH-POS and TH-NEG neurons, T-channels strongly contribute to the hyperpolarization-induced rebound spiking as demonstrated by the ability of the specific T-channel blocker TTA-P2 to block the effects of rebound spiking. In addition, we showed that properties of T-currents in WT mice are generally similar to the currents in WT rats, and that global $\text{Ca}_v3.1$ KO mice have virtually no T-currents in the VTA.

Our choice of TTA-P2 for this study relied on our previous studies that have extensively characterized its effects in other native preparations *in vitro* and *in vivo* (Choe et al., 2011; Eckle et al., 2012; DiGrucio et al., 2015; Joksimovic et al., 2017). A series of previous experiments has shown that TTA-A2, another selective T-channel blocker and analog of TTA-P2, was capable of blocking drug reward-related behaviors in multiple animal models of addiction. For example, when TTA-A2 was administered in conjunction with cocaine or amphetamine to mice previously given repeated daily injections of either amphetamine or cocaine, expression of locomotor sensitization was blocked (Gangarossa et al., 2014). Further, in a nicotine self-administration model, rats given TTA-A2 showed reduced motivation to self-administer nicotine and motivation was not reinstated when either a priming dose of nicotine or a nicotine associated cue was presented (Uslaner et al., 2010). In addition, TTA-A2 appeared to selectively block drug-related behaviors in the tasks tested without impairing the response to a natural food reward. In a follow-up study, systemic administration of TTA-A2 reduced the degree of amphetamine-induced hyperactivity in the nucleus accumbens (NAC) as measured by c-fos expression levels; and microdialysis revealed that TTA-A2 was capable of blocking increases in glutamatergic efflux in the NAC when systemic administration of MK-801 was compared to vehicle (Uslaner et al., 2012).

Most classes of abused drugs show acute locomotor enhancing effects at specific dosage ranges, including NMDA antagonists (Caixeta et al., 2013). Here, we utilized a potent and highly selective NMDA antagonist MK-801 with WT and $\text{Ca}_v3.1$ KO mouse lines to specifically examine the contribution of $\text{Ca}_v3.1$ T-channel isoform to this drug-induced behavioral phenotype. Our results with systemic injection of MK-801 into WT and $\text{Ca}_v3.1$ KO indicate that indeed, this T-channel isoform is required for MK-801-induced acute hyperlocomotion. Previous *in vivo* studies have documented that systemic administration of MK-801 induced hyperlocomotion and dopamine release in the NAC are largely mediated by enhanced excitability of VTA neurons (Mathé et al., 1998). Furthermore, molecular studies have demonstrated that mRNA for $\text{Ca}_v3.1$ is abundant in VTA neurons but below detectable levels in NAC (Talley et al., 1999). This strongly suggests that $\text{Ca}_v3.1$ channels in VTA neurons played an important role in the MK-801-induced hyperlocomotion in our

experiments. Future studies with VTA-specific $\text{Ca}_v3.1$ knock-out mice would be useful to further implicate these channels in behavioral responses induced by systemic administration of various drugs of abuse including NMDA antagonists.

To the authors' knowledge, only one other independent study (Philippart et al., 2016) utilized TTA-P2 as well as a number of other antagonists to delineate the overall contribution of multiple subtypes of VGCCs to the total calcium current in VTA neurons. They showed that about 20% of the total inward calcium current measured was due to contributions from T-type channels. However, the voltage-clamp stimulus waveform utilized to produce the signal was not suitable for maximal activation of T- channels as they were holding cells at -100 mV and moving to $+10$ mV since they examined all subtypes of VGCCs. In contrast, our findings demonstrated that isolated T-type currents in the VTA were maximally activated while holding at -120 mV and moving to -50 mV. Regardless, the observation that even in these circumstances 20% of the total calcium current in the VTA could be attributed to T-type channels is intriguing and suggests in more specific electrophysiological circumstances, T-type channels may play an even greater role in calcium flux.

Our data established that the $\text{Ca}_v3.1$ isoform is a major contributor of T-channels in the mouse VTA. Our findings are in agreement with an *in-situ* hybridization study, which identified high levels of expression of mRNA for $\text{Ca}_v3.1$ in the VTA (Talley et al., 1999). In general, kinetic properties of T-currents in the VTA are equivalent to other brain regions enriched in $\text{Ca}_v3.1$ such as the ventrobasal nucleus of the thalamus (Cope et al., 2005; Dreyfus et al., 2010; Eckle et al., 2012), subiculum (Joksimovic et al., 2017), and cerebellar Purkinje neurons (Hildebrand et al., 2009). Furthermore, the $\text{Ca}_v3.1$ isoform of T-channels was reported to play an important role in the plasticity of dopaminergic neurons in the substantia nigra (Poetschke et al., 2015). Another recent interesting study described the role of T-type channels in supporting LTS and burst firing in calbindin-negative but not calbindin-positive dopaminergic neurons within substantia nigra (Evans et al., 2017). It remains possible that T-channels and LTS are expressed only in functionally-distinct subtype of dopaminergic neurons of VTA as well.

While T-channels and specifically the $\text{Ca}_v3.1$ isoform have not been previously described in the VTA, there is ample evidence that VTA neurons fire rebound burst APs that are implicated in both physiological processes (Chen and Lodge, 2013) and disorders of the mesolimbic reward system, including animal models of schizophrenia (Krabbe et al., 2015). For example, even a single exposure to cocaine produces a lasting increase in bursting of dopaminergic VTA neurons *via* plasticity of glutamatergic receptors and calcium-activated small-conductance calcium-dependent potassium (SK) channels (Creed et al., 2014). Furthermore, the addictive substance and ketamine analogue, methoxetamine, induces self-administration behavior in rats and increases burst firing in the NAC projecting dopaminergic neurons of the VTA (Mutti et al., 2016). Additional evidence for the role of a burst firing pattern in the VTA in pathological processes was reported for neuropathic pain caused by spared nerve injury in rats (Sagheddu et al., 2015). Our results of *in vivo* and *in vitro* studies presented herein strongly suggest a substantial role for pharmacological targeting of T-channels in various disorders of the mesolimbic reward system.

Overall, our results establish for the first time that there is a major role for T-channels in both dopaminergic and non-dopaminergic neurons within the VTA that affect their rebound excitability and burst firing. Furthermore, our study opens new avenues for future investigations which may delineate whether there are specific and more specialized roles for T-currents in the neurons which exhibit hyperpolarization-induced rebound spiking. Questions include whether dopaminergic neurons with rebound spiking project to major ascending regions of the brain such as the NAC or the prefrontal cortex; or what possible role may T-type channels play in the VTA in terms of mediating temporally discrete signaling? Some studies have also implicated hyperpolarization-activated current (I_h) in rebound spiking of both dopaminergic and non-dopaminergic neurons in the VTA (Tateno and Robinson, 2011). While the presence of T-channels and I_h in both dopaminergic and non-dopaminergic neurons strongly suggest their complex role within the VTA, our results provide an important first step that lays the groundwork for future experiments within this heterogeneous region. For example, transgenic animals could determine if T-channels are expressed in inhibitory interneurons of the VTA, which represent the most abundant subpopulation of non-dopaminergic neurons in VTA (Morales and Margolis, 2017). This is very likely, given that our results indicate robust rebound bursting in the majority of studied TH-NEG neurons. However, due to the heterogeneous populations of neuronal subtypes within the VTA, examining whether T-channels are present on either glutamatergic, GABAergic or cholinergic subpopulations would be an important next step.

Overall, our results confirm the idea that T-channels critically support rebound burst firing following periods of membrane hyperpolarization, similar to those that occur during inhibitory synaptic potentials in the VTA. Further, our behavioral results indicate that deletion of $Ca_v3.1$ channels disrupts the hyperlocomotion that is well established among drugs of abuse and disorders like schizophrenia. While additional experiments are necessary to establish if other individual isoforms of T-channels within the mesolimbic pathway may also contribute to this effect, the implication that $Ca_v3.1$ channels are necessary for expression of this behavioral effect provides strong evidence for further examining their role in reward-related circuitry. We conclude that $Ca_v3.1$ isoform of T-type channels may represent a new unexplored target for pharmacological therapies in treating disorders that result from dysfunction of the mesolimbic reward system.

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Abbreviations

LVA	low-voltage-activated
TEA-OH	tetraethylammonium hydroxide
TMA-OH	tetramethylammonium hydroxide
TTX	tetrodotoxin

TTA-P2	3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide
DMSO	dimethylsulfoxide
EGTA	ethylene glycol tetraacetic acid
GTP	guanosine three phosphate
ATP	adenosine three phosphate
MTA	ventral tegmental area
WT	wild type
TH	tyrosine hydroxylase
eGFP	enhanced green fluorescent protein
KI	knock-in
KO	knock-out
VGCC	voltage-gated calcium channels
DAB	diaminobenzidine tetrahydrochloride
PBS	phosphate buffer saline
AP	action potential
ADP	after-depolarizing potential
HF	hydrofluoric acid
LTS	low threshold spike
RMP	resting membrane potential
Rin	input resistance
MK-801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, Dizocilpine hydrogen maleate)

References

- Belardetti F, Ahn S, So K, Snutch TP, Phillips AG. Block of voltage-gated calcium channels stimulates dopamine efflux in rat mesocorticolimbic system. *Neuropharmacology*. 2009; 56:984–993. DOI: 10.1016/j.neuropharm.2009.02.001 [PubMed: 19371580]
- Cain SM, Snutch TP. Contributions of T-type calcium channel isoforms to neuronal firing. *Channels (Austin)*. 2010; 4:475–82. DOI: 10.4161/chan.4.6.14106 [PubMed: 21139420]
- Caixeta FV, Cornélio AM, Scheffer-Teixeira R, Ribeiro S, Tort ABL. Ketamine alters oscillatory coupling in the hippocampus. *Sci Rep*. 2013; 3:2348.doi: 10.1038/srep02348 [PubMed: 23907109]
- Chen L, Lodge DJ. The lateral mesopontine tegmentum regulates both tonic and phasic activity of VTA dopamine neurons. *J Neurophysiol*. 2013; 110:2287–94. DOI: 10.1152/jn.00307.2013 [PubMed: 24004527]

- Choe W, Messinger RB, Leach E, Eckle VS, Obradovic A, Salajegheh R, Jevtovic-Todorovic V, Todorovic SM. TTA-P2 is a potent and selective blocker of T-type calcium channels in rat sensory neurons and a novel antinociceptive agent. *Mol Pharmacol*. 2011; 80:900–10. DOI: 10.1124/mol.111.073205 [PubMed: 21821734]
- Cope DW, Hughes SW, Crunelli V. GABAA Receptor-Mediated Tonic Inhibition in Thalamic Neurons. *J Neurosci*. 2005; 25:11553–11563. DOI: 10.1523/JNEUROSCI.3362-05.2005 [PubMed: 16354913]
- Creed MC, Ntambi NR, Tan KR. VTA GABA neurons modulate specific learning behaviors through the control of dopamine and cholinergic systems. *Front Behav Neurosci*. 2014; 8:8.doi: 10.3389/fnbeh.2014.00008 [PubMed: 24478655]
- Dreyfus FM, Tscherter A, Errington AC, Renger JJ, Shin HS, Uebele VN, Crunelli V, Lambert RC, Leresche N. Selective T-type calcium channel block in thalamic neurons reveals channel redundancy and physiological impact of I(T)window. *J Neurosci*. 2010; 30:99–109. DOI: 10.1523/JNEUROSCI.4305-09.2010 [PubMed: 20053892]
- Eckle VS, DiGrucio MR, Uebele VN, Renger JJ, Todorovic SM. Inhibition of T-type calcium current in rat thalamocortical neurons by isoflurane. *Neuropharmacology*. 2012; 63:266–273. DOI: 10.1016/j.neuropharm.2012.03.018 [PubMed: 22491022]
- Evans RC, Zhu M, Khaliq ZM. Dopamine Inhibition Differentially Controls Excitability of Substantia Nigra Dopamine Neuron Subpopulations through T-Type Calcium Channels. *J Neurosci*. 2017; 37:3704–3720. DOI: 10.1523/JNEUROSCI.0117-17.2017 [PubMed: 28264982]
- Gangarossa G, Laffray S, Bourinet E, Valjent E. T-type calcium channel Cav3.2 deficient mice show elevated anxiety, impaired memory and reduced sensitivity to psychostimulants. *Front Behav Neurosci*. 2014; 8:92.doi: 10.3389/fnbeh.2014.00092 [PubMed: 24672455]
- Grace AA. Regulation of spontaneous activity and oscillatory spike firing in rat midbrain dopamine neurons recorded in vitro. *Synapse*. 1991; 7:221–34. DOI: 10.1002/syn.890070307 [PubMed: 1882331]
- Hildebrand ME, Isope P, Miyazaki T, Nakaya T, Garcia E, Feltz A, Schneider T, Hescheler J, Kano M, Sakimura K, Watanabe M, Dieudonné S, Snutch TP. Functional coupling between mGluR1 and Cav3.1 T-type calcium channels contributes to parallel fiber-induced fast calcium signaling within Purkinje cell dendritic spines. *J Neurosci*. 2009; 29:9668–82. DOI: 10.1523/JNEUROSCI.0362-09.2009 [PubMed: 19657020]
- Iacovitti L, Wei X, Cai J, Kostuk EW, Lin R, Gorodinsky A, Roman P, Kusek G, Das SS, Dufour A, Martinez TN, Dave KD. The hTH-GFP Reporter Rat Model for the Study of Parkinson's Disease. *PLoS One*. 2014; 9:e113151.doi: 10.1371/journal.pone.0113151 [PubMed: 25462571]
- Johnson SW, North RA. Two types of neurone in the rat ventral tegmental area and their synaptic inputs. *J Physiol*. 1992; 450:455–68. [PubMed: 1331427]
- Joksimovic SM, Eggan P, Izumi Y, Joksimovic SL, Tesic V, Dietz RM, Orfila JE, DiGrucio MR, Herson PS, Jevtovic-Todorovic V, Zorumski CF, Todorovic SM. The role of T-type calcium channels in the subiculum: to burst or not to burst? *J Physiol*. 2017; doi: 10.1113/JP274565
- Joksovic PM, Nelson MT, Jevtovic-Todorovic V, Patel MK, Perez-Reyes E, Campbell KP, Chen CC, Todorovic SM. CaV3.2 is the major molecular substrate for redox regulation of T-type Ca²⁺ channels in the rat and mouse thalamus. *J Physiol*. 2006; 574:415–30. DOI: 10.1113/jphysiol.2006.110395 [PubMed: 16644797]
- Krabbe S, Duda J, Schiemann J, Poetschke C, Schneider G, Kandel ER, Liss B, Roeper J, Simpson EH. Increased dopamine D2 receptor activity in the striatum alters the firing pattern of dopamine neurons in the ventral tegmental area. *Proc Natl Acad Sci U S A*. 2015; 112:E1498–506. DOI: 10.1073/pnas.1500450112 [PubMed: 25675529]
- Licata SC, Freeman AY, Pierce-Bancroft AF, Pierce RC. Repeated stimulation of L-type calcium channels in the rat ventral tegmental area mimics the initiation of behavioral sensitization to cocaine. *Psychopharmacology (Berl)*. 2000; 152:110–8. [PubMed: 11041323]
- Lin D, Koob GF, Markou A. Differential effects of withdrawal from chronic amphetamine or fluoxetine administration on brain stimulation reward in the rat—interactions between the two drugs. *Psychopharmacology (Berl)*. 1999; 145:283–294. [PubMed: 10494577]

- Liu Y, Chen X. Cholinergic excitation of dopaminergic cells depends on sequential activation of protein kinase C and the L-type calcium channel in ventral tegmental area slices. *Brain Res.* 2008; 1245:41–51. DOI: 10.1016/j.brainres.2008.09.062 [PubMed: 18929546]
- Liu Y, Dore J, Chen X. Calcium Influx through L-type Channels Generates Protein Kinase M to Induce Burst Firing of Dopamine Cells in the Rat Ventral Tegmental Area. *J Biol Chem.* 2007; 282:8594–8603. DOI: 10.1074/jbc.M610230200 [PubMed: 17237234]
- Liu Y, Harding M, Pittman A, Dore J, Striessnig J, Rajadhyaksha A, Chen X. Cav1.2 and Cav1.3 L-type calcium channels regulate dopaminergic firing activity in the mouse ventral tegmental area. *J Neurophysiol.* 2014; 112:1119–30. DOI: 10.1152/jn.00757.2013 [PubMed: 24848473]
- Mathé JM, Nomikos GG, Blakeman KH, Svensson TH. Differential actions of dizocilpine (MK-801) on the mesolimbic and mesocortical dopamine systems: role of neuronal activity. *Neuropharmacology.* 1999; 38:121–8. [PubMed: 10193903]
- Mathé JM, Nomikos GG, Schilström B, Svensson TH. Non-NMDA excitatory amino acid receptors in the ventral tegmental area mediate systemic dizocilpine (MK-801) induced hyperlocomotion and dopamine release in the nucleus accumbens. *J Neurosci Res.* 1998; 51:583–592. DOI: 10.1002/(SICI)1097-4547(19980301)51:5<583::AID-JNR5>3.0.CO;2-B [PubMed: 9512002]
- Morales M, Margolis EB. Ventral tegmental area: cellular heterogeneity, connectivity and behaviour. *Nat Rev Neurosci.* 2017; 18:73–85. DOI: 10.1038/nrn.2016.165 [PubMed: 28053327]
- Mutti A, Aroni S, Fadda P, Padovani L, Mancini L, Collu R, Muntoni AL, Fattore L, Chiamulera C. The ketamine-like compound methoxetamine substitutes for ketamine in the self-administration paradigm and enhances mesolimbic dopaminergic transmission. *Psychopharmacology (Berl).* 2016; 233:2241–2251. DOI: 10.1007/s00213-016-4275-0 [PubMed: 27020786]
- Narayanan S, Willins D, Dalia A, Wallace L, Uretsky N. Role of dopaminergic mechanisms in the stimulatory effects of MK-801 injected into the ventral tegmental area and the nucleus accumbens. *Pharmacol Biochem Behav.* 1996; 54:565–73. [PubMed: 8743630]
- National Research Council. Guide for the care and use of laboratory animals. 8th. National Academies Press; Washington, D.C.: 2011.
- Negus SS, Miller LL. Intracranial Self-Stimulation to Evaluate Abuse Potential of Drugs. *Pharmacol Rev.* 2014; 66:869–917. DOI: 10.1124/pr.112.007419 [PubMed: 24973197]
- Paxinos, G., Franklin, KBJ. *The Mouse Brain in Stereotaxic Coordinates.* 2nd. Academic Press; San Diego: 2001.
- Perez-Reyes E. Molecular Physiology of Low-Voltage-Activated T-type Calcium Channels. *Physiol Rev.* 2003; 83:117–161. DOI: 10.1152/physrev.00018.2002 [PubMed: 12506128]
- Petrenko AB, Tsujita M, Kohno T, Sakimura K, Baba H. Mutation of alpha1G T-type Calcium Channels in Mice Does Not Change Anesthetic Requirements for Loss of the Righting Reflex and Minimum Alveolar Concentration but Delays the Onset of Anesthetic Induction. *Anesthesiology.* 2007; 106:1177–1185. DOI: 10.1097/01.anes.0000267601.09764.e6 [PubMed: 17525593]
- Philippart F, Destreel G, Merino-Sepúlveda P, Henny P, Engel D, Seutin V. Differential Somatic Ca²⁺ Channel Profile in Midbrain Dopaminergic Neurons. *J Neurosci.* 2016; 36:7234–45. [PubMed: 27383597]
- Poetschke C, Dragicevic E, Duda J, Benkert J, Dougalis A, DeZio R, Snutch TP, Striessnig J, Liss B. Compensatory T-type Ca²⁺ channel activity alters D2-autoreceptor responses of Substantia nigra dopamine neurons from Cav1.3 L-type Ca²⁺ channel KO mice. *Sci Rep.* 2015; 5:13688.doi: 10.1038/srep13688 [PubMed: 26381090]
- Sagheddu C, Aroni S, De Felice M, Lecca S, Luchicchi A, Melis M, Muntoni AL, Romano R, Palazzo E, Guida F, Maione S, Pistis M. Enhanced serotonin and mesolimbic dopamine transmissions in a rat model of neuropathic pain. *Neuropharmacology.* 2015; 97:383–393. DOI: 10.1016/j.neuropharm.2015.06.003 [PubMed: 26113399]
- Shipe WD, Barrow JC, Yang ZQ, Lindsley CW, Yang FV, Schlegel KAS, Shu Y, Rittle KE, Bock MG, Hartman GD, Tang C, Ballard JE, Kuo Y, Adarayan ED, Prueksaritanont T, Zrada MM, Uebele VN, Nuss CE, Connolly TM, Doran SM, Fox SV, Kraus RL, Marino MJ, Graufelds VK, Vargas HM, Bunting PB, Hasbun-Manning M, Evans RM, Koblan KS, Renger JJ. Design, synthesis, and evaluation of a novel 4-aminomethyl-4-fluoropiperidine as a T-type Ca²⁺ channel antagonist. *J Med Chem.* 2008; 51:3692–5. DOI: 10.1021/jm800419w [PubMed: 18540666]

- Talley EM, Cribbs LL, Lee JH, Daud A, Perez-Reyes E, Bayliss DA. Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. *J Neurosci*. 1999; 19:1895–911. [PubMed: 10066243]
- Tateno T, Robinson HPC. The mechanism of ethanol action on midbrain dopaminergic neuron firing: a dynamic-clamp study of the role of I(h) and GABAergic synaptic integration. *J Neurophysiol*. 2011; 106:1901–22. DOI: 10.1152/jn.00162.2011 [PubMed: 21697445]
- Todorovic SM, Lingle CJ. Pharmacological properties of T-type Ca²⁺ current in adult rat sensory neurons: effects of anticonvulsant and anesthetic agents. *J Neurophysiol*. 1998; 79:240–52. [PubMed: 9425195]
- Tricklebank MD, Singh L, Oles RJ, Preston C, Iversen SD. The behavioural effects of MK-801: a comparison with antagonists acting non-competitively and competitively at the NMDA receptor. *Eur J Pharmacol*. 1989; 167:127–35. [PubMed: 2550253]
- Uslaner JM, Smith SM, Huszar SL, Pachmerhiwala R, Hinchliffe RM, Vardigan JD, Nguyen SJ, Surles NO, Yao L, Barrow JC, Uebele VN, Renger JJ, Clark J, Hutson PH. T-type calcium channel antagonism produces antipsychotic-like effects and reduces stimulant-induced glutamate release in the nucleus accumbens of rats. *Neuropharmacology*. 2012; 62doi: 10.1016/j.neuropharm.2010.11.015
- Uslaner JM, Vardigan JD, Drott JM, Uebele VN, Renger JJ, Lee A, Li Z, Lê AD, Hutson PH. T-Type Calcium Channel Antagonism Decreases Motivation for Nicotine and Blocks Nicotine- and Cue-Induced Reinstatement for a Response Previously Reinforced with Nicotine. *Biol Psychiatry*. 2010; 68:712–718. DOI: 10.1016/j.biopsych.2010.05.004 [PubMed: 20579972]

Highlights

1. Subpopulation of neurons of the ventral tegmental area (VTA) express prominent T-type calcium currents.
2. T-currents amplitudes and kinetics were similar in wild-type mice and wild-type rats but were abolished in $\text{Ca}_v3.1$ knock-out mice.
3. T-channels support rebound burst firing in a subset of both dopaminergic and non-dopaminergic VTA neurons.
4. Pan-selective T-channel blocker TTA-P2 completely inhibited rebound bursting in VTA neurons.
5. MK-801 increased locomotor activity in WT but not in $\text{Ca}_v3.1$ KO mice, demonstrating a crucial role of the $\text{Ca}_v3.1$ isoform of T-type channels in mediating behavioral effects of an NMDA antagonist.
6. Pharmacological targeting of $\text{Ca}_v3.1$ isoform of T-channels may be a novel therapeutic approach for the disorders of the mesolimbic reward system.

Figure 1. Rebound burst firing in a subpopulation of VTA neurons in WT rats

(A) Depiction of two current injection traces of 50 pA (red trace) and 100 pA (black trace) from the dual step protocol utilized to elicit depolarization-induced firing mode and after-hyperpolarization-induced rebound spiking. The procedure begins with an injection of positive current at successive steps for 200 ms, followed by a current injection of negative current for 500 ms to attempt to elicit a series of rebound spiking. (B) Representative traces from the dual step protocol of a neuron in the VTA which produces depolarization-induced firing and hyperpolarization-induced rebound spiking. (C) Representative trace from another neuron that fired only after injections of depolarizing currents, but did not produce hyperpolarization-induced rebound spiking. Recorded neurons in B and C were labeled with biocytin (b and c, respectively), which was included in the recording pipette (scale bar = 15 microns).

Figure 2. Selective inhibition of T-channels abolished rebound bursting in rat VTA neurons (A) The stimulus waveform for the dual step protocol (top panel) overlapped with baseline control traces (middle panel) and ten minutes after application of 5 μ M of TTA-P2 in the same neuron (bottom panel). A barrage of depolarization-induced APs and prominent hyperpolarization-induced rebound bursting can be seen. Very little interaction in depolarization-induced firing of APs is evident 10 min after exposure to the specific T-type channel blocker, however the rebound spikes are completely ablated. (B–D) Aggregation of eleven observations following exposure to TTA-P2 from seven WT rats were averaged and compared to baseline control for statistical significance. (B) TTA-P2 (red filled square symbols) produced a significant interaction in hyperpolarization-induced rebound spiking [$F(10, 100) = 8.875$, $***p=0.001$ (Two-way mixed-design ANOVA)] resulting in decreased spiking from 30–100% of maximal current intensity when compared to baseline controls (open black circle symbols). (C) TTA-P2 failed to produce a significant change in the depolarization-induced firing frequency in the same neurons. (D) Application of TTA-P2 produced a significant interaction when compared to baseline control (open circles) [$F(20, 180) = 3.008$, $***p=0.001$] resulting in an increase in input resistance at 10–100% of maximal current at the both 5 min (red squares) and 10 min (black triangles) time points.

Figure 3. Characterization of voltage-dependent activation kinetics of T-currents in rat VTA neurons

(A) Voltage-clamp stimulus waveform and representative current traces (V_h -90 mV, V_t -80 through -20 mV) for the current-voltage activation protocol in WT rats. (B) Analysis of peak current for each trace across subjects was aggregated and normalized to determine an estimated V_{50} of -59.6 ± 0.4 mV and slope factor of 4.3 ± 0.3 mV for the voltage-dependent activation of well isolated T-currents. Individual data points are in 2.5 mV incremental steps and are averages from multiple determinations. Red solid line on the graph is best fit of data points using Boltzmann equation. (C) The same data points from graph B plotted as current density (peak current amplitude divided by cell's capacitance) against test potential for the current-voltage activation protocol. (D) Inactivation time constants at different points ranging from -55 to -20 mV plotted against test potential from the current-voltage relationship from the same cells presented on panel B of this figure. Note that inactivation tau becomes progressively faster with stronger depolarizing steps and becomes largely voltage-independent at more positive potentials.

Figure 4. Characterization of voltage-dependent inactivation kinetics of T-currents in rat VTA neurons

(A) Voltage-clamp stimulus waveform and representative current traces for the voltage-dependent inactivation protocol. (B) Analysis of peak current for each trace across subjects was aggregated and normalized to determine a V_{50} of -94.4 ± 0.6 mV and a slope factor of 5.8 ± 0.6 mV for the voltage-dependent steady-state inactivation in rat VTA neurons. Individual data points are in 5 mV incremental steps and are averages from multiple determinations. Red solid line on the graph is best fit of data points using Boltzmann equation. (C) Normalized current and conductance plotted against test potential for both the inactivation (black) and activation (red) protocol, respectively show the overlapping region, termed T-type “window” current.

Figure 5. Diminished T-current amplitudes in Cav3.1 knock-out (KO) mice when compared to wild-type mice

(A) Representation of the voltage-clamp stimulus waveform for the single step activation protocol. (B) Representative sweeps from traces of neurons in the VTA for a wild-type (black trace) and Cav3.1 KO mouse (red trace). (C) Aggregation of T-current peaks across 10 observations of wild-type mice from 4 animals and 10 observations of Cav3.1 KO mice from 3 animals were averaged and compared for statistical significance; t-test, two-tailed. Current amplitudes were greatly diminished in KO mice (3.7 ± 1.7 pA) when compared to WT mice (91.3 ± 15.6 pA) (***, $p < 0.001$).

Figure 6. Immunohistochemistry of VTA neurons from TH-eGFP transgenic rats

(A) Double immunofluorescence image of a brain slice showing co-localization of tyrosine-hydroxylase-eGFP positive neurons (green) and NeuN positive neurons (red) in the VTA. **(B)** Magnified images of the white square in (A). Upper panel shows TH-eGFP positive neurons (green), bottom panel with NeuN positive neurons (red) and the middle panel shows merged image. Red nuclei lacking a green signal demonstrates not all of the neurons in VTA are dopaminergic. Scale bar = 150 μ m.

Figure 7. Comparison of depolarization-induced and rebound burst firing properties in TH-positive and TH-negative VTA neurons of transgenic TH-eGFP knock-in rat

(A) Representative sweep of current injection protocol used to study AP firing patterns in VTA neurons (top panel). Family of sweeps from traces from a TH-positive (TH-POS, green) and TH-negative (TH-NEG, red) neuron, which both produced depolarization-induced firing of APs and rebound burst firing, are depicted in the middle and bottom panel, respectively. (B) The input-output curve as a function of the number of rebound APs presented as the percentage of maximal current injection between TH-POS and TH-NEG neurons. TH-NEG neurons fired significantly more APs than TH-POS for the 70%, 90%, and 100% data points ($p < 0.05$). Two-way ANOVA showed borderline interaction $p=0.092$

and treatment $p=0.057$. Hence, we performed multiple comparisons (Benjamini, Krieger and Yekutieli method, Graph-Pad Prism) which showed significant $p = 0.015$ at 70%, $p = 0.001$ at 90% and $p = 0.019$ at 100% normalized current injections. **(C)** However, for the initial depolarizing phase there was no statistical difference in the maximal firing frequency between TH-POS and TH-NEG neurons. **(D)** TTA-P2 at 5 μM (gray trace) completely inhibited one rebound AP present at control baseline (green trace) in this TH-POS neuron (the same neuron shown in the middle of panel A). **(E)** TTA-P2 at 5 μM (gray trace) completely inhibited two rebound APs present at control baseline (red trace) in this TH-NEG neuron (the same neuron shown in the bottom of panel A).

Figure 8. MK-801-induced hyperlocomotion

(A) The effects of $\text{Ca}_v3.1$ T-channels on hyperlocomotion induced by 0.1 mg/kg MK-801. The baseline locomotor activity and activity after saline injection was not significantly different between WT and $\text{Ca}_v3.1$ KO mice (two-way mixed-design ANOVA). When monitoring the mice for an hour immediately after MK-801 injection (5 min time-bins), WT but not $\text{Ca}_v3.1$ KO mice exhibited a dramatic increase in locomotor activity. (B) Bar graphs show a significant decrease in the total moved distance in $\text{Ca}_v3.1$ KO, as compared to WT mice, following MK-801 challenge. * $p < 0.05$

Table 1

Active and passive membrane properties of TH-positive neurons (TH-POS) compared to TH-negative neurons (TH-NEG) in the VTA of TH-eGFP knock-in rats

Membrane property	Neuron Type		TH-POS vs TH-NEG p-value
	TH-POS ^a SEM ± Mean	TH-NEG ^b SEM ± Mean	
Membrane potential (mV)	-50.87 ± 1.03	-51.56 ± 0.76	0.588 ^c
Input resistance (MΩ)	443.76 ± 63.39	636.17 ± 91.47	0.233 ^d
Rheobase (pA)	228.30 ± 42.73	135.90 ± 18.67	0.018 ^c
Latency to spike (msec)	38.23 ± 10.64	195.15 ± 78.34	0.132 ^d
Threshold hyperpolarizing potential (mV)	-85.70 ± 11.35	-70.51 ± 10.78	0.295 ^c

^aTH-POS = 12 neurons, 12 TH-eGFP rats.

^bTH-NEG = 17 neurons, 11 TH-eGFP rats.

All data are grouped by neurons and are presented as means of multiple determinations. Data are compared using unpaired two-tailed t-test^c or Mann-Whitney U-test^d.