Differential modulation of ventral tegmental area circuits by the nociceptin/orphanin FQ system

Joseph R. Driscoll[1,2]; Tanya L. Wallace[1]; William J. Martin[1]; Elyssa B. Margolis[2]

[1]BlackThorn Therapeutics
780 Brannan Street
San Francisco, CA 94103

[2]Department of Neurology
Wheeler Center for the Neurobiology of Addiction
University of California, San Francisco
675 Nelson Rising Lane
Box 0444
San Francisco, CA 94143, USA

JRD, TLW, WJM and EBM designed the studies; JRD and EBM collected the data; JRD analyzed the data; JRD, TLW, WJM and EBM wrote the manuscript.

Corresponding Author:
Elyssa B. Margolis
elyssa.margolis@ucsf.edu
University of California, San Francisco
675 Nelson Rising Lane
Box 0444
San Francisco, CA 94143, USA
Phone: (415) 353-4793

Abstract

The neuropeptide nociceptin/orphanin FQ (N/OFQ) system can be activated under conditions of stress and is associated with disorders of emotional regulation and reward processing. N/OFQ and its cognate receptor, NOP, are highly enriched in dopaminergic pathways, and intra-ventricular agonist delivery decreases dopamine levels in the dorsal striatum, nucleus accumbens (NAc), and the ventral tegmental area (VTA). We used whole cell electrophysiology in acute rat brain slices to investigate synaptic actions of N/OFQ in VTA neurons. Consistent with previous reports, we found that N/OFQ is primarily inhibitory, causing outward currents in both immunocytochemically identified dopaminergic (tyrosine hydroxylase positive (TH(+)) and non-dopaminergic (TH(-)) VTA neurons (effect at 1 μM: 20 ± 4 pA). A smaller population of VTA neurons responded to low dose N/OFQ with small inward currents (effect at 10 nM: -11 ± 2 pA). Application of 100 nM N/OFQ resulted in a markedly diminished response to a second N/OFQ application, consistent with receptor desensitization (14 ± 10% of first response). N/OFQ generated outward currents in VTA neurons that project to the medial prefrontal cortex (mPFC), but inward currents in a subset of VTA neurons that project to posterior anterior cingulate cortex. Interestingly, while N/OFQ inhibited VTA cell bodies that project to the NAc, it had little effect on electrically or optogenetically evoked dopamine released from terminals in the NAc measured *ex vivo* with fast scan cyclic voltammetry. These results extend our understanding of the N/OFQ system in dopaminergic pathways implicated in many neurobehavioral disorders.

Introduction

Nociceptin/Orphanin FQ (N/OFQ) and its receptor (NOP) make up a neuropeptide system de-orphaned in 1995 (Meunier et al. 1995; Reinscheid et al. 1995) that is engaged under conditions of stress (Ciccocioppo et al. 2000; Devine et al. 2001; Fernandez et al. 2004; Green et al. 2007; Green and Devine 2009; Leggett et al. 2006, 2007; Nativio et al. 2012; Nicholson et al. 2002). The NOP is a G-protein coupled 7-transmembrane domain receptor that canonically signals through Gi/o proteins, post-synaptically activating G-protein coupled inward-rectifying potassium channels (GIRKs), or pre-synaptically reducing probability of neurotransmitter release via inhibition of N-type calcium channels (Hawes et al. 2000; Knoflach et al. 1996; New and Wong 2002; Vaughan and Christie 1996). While amino acid sequence homology has led some to categorize the NOP as an opioid receptor (Bunzow et al. 1994; Meunier et al. 1995; Mollereau et al. 1994; Wang et al. 1994), NOP activation is not blocked by naloxone, a non-selective opioid receptor antagonist that was originally used to classify responses as belonging to the opioid family, blocking activation at mu, delta, and kappa opioid receptors (MOPs, DOPs, and KOPs, respectively) (Gintzler et al. 1997; Mogil and Pasternak 2001; Reinscheid et al. 1995, 1996). Furthermore, the endogenous opioid ligands (dynorphins, enkephalins, and endorphins) do not bind to the NOP, and N/OFQ does not bind to the MOP, DOP, or KOP (Ma et al. 1997; Meng et al. 1996; Sim et al. 1996). Because of these distinct pharmacological properties, N/OFQ and the NOP are most appropriately subclassified as non-classical members of the opioid family (Cox et al. 2015; Toll et al. 2016).

N/OFQ and the NOP are highly enriched in the ventral tegmental area (VTA), dorsal striatum, nucleus accumbens (NAc), medial prefrontal cortex (mPFC), and central nucleus of the amygdala (Berthele et al. 2003; Neal et al. 1999; Parker et al. 2019). The VTA is the major

source of dopamine to limbic forebrain regions and plays a key role in brain networks that coordinate motivation and learned appetitive behaviors (Fields et al. 2007). Activity of VTA dopamine neurons is associated with salience and reward prediction, while destruction of these neurons results in motivation deficits (Fields et al. 2007; Kim et al. 2012; Mohebi et al. 2019; Morales and Margolis 2017; Tsai et al. 2009; Ungerstedt 1971; Wise 2005; Witten et al. 2011). Intracerebroventricular (ICV) injections of N/OFQ produce a decrease in extracellular dopamine in the dorsal striatum and NAc, and some midbrain putative dopamine cell bodies are inhibited by NOP activation (Di Giannuario and Pieretti 2000; Lutfy et al. 2001; Murphy et al. 1996; Murphy and Maidment 1999; Vazquez-DeRose et al. 2013; Zheng et al. 2002).

Dysregulation of the N/OFQ system has been associated with disorders of motivated responding (Civelli 2008), and the N/OFQ system has been investigated as a novel target for major depressive disorder and alcohol use disorder (Witkin et al. 2019), however understanding the involvement of the N/OFQ system in these behaviors remains a challenge. In fact, in some cases, activation and inhibition of NOPs paradoxically produce the same behavioral outcomes, such as with alcohol consumption (Ciccocioppo et al. 2014; Kuzmin et al. 2007; Rorick-Kehn et al. 2016) and anxiety-related behaviors (Dautzenberg et al. 2001; Fernandez et al. 2004; Gavioli et al. 2002; Green et al. 2007; Jenck et al. 1997; Kamei et al. 2004; Varty et al. 2008; Vitale et al. 2006). Such observations may be explained by off-target effects of N/OFQ, activation of N/OFQ sensitive neural circuits that compete for behavioral control, or receptor desensitization.

Here we investigated the basic physiology of N/OFQ responses in VTA neurons to better characterize how N/OFQ may contribute to reward processing. To confirm that our physiological responses to N/OFQ were due to NOP activation we utilized the selective NOP antagonist BTRX-246040 (Toledo et al. 2014) to block N/OFQ responses. We observed similar N/OFQ effects on both dopamine and non-dopamine VTA neurons, and provide neurophysiological evidence that moderate doses of N/OFQ cause functional desensitization of the NOP, preventing responding to subsequent agonist application. Importantly, we found that responses to N/OFQ differ depending upon the projection target of the VTA neuron. Furthermore, although NAc-projecting cell bodies were inhibited by NOP activation, N/OFQ did not inhibit dopamine release at their terminals in the NAc. In addition, N/OFQ induced small inward currents in the VTA neurons that project to posterior anterior cingulate cortex (pACC). Each of these actions must be considered to understand the biological relevance of the nociceptin/NOP system and to guide further research.

Materials and Methods

Electrophysiology: Male Sprague Dawley rats, p22 – p36, were anesthetized with isoflurane, and brains were removed. Horizontal brain slices (150 μm thick) containing the VTA were prepared using a Vibratome (Leica Instruments, Nussloch, Germany). Slices were submerged in Ringer's solution containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose saturated with 95% O_2–5% CO_2 and allowed to recover at 35°C for at least 1 hr before recordings were initiated.

Individual slices were visualized under an Olympus BX50WI microscope (Olympus Life Science Solutions, Waltham, Massachusetts) with differential interference contrast optics and near infrared illumination, using an Andor xIon+ camera, and Andor Solis imaging software (Andor Technology Ltd, Belfast, Northern Ireland). Whole-cell patch-clamp recordings were

89 made at 33°C using 2.5– 4M pipettes containing (in mM): 123 K-gluconate, 10 HEPES, 0.2
90 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na_3GTP, pH 7.2, osmolarity adjusted to 275 mOsm. Biocytin
91 (0.1%) was added to the internal solution for post hoc identification.
92 Recordings were made using an Axopatch 1-D (Axon Instruments, Union City, CA),
93 filtered at 2 kHz, and collected at 20 kHz using IGOR Pro (Wavemetrics, Lake Oswego, OR).
94 Liquid junction potentials were not corrected during recordings. Hyperpolarization-activated
95 cation currents (I_h) were recorded by voltage clamping cells and stepping from -60 to -40, -50, -
96 70, -80, -90, -100, -110, and - 120 mV. The I_h magnitude was measured as the difference
97 between the initial response to the voltage step after the capacitive peak and the final current
98 response.
99 Pharmacology experiments were completed in voltage-clamp mode (V = -60 mV) to
100 measure changes in membrane current. Series resistance was monitored online by measuring
101 the peak of the capacitance transient in response to a −4 mV voltage step applied at the onset
102 of each sweep. Input resistance was measured using the steady state response to the same
103 voltage step. Upon breaking into the cell, at least 10 min was allowed for the cell to stabilize
104 and for the pipette internal solution to dialyze into the cell. Drugs were applied via bath perfusion
105 at a flow rate of 2 mL/min or pressure ejection nearby the recorded cell (within ~200 μm) using a
106 SmartSquirt micro-perfusion system (AutoMate Scientific, Berkeley, CA). N/OFQ (1 nM to 10
107 μM) was bath applied (5-7 min) only after a 5-min stable baseline was achieved. To test that
108 observed N/OFQ-mediated effects were specific to NOP, the selective NOP antagonist BTRX-
109 246040 (10 or 100 nM) was applied for 10 min prior to N/OFQ.
110 Stock solutions of drugs were made in advance, stored at -20°C, and diluted into aCSF
111 immediately before application. N/OFQ was obtained from Tocris (Minneapolis, MN) and diluted
112 to a 100 μM stock solution in ddH_20. Stock BTRX-246040 was obtained from BlackThorn
113 Therapeutics and diluted in DMSO (10 mM).
114 *Retrograde Tracer Injections*: Male Sprague Dawley rats, 21–100 d old, were
115 anesthetized with isoflurane. A glass pipette (30- to 50-μm tip) connected to a Nanoject
116 II/Nanoliter 2000 microinjector (Drummond Scientific Co.) was stereotaxically placed in the
117 mPFC (from bregma [in mm]: anteroposterior [AP], +2.6; mediolateral [ML], ±0.8; ventral [DV],
118 −4.0 from skull surface), the pACC (AP, -1.6; ML, ± 0.6; V, −3.5), or the NAc (AP, +1.5; ML, ±
119 0.8; V, −6.7). Neuro-DiI (7% in ethanol; Biotium) was slowly injected, 50.6 nL per side. Animals
120 were allowed to recover for 5 to 7 days while the retrograde tracer transported back to the cell
121 bodies. On the day of recording, the experimenter was blinded to the location of retrograde
122 tracer injection (mPFC, pACC, or NAc) and slices were prepared as above. Projection neurons
123 were chosen by selecting cells observed as labeled using epiflorescent illumination. All injection
124 sites were histologically confirmed by a third party blinded to the results to avoid bias. N/OFQ
125 responses were analyzed prior to unblinding. Animals with improper injection placements or
126 significant diffusion outside of the target region were rejected.
127 *Immunohistochemistry*: Slices were pre-blocked for 2 h at room temperature in PBS with
128 0.2% BSA and 5% normal goat serum, then incubated at 4°C with a rabbit anti-TH polyclonal
129 antibody (1:100). Slices were then washed thoroughly in PBS with 0.2% BSA before being
130 agitated overnight at 4°C with Cy5 anti-rabbit secondary antibody (1:100) and FITC streptavidin
131 (6.5 μL/mL). Sections were rinsed and mounted on slides using Bio-Rad Fluoroguard Antifade
132 Reagent mounting media and visualized with an Axioskop FS2 Plus microscope with an

133 Axiocam MRm running Neurolucida (MBF Biosciences). Neurons were only considered TH(−) if
134 there was no colocalization of biocytin with TH signal and the biocytin soma was in the same
135 focal plane as other TH(+) cell bodies. Primary antibodies were obtained from Millipore
136 Bioscience Research Reagents or Millipore, secondary antibodies were obtained from Jackson
137 ImmunoResearch Laboratories, and all other reagents were obtained from Sigma Chemical.
138 *Fast Scan Cyclic Voltammetry*: Male Sprague Dawley rats, 21–26 d old, or *Th::Cre*
139 transgenic rats (Witten et al. 2011), 46-51 d old at the time of virus injection, were used in these
140 studies. Th::Cre rats were injected with the Cre dependent ChR2 expressing virus (AAV2-Ef1a-
141 DIO-hChR2(H134R)-mCherry, titer $5.1X10^{12}$ viral particles/mL, UPenn viral core) bilaterally into
142 the VTA 500 nL per side (AP, −5.3; ML, ± 0.4; DV, −8.2 mm from bregma). Five weeks later,
143 coronal slices (400 μm) containing the NAc were prepared for voltammetry measurements. The
144 use of Cre dependent ChR2 expression allowed selective optical control of VTA dopamine
145 terminals in the NAc.
146 Extracellular dopamine release was achieved using either electrical (in wild-type
147 Sprague Dawley rats) or 488 nm light (in Th::Cre rats) stimulation. Stimulation parameters were
148 the same for both electrical and optical stimulation (10 Hz, 2 pulses, 4 ms). Electrochemical
149 recordings were made using carbon fiber electrodes fabricated from T-650 carbon fiber (7 μm
150 diameter, gift from Dr. Leslie Sombers (NCSU)) that was aspirated into a borosilicate glass
151 capillary (0.6 × 0.4 mm or 1.0 × 0.5 mm diameter, King Precision Glass Inc., Claremont, CA)
152 and pulled using a PE-22 puller (Narishige, Tokyo, Japan). Carbon fiber electrodes were
153 positioned 80 μm into the tissue either between the bipolar tips of the stimulating electrode or
154 directly in front of an optical fiber connected to an LED emitting 488 nm light (7-10 mW). The
155 potential of the carbon fiber electrode was held at -0.4 V relative to the Ag/AgCl reference
156 electrode. A triangle wave form was passed through the carbon fiber driving the potential from -
157 0.4 V to +1.3 V and back to -0.4 V at a rate of 400 V/s, at 60 Hz for conditioning and 10 Hz for
158 data collection. Data were collected with a WaveNeuro FSCV potentiostat (Pine Research,
159 Durham, NC) using HDCV acquisition software package (freely available through UNC
160 Department of Chemistry). HDCV Acquisition Software was used to output the electrochemical
161 waveform and for signal processing (background subtraction, signal averaging, and digital
162 filtering (4-pole Bessel filter, 2.5 kHz)). Dopamine release was stimulated at 2 min intervals for
163 electrical stimulation and 3 min intervals for optical stimulation to minimize rundown of the
164 dopamine release signal. Mean background currents from 1 sec of data prior to stimulation were
165 removed by subtraction of cyclic voltammograms for each trial.
166 *Data Analysis*: For electrophysiology, effects of N/OFQ were statistically evaluated in
167 each neuron by binning data into 30 s data points and comparing the last eight baseline binned
168 points to the last eight binned points drug application using Student's unpaired *t* test. $P < 0.05$
169 was required for significance in all analyses. Violin plots were constructed by calculating the
170 kernal density estimate, made using a Scott estimator for the kernal bandwidth estimation. The
171 kernel size was determined by multiplying the Scott bandwidth factor by the standard deviation
172 of the data within each bin. Each individual violin plot was normalized to have an equal area
173 under the curve. Time course figures are averages of the binned current traces for all cells time
174 locked to the start of drug application. Results are presented as mean and standard error of the
175 mean (SEM). Differences between neuron populations were tested using two-tailed permutation
176 analyses unless otherwise indicated.

Results

N/OFQ effects on holding current in VTA dopamine and non-dopamine neurons

To test the postsynaptic responses of VTA neurons to N/OFQ, we made *ex vivo* whole cell voltage clamp recordings (V_m = -60 mV). N/OFQ application changed the holding current in 71% (60/85) of neurons tested in the VTA (10 nM; Fig. 1A,B). The majority of responses were relatively small outward currents (73% of responsive neurons, 44/60; 54% of all neurons tested, 44/85; mean response = 15 $\pm$ 2 pA; Fig. 1D), consistent with the opening of inhibitory GIRK channels. Using post-hoc immunocytochemistry, we analyzed TH content in each recovered neuron and found that N/OFQ inhibited both confirmed dopamine and non-dopamine neurons in similar proportions (of 44 inhibited neurons, 23 were identified: TH(+): 10/23; TH(-): 13/23). The magnitudes of responses were also similar between confirmed dopamine and non-dopamine neurons (TH(+): 12 $\pm$ 2 pA (n = 10); TH(-): 10 $\pm$ 2 pA (n = 13); Fig. 1C). The EC_{50} for these outward currents is in the low nM range (8 $\pm$ 6 nM; Fig. 1E).

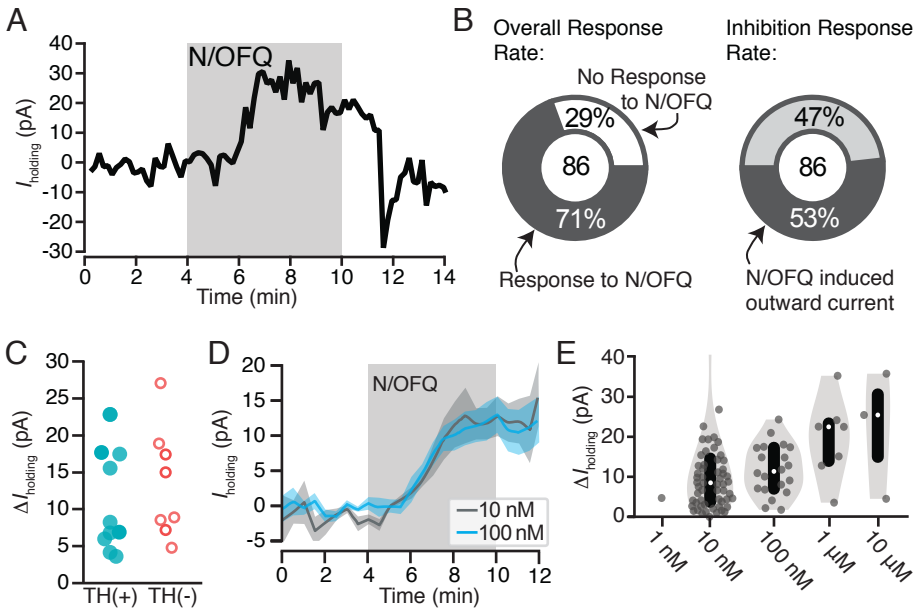

Figure 1: N/OFQ induced outward currents in a subset of VTA neurons. A: Example voltage clamp recording ($V_{holding}$ = -60 mV) of a VTA neuron that responded to N/OFQ with an outward current. B: Across recordings in control rats (46/86), the majority of VTA neurons responded to 10 nM N/OFQ application. Approximately 2/3s of these responses were outward currents. C: A subset of recorded neurons were recovered following whole cell recording and immunocytochemically identified for TH content, a marker for dopamine neurons. Outward currents of similar magnitudes were observed in TH(+) and TH(-) neurons. D: The time courses and maximal effects of bath application of 10 nM and 100 nM N/OFQ were similar. E: Concentration response relationship for VTA neurons showing a positive change, both significant and not significant, in holding current with N/OFQ application (grey dots include all neurons with a change > 0 pA; median shown in white dots; black bars show 25 and 75 percentiles; 1 nM: n = 1/6; 10 nM: n = 55/80; 100 nM: n = 20/25; 1 μM: n = 7/7; 10 μM: n = 3/3).

To confirm responses were due to activation of the NOP and were not off-target effects, we tested whether these inhibitions were blocked by the selective NOP antagonist BTRX-246040. In neurons responding to N/OFQ with an outward current (10 nM mean response = 14

194 ± 3 pA) BTRX-246040 (100 nM) was applied for 10 min followed by N/OFQ application again.
195 BTRX-246040 consistently blocked N/OFQ-induced outward currents (baseline N/OFQ
196 response: 14 ± 3 pA; N/OFQ response in BTRX-246040: -1 ± 2 pA; n = 15; p<0.05; Fig. 2).

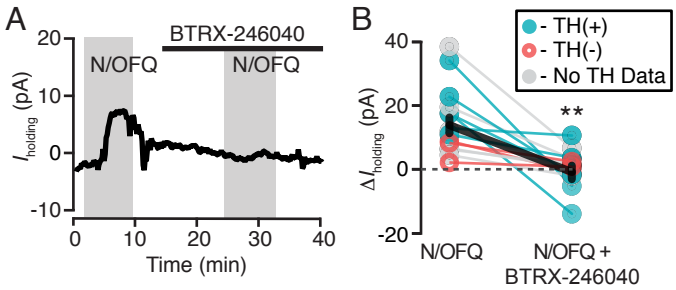

Figure 2: BTRX-246040 consistently blocks N/OFQ induced currents. A: Example recording of a N/OFQ (10 nM) responsive neuron where the selective NOP antagonist BTRX-246040 (100 nM) blocked the response to a subsequent N/OFQ application. B: BTRX-246040 blocked N/OFQ responses across VTA neurons, including both TH(+) and TH(-) neurons (n = 6 and 3, respectively; n = 6 no TH data; mean ± SEM in black). $^{**}P < 0.01$.

197
198 We also observed a subpopulation of neurons that responded to N/OFQ application with
199 a small inward current, consistent with an excitatory effect (10 nM mean response = -16 ± 6 pA)
200 (Fig. 3A,B). Inward currents were observed in approximately 27% (16/60) of the neurons that
201 were responsive to N/OFQ (1-100 nM) and 19% of all tested VTA neurons (16/85) (Fig. 3C,D).
202 Among neurons responding to N/OFQ with an inward current and immunocytochemically
203 identified, 4/12 were TH(+) and 8/12 were TH(-) (Fig. 3E). These N/OFQ evoked excitatory
204 responses were only observed at low concentrations (< 100 nM; Fig. 3D); at higher
205 concentrations inhibitory outward currents were observed (Fig. 1E, 3D).
206
207 *Concentration dependent desensitization of NOP*
208 Given the inconsistencies in the reports of behavioral effects of NOP agonists and
209 antagonists, we tested whether N/OFQ causes rapid NOP desensitization at moderate doses.
210 We observed a concentration-dependent diminished response to a second application of
211 N/OFQ when the first application of N/OFQ was ≥ 100 nM (n = 12; p = 0.00003; Fig. 4A,B). This
212 is consistent with NOP desensitization, and observed in both TH(+) and TH(-) neurons (Fig. 4B).
213 In contrast, following administration of 10 nM N/OFQ, no difference in response was observed
214 between the first and second applications (n = 10; p = 0.13; Fig. 4C,D). Therefore, the
215 remaining experiments were conducted using a concentration of 10 nM N/OFQ to avoid
216 confounds from desensitization.
217
218 *N/OFQ effects on VTA neurons vary with projection target*
219 As described above, we observed heterogeneity in responses of VTA neurons to
220 N/OFQ. Given that other pharmacological responses of VTA neurons, including to kappa opioid
221 receptor activation (Ford et al. 2006; Margolis et al. 2006) vary with projection target, we
222 investigated whether the N/OFQ responses would be more consistent within subpopulations of
223 VTA neurons that share a projection target. Accordingly, we recorded N/OFQ (10 nM)
224 responses in VTA neurons that were retrogradely labeled by tracer injections into mPFC, pACC,
225 or medial NAc (Fig. 5A,B). Recordings were conducted with the investigator blinded to the
226 injection site.
227 The majority of mPFC-projecting VTA neurons, 67% (8/12), were significantly inhibited
228 by N/OFQ (Fig. 5C), responding with an outward current (11 ± 3 pA; Fig. 5D). No excitations

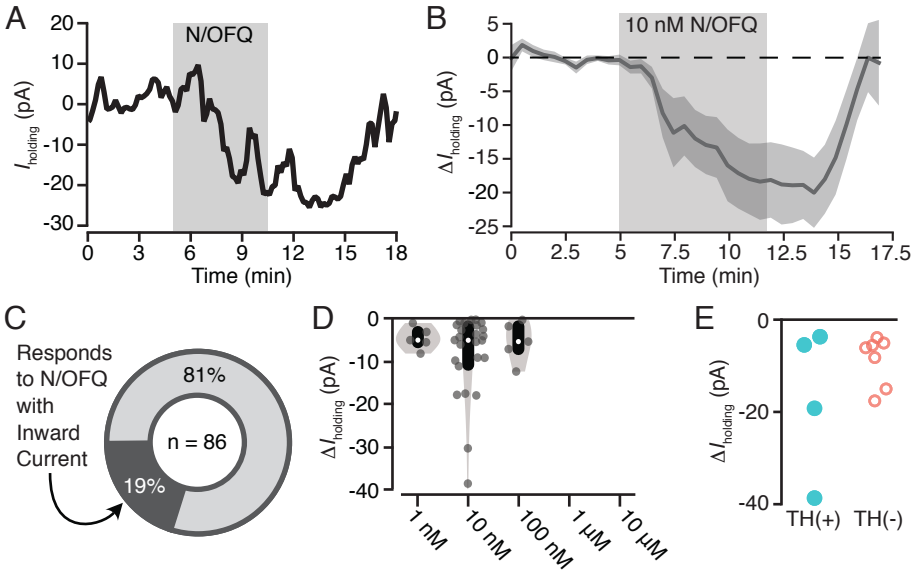

Figure 3: Low dose N/OFQ induced small inward currents in a subset of VTA neurons. A: Example voltage clamp recording ($V_{holding}$ = -60 mV) of a VTA neuron that responded to N/OFQ with an inward current. B: The mean ± SEM time course across neurons with inward currents shows the onset of this response is time locked to the initiation of drug application (n = 13). C: Across all VTA neurons from control rats that were tested for 10 nM N/OFQ responses, 19% responded with a significant inward current. D: Grey dots indicate each neuron showing a positive change, both significant and not significant, in holding current with N/OFQ application (grey dots include all neurons with a change < 0 pA; median shown in white dots; black bars show 25 and 75 percentiles; 1 nM: n = 5/6; 10 nM: n = 25/80; 100 nM: n = 5/25; 1 µM: n = 0/7; 10 µM: n = 0/3) Significant inward currents were observed at 10 nM, while higher concentrations only generated outward currents (see Fig. 1E). E: Inward currents were observed in both immunocytochemically identified TH(+) and TH(-) neurons.

were observed in mPFC-projecting neurons. Five mPFC-projecting neurons were recovered and processed for TH immunoreactivity; two were TH(+), and 3 were TH(-); all of these responded to N/OFQ with an outward current (Fig. 5D).

VTA projections to different cortical targets, including the pACC, arise from largely different VTA neurons (Chandler et al. 2013). The pACC-projecting neurons are concentrated in different parts of the VTA, and fewer of them are dopaminergic compared to the projection to mPFC (Breton et al. 2019). Interestingly, many of the VTA neurons comprising this projection responded to N/OFQ with an inward current (4/6 inward current, -24 ± 12 pA, 1/6 outward current; Fig. 5C,D). These N/OFQ excited, pACC-projecting VTA neurons included both TH(+) and TH(-) cells (Fig. 5D).

Half of NAc-projecting VTA neurons (7/14) responded to N/OFQ with outward currents (9 ± 1 pA; Fig. 5C,D). No excitations were observed in this projection. Of the 7 NAc-projecting neurons that responded to N/OFQ, 2 were confirmed TH(+) and 3 were TH(-) (Fig. 5D). Together, these data indicate that similar N/OFQ inhibitory effects occur in VTA neurons that project to mPFC and NAc, but these effects are opposed to those on VTA projections to pACC, some of which responded to N/OFQ with an inward current.

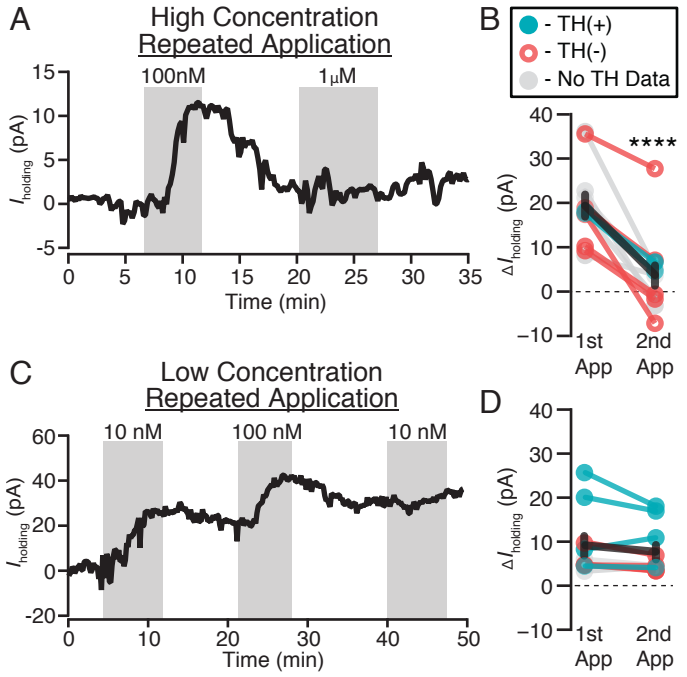

Figure 4: Moderate doses of N/OFQ cause functional desensitization in VTA neurons. A: Example voltage clamp recording ($V_{holding}$ = -60 mV) where 100 nM is sufficient to prevent a subsequent response to 1 μM application of N/OFQ. B: A summary across VTA neurons where the first N/OFQ application was ≥ 100 nM, the response to the second application was consistently smaller (p = 0.00003), in both TH(+) and TH(-) neurons (n = 2 and 5, respectively; n = 5 no TH data). C: Example voltage clamp recording ($V_{holding}$ = -60 mV) showing that 10 nM N/OFQ does not impair responses to subsequent N/OFQ application. In the same cell, 100 nM did prevent additional responding. D: Summary across VTA neurons shows similar magnitudes of responses to the second application of N/OFQ when the first application was 10 nM (p = 0.13; TH(+) n = 4, TH(-) n = 2, TH no data n = 2).

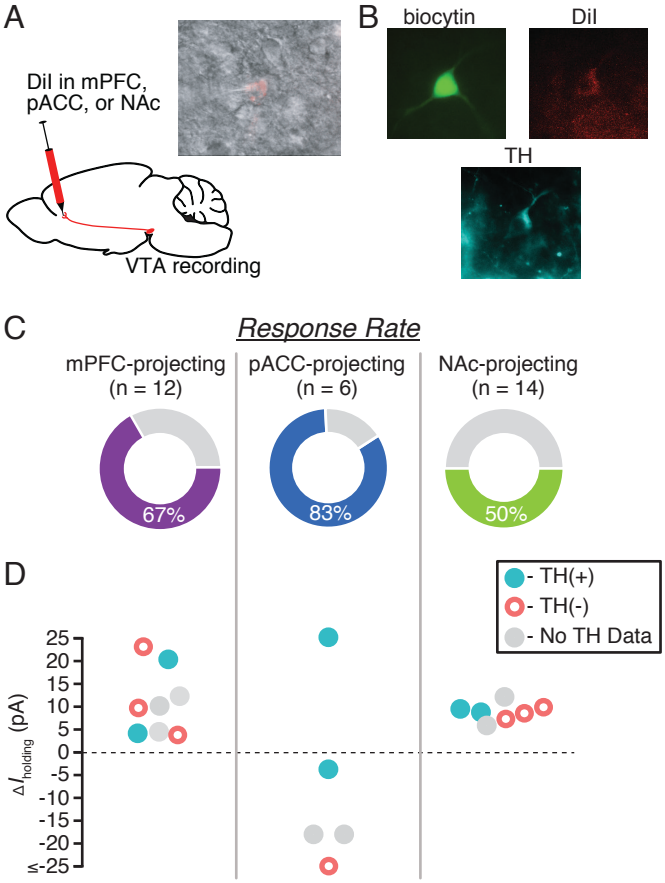

Figure 5: N/OFQ effects in VTA neurons vary with projection target. A: Cartoon showing the experimental approach: 7 days prior to recording, the retrograde tracer DiI was stereotaxically injected into mPFC, pACC, or medial NAc. DiI neurons were identified during whole cell recordings (inset). B: Example image of a neuron filled with biocytin during recording (color), the retrograde tracer (red) and was immunocytochemically identified as TH(+) (green). (fill in after pACC data added) C: The overall percentage of neurons that responded to N/OFQ was greatest among pACC-projecting neurons and lowest among NAc-projecting neurons. D: Graph of magnitudes of significant N/OFQ responses, showing that only pACC-projecting neurons respond to N/OFQ with an inward current.

N/OFQ has little effect on terminal dopamine release in the NAc

ICV or intra-VTA N/OFQ decreases dopamine levels in the NAc (Murphy et al. 1996; Murphy and Maidment 1999). Consistent with this result, we found that N/OFQ directly inhibits

250 a subset of the VTA dopamine NAc-projecting somata. N/OFQ may also inhibit dopamine
251 release in the NAc at the terminals; to test if NOPs on dopamine terminals in the NAc also
252 contribute to an N/OFQ-induced decrease in NAc dopamine levels, we used FSCV to detect
253 changes in stimulated dopamine release in NAc slices (Fig. 6A). In tissue from control SD rats,
254 we stimulated dopamine release with a bipolar electrode. To limit stimulation to dopaminergic
255 axons, we expressed ChR2 in Th::Cre rats and stimulated with 488 nm light pulses. N/OFQ (10
256 nM or 100 nM) had a marginal effect on evoked dopamine release in the NAc (10 nM N/OFQ
257 effect on electrically evoked dopamine release: 90 $\pm$ 4% of baseline, n = 6; 100 nM N/OFQ
258 effect on electrically evoked dopamine release: 94 $\pm$ 8% of baseline in N/OFQ, n = 7; 100 nM
259 N/OFQ effect on optically evoked dopamine release: 77 $\pm$ 5% of baseline in N/OFQ, n = 3; Fig.
260 6B). As a positive control, we applied the selective kappa opioid receptor agonist U69593 (1
261 μM), previously shown to inhibit dopamine release in the NAc (Di Chiara and Imperato 1988a;
262 Ebner et al. 2010; Karkhanis et al. 2016; Spanagel et al. 1992; Werling et al. 1988), at the end
263 of each experiment, after N/OFQ. U69593 caused a substantial decrease in stimulated
264 dopamine release (electrical: 66 $\pm$ 15% of baseline (in N/OFQ), n = 10, and optical: 52 $\pm$ 3% of
265 baseline (in N/OFQ), n = 3, respectively; Fig. 6B). Therefore, the NOP modulation of this
266 dopaminergic circuit is predominantly in the somadendritic region and the terminals in the NAc
267 are relatively insensitive to NOP activation. These results contrast with the kappa opioid control
268 of these neurons, which strongly inhibits release but does not directly hyperpolarize the cell
269 bodies of these neurons (Fig. 6C).
270
271 **Discussion**
272 The results presented here demonstrate that N/OFQ affects both dopaminergic and non-
273 dopaminergic VTA neurons, through activation of the NOP, and primarily causes inhibitory
274 outward currents. These data are consistent with an early report by Zheng et al. 2002, who
275 showed N/OFQ inhibited putative dopamine and putative GABA neurons in midbrain slice
276 recordings. In a small population of neurons, low concentrations of N/OFQ induced an inward
277 current consistent with excitation. N/OFQ effects in these neurons were blocked by the NOP
278 selective antagonist BTRX-246040, confirming its action at NOP. Importantly, neuronal
279 responses to N/OFQ desensitized at concentrations $\geq$ 100 nM. In addition to providing a basic
280 characterization of the range of postsynaptic N/OFQ responses in VTA neurons, we
281 demonstrated differential responding of subsets of VTA neurons to NOP activation related to
282 projection target: mPFC-projecting and NAc-projecting VTA neurons responded to N/OFQ with
283 outward currents (inhibitory), while pACC-projecting VTA neurons responded with mostly inward
284 currents (excitatory). Within the dopaminergic projection to the NAc, N/OFQ caused outward
285 currents at the somatodendritic region of these neurons, and release at the terminals was not
286 inhibited by NOP activation. Together, these data show that N/OFQ effects in VTA neurons
287 differ depending upon their projection target, and that at higher concentrations of N/OFQ only
288 inhibitions are observed, followed by desensitization of NOP function.
289 Unexpectedly, a small population of neurons in the VTA, both TH(+) and TH(-),
290 responded to low concentrations of N/OFQ with an inward current, consistent with excitation.
291 This finding presents a novel mechanism by which N/OFQ could selectively activate specific
292 VTA circuits, while inhibiting the majority of VTA outputs. This effect was only observed in VTA
293 neurons projecting to the pACC, but not those projecting to the NAc or mPFC, consistent with

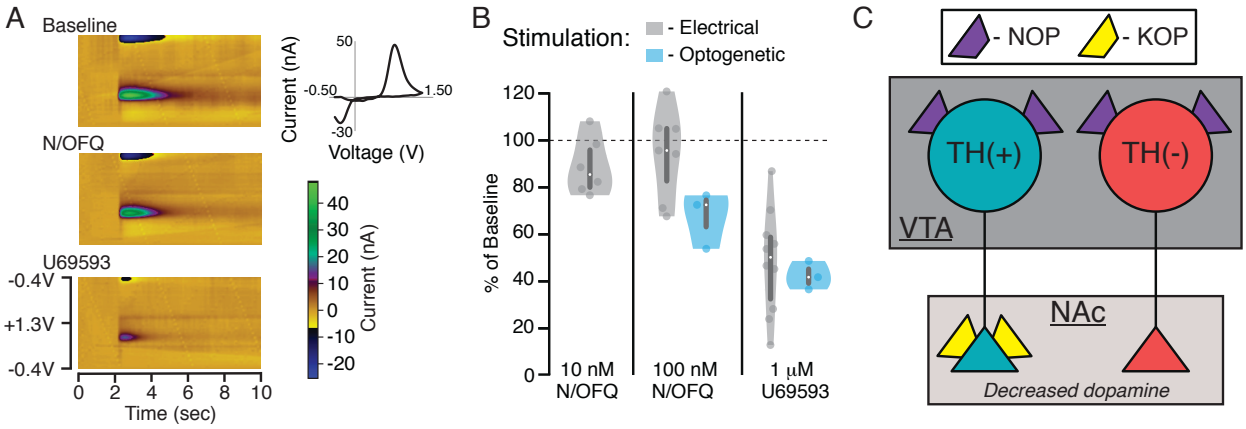

Figure 6: N/OFQ does not inhibit dopamine release at NAc terminals. We used FSCV in acute, coronal slices containing the NAc to test for N/OFQ effects on terminal release of dopamine. Dopamine release was evoked in slices from control rats with bipolar electrodes locally in the NAc. Recordings were made on the NAc shell-core border. Alternatively, to limit stimulation to dopamine fibers, Th::Cre rats were injected with (AAV2-Ef1a-DIO-hChR2(H134R)-mCherry) in the VTA at least 4 weeks prior to recordings, during which 488 nm light pulses were used to stimulate dopamine release. A: Example color plots of FSCV measurement of electrically evoked dopamine release. Inset: background subtracted cyclic voltammogram at peak of putative dopamine release. B: Across slices, 10 and 100 nM N/OFQ had minimal effects on either electrically or light evoked dopamine release in the NAc. In contrast, the kappa opioid receptor agonist U69593 (1 μM) effectively inhibited evoked dopamine release in the same recording slices. White dots represent median values. C: Summary diagram shows the contrast between NOP and KOP function in NAc-projecting VTA dopamine neurons. While NOP activation inhibits the somatodendritic compartment only, KOP induced inhibition is limited to dopaminergic axon terminals in these neurons. Further, NOP activation inhibits NAc-projecting non-dopaminergic VTA cell bodies, which are insensitive to KOP activation.

this circuit-selection proposition. The fact that this effect was only observed at low doses indicates that very robust N/OFQ release into the VTA, on the other hand, would likely have a broad inhibitory effect on the vast majority of VTA neurons, regardless of circuit. While the canonical response of NOPs is inhibitory through activation of Gi/o proteins, there is a growing literature suggesting that classic opioid receptors can signal through a variety of mechanisms, and these phenomena may encompass the NOP as well. Activation of postsynaptic mu or delta opioid receptors results in a $Ca_v2.1$ channel-mediated depolarization in subsets of VTA neurons (Margolis et al. 2014, 2017). Further, the mu opioid receptor agonist DAMGO increases $Ca_v2.1$ currents in cerebellar Purkinje neurons (Iegorova et al. 2010) and morphine activates adenylyl cyclase in the corpus striatum and olfactory bulb (Onali and Olianas 1991; Puri et al. 1975). While this is the first report of N/OFQ-mediated excitations in an acute brain slice preparation, intracellular increases in Ca^{2+} have been observed in a cultured human neuroblastoma cell line in response to N/OFQ in the presence of the cholinergic agonist carbachol (Connor et al. 1996). Therefore, while there are few reports of excitatory actions of N/OFQ, the observation is not unprecedented.

We also found that in neurons treated with a higher concentration of N/OFQ (≥ 100 nM) no longer responded to subsequent applications of N/OFQ. This finding indicates that in the VTA, N/OFQ may act as a functional antagonist at NOP by desensitizing these responses when higher concentrations of N/OFQ are present. This is different from postsynaptic responses to

313 agonists at the mu and delta opioid receptor in VTA neurons, where repeated application of
314 saturating doses of selective agonists generate responses of similar magnitudes (Margolis et al.
315 2014, 2017). The apparent NOP desensitization observed here is consistent with previous
316 studies showing that high concentrations or repeated sustained exposure to NOP agonists
317 causes desensitization in cell culture (Connor et al. 1996; Mandyam et al. 2000, 2002; Thakker
318 and Standifer 2002). In addition, NOPs internalize fairly rapidly (Corbani et al. 2004;
319 Spampinato et al. 2001, 2002; Zhang et al. 2012) at the same concentrations that we observed
320 desensitization. *In vivo*, N/OFQ administration can result in dose dependent performance
321 changes in behavioral spatial memory, locomotor, and anxiety tasks, with low concentration
322 N/OFQ having the opposite effects compared to high doses (Florin et al. 1996; Jenck et al.
323 1997; Sandin et al. 2004). One possible explanation for these opposing behavioral outcomes is
324 that N/OFQ may be acting as an agonist at low concentrations and a functional antagonist at
325 high concentrations.
326 N/OFQ inhibited both dopamine and non-dopamine neurons in the VTA that project to
327 the NAc. This finding is consistent with the observation that N/OFQ administered ICV or into the
328 VTA results in a decrease in extracellular dopamine in the NAc (Murphy et al. 1996; Murphy and
329 Maidment 1999). A prominent proposal in the literature is that a decrease in NAc dopamine
330 produces aversion (McCutcheon et al. 2012). Therefore, one would expect ICV injection of
331 N/OFQ to be aversive. However, this manipulation generates no response in the place
332 conditioning paradigm (Devine et al. 1996). On the other hand, optogenetic or chemogenetic
333 stimulation of N/OFQ containing inputs to the VTA can be aversive and decrease reward
334 seeking (Parker et al. 2019). One possible explanation for this lack of clear motivational effect is
335 the combination of inhibition of both dopamine and non-dopamine neurons: dopamine and non-
336 dopamine neurons originating in the VTA synapse onto different types of neurons in the NAc,
337 therefore affecting behavior in different ways. For instance, VTA glutamate neurons synapse
338 onto parvalbumin containing interneurons in the NAc and optogenetic activation of these NAc-
339 projecting glutamate neurons is aversive (Qi et al. 2016). Activation of NAc-projecting VTA
340 GABA neurons causes a pause in cholinergic interneuron activity (Brown et al. 2012). These
341 neurons modulate associative learning but are insufficient to drive preference or aversion
342 independently (Collins et al. 2019). N/OFQ inhibition of dopamine, GABA, and glutamate
343 neurons projecting to the NAc, therefore, may result in no net hedonic value and a lack of
344 preference in a place preference paradigm. Further, various reports indicate that decreasing
345 activity at dopamine receptors in the NAc with microinjections of antagonists does not produce
346 aversion (Baker et al. 1996, 1998; Fenu et al. 2006; Josselyn and Beninger 1993; Laviolette and
347 van der Kooy 2003; Morutto and Phillips 1998; Spina et al. 2006) but see (Shippenberg et al.
348 1991), and aversive outcomes can even be observed following manipulations that increase
349 dopamine levels in the NAc (Devine et al. 1993b; Shippenberg and Bals-Kubik 1995). Add to
350 this the N/OFQ effects on other circuits following ICV injection, including other VTA neurons,
351 and the possibility that the most robust, long lasting effect is receptor desensitization at higher
352 doses of agonist, together making it potentially less surprising that ICV N/OFQ was not reported
353 to generate aversion.
354 N/OFQ's effect on the VTA to NAc circuit provides an interesting point of comparison for
355 how the NOP may be functionally distinct from the structurally related KOP. *In vivo*, systemic or
356 ICV administration of N/OFQ or a KOP agonist each causes a decrease in extracellular

357 dopamine in the NAc (Devine et al. 1993a; Di Chiara and Imperato 1988b; Murphy et al. 1996;
358 Murphy and Maidment 1999). However, these two receptors function very differently in the
359 dopamine neurons that project to the NAc. We show here that N/OFQ inhibits VTA cell bodies
360 that project to the NAc, but has little effect on the dopamine terminals within the NAc. Kappa
361 opioid receptor activation, on the other hand, has no effect on the cell bodies of NAc-projecting
362 VTA dopamine neurons, but strongly inhibits dopamine release at the terminals in the NAc (Britt
363 and McGehee 2008; Margolis et al. 2006) (Fig. 5C). One implication for this organization is that
364 whether or not the respective endogenous peptides, N/OFQ and dynorphin, affect NAc-
365 projecting dopamine neurons will depend upon the brain region of peptide release. There is
366 also evidence for dopamine release in the NAc that is independent of action potential firing in
367 midbrain dopamine neurons (Cachope et al. 2012; Mohebi et al. 2019). In this organization of
368 differential receptor effects localized to somadendritic regions vs terminals, dynorphin has
369 control over this terminal activity while N/OFQ does not. Together these observations bring into
370 focus the critical importance of understanding precisely where receptors are functional in brain
371 circuits and their specific actions at each site.
372 We found opposing effects of N/OFQ on the VTA projections to mPFC and pACC, which
373 may contribute to the reported N/OFQ impact on behavioral measures associated with cortical
374 dopamine function such as working memory, learning, and behavioral flexibility (Gonzalez et al.
375 2014; Huang et al. 2018; Ott and Nieder 2019; Puig et al. 2014; Tzschentke 2001; Winter et al.
376 2009). Our results also show that the non-dopamine VTA projection to cortical regions are
377 affected by N/OFQ as well; while the majority of the VTA neurons that project to these cortical
378 regions are not dopaminergic (Breton et al. 2019), little is currently known regarding their
379 contribution to behavior. Preclinical studies show that ICV administration of N/OFQ impairs
380 working memory (Hiramatsu and Inoue 1999) and associative learning and memory (Goeldner
381 et al. 2009), and blocking NOP with an antagonist or genetic knockout enhances both working
382 memory and learning (Jinsmaa et al. 2000; Nagai et al. 2007; Noda et al. 1999). How and why
383 such an ongoing break on learning and memory by N/OFQ contributes to normal behavioral
384 adaptation, and whether dopamine or other VTA outputs play a role, remains to be determined.
385 One provocative possibility is that it is this degradation of working memory function that is the
386 primary mechanism underlying the lack of place conditioning in response to central N/OFQ,
387 rather than that this treatment is affectively neutral. This interpretation is consistent with work
388 showing that N/OFQ blocks opioid induced conditioned place preference yet has no effect on
389 opioid self-administration as well (Sakoori and Murphy 2004; Walker et al. 1998).
390 The results of this study extend our understanding of the NOP system biology and
391 provide considerations for additional investigation into NOP function within limbic circuits.
392 These findings clarify that strong NOP desensitization occurs in neurons at moderate
393 concentrations of the endogenous agonist N/OFQ. Importantly, not only does the nature of the
394 NOP response vary with the projection target of VTA neurons, but the NOP function is largely
395 sequestered to the somatodendritic compartment of VTA dopamine neurons that project to the
396 NAc, demonstrating two different kinds of circuit level organization of this receptor system.
397 Building on this groundwork, future studies of these VTA circuits during different behavioral
398 states and tasks related to motivation and cognition will help to elucidate the differences
399 between the normal and dysfunctional NOP-N/OFQ system, improving the potential for
400 therapeutic targeting.

Acknowledgements

We would like to thank Benjamin J. Snyder, Kasra Mansourian, and Venkat Ganesh for their technical assistance. We also thank Howard L. Fields for helpful comments on the manuscript.

Conflict of interest

Yes, TLW and WJM are employees of and shareholders in BlackThorn Therapeutics, which is evaluating BTRX-246040 for the treatment of neurobehavioral disorders.

Funding

This work was supported through a sponsored research agreement from BlackThorn Therapeutics, Inc. to the University of California, San Francisco.

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