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Hypocretin / Orexin Receptor 1 Knockdown in GABA or Dopamine Neurons in the Ventral Tegmental Area Differentially Impact Mesolimbic Dopamine and Motivation for Cocaine

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Abstract

The hypocretins/orexins (HCRT) have been demonstrated to influence motivation for cocaine through actions on dopamine (DA) transmission. Pharmacological or genetic disruption of the hypocretin receptor 1 (Hcrtr1) reduces cocaine self-administration, blocks reinstatement of cocaine seeking, and decreases conditioned place preference for cocaine. These effects are likely mediated through actions in the ventral tegmental area (VTA) and resulting alterations in DA transmission. For example, HCRT drives VTA DA neuron activity and enhances the effects of cocaine on DA transmission, while disrupting Hcrtr1 attenuates DA responses to cocaine. These findings have led to the perspective that HCRT exerts its effects through Hcrtr1 actions in VTA DA neurons. However, this assumption is complicated by the observation that Hcrtr1 are present on both DA and GABA neurons in the VTA and HCRT drives the activity of both neuronal populations. To address this issue, we selectively knocked down Hcrtr1 on either DA or GABA neurons in the VTA and examined alterations in DA transmission and cocaine self-administration in female and male rats. We found that Hcrtr1 knockdown in DA neurons decreased DA responses to cocaine, increased days to acquire cocaine self-administration, and reduced motivation for cocaine. Although, Hcrtr1 knockdown in GABA neurons enhanced DA responses to cocaine, this manipulation did not affect cocaine self-administration. These observations indicate that while Hcrtr1 on DA versus GABA neurons exert opposing effects on DA transmission, only Hcrtr1

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Supplementary materials

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

on DA neurons affected acquisition or motivation for cocaine – suggesting a complex interplay between DA transmission and behavior.

Keywords

Addiction; Voltammetry; Mesolimbic; Motivation; Nucleus accumbens; mRNA

1. Introduction

The hypocretin/orexin (HCRT) system is known to influence reward and reinforcement processes across various drugs of abuse [1–12]. For example, hypocretin receptor 1 (Hcrtr1) antagonists reduce conditioned place preference for morphine [13], alcohol [14], and cocaine [9, 11], and reduce self-administration of cocaine on high effort schedules of reinforcement [1, 3, 5, 7, 10]. These effects of HCRT manipulations are consistent with the effects of genetic disruption of HCRT signaling, as HCRT peptide knockout mice display a decrease in preference for morphine [13] and cocaine [15]. Additionally, animals with Hcrtr1 knockout demonstrate decreases in cocaine [16] and synthetic cannabinoid self-administration [17], while those with Hcrtr1 knockdown display decreases in motivation for cocaine [18]. When considered together, these observations indicate that the HCRT system – especially through actions at Hcrtr1 – contributes to reward and reinforcement processes.

The effects of HCRT manipulations on drug-associated behaviors appear to be mediated, in part, through actions on the mesolimbic dopamine (DA) system. HCRT neurons have robust projections to the ventral tegmental area (VTA) [19] where HCRT acts through both Hcrtr1 and HCRT receptor 2 to modulate excitatory drive on DA neurons [20–24]. Consistent with this, intra-VTA infusions of HCRT-1 peptide increase DA in the nucleus accumbens (NAc) core at baseline [13, 22] and promote DA responses to cocaine [5]. By comparison, intra-VTA infusions of the Hcrtr1 antagonist, SB-334867, decrease excitation of DA neurons [21, 25], reduce baseline DA signaling in the NAc [5], and disrupt DA responses to cocaine [5, 10]. Genetic manipulations of the HCRT system also reveal the importance of HCRT peptide and receptors on DA transmission. For example, HCRT peptide knockout mice display disrupted DA signaling in the NAc at baseline and in response to cocaine [15] while rats with Hcrtr1 knockdown in the VTA display reduced baseline and cocaine-induced DA transmission in the NAc [18].

Hypocretin receptor 1 modulation of DA transmission is often assumed to occur through direct actions on VTA DA neurons. However, this assumption is complicated by the observation that the VTA contains a heterogeneous population of neurons, including DA, GABA, and glutamate neurons, as well as neurons that co-release more than one of these transmitters [26–38]. The two largest neuronal populations in the VTA are DA neurons containing tyrosine hydroxylase (TH) [28] and GABA neurons containing glutamate decarboxylase (GAD), comprising 55% and 23–35% of the VTA population, respectively [30, 32]. Both DA and GABA neurons in the VTA send projections to the NAc [34, 39–42] and express Hcrtr1 [20]. As such, previous studies employing non-selective pharmacological

or genetic approaches have not allowed for consideration of Hcrtr1 modulations on DA versus GABA neurons.

To address this gap in knowledge, we employed a combinatorial viral approach to selectively knockdown Hcrtr1 on either DA or GABA neurons of the VTA. With this specificity, we evaluated the effects of Hcrtr1 knockdown on baseline and cocaine-induced DA dynamics in the NAc. Additionally, we examined whether Hcrtr1 knockdown on DA or GABA neurons altered motivation to self-administer cocaine.

2. Methods

2.1. Animals

Female (200–250g) and male (300–350g) Sprague-Dawley rats (Envigo, Frederick MD) were housed on a reverse light/dark cycle and given ad-libitum access to food and water. Rats were pair-housed prior to receiving virus infusions, after which they were individually housed for the remainder of the study.

2.2. Preparation of Viral Vectors

We utilized AAVs to knock down Hcrtr1 mRNA specifically in DA or GABA neurons. An AAV expressing Cre recombinase under the TH promotor (AAV-TH-Cre) [43] was used to target Cre recombinase expression selectively in DA neurons while an AAV expressing Cre recombinase under the GAD1 promotor (AAV-GAD1-Cre) [44] was used to target Cre recombinase expression selectively in GABA neurons. The AAV-TH-Cre contains a human beta-globin (hBG) intron used to enhance gene expression [45]. While the AAV-GAD1-Cre contains a combined SV40 intron/polyA termination sequence. To knock down Hcrtr1, we developed novel AAVs expressing Cre-dependent shRNAs (AAV-SICO-Hcrtr1shRNA) through incorporation of a pSico plasmid promotor [46] and driven by the mouse U6 promotor. By designing these viruses in a Cre-dependent fashion, we ensured that shRNA expression would only occur in TH or GAD containing neurons, depending on what viral combination was used. The Cre dependent shRNA expression occurs when a CMV-EGFP stop cassette is excised by the activity of Cre at flanking TATA lox sites, bringing the shRNA sequence close to and in alignment with the U6 promoter. In the absence of Cre, the loxP sites will not recombine and the shRNA will not be expressed. However, EGFP will be expressed from the CMV-EGFP cassette, thus the EGFP signal allows for visualization of cells that do not express the shRNA. With this method, we were able to target neurons in a highly selective manner [47] and identify virus infusion placements. We used a cocktail of 3 viruses containing unique shRNAs to knockdown Hcrtr1 in combination with either the AAV-TH-Cre or AAV-GAD1-Cre virus. Each shRNA was designed to target a specific portion of Hcrtr1 mRNA (target sequences: TGGTGCGGAACTGGAAGCGA, TGGCGCGATTATCTCTATCCG, and TAGCCAATCGCACACGGCTCT). The combination of the 3 shRNAs in the Hcrtr1 knockdown condition was used because previous research suggests that such an approach can produce a stronger cumulative effect than using one target alone [48]. Further, the efficiency of Hcrtr1 mRNA knockdown in the VTA was demonstrated in a previous finding, although this was not selective for DA or GABA neurons [18]. As viral controls,

we designed a scramble virus (AAV-SICO-SCRM) that does not target any known RNA sequence in the rat genome, and delivered the scramble virus in combination with either the AAV-TH-Cre or AAV-GAD1-Cre. All viruses were packaged using the triple transfection protocol to generate pseudotyped AAV2/10 viruses, in which the AAV2 replicase was used to package the recombinant viral genome into AAV10 capsids [49].

2.3. Virus Infusion

Rats were anesthetized with isoflurane, received ketoprofen (5mg/kg, s.c.) and baytril (5mg/kg, s.c.), and were then placed into a stereotaxic frame, with the skull flat. To target the VTA, a 1 mm hole was drilled in the skull (−5.3 mm A/P, ± 1.0–0.9 mm M/L relative to bregma) and an infusion needle was lowered into the VTA, reaching a final depth of 7.6–7.8 mm ventral to the brain surface. This region of the VTA has previously been shown to express moderate levels of Hcrtr1 [24, 50] and sensitivity to Hcrtr1 manipulations [5, 18, 51]. A total of 0.5 μl of the AAV-TH-Cre or AAV-GAD1-Cre viruses combined with the AAV-SICO-Hcrtr1shRNA or AAV-SICO-SCRM viruses in a 1:3 ratio, respectively, was injected into each hemisphere of the VTA over 10 min using an infusion pump. The infusion needle remained in place for another 10 min following completion of the infusion to allow the virus to diffuse. Rats again received ketoprofen (5mg/kg, s.c.) and baytril (5mg/kg, s.c.) 12–24 hr after surgery. Four cohorts of rats were tested for PCR, fast scan cyclic voltammetry, western blotting, and behavior.

2.4. Quantitative Real-Time PCR (qRT-PCR)

Two weeks after virus infusion, rats were anesthetized with isoflurane and rapidly decapitated. Brains were extracted over ice and placed into a brain matrix slicing guide with the ventral surface visible. Two coronal cuts were made, with the rostral cut at the caudal portion of the mammillary bodies, and the second cut 2.0 mm caudal to the first. This yielded a single brain slice (−4.4 to −6.4 mm A/P relative to bregma) which encompassed most of the VTA. The VTA was dissected out as the area ventral to the mesencephalic reticular nucleus, and medial to the substantia nigra. Immediately following dissection, the tissue was placed in 75 μl of RNAlater (Qiagen Inc., Valenia, CA) and stored at −20°C until processing.

As previously described [18, 52], total RNA from each brain sample was extracted using an RNeasy Mini Kit (Qiagen Inc.) and DNA was removed using RNase-free DNase 1 (Qiagen Inc.). The yield was quantified with a NanoDrop Lite spectrophotometer (Thermo Electron North America LLC, Madison, WI) with high purity indicated by A_{260}/A_{280} ratios between 1.88 and 2.19. The cDNA was reverse transcribed using SuperScript® VILO™ Master Mix (Invitrogen, Grand Island, NY) in a SimpliAmp™ Thermal Cycler (Applied Biosystems, Waltham, MA), using 1 μg of RNA from each sample. The qRT-PCR used a SYBR Green PCR core reagents kit (Applied Biosystems, Grand Island, NY), with 12.5 ng of cDNA template in a 25 μl reaction volume in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosystems). A StepOnePlus Real-Time PCR System (Applied Biosystems) was used to carry out the reaction, which used the conditions of 2 min at 50°C (primer annealing), 10 min at 95°C (polymerase activation and sequence extension), and 40 cycles of 15 sec at 95°C (denaturation), plus 1 min at 60°C (annealing and extension). Each

sample was run in triplicate. Expression of Hcrtr1 was quantified relative to cyclophilin-A with the relative purification method ($\Delta\Delta C_T$). Primers for Hcrtr1 and cyclophilin-A that were previously validated [18] and purchased from Invitrogen at ThermoFisher Scientific (Grand Island, NY) were used for qRT-PCR. In all cases, rats with Hcrtr1 knockdown in DA neurons were compared with TH-specific scramble controls, while those with Hcrtr1 knockdown in GABA neurons were compared with GAD-specific scramble controls.

2.5. Fast Scan Cyclic Voltammetry

Two weeks after virus infusion, rats were anesthetized with 2.5% isoflurane for 5 min and decapitated. Previous studies have shown that these anesthesia parameters have no effect on DA uptake [3]. Brains were rapidly removed and transferred to oxygenated, ice-cold artificial cerebral spinal fluid (aCSF) containing NaCl (126 mM), KCl (2.5 mM), NaH_2PO_4 (1.2 mM), $CaCl_2$ (2.4 mM), $MgCl_2$ (1.2 mM), $NaHCO_3$ (25 mM), glucose (11 mM), L-ascorbic acid (0.4 mM), and pH adjusted to 7.4. The portion of the brain containing the VTA was removed for later slicing and virus placement confirmation (Supplemental Fig. 1). A vibrating microtome was used to produce 400 μm sections containing the NAc core. Sections were then dissected into two slices, each containing one hemisphere, and slices were kept at room temperature for 1 hr before being transferred into a recording chamber flushed with aCSF (32°C).

A bipolar stimulating electrode (Plastics One, Roanoke, VA) was placed on the surface of the tissue and a carbon fiber microelectrode was implanted between the two stimulating electrode leads. Dopamine release was evoked with a single electrical pulse every 3 min (~400 μA, 4 ms, monophasic) and measured using Demon Voltammetry and Analysis software [53]. Given that each hemisphere of the brain received a distinct virus infusion, slices containing the NAc core from each hemisphere were treated as separate data points. Stable baseline DA release and uptake recordings were obtained from each slice (3 stimulations with < 10% variation). To examine DA responses to cocaine, the same NAc slices were superfused with 5 increasing concentrations of cocaine (0.3 – 30 μM) as previously described [3, 54–56].

Dopamine concentrations were calculated by comparing currents at peak oxidation potential for DA with electrode calibrations determined using an *in-situ* calibration method described previously [55, 57–59]. To determine if Hcrtr1 knockdown in DA or GABA neurons influenced DA transmission, we assessed stimulated DA release, DA uptake rate (V_{max}), and cocaine-induced DA uptake inhibition (app K_m) using a Michaelis-Menton based model [54, 56]. Baseline uptake was determined by setting K_m values to 0.2 μM while all cocaine-induced alterations in uptake were attributed to changes in apparent K_m.

2.6. Western Blotting

Two weeks after virus infusion, a separate cohort of rats was anesthetized with isoflurane and rapidly decapitated. Following decapitation, the NAc was dissected and stored at −80°C until preparation. Synaptosomes were prepared, and previously published procedures were modified for performance of membrane fractionation [58–60]. Tissue homogenization occurred in ice-cold lysis buffer (1000ml, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 320 mM

sucrose) with 1x protease inhibitor cocktail, 1x phosphatase inhibitor cocktail, and 1 mM PMSF. The homogenate was centrifuged at 1,000x g for 5 min at 4°C and the extracted supernatant was recentrifuged at 10,000x g for 20 min at 4°C. The resulting synaptosomal pellet was then resuspended with 300ml lysis buffer for Western blot experiments. Immunoblotting was performed with rabbit anti dopamine transporter (DAT) polyclonal antibody (1:1000, AB2231, EMD Millipore Corp, RRID:AB_1586991), rabbit anti phospho-DAT polyclonal antibody (1:1000, P435–53, PhosphoSolutions, RRID:AB_2492078), and peroxidase-conjugated goat anti-rabbit IgG (H1 L) (1:5000, Jackson Immuno Research, RRID:AB_2313586). The membrane protein control, GAPDH, was determined with rabbit anti-GAPDH polyclonal antibody (1:5000, PA1–987, Invitrogen, RRID: AB_2107311). Total DAT (tDAT), phosphorylated DAT at threonine53 (pDAT), and GAPDH immunoblots were quantified by densitometry with ImageQuant LAS4000 (GE Healthcare Bio-Sciences). Data were analyzed and presented as a ratio of each protein of interest to GAPDH, as previously reported [58, 60].

2.7. Cocaine Self-Administration

At the time of virus infusion surgery, a separate cohort of rats was also implanted with intravenous silastic jugular catheters as previously described [3, 5, 10]. One week after surgery, rats were placed in operant behavioral chambers where they were housed for the duration of the experiment. Intravenous catheters were connected through a stainless-steel spring to a counter balanced swivel (Instech Laboratories, Plymouth Meeting, PA, USA). Rats were placed on a fixed ratio 1 (FR1) schedule of reinforcement wherein a single active lever response resulted in a 0.75 mg/kg infusion of cocaine (in saline; National Institute on Drug Abuse), retraction of the levers, illumination of a cue-light, and a 20 sec time out period. Presses on the inactive lever were without consequence. Rats were given up to 6 hr of daily access to the levers with a maximum of 20 injections per session, before the session was terminated. Acquisition of self-administration behavior was defined as the first day of three consecutive days in which rats reached 20 injections of cocaine in a single session. After acquiring, rats remained on the FR1 schedule until reaching stable intake (< 20% decrease in intake rate) for at least 3 sessions of 20 injections per session. After meeting these criteria, rats were switched to a progressive ratio (PR) schedule of reinforcement, during which rats had 6 hr access to levers and single 0.75 mg/kg cocaine delivery was dependent on an exponentially increasing number of lever responses [61]. Completing the required number of lever presses resulted in cocaine delivery, retraction of the levers, illumination of the cue-light, and a 20 sec time out period. Presses on the inactive lever were without consequence. The 0.75 mg/kg dose was selected for comparison with previous observations [3, 5, 10, 18]. The average breakpoint was recorded for 3 days. Brains were collected following completion of self-administration experiments and sections containing the VTA were sliced and examined for confirmation of virus infusion placement (Supplemental Fig. 2).

Importantly, all rats began cocaine self-administration on the FR1 schedule after 1 week of virus injection, which differs from the 2-week virus incubation period used for the qRT-PCR, voltammetry, and western blotting experiments. This experimental design was intentional, as we needed to examine whether Hcrtr1 knockdown impacted high-effort

motivation to self-administer cocaine on the PR schedule. Therefore, for the 2-week virus incubation period to approximately coincide with the beginning of the PR experiments, rats were allowed to obtain the necessary training for cocaine self-administration on the FR1 schedule after just one week of virus injection.

2.8. Statistical Analysis

Analyses to detect sex differences are presented in Supplemental Tables 1 and 2. We did not observe any interactions between sex and virus group (KD or SCRM), indicating that the effects of Hcrtr1 knockdown were not dependent on sex. Therefore, female and male data were combined, as recommended by previous studies [62, 63]. We did not conduct analyses for sex differences for the western blot studies due to low sample sizes.

Experiments were designed to enable statistical comparison between the effects of the knockdown and scramble treatments within either the TH- or GAD-specific groups. As such, animals with TH- and GAD-specific manipulations comprise distinct cohorts and could be statistically compared. Therefore, for all experiments, data for AAV-TH-Cre and AAV-GAD1-Cre were analyzed separately. To assess the effects of Hcrtr1 knockdown on VTA mRNA levels, DA release and uptake, days to acquire, breakpoints, tDAT, and pDAT we compared groups using Student's *t*-tests. To examine the impact of Hcrtr1 knockdown on the effects of cocaine on DA transmission, we used two-way repeated measures ANOVAs with virus as the between subjects variable and cocaine concentration as the within subjects variable. When sphericity was violated, the Greenhouse-Geisser correction was applied. Data were analyzed using GraphPad Prism 9.1.1 (San Diego, CA).

3. Results

3.1. Combinatorial viral approach reduced Hcrtr1 mRNA

To assess the effectiveness of the combinatorial virus approach on Hcrtr1 knockdown in DA neurons, rats were injected intra- VTA with AAV-TH-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 12$) or AAV-SICO-SCRM ($n = 11$). After two weeks of viral incubation, AAV-SICO-Hcrtr1shRNA resulted in significant knockdown of VTA Hcrtr1 mRNA by approximately one third compared to AAV-SICO-SCRM levels ($t_{(21)} = 2.687$, $p < 0.05$; Fig. 1). To assess the effectiveness of Hcrtr1 knockdown in GABA neurons, rats were injected intra- VTA with AAV-GAD1-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 10$) or AAV-SICO-SCRM ($n = 10$). After two weeks of viral incubation, AAV-SICO-Hcrtr1shRNA resulted in significant knockdown of VTA Hcrtr1 by approximately one third compared to AAV-SICO-SCRM levels ($t_{(18)} = 3.662$, $p < 0.01$; Fig. 1).

3.2. Hcrtr1 knockdown in DA neurons reduced baseline DA uptake rate and attenuated DA responses to cocaine

To assess the effects of Hcrtr1 knockdown in DA neurons on DA transmission in the NAc core, rats were injected intra-VTA with AAV-TH-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 12$) or AAV-SICO-SCRM ($n = 11$). Two weeks after virus infusion, we examined DA transmission using fast scan cyclic voltammetry in brain slices containing the NAc core. We observed that Hcrtr1 knockdown did not affect stimulated DA release ($t_{(21)} =$

1.251, $p \leq 0.2246$), but it significantly decreased maximal DA uptake rate ($t_{(21)} = 4.123$, $p < 0.01$; Fig. 2). After assessing baseline release and uptake, we examined the effect of Hcrtr1 knockdown on DA responses to increasing concentrations of cocaine (0.3 – 30 μM) in the same NAc slices. A two-way repeated measures ANOVA with virus as the between subjects variable and cocaine as the within subjects variable revealed a significant effect of cocaine concentration ($F_{(1.887, 39.63)} = 43.411$, $p < 0.0001$), but no effect of virus ($F_{(1,21)} = 0.002352$, $p \leq 0.9618$) or a virus x cocaine interaction ($F_{(4, 84)} = 0.1720$, $p \leq 0.9521$) on DA release. A two-way repeated measures ANOVA with virus as the between subjects variable and cocaine as the within subjects variable revealed a significant effect of virus ($F_{(1, 21)} = 14.47$, $p < 0.05$), cocaine concentration ($F_{(1.092, 22.93)} = 185.1$, $p < 0.0001$), and a significant virus x cocaine interaction ($F_{(4, 84)} = 7.899$, $p < 0.0001$) on inhibition of DA uptake. Holm-Bonferroni post hoc analysis revealed that cocaine was significantly less effective at inhibiting DA uptake in rats with Hcrtr1 knockdown at 0.3 μM cocaine ($p \leq 0.001$), 1.0 μM cocaine ($p < 0.0001$), 3.0 μM cocaine ($p < 0.0001$), 10 μM cocaine ($p < 0.01$), and 30 μM cocaine ($p < 0.01$) compared to scramble controls (Fig. 3).

3.3. Hcrtr1 knockdown in GABA neurons increased baseline DA uptake rate and enhanced DA responses to cocaine

To assess the effects of Hcrtr1 knockdown in GABA neurons on DA transmission in the NAc core, rats were injected intra-VTA with AAV-GAD1-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 11$) or AAV-SICO-SCRM ($n = 9$). Two weeks after virus infusion, we observed no significant effect of Hcrtr1 knockdown on stimulated DA release ($t_{(18)} = 0.06551$, $p \leq 0.9485$), but Hcrtr1 knockdown significantly increased maximal DA uptake rate ($t_{(18)} = 2.259$, $p < 0.05$; Fig. 4). After assessing baseline release and uptake, we examined the effect of Hcrtr1 knockdown on DA responses to increasing concentrations of cocaine (0.3 – 30 μM) in the same NAc slices. A two-way repeated measures ANOVA with virus as the between subjects variable and cocaine as the within subjects variable revealed a significant effect of cocaine concentration ($F_{(1.754, 32.06)} = 45.63$, $p < 0.0001$), a virus x cocaine interaction significant only at the 0.0602 level ($F_{(4, 72)} = 2.373$, $p \leq 0.0602$), and no effect of virus ($F_{(1, 18)} = 1.596$, $p \leq 0.2226$) on DA release. A two-way repeated measures ANOVA with virus as the between subjects variable and cocaine as the within subjects variable revealed a significant effect of virus ($F_{(1, 18)} = 9.950$, $p < 0.01$), cocaine concentration ($F_{(1.481, 26.65)} = 127.8$, $p < 0.0001$), and a significant virus x cocaine interaction ($F_{(4, 72)} = 7.426$, $p < 0.0001$) on inhibition of DA uptake. Holm-Bonferroni post hoc analysis revealed that cocaine was significantly more effective at inhibiting DA uptake in rats with Hcrtr1 knockdown at 0.3 μM cocaine ($p < 0.05$), 1.0 μM cocaine ($p < 0.05$), 10 μM cocaine ($p < 0.041$), and 30 μM cocaine ($p < 0.033$) compared to scramble controls (Fig. 5).

3.4. Hcrtr1 knockdown in DA neurons did not affect DAT expression or phosphorylation

Our previous work indicates that acute blockade of Hcrtr1 reduces DA uptake rate and attenuates DA responses to cocaine [5, 7, 10]. Further, we have also demonstrated that these effects may be linked to changes in DAT expression or phosphorylation [60]. To examine whether the effects of Hcrtr1 knockdown in DA neurons on DA transmission were associated with changes in total DAT (tDAT) or phosphorylated DAT at the threonine

53 site (pDAT), rats were injected intra-VTA with AAV-TH-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 6$) or AAV-SICO-SCRM ($n = 6$). Two weeks after virus infusion, we performed western blotting on NAc tissue. As shown in Fig. 6, we observed that Hcrtr1 knockdown on DA neurons had no effect on tDAT ($t_{(10)} = 0.248$, $p < 0.809$), or pDAT expression ($t_{(10)} = 0.026$, $p < 0.979$) relative to scramble controls.

3.5. Hcrtr1 knockdown in GABA neurons did not affect DAT expression or phosphorylation

To assess the effects of Hcrtr1 knockdown in GABA neurons on DAT expression and phosphorylation rats were injected intra-VTA with AAV-GAD1-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 6$) or AAV-SICO-SCRM ($n = 6$). Two weeks after virus infusion, we observed that Hcrtr1 knockdown on GABA neurons had no effect on either tDAT ($t_{(10)} = 0.342$, $p < 0.74$) or pDAT ($t_{(10)} = 0.665$, $p < 0.52$) expression in the NAc relative to scramble controls (Fig. 6).

3.6. Hcrtr1 knockdown in DA neurons impacted cocaine self-administration

To assess the effect of Hcrtr1 knockdown in VTA DA neurons on cocaine self-administration, rats were implanted with a jugular catheter and injected intra-VTA with AAV-TH-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 11$) or AAV-SICO-SCRM ($n = 10$). One week after virus infusion, rats were first trained to self-administer 0.75 mg/kg cocaine on an FR1 schedule. We observed that rats with DA-specific Hcrtr1 knockdown took significantly more days to acquire cocaine self-administration compared to scramble controls ($t_{(19)} = 2.184$, $p < 0.05$). However, no differences were observed in average response interval ($t_{(19)} = 0.5186$, $p \leq 0.6100$) or rate of intake ($t_{(19)} = 0.7448$, $p \leq 0.4655$) between Hcrtr1 knockdown and scramble groups. To assess the effects of Hcrtr1 knockdown in DA neurons on motivation for cocaine, rats were then switched to a PR schedule of reinforcement. We observed that Hcrtr1 knockdown significantly decreased breakpoints ($t_{(19)} = 3.551$, $p < 0.01$) compared to scramble controls (Fig. 7).

3.7. Hcrtr1 knockdown in GABA neurons did not impact cocaine self-administration

To assess the effect of Hcrtr1 knockdown in VTA GABA neurons on cocaine self-administration, rats were implanted with a jugular catheter and injected intra-VTA with AAV-GAD1-Cre and either AAV-SICO-Hcrtr1shRNA ($n = 10$) or AAV-SICO-SCRM ($n = 8$). Rats were trained to self-administer cocaine on an FR1 schedule. We observed no significant difference in days to acquire self-administration, ($t_{(16)} = 0.2566$, $p \leq 0.8008$), average response interval ($t_{(16)} = 0.7852$, $p \leq 0.4438$), or rate of intake ($t_{(16)} = 0.2568$, $p \leq 0.8006$) between Hcrtr1 knockdown and scramble controls. To assess the effect of Hcrtr1 knockdown in VTA GABA neurons on motivation to self-administer cocaine, rats were then switched to a PR schedule of reinforcement. We observed that Hcrtr1 knockdown had no effect on breakpoints ($t_{(16)} = 0.1117$, $p \leq 0.9124$) compared to scramble controls (Fig. 8).

4. Discussion

In these experiments, we used a novel combinatorial viral approach to selectively knockdown Hcrtr1 in DA or GABA neurons of the VTA. We observed that selective

knockdown of Hcrtr1 in DA neurons of the VTA decreased baseline DA uptake rate, attenuated the effects of cocaine at inhibiting DA uptake, and decreased motivation for cocaine. By comparison, knockdown of Hcrtr1 in GABA neurons of the VTA increased baseline DA uptake rate, enhanced the effects of cocaine at inhibiting DA uptake, but had no effect on cocaine self-administration. These observations suggest that Hcrtr1 knockdown in DA neurons of the VTA impacts DA transmission in the NAc in a manner consistent with reductions in cocaine self-administration. However, Hcrtr1 knockdown in GABA neurons of the VTA affected DA transmission in the NAc without influencing cocaine self-administration behavior.

4.1. Hcrtr1 on VTA DA neurons impacted baseline and cocaine-induced DA uptake in the NAc

Our observation that Hcrtr1 knockdown in VTA DA neurons reduced DA uptake in the NAc, is in line with previous reports demonstrating that intra-VTA Hcrtr1 knockdown or antagonism decreases DA uptake in the NAc [5, 18]. Previous studies suggest that *in vitro* changes in membrane potential lead to alterations in DA uptake [65, 66], as a result of changes in DAT function [67]. Further, it has recently been demonstrated that intra-VTA inhibition of DA neurons induced by G_i DREADDs, leads to putative changes in DAT conformational state that may contribute to lower DA uptake in the NAc [60]. Given that HCRT has been shown to drive DA neuron activity through Hcrtr1 [20, 21, 25], decreasing Hcrtr1 expression in DA neurons of the VTA is expected to disrupt HCRT-induced DA neuron activity. When viewed in combination with other work suggesting a lack of appreciable expression of Hcrtr1 in the NAc core [24, 50, 68], our findings of reduced DA uptake in the NAc following Hcrtr1 knockdown could be explained by somatodendritic actions that result in alterations in DAT function in DA terminals in the NAc. While this depolarization-based mechanism can account for rapid changes in DA uptake [60, 69], it should be noted that the viral manipulations used in our experiments likely confer longer lasting neuronal changes that would be present even following disconnection of VTA cell bodies. Such changes in mesolimbic signaling may be influenced by second messenger signaling cascades that alter the function and surface presence of the DAT [70, 71]. Specifically, second messenger signaling cascades such as protein kinase C and calmodulin-dependent protein kinase II regulate phosphorylation and glycosylation of the DAT, and HCRT has been shown to modulate both of these processes [72, 73], thereby influencing DAT surface expression and function [74, 75]. Therefore, we posit that our observed reductions in DA uptake following Hcrtr1 knockdown in VTA DA neurons may be due to HCRT-induced alterations in DAT function through a combination of altered second messenger signaling and reductions in membrane depolarization.

In addition to changes in baseline DA uptake, Hcrtr1 knockdown also influenced DA responses to cocaine. We observed that Hcrtr1 knockdown in VTA DA neurons reduced cocaine-induced uptake inhibition. This finding is consistent with previous reports demonstrating that intra- VTA Hcrtr1 knockdown or antagonism attenuate cocaine-induced inhibition of DA uptake in the NAc [5, 18]. Indeed, modifications that influence NAc terminal expression of DAT and alter baseline DA uptake rates have also been shown to alter the potency of psychostimulants [76–78]. Further, given that changes in membrane

depolarization influence the functional state of the DAT [65, 66], likely altering the availability of the cocaine-binding site, it is possible that HCRT-induced changes in DA neuron activity may also contribute to reduced inhibition of DA uptake.

4.2. Hcrtr1 knockdown in VTA GABA neurons enhanced DA terminal dynamics and cocaine-induced inhibition of DA uptake in the NAc

In addition to specific knockdown of Hcrtr1 in DA neurons, we also utilized the combinatorial virus approach to decrease Hcrtr1 expression in GABA neurons. Knockdown of Hcrtr1 in VTA GABA neurons increased baseline DA uptake rate and enhanced cocaine-induced inhibition of DA uptake. These changes in DAT function in the NAc likely occur as a result of Hcrtr1 knockdown-induced alterations in VTA GABA neuron activity. Indeed, previous studies demonstrated that activation of VTA GABA neurons attenuates the activity of nearby VTA DA neurons [34], suggesting that a decrease in the Hcrtr1-mediated drive on VTA GABA neurons would result in disinhibition of VTA DA neurons, thereby leading to increased VTA DA neuron activity. As discussed above, such changes in VTA DA neuron activity and/or second messenger signaling over the course of the two-week viral incubation period are thought to alter DAT function in the NAc. Our findings of enhanced DA uptake and cocaine potency following Hcrtr1 knockdown in VTA GABA neurons demonstrate that DA dynamics in the NAc are modulated through Hcrtr1 on GABA neurons in the VTA in a manner opposing what is observed with Hcrtr1 on DA neurons.

Additionally, the increase in DA uptake observed following Hcrtr1 knockdown in VTA GABA neurons opposes the decrease in DA uptake observed following knockdown in VTA DA neurons. These findings suggest that non-specific disruption of Hcrtr1 in both of these VTA neuron populations would counterbalance, resulting in no net changes in DA transmission. However, this is not what has been documented previously, as generic Hcrtr1 knockdown and antagonism of Hcrtr1 in the VTA both produce reductions in DA uptake [7, 18], similar to what we observed following Hcrtr1 knockdown specifically in DA neurons. Although there is no clear topographical organization of VTA GABA neurons [32], some studies suggest that neurons containing GAD may be more commonly observed in lateral regions of the VTA [38]. Given that our infusion placements, particularly in rats receiving GAD-targeted virus infusions prior to voltammetry, tended to be slightly more lateral in nature (see Supplemental Fig. 1B), it is possible there was a more substantial knockdown of Hcrtr1 in GABA neurons than in DA neurons in the voltammetry cohort. As a result, it is possible that the effects of Hcrtr1 knockdown in GABA neurons were more robust in our experiments than in prior work.

4.3. Hcrtr1 knockdown in DA or GABA neurons did not influence stimulated DA release

Hypocretin actions on Hcrtr1 tend to be excitatory as Hcrtr1 is a G_q -coupled receptor. HCRT has been shown to drive both VTA DA [20–22, 25] and GABA neuron activity, likely in a Hcrtr1-dependent manner [20]. Therefore, knockdown of Hcrtr1 on DA or GABA neurons in the VTA would be expected to disrupt the activity of both DA and GABA neurons.

Alterations in VTA GABA neuron activity have been shown to impact the activity of DA neurons [79], with optogenetic activation of VTA GABA neurons attenuating the activity of nearby VTA DA neurons [34]. With this in mind, it follows that Hcrtr1 knockdown in either DA or GABA neurons should affect DA release. In the case of Hcrtr1 knockdown in VTA DA neurons, these DA neurons would be expected to receive reduced HCRT peptide influence, resulting in a hypothesized decrease in DA release. Conversely, when Hcrtr1 is decreased in GABA neurons, the inhibitory influence of GABA onto VTA DA neurons would be attenuated, and DA release should increase.

Notably, no changes in stimulated DA release in the NAc were observed with either Hcrtr1 knockdown in DA or GABA neurons in our experiments. Interestingly, prior reports indicate decreased stimulated DA release following HCRT knockout or Hcrtr1 knockdown or antagonism [5, 15, 18]. However, those studies were conducted in intact, whole animal preparations which preserves the VTA to NAc circuit. In our experiments, NAc slices were used, resulting in separation of NAc DA terminals from cell bodies in the VTA. Any stimulated release observed under these circumstances is therefore unaffected by ongoing changes in VTA neuron activity. Thus, although stimulated DA release was not altered in these experiments, it is probable that alterations in stimulated DA release would be observed with intact VTA neurons. Future studies should examine the effects of Hcrtr1 knockdown on DA release using intact, whole animal preparations.

4.4. Hcrtr1 knockdown on either DA or GABA neurons had no effect on DAT expression or phosphorylation

Previous observations indicate that DAT expression and phosphorylation are critical factors in the regulation of DA uptake, DAT sensitivity to psychostimulants, and cocaine-associated behaviors [80, 81]. For example, low DAT expression is associated with slower DA uptake rate [82–88], and reduced DAT sensitivity to cocaine [7, 83–86, 88]. Furthermore, there is evidence to suggest that low DAT expression is associated with reduced behavioral responses to psychostimulants [82, 87, 89], though the direction of effect varies from study to study [90–93]. In our studies, we did not find differences in total DAT expression with Hcrtr1 knockdown in either DA or GABA neurons suggesting that observed changes in DA transmission are not likely to be associated with DAT expression on the membrane.

In addition to potential changes in total membrane DAT expression, there is accumulating evidence suggesting that phosphorylation of the DAT at threonine 53 influences DA transmission [56, 60, 94, 95], with lower pDAT tied to reduced DA uptake and DA responses to cocaine. In fact, we previously showed that acute Hcrtr1 blockade preferentially reduces pDAT expression in the NAc, which was associated with both reduced DA uptake rate and attenuated DAT sensitivity to cocaine [59]. Based on these findings, we hypothesized that the changes in DA transmission observed herein would be tied to alterations in pDAT expression. Surprisingly, however, knockdown of Hcrtr1 on either DA or GABA neurons did not affect pDAT expression.

Beyond changes in membrane DAT expression or phosphorylation at threonine 53, there are several post-translational modifications that could explain our Hcrtr1 knockdown effects. For example, several reports suggest that changes in basal DA uptake rate may be related

to alterations in the balance of inward/outward facing DATs [96], dimerization of the DAT with sigma receptors [97], shifts in oligomer/monomer ratios [98, 99], or changes in phosphorylation of serine sites on the DAT [100, 101]. Given we did not find a relationship between DAT expression or pDAT, future studies will be required to identify the mechanisms mediating the effects of Hcrtr1 knockdown on DA transmission.

4.5. Hcrtr1 knockdown on VTA DA neurons disrupted cocaine self-administration

We observed that Hcrtr1 knockdown in VTA DA neurons increased days to acquire cocaine self-administration on a low effort, FR1 schedule of reinforcement. Extensive previous work indicated that Hcrtr1 antagonism or knockdown did not influence low effort self-administration of cocaine [1, 3, 5] (but see [102]). Uncovering an alteration in days to acquire cocaine self-administration on a low effort, FR1 schedule is notable because it suggests that previous non-specific approaches to disrupt Hcrtr1 may have impacted Hcrtr1 on multiple neuronal subtypes in the VTA, potentially masking delays in time to acquire cocaine self-administration observed with Hcrtr1 knockdown selectively in DA neurons.

In addition to effects on time to acquire self-administration, we also observed that Hcrtr1 knockdown in VTA DA neurons decreased breakpoints on a PR schedule. While PR does not encompass all aspects of reinforcement, it is a useful measure to assess reinforcer efficacy or motivation [103]. A lower breakpoint suggests reduced motivated to self-administer cocaine in rats with Hcrtr1 knockdown in DA neurons. This finding is in support of previous observations indicating that motivation to self-administer cocaine on a PR schedule of reinforcement is decreased with antagonism or knockdown of Hcrtr1 [1, 3, 5, 7, 10, 18]. Further, combined with the decrease in DAT sensitivity to cocaine following Hcrtr1 knockdown in DA neurons, these results suggest that Hcrtr1 on VTA DA neurons are an important mediator of motivation for cocaine.

4.6. Dissociation between the neurochemical and behavioral effects of Hcrtr1 knockdown in GABA neurons

Although we observed that Hcrtr1 knockdown in VTA GABA neurons enhanced baseline and cocaine-induced DA uptake in the NAc, contrary to our predictions, we observed no behavioral change in acquisition or motivation to self-administer cocaine. The hypothesis that Hcrtr1 knockdown in VTA GABA neurons would result in increased sensitivity to cocaine and increased motivation to self-administer cocaine is based upon the assumption that an increase in sensitivity to cocaine in the NAc would be sufficient to increase motivation for cocaine [54, 55]. In other words, that enhancing the DA response to cocaine in the NAc would mimic the effects of increasing cocaine dose, which for the PR schedule results in greater reinforcing efficacy of cocaine [61, 103]. Consistent with this tenant, prior studies demonstrate that intra-VTA delivery of HCRT peptide both increased DAT sensitivity to cocaine in the NAc, and produced a significant, albeit modest, increase in motivation for cocaine [51]. Despite this evidence, and the expectation that an increase in DA responses to cocaine would manifest as greater motivation for cocaine, our results did not support our hypothesis. Rather, knockdown of Hcrtr1 in GABA neurons increased DAT sensitivity to cocaine, but there was no corresponding increase in motivation to self-administer cocaine.

While the experiments presented here cannot easily reconcile these discrepant findings, it is important to note that unlike the well-described role of DA neuron firing in reward-related behaviors [104, 105–111], there is considerably less evidence for involvement of GABA neuron firing in such processes. Indeed, GABA neuron activity has only recently begun to be studied in this context and results are somewhat inconclusive. For example, some studies demonstrate increased VTA GABA neuron activity in response to rewards [112] or to reward-predicting cues [113], which is the opposite of what would be expected if DA neurons are primarily mediating reward processes. In contrast, chemogenetic activation of VTA GABA neurons has been shown to decrease responses to incentive cues as well as latencies to respond to the cue, suggesting potential VTA GABA-mediated decreases in motivation for reward [44]. Further, numerous other studies indicate that GABA neurons increase activity in response to aversive stimuli or cues predicting aversive stimuli [112–115]. As such, a lack of understanding surrounding the role of GABA neuron activity in reward and motivational processing makes it challenging to confidently predict any possible interactions between the potential effects of Hcrtr1 knockdown on GABA neuron activity and observed increases in sensitivity to cocaine in the NAc, as well as how this might impact behavior. These kinds of questions are an excellent premise for future studies, as it is likely that GABA neuron responses during drug-associated behaviors are more complex than previously expected.

Another consideration is the presence of both GABA interneurons and projection neurons in the VTA. At present, the distribution of Hcrtr1 between these different GABA neurons is unknown, and as such, neurons targeted by the AAV-GAD1-Cre virus in the VTA may be either of these neuronal subtypes. Given that these different types of GABA neurons have different characteristics and effects on potential targets, it is possible that Hcrtr1 knockdown on these neuron types may result in different neurobiological and behavioral outcomes. While GABA projection neurons only comprise approximately 25% of VTA GABA neurons [28], it is possible that Hcrtr1 knockdown could be occurring primarily on these projection neurons. These neurons preferentially project to cholinergic interneurons and firing of these projection neurons results in a pause of cholinergic interneuron firing in the NAc [116]. In the context of our experiments, Hcrtr1 knockdown would likely reduce VTA GABA neuron firing, resulting in more continuous firing of cholinergic interneurons. In addition to potential increases in DA release and in uptake rate in the NAc [117–119], enhanced cholinergic activity could lead to a difficulty in forming stimulus-outcome associations [116]. While behavioral findings in this study do not necessarily suggest the involvement of GABA neurons projecting to cholinergic interneurons, our finding of increased DA uptake rate following Hcrtr1 knockdown in GABA neurons is consistent with potential effects on GABA projection neurons.

In contrast to projection neurons, the majority of VTA GABA neurons are interneurons which make local inhibitory connections within the VTA [28]. Prior work demonstrated that optogenetic activation of VTA GABA neurons attenuates excitability and activity of neighboring VTA DA neurons in addition to disrupting reward consumption [34]. These findings would suggest that reducing the activity of VTA GABA interneurons would enhance VTA DA neuron activity and promote reward consumption. Though this concept is not supported by our experimental results, it is important to note that studies

investigating behaviors following VTA GABA interneuron activation have mixed results. As mentioned above, some studies show that activation of GABA neurons is tied to prediction of appetitive rewards [113] while others suggest that activation causes conditioned place aversion [114]. Thus, while VTA GABA interneurons may be responsible for a variety of behaviors, it is still unclear how Hcrtr1 knockdown on these neurons would alter cocaine self-administration as measured herein.

With the newly uncovered influence of Hcrtr1 on VTA GABA neurons on DA signaling, future studies should aim to discern the contribution from GABA projection neurons versus GABA interneurons. Though our experiments were limited by the intra-VTA combination of Hcrtr1shRNA and cre-dependent GAD-1 AAVs, future investigation can build upon our work with more complex manipulations. For example, the effects of Hcrtr1 knockdown in VTA-NAc GABA projection neurons can be examined through injection of a retrograde AAV-GAD1-Cre virus into the NAc in combination with injection of Hcrtr1shRNA in the VTA. With this virus combination, any alterations in NAc DA dynamics or cocaine self-administration that follow would allow for the disambiguation of the effects of Hcrtr1 knockdown in VTA GABA projection neurons from VTA GABA interneurons.

5. Conclusion

Our findings indicate that Hcrtr1 knockdown has differing effects on VTA DA compared to GABA neurons. These effects are hypothesized to be mediated through changes in DA and GABA neuron activity that fundamentally influence DA dynamics and – in the case of DA neurons – the behavioral response to cocaine. These results highlight the importance of the impact of the HCRT system on specific populations of neurons and suggest that efforts to develop future pharmacotherapies should utilize drugs acutely restricted by tethering, focusing on Hcrtr1 manipulations specifically on DA neurons in the VTA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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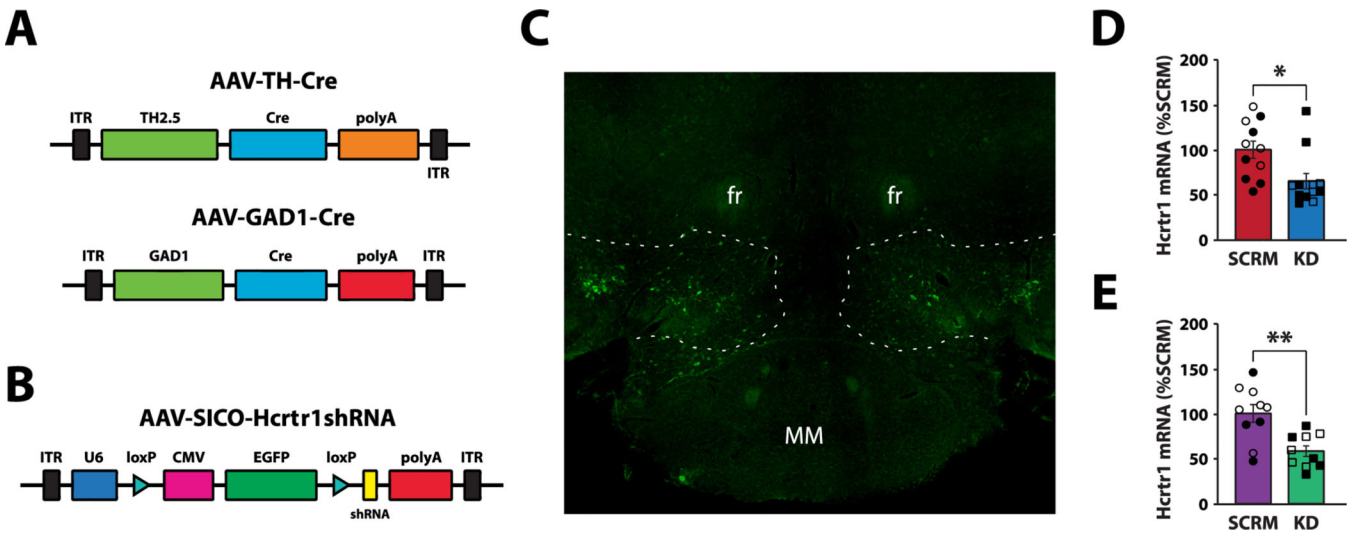

Fig. 1.

Hcrtr1 mRNA levels are decreased in the VTA following Hcrtr1 knockdown. **(A)** AAV-TH-Cre or AAV-GAD1-Cre was paired with either **(B)** AAV-SICO-Hcrtr1shRNA (KD) or AAV-SICO-SCRM (SCRM; not depicted) in the VTA. **(C)** Example virus infusion placement in the VTA. Dashed lines depict approximate boundaries of the VTA [64]. MM, mamillary body; fr, fornix. Hcrtr1 mRNA expressed as a percent of SCRM controls for Hcrtr1 knockdown in **(D)** DA or **(E)** GABA neurons. Data are shown as mean ± SEM. Females - closed symbols; Males - open symbols. Students' *t*-test: $*p < 0.05$, $**p < 0.01$. DA SCRM ($n = 11$); DA Hcrtr1 KD ($n = 12$); GABA SCRM ($n = 10$); GABA Hcrtr1 KD ($n = 10$).

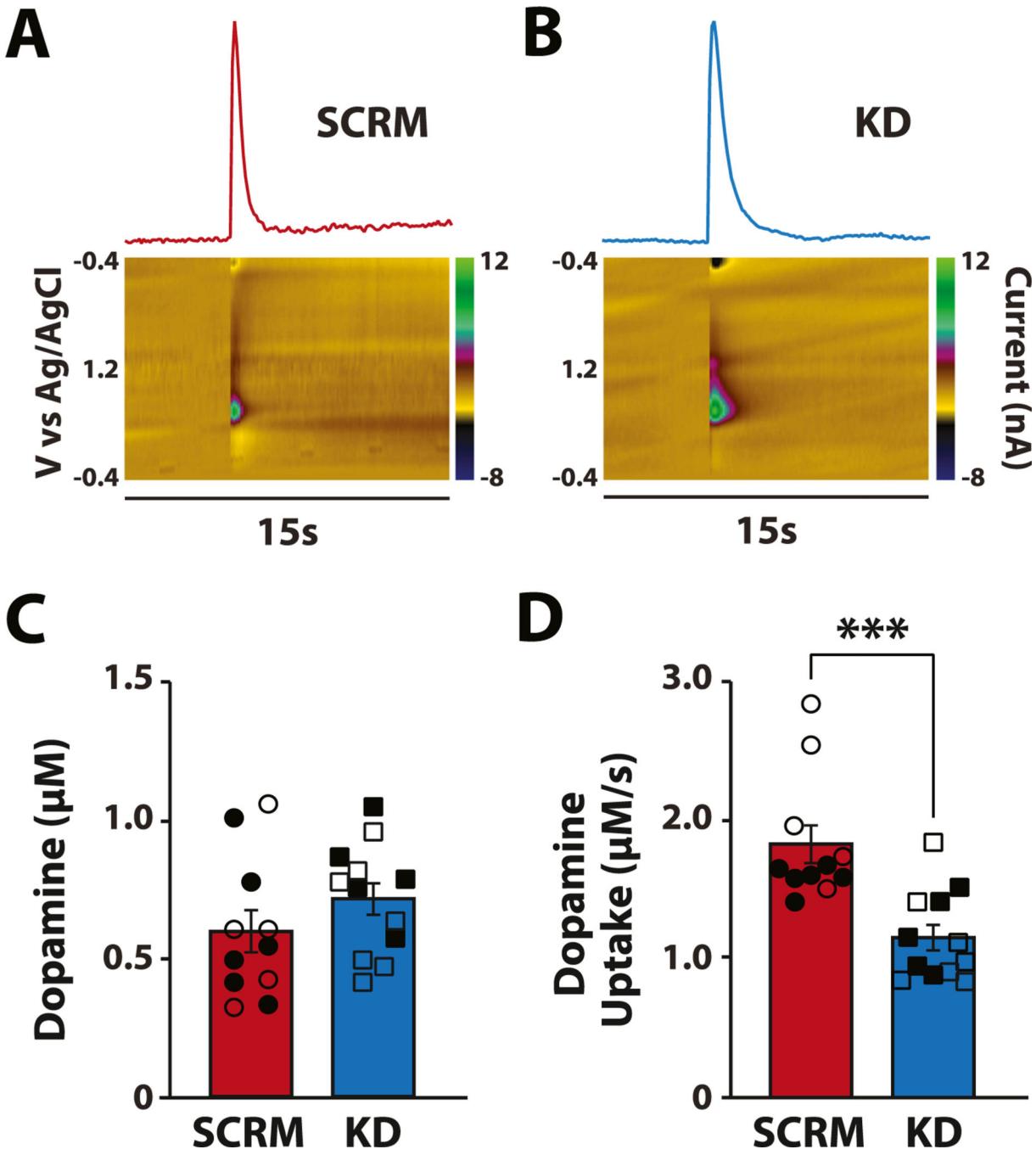

Fig. 2.

Hcrtr1 knockdown on VTA DA neurons decreased DA uptake. Example current vs time plots and pseudo-color plots showing stimulated DA release and uptake from **(A)** a scramble (SCRM) and **(B)** a Hcrtr1 knockdown (KD) rat. **(C)** DA release and **(D)** DA uptake for SCRM and KD rats. Data are shown as mean ± SEM. Females - closed symbols; Males - open symbols. Students' *t*-test: ***$p < 0.001$. SCRM (n = 11); Hcrtr1 KD (n = 12).

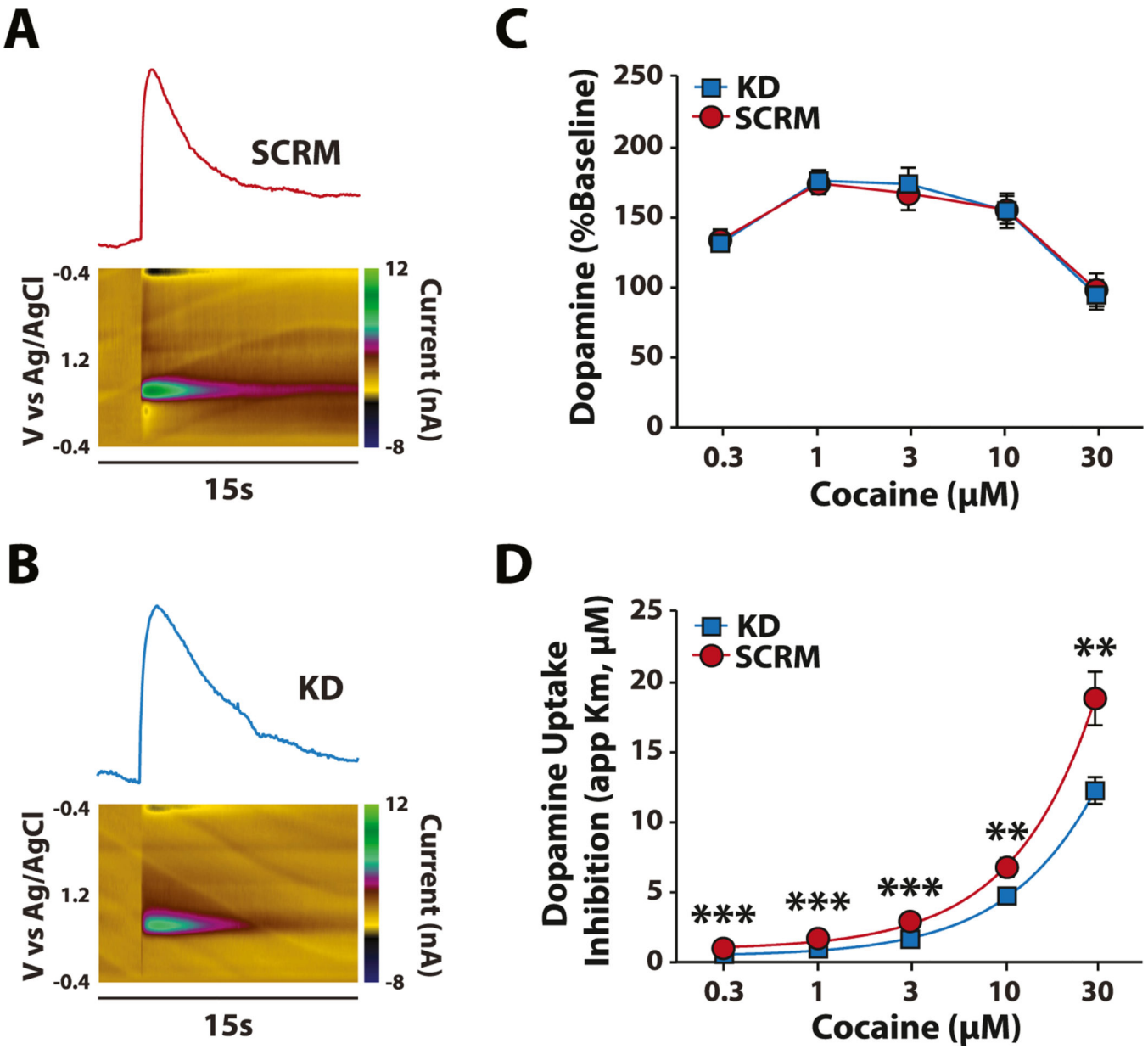

Fig. 3.

Hcrtr1 knockdown in VTA DA neurons decreased cocaine-induced DA uptake inhibition. Example current vs time plots and pseudo-color plots showing stimulated DA release and uptake following 30μM cocaine from **(A)** a SCRM and **(B)** a Hcrtr1 KD rat. **(C)** DA release at increasing concentrations of cocaine. **(D)** Inhibition of DA uptake (app Km) at increasing concentrations of cocaine. Data are shown as mean ± SEM. Holm-Bonferroni post-hoc: *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$. SCRM (n = 11); Hcrtr1 KD (n = 12).

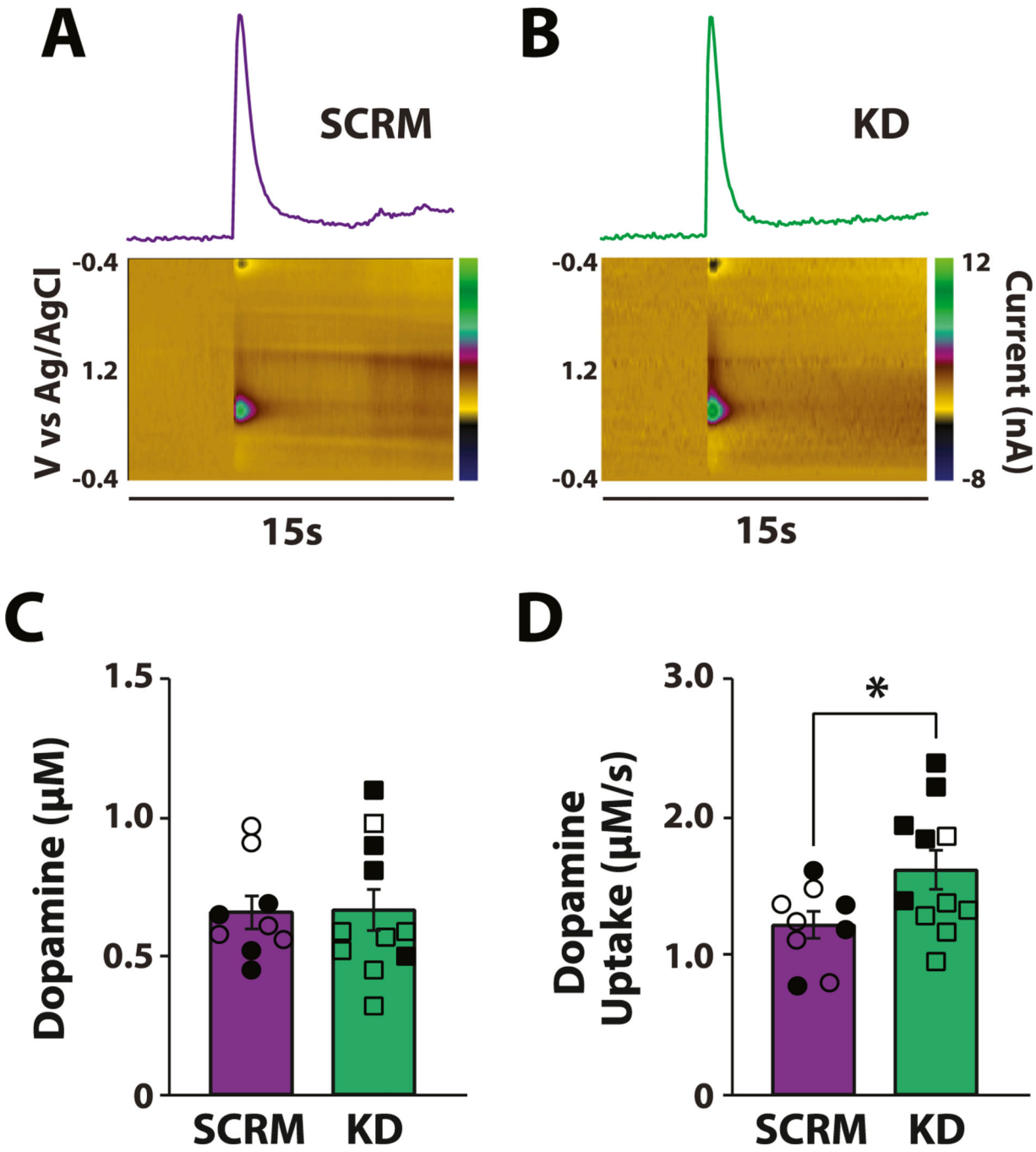

Fig. 4.

Hcrtr1 knockdown in VTA GABA neurons increased DA uptake. Example current vs time plots and pseudo-color plots showing stimulated DA release and uptake from **(A)** a scramble (SCRM) and **(B)** Hcrtr1 knockdown (KD) rat. **(C)** DA release and **(D)** DA uptake for SCRM and KD rats. Data are shown as mean ± SEM. Females closed symbols; Males - open symbols. Students' *t*-test: $*p < 0.05$. SCRM (n = 9); Hcrtr1 KD (n = 11).

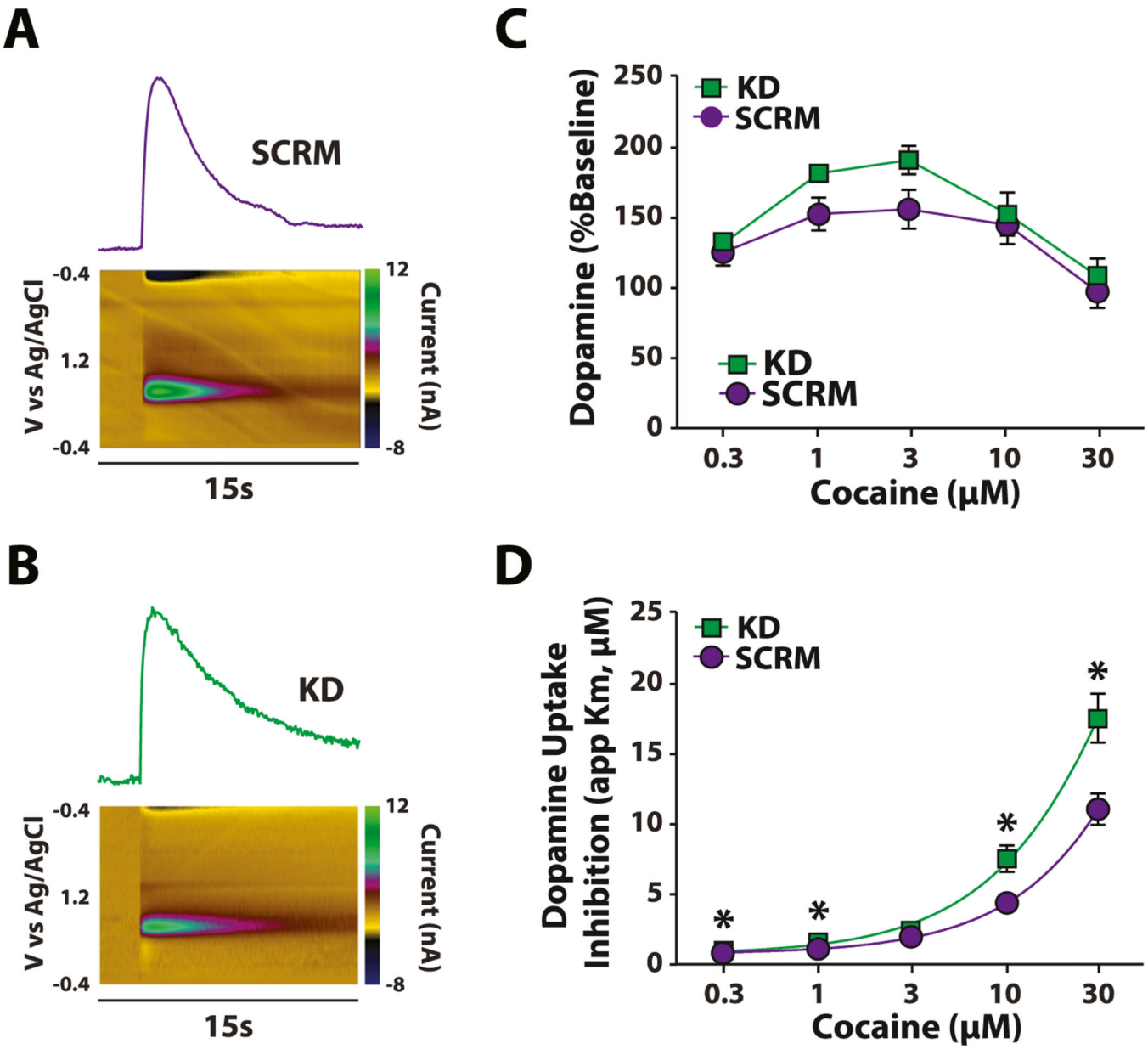

Fig. 5.

Hcrtr1 knockdown in VTA GABA neurons increased cocaine-induced DA uptake inhibition. Example current vs time plots and pseudo-color plots showing stimulated DA release and uptake following 30μM cocaine from **(A)** a SCRM and **(B)** a Hcrtr1 KD rat. **(C)** DA release at increasing concentrations of cocaine. **(D)** Inhibition of DA uptake (app Km) at increasing concentrations of cocaine. Data are shown as mean ± SEM. Holm-Bonferroni post-hoc: $*p < 0.05$. SCRM (n = 9); Hcrtr1 KD (n = 11).

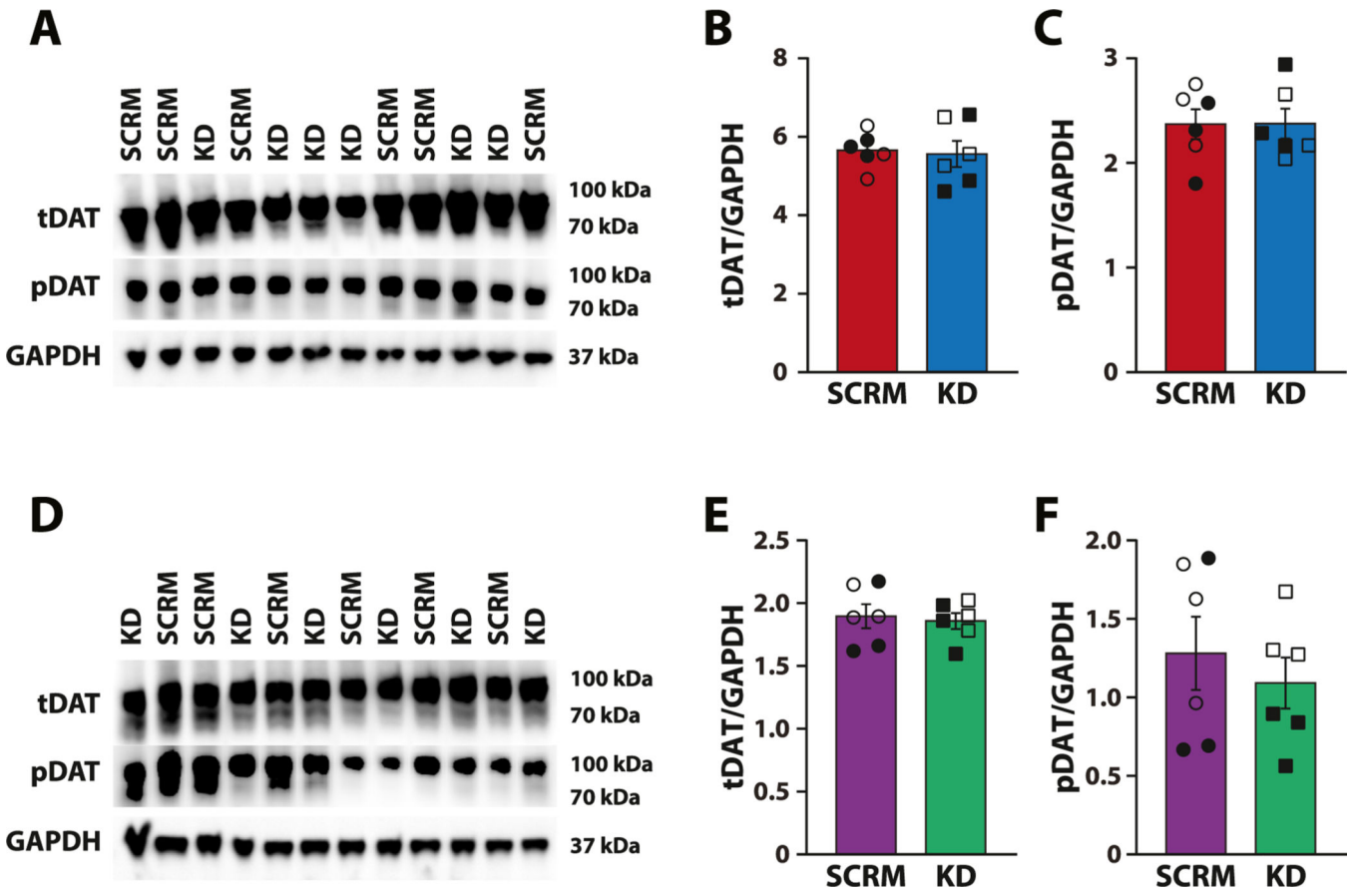

Fig. 6.

Hcrtr1 knockdown did not affect DAT expression or phosphorylation in the NAc. **(A)** Example western blots for scramble (SCRM) and Hcrtr1 knockdown (KD) in DA neurons. **(B)** Quantification of total DAT (tDAT) and **(C)** phosphorylated DAT (pDAT) normalized to GAPDH expression. **(D)** Example western blots for SCRM and Hcrtr1 KD in GABA neurons. **(E)** Quantification of total tDAT and **(F)** pDAT normalized to GAPDH expression. Data shown as mean ± SEM. Females - closed symbols; Males - open symbols. SCRM (n =6); Hcrtr1 KD (n = 6).

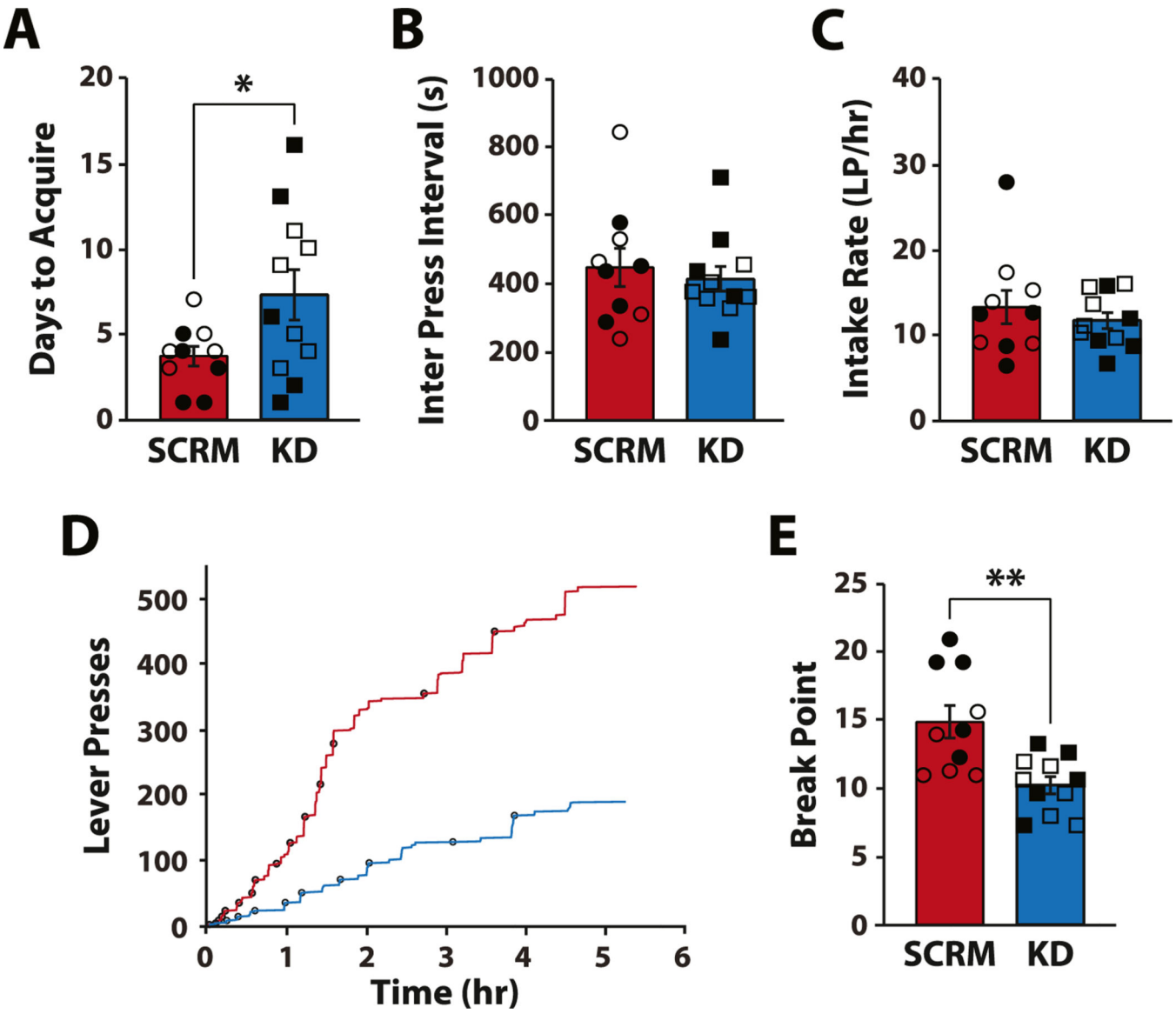

Fig. 7.

Hcrtr1 knockdown in VTA DA neurons disrupted cocaine self-administration. **(A)** Days to acquire, **(B)** inter press interval, and **(C)** intake rate for FR1 self-administration for scramble (SCRM) and Hcrtr1 knockdown (KD). **(D)** Example cumulative response plots and **(E)** breakpoints for SCRM and Hcrtr1 KD rats. Data are shown as mean ± SEM. Females - closed symbols; Males - open symbols. Student's *t*-test: $*p < 0.05$, $**p < 0.01$. SCRM ($n = 10$); Hcrtr1 KD ($n = 11$).

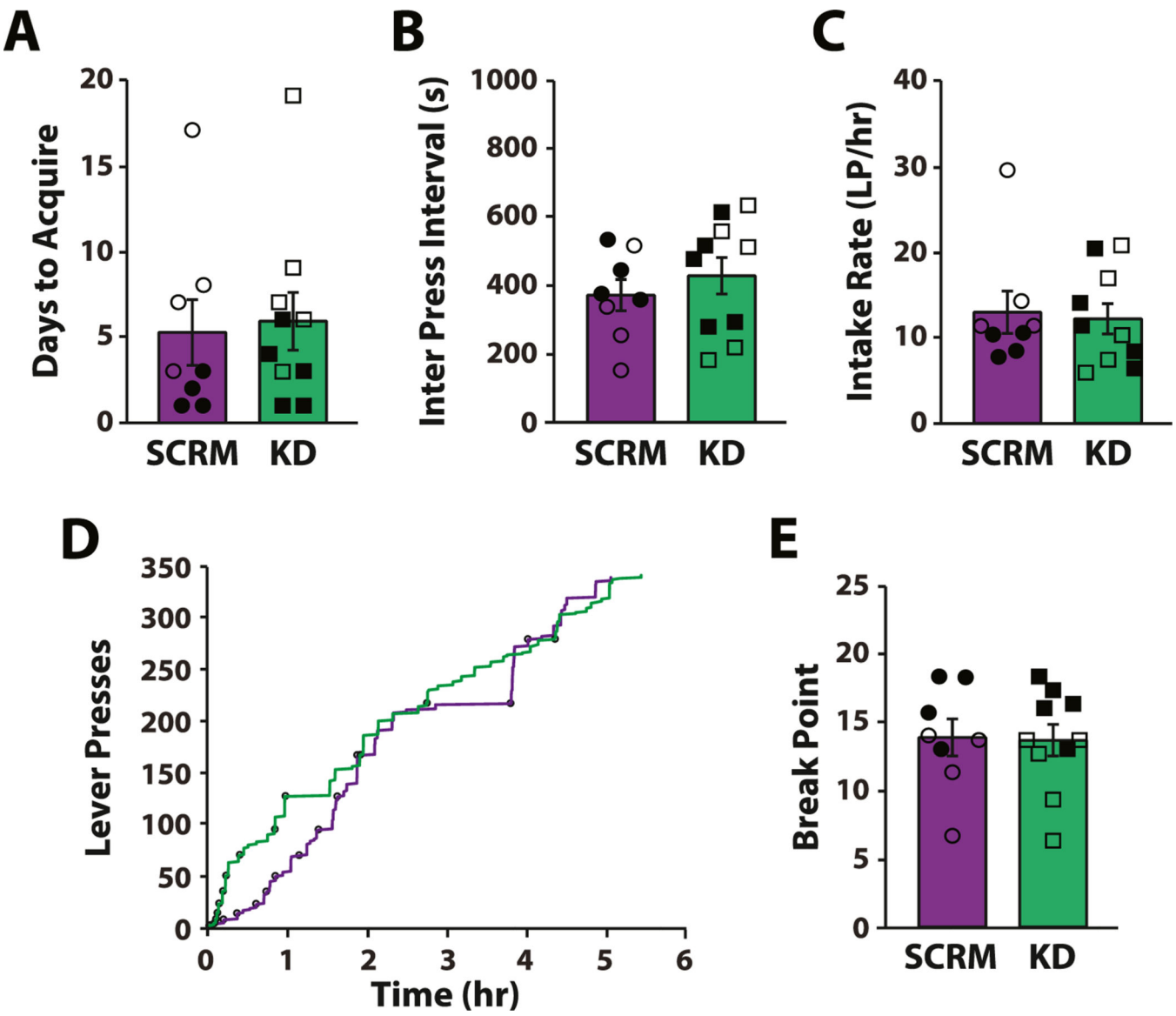

Fig. 8.

Hcrtr1 knockdown in VTA GABA neurons had no effect on cocaine self-administration. **(A)** Days to acquire, **(B)** inter press interval, and **(C)** intake rate for FR1 self-administration for scramble (SCRM) and Hcrtr1 knockdown (KD). **(D)** Example cumulative response plots and **(E)** breakpoints for SCRM and Hcrtr1 KD rats. Data are shown as mean ± SEM. Females - closed symbols; Males - open symbols. Student's *t*-test. SCRM ($n = 8$); Hcrtr1 KD ($n = 10$).