Published in final edited form as:

ACS Chem Neurosci. 2019 April 17; 10(4): 1935–1940. doi:10.1021/acschemneuro.8b00437.

Local mu-opioid receptor antagonism blunts evoked phasic dopamine release in the nucleus accumbens of rats

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Abstract

Mu-opioid receptors (MORs) in the nucleus accumbens (NAc) can regulate reward-related behaviors that are dependent on mesolimbic dopamine, but the precise mechanism of this MOR regulation is unknown. We hypothesized that MORs within the NAc core regulate dopamine release. Specifically, we infused the MOR antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2) into the NAc core while dopamine release was evoked by electrical stimulation of the ventral tegmental area and measured by fast-scan cyclic voltammetry. We report that CTAP dose-dependently inhibited evoked dopamine release, with full blockade achieved with the 8 μg infusion. In contrast, evoked dopamine release increased after nomifensine infusion and was unchanged after vehicle infusion. These findings demonstrate profound local control of dopamine release by MORs within the NAc core, which has implications for regulation of reward processing.

Graphical Abstract

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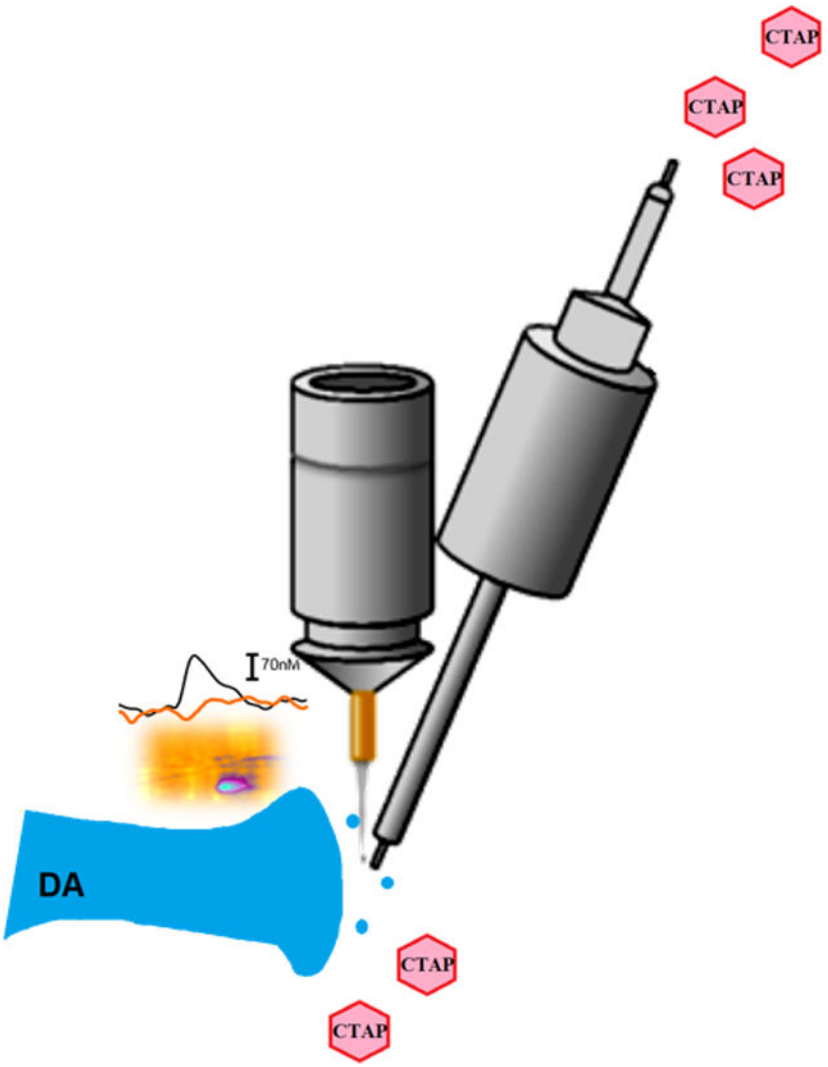
Author contributions: TAS, DLR, LAS and SMN designed the studies; TAS, AGA, ELB and HMB collected and analyzed the data; LAS developed the infusion technique and provided training; DLR, AGA and SMN interpreted the data; AGA, HMB, TAS, SMN and DLR wrote the manuscript; all authors edited and approved the manuscript.

Conflict of interest: The authors declare no real or perceived conflict of interest associated with this research.



Keywords

Mu opioid receptor; dopamine; accumbens; phasic; electrochemistry; local infusion

The nucleus accumbens (NAc) is in the ventral extent of the striatum and constitutes a main input nucleus of the basal ganglia. Multiple neurotransmitter systems participate in the function of this nucleus; however, dopamine projections from the ventral tegmental area (VTA) are key modulators of the NAc.[1–2] Mesolimbic dopamine is critical for reward learning and motivational processing,[3] and its dysregulation is associated with disorders such as addiction and schizophrenia.[e.g., 4–5]

In the NAc, mu-opioid receptors (MORs) are located on both cholinergic and GABA interneurons and GABA medium spiny neurons.[6–7] MOR activity affects reward-related behavior, as seen with direct infusion of opioid drugs to the NAc. For example, infusion of MOR agonists in the NAc promotes both hedonic taste reactions to sucrose[8] and consumption of palatable foods, particularly those high in fat.[9–14] Intriguingly, however,

infusion of MOR antagonists into the NAc does not reliably reduce palatable food consumption, with some studies reporting reductions whereas others show no or smaller effects.[15–19] In contrast, blockade of MORs in the NAc reduces dopamine-dependent appetitive behaviors in several paradigms, including progressive ratio for food reward,[20] a runway task,[21] operant responding during a Pavlovian-instrumental transfer test,[22] cocaine-induced place preference and hyperactivity,[23] and cued approach to a receptacle where cream reward is available.[24] These results raise the hypothesis that endogenous opioids in the NAc promote reward-seeking behavior by increasing the release of dopamine from dopamine terminals. Consistent with this idea, NAc MOR activation can increase dopamine levels in the NAc as measured by microdialysis,[25–29] perhaps by inhibition of GABA release onto cholinergic neurons that stimulate dopamine release via nicotinic receptor-mediated excitation of dopamine terminals.[30–32]

Although much evidence suggests that activation of MORs within the NAc is sufficient to enhance tonic dopamine, it is presently unclear whether endogenous opioids promote phasic dopamine release. We hypothesized that local infusion of the MOR antagonist CTAP (2, 4 and 8 μg) would decrease electrically evoked dopamine release in a dose-dependent fashion. To test that, we used fast scan cyclic voltammetry (FSCV), an electrochemical technique with a high anatomical and temporal resolution,[33] to measure evoked dopamine transients before and after the local infusion of CTAP into the NAc. Unlike systemic administration, local infusion circumvents the possibility that the drug acts elsewhere in the brain or body, and specifically tests how local modulation of MORs influence dopamine dynamics.

To apply drugs directly to the electrochemical recording site *in vivo*, we employed an infusion technique adapted from previous studies.[34–35] This method combined an infusion cannula with a guide cannula for a carbon-fiber microelectrode, such that the tip of the injector was positioned approximately 150 μm from the active surface of the electrode (Fig. 1A). Estimated electrode/injector placements for the CTAP infusions (described below) are depicted in Fig. 1B.

To characterize this technique, we first infused nomifensine (NOM), a dopamine transporter inhibitor with well characterized effects on extracellular dopamine: it enhances evoked dopamine release and slows subsequent clearance.[36] Each rat was anesthetized and secured in a stereotaxic frame, and the pre-assembled guide cannula ensemble was secured on the skull to target the NAc core. Next, a carbon-fiber microelectrode was lowered into the NAc via a manipulator inserted in the guide cannula; each turn of the manipulator wheel pushed the electrode 300 μm into the tissue. The electrode was lowered to a predetermined position near the infusion cannula. There, a range of potentials was applied to the electrode using a triangle waveform (−0.4V to 1.3V to −0.4V, 400V/s, 10Hz) while dopamine neurons were periodically activated via electrical stimulation to the VTA (125nA, 24 pulses at 60Hz, biphasic, 2ms/phase). Dopamine release time-locked to the electrical stimulation was electrochemically confirmed via the background-subtracted cyclic voltammogram containing oxidative and reductive current peaks characteristic for catecholamines. Next, the infusion injector was slowly inserted and evoked dopamine was again confirmed.

Locally applied NOM (40 μM, 0.5 μl over 2 minutes) enhanced VTA-evoked dopamine release in the NAc core of anesthetized rats (n=4). Representative dopamine signals obtained before and after NOM application in an individual rat are shown in Fig. 2A. The color plots show current (color) at each applied potential (y-axis) over a 10-second scan (x-axis). Current at the peak oxidation potential of dopamine, converted to dopamine concentration using *in vitro* electrode calibrations, is depicted in the line graph above the color plot. Composite data (Fig. 2B) show that NOM significantly increased evoked dopamine within 5 minutes after the start of infusion (one-way RM ANOVA: $F_{3,12}$=3.9, p<0.001). Pre-infusion, evoked $[DA]_{max}$ was approximately 65 nM, and this increased 5-fold at 15 min post-infusion. Post-hoc analysis revealed that $[DA]_{max}$ was significantly higher at each time point post-infusion (all t's > 2.3, all p's < 0.03). These results, along with those from the saline group, indicate that drugs delivered via this infusion technique could quickly alter local dopamine release.

With this validated infusion technique, we next aimed to explore the local effect of MOR antagonism on evoked DA release. In separate groups of rats (n=4–5 rats per dose), CTAP (2, 4 or 8 μg in 0.5 μl) was infused over 2 minutes and compared to vehicle infusions (0.5 μl saline). Data from individual rats (Fig. 3A, as described for Fig. 2A) illustrate that saline did not alter evoked dopamine release, while CTAP diminished it. The left panels illustrate dopamine release after electrical stimulation (at 5 seconds) under baseline conditions; note the individual variability in release due to factors such as the stimulating electrode placement and carbon-fiber length. The right panels show evoked dopamine release in the same rats 30 min after saline, 4 or 8 μg CTAP, and CTAP reduced release while saline did not. The dose-dependent effect of CTAP across rats is depicted in Fig. 3B, as the change in dopamine release from baseline across all doses is presented over time. While saline and 2 μg CTAP minimally affected evoked dopamine release, both 4 and 8 μg CTAP reduced the dopamine signal. Statistics were calculated on the average change in dopamine over 60 minutes post-infusion (Fig. 3C). A one-way ANOVA revealed a main effect of group ($F_{3,15}$=11.7, p<0.001) and Holm-Sidak post-hoc comparisons found that both 4 and 8 μg CTAP significantly reduced evoked dopamine release compared to saline (both t's>4.6, p's≤0.002), while the 8 μg dose was also different from the 2 μg dose (t=3.3, p<0.02).

One potential explanation for the reduction in evoked dopamine signal after CTAP application is that CTAP fouled the electrode, reducing its sensitivity for dopamine. To test this, fresh, unused electrodes (n=8) were calibrated with 1 μM dopamine before and after immersion in 16 μg/μl CTAP solution (the concentration used for 8 μg infusions) for 10 minutes. The average current from dopamine oxidation was initially 14±3 nA. Next, electrodes were immersed in the CTAP solution while we applied the same potentials to the carbon-fiber electrode as used *in vivo*. The 10-minute period was chosen to ensure any potential CTAP effect; however, during experiments the infusion occurred over a 2-minute period followed by drug diffusion and subsequent decrease in CTAP concentration around the electrode. The average current from dopamine oxidation obtained after CTAP application was unchanged, at 12±2 nA (paired t-test, t_7=1.4, p=0.207).

Together, these data clearly demonstrate that MOR antagonism can reduce evoked dopamine release, indicating a role for endogenous opioids in the NAc to regulate mesolimbic

dopamine. This finding is consistent with studies reporting that NAc MOR activation increases dopamine levels in the NAc.[25, 29–32] As dopamine terminals express kappa-opioid receptors and delta-opioid receptors, but not MORs,[37–39] CTAP is not acting directly on dopamine terminals. However, MORs are expressed on GABA interneurons,[37] cholinergic interneurons[46] and medium spiny neurons expressing dopamine D1 receptors (D1-MSNs),[38] and together these cells appear to modulate neuronal activity of the whole striatal network.[40] Thus, CTAP would act on several different targets into the NAc changing local and circuital dynamics, and the observations reported here likely result from a combination of actions. For example, it is well known that acetylcholine (ACh) locally regulates dopamine release via nicotinic ACh receptors.[39–42] In this way, nicotinic ACh receptor activation on dopamine terminals can act as a low-frequency pass filter, enhancing initial dopamine release probability but reducing phasic transients, while inactivation of nicotinic ACh receptors – either by reduction of ACh release or desensitization of the receptors – acts as a high-frequency pass filter to blunt low-frequency stimulated dopamine release but augment burst-like stimulated dopamine release. Thus, CTAP, by blocking the inhibitory effects of MORs on ACh release, might enhance these effects of ACh on dopamine terminals. On the other hand, GABA interneurons also express MORs, and by the same reasoning, CTAP antagonism at those receptors would enhance GABA inhibition of cholinergic interneurons and subsequent inhibition of ACh release. Also, other players involved in local activity include M2/M4 metabotropic autoreceptors that modulate the release of ACh,[43–44] GABA-B receptors expressed on dopamine terminals that inhibit dopamine release,[45] and glutamatergic terminals that are regulated by ACh via nicotinic ACh receptors and in turn stimulate dopamine terminals via ionotropic glutamate receptors.[46] Thus, decreased dopamine release after CTAP could be the result of multiple, different interactions in the NAc.

Of course, CTAP effects on striato-tegmental circuits may have contributed to the present results. MORs are present on direct-pathway D1-MSNs, and there is evidence that MSNs form monosynaptic connections with dopamine neurons in the VTA.[47–49] In this case, if CTAP binds to MORs expressed on these D1-MSNs, GABA release would increase and, consequently, inhibit VTA dopamine neurons. On the other hand, electrophysiological evidence indicates that the majority of MSN input from the NAc to the VTA is to GABA neurons, including interneurons.[50–52] In that case, one would predict the opposite effect of CTAP in the NAc, as enhanced GABA release would inhibit VTA interneurons and disinhibit dopamine neurons.[51] However, by locally infusing CTAP into the NAc and delivering current to stimulate the dopamine neurons in the VTA, it is less likely that circuit-based pharmacological effects were the main contributor to the findings reported here.

As several different variables are involved in regulating dopamine dynamics, the complexity of the variables makes it difficult to give more weight to one particular factor over the others. In fact, the results we are presenting here could arise from a complex interaction between different variables (as mentioned) and possibly others not included. The nature of such interaction remains to be determined. At present we cannot rule out the possibility that CTAP had non-specific effects on receptors other than MOR receptors, although we are confident that our results are not due to fouling of the carbon-fiber electrodes.

In conclusion, this study determined that the MOR antagonist CTAP dose-dependently reduced evoked DA release in the NAc via local mechanisms. Specifically, we found that while 2 μg CTAP did not alter dopamine release, 4 and 8 μg CTAP immediately reduced [DA]max following infusion. These findings provide a reliable method to measure effects of a drug directly infused at the electrode and further the understanding of the role of μ-opioid receptor antagonism on dopamine regulation in the NAc. Our results offer support for behavioral studies that showed reduction of dopamine-dependent appetitive behaviors[20] after blockade of MORs in the NAc, suggesting that the reported behavioral effects are due to reductions in dopamine release. However, the mechanism underlying the observed effects is still unknown and additional studies are necessary to completely understand those mechanisms.

Methods

Animals

Adult, male Long-Evans rats were purchased from Envigo Laboratories (Indianapolis, IN) at 225–250g; we chose these rats as male Long Evan rats were also used in studies demonstrating that CTAP infused into the NAc reduced cue-evoked reward seeking.[24] All animals were pair-housed in a facility with a 12-hr light, 12-hr dark cycle with rat chow and water available ad libitum. All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals with procedures approved by the Institutional Animal Care and Use Committee of the University of North Carolina.

Fast scan cyclic voltammetric measurements

Urethane (50% w/w solution in saline, 1.5g/kg, IP) was used to anesthetize the rats throughout the experiment. During surgery, a reference electrode was placed in the left hemisphere and secured with a stainless steel screw and dental acrylic. Then, a stimulating electrode was placed in the VTA at the coordinates (from bregma) AP −5.2mm, ML +1.0mm and DV −8.0mm; the DV coordinate was adjusted in each rat to elicit an evoked dopamine signal with S:N > 30. Finally, an assembled guide cannula ensemble was placed in the NAc core with coordinates AP +1.6mm and ML +1.7mm. A schematic of the guide cannula ensemble is shown in Fig. 1. The ensemble consisted of a guide cannula for the carbon-fiber electrode (left) and a guide cannula for the injector (right) used to deliver the drug during the experiment. As demonstrated in the figure, the two cannulae were arranged to achieve an approximate distance of ~150 μm between the carbon-fiber and end of the injector. The assembly of the two guides was performed prior to the experiments and was done under the microscope to ensure the distance between the electrode and site of drug infusion.

FSCV at carbon-fiber electrodes (cylinders, 6–7μm diameter, 86±13μm active length, insulated in glass) was used to measure electrically evoked DA release in the NAc core as previously described.[53–54] Measurements were taken every 100 ms with an applied potential from −0.4V to +1.3V and back to −0.4V at a rate of 400 V/s versus the Ag/AgCl reference electrode. In order to mimic phasic dopamine release, 24 pulses of 125–300μA (60 Hz, biphasic, 2 ms/phase) were delivered to the VTA using a bipolar stimulating electrode (Plastics One, Inc., Roanoke, VA). TarHeel CV (UNC Department of Chemistry) was used

to collect and analyze the electrochemical data. The current associated with dopamine oxidation was used to evaluate dopamine release.

During each experiment, the carbon-fiber electrode was lowered into the NAc using a micromanipulator to the predetermined depth for that cannula ensemble (approximately DV −6.5 to −7.0mm from bregma), and electrically-evoked dopamine release was measured with FSCV. The stimulating electrode was lowered at increments of 0.2 mm until an evoked dopamine signal at appropriate signal:noise was found. Next, the injection apparatus was set up, the injector was filled with a drug or saline solution, and the injector was inserted into the guide cannula. At least 15 minutes elapsed to allow the tissue to adjust to the injector insertion. Three basal level measurements and twelve post-drug infusion measurements of electrically evoked dopamine release were collected with 5 minutes between each stimulation.

Drugs and Injection Procedure

NOM and CTAP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in saline, which was used as a vehicle control solution. Drugs were injected in a volume of 0.5 µl over a period of 2 minutes, and at least 2 additional minutes were allowed for diffusion before the next electrical stimulation. Independent groups of rats received different doses of CTAP (0, 2, 4 and 8 µg). Measurements continued for up to 60 minutes post-infusion with 5 minutes between stimulations.

In vitro calibration

Electrodes were calibrated *in vitro* after each experiment. A flow cell was used such that the carbon fiber electrode and reference electrode were constantly submerged in flowing TRIS buffer (32.5 mM KCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20mM Na_2SO_4, 12.5 mM NaH_2PO_4, 1.45 M NaCl, 150 MM TRIS). A ValveLink 8.2 controller (AutoMate Scientific, Berkeley, CA) was used to switch from buffer to 1 µM dopamine in buffer for a period of 5 seconds, then back to buffer.

To evaluate a potential fouling effect of CTAP application on the sensitivity of electrodes to dopamine, an *in vitro* experiment was performed. Freshly made carbon-fiber electrodes with lengths of 75±20 µm were soaked in isopropyl alcohol for 20 minutes,[55] and then calibrated as described. After initial calibration, electrodes were placed in 8 µg CTAP solution along with a reference electrode with the FSCV waveform continuously applied at 10 Hz for a period of 10 minutes. Immediately afterward, the electrodes were recalibrated in the flow cell using the same method as described above.

Acknowledgments

Funding sources: This research was funded by a Klarman Family Foundation grant to SMN and by the UNC Bowles Center for Alcohol Studies. ELB was supported by a Summer Undergraduate Research Fellowship from the Office for Undergraduate Research at the University of North Carolina at Chapel Hill.

Abbreviations

ACh	acetylcholine

CTAP	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2, MOR antagonist
FSCV	fast scan cyclic voltammetry
MOR	mu opioid receptor
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NOM	nomifensine, dopamine transporter blocker

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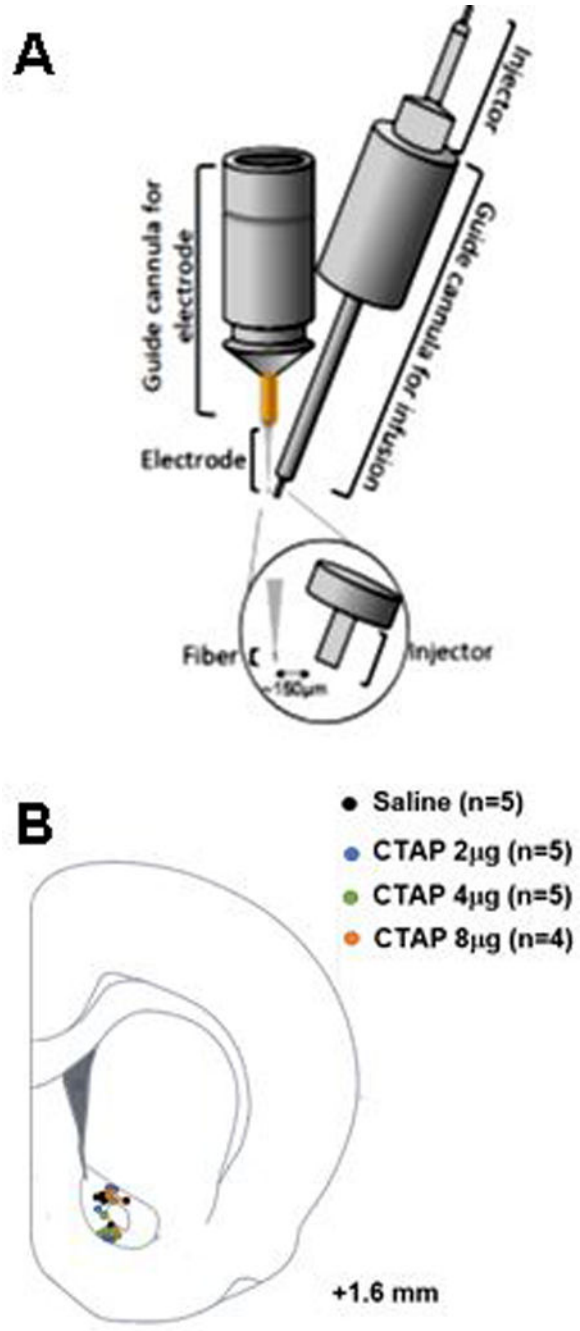

Figure 1.

Schematic representation of the guide cannula ensemble and anatomical placements for simultaneous *in vivo* voltammetric measurement of evoked dopamine release and local application of a drug. (A) Guide cannula ensemble: The electrochemical measurements were performed at carbon-fiber microelectrodes lowered via the guide cannula on the left. The drug applications were performed via the injector inserted into the guide cannula on the right. Both cannulae were positioned and cemented together under a microscope prior to any experiment to ensure an approximate distance of 150 µm between the electrode and end of

the injector. (B) Representations of electrode/injector placements within nucleus accumbens core.

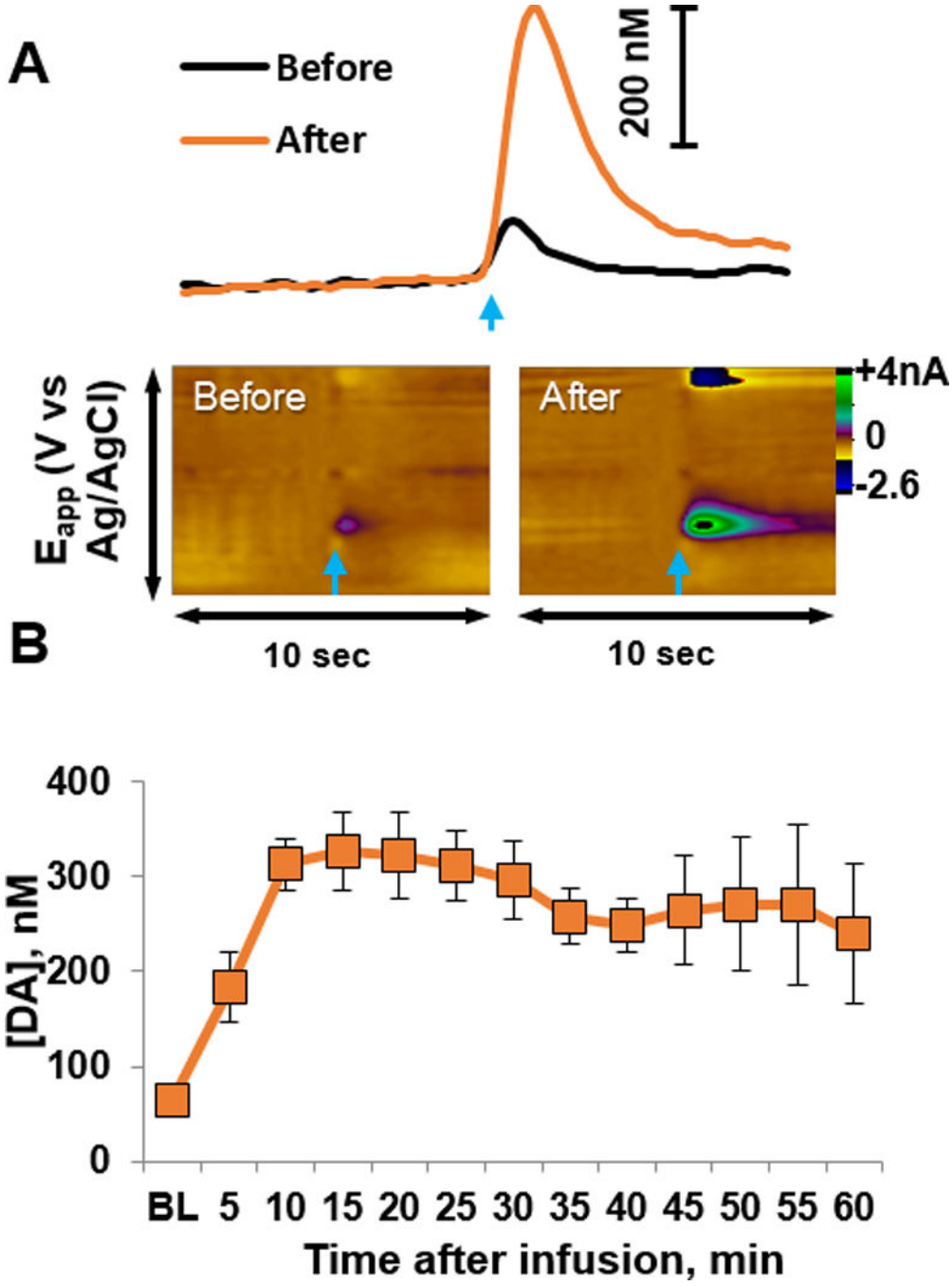

Figure 2.

Locally applied nomifensine enhanced evoked dopamine transients. (A) Current versus time traces (top) and color plots (bottom) of evoked dopamine release from an individual rat before and after nomifensine infusion. The time of electrical stimulation is indicated by blue arrows. In the color plots, current (color) is depicted at the different applied potentials (y-axis) over time (x-axis). Dopamine oxidation is evident as positive current at ~ 0.65 V and reduction is evident as negative current at ~ −0.25 V. (B) Composite data (n=4 rats) show

that nomifensine enhanced evoked $[DA]_{max}$ within 5 minutes, with a peak response at 15 minutes. * different from baseline (BL), $p<0.03$.

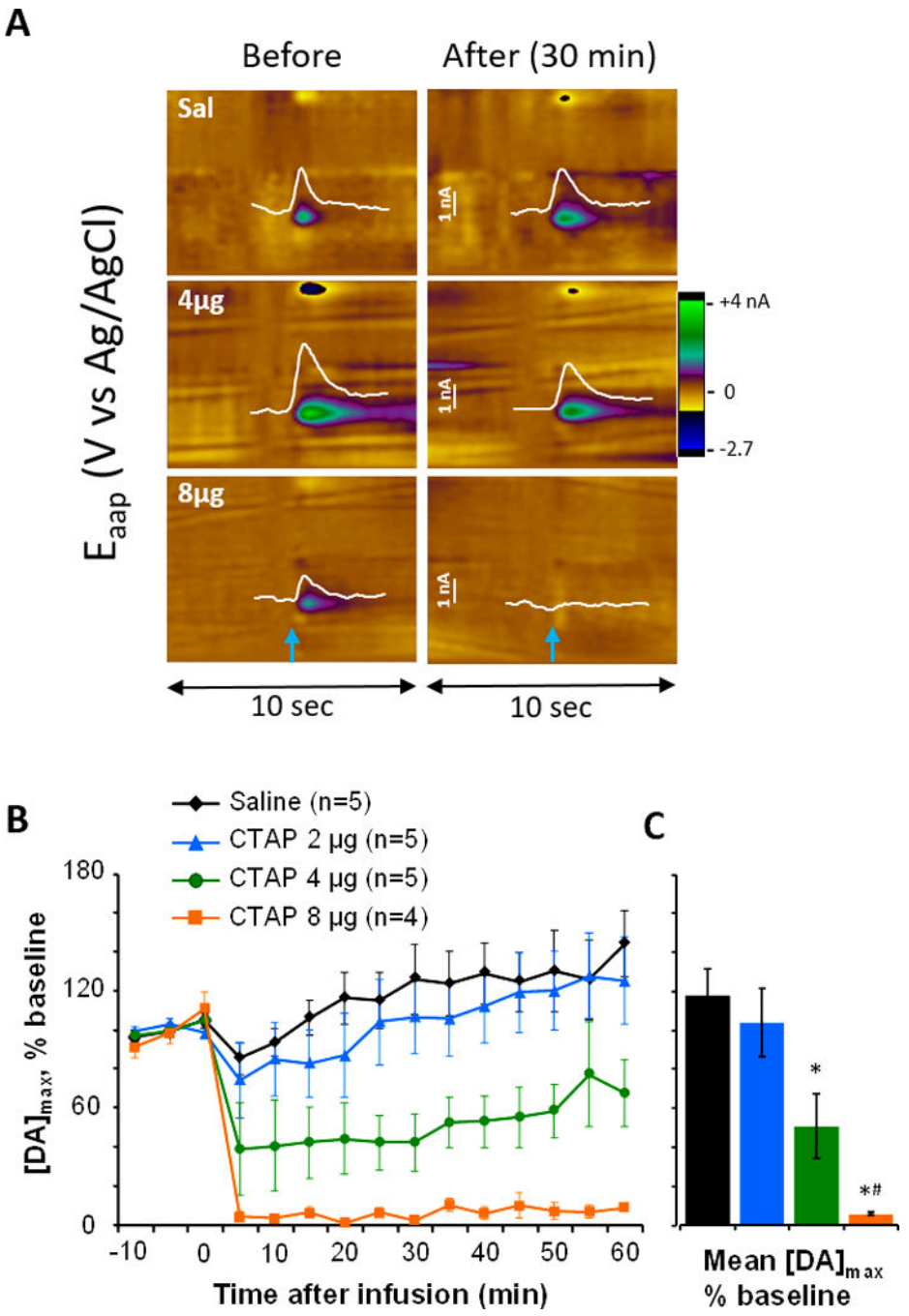

Figure 3.

Infusion of CTAP to the nucleus accumbens core dose-dependently reduces evoked dopamine release. The selective MOR antagonist CTAP (2, 4, and 8 µg) or saline was infused to the area of dopamine measurement via an infusion cannula approximately 150 µm from the voltammetric electrode. (A) Current-versus-time traces at the oxidation potential of dopamine (white, 7 seconds) are overlaid on color plots of evoked dopamine release from individual rats before and after saline, 4 µg and 8 µg CTAP infusion. The time of electrical stimulation is indicated by blue arrows. Infusion of 4 µg CTAP partially blunted electrically-

evoked dopamine release while 8 μg CTAP blocked it. (B) and (C): Composite data show the dose-dependent effects of CTAP on evoked dopamine release over time (B) and averaged across post-infusion time points (C). Electrically-evoked dopamine was unchanged by saline and 2 μg CTAP, but reduced by 4 and 8 μg CTAP. Statistics were calculated on the data in panel C: * different from Saline, $p<0.05$; # different from 2 μg CTAP, $p<0.05$.