Supporting Information

Surgical procedure

Rats were anesthetized with isoflurane, and a polyurethane catheter (Instech Laboratories, Plymouth Meeting, PA) was implanted into the right jugular vein. The catheter was connected to a vascular access button (Instech Laboratories) which was located on the back just caudal to the scapula. Rats were allowed to recover from surgery for 7 days and during which time catheters were flushed daily with Baytril (0.23 mg/ml) followed by 0.1 mL of heparinized saline (30 IU/mL) to maintain catheter patency and prevent infection. Carprofen (5 mg/kg, s.c.) was given post-operatively for 7 days for analgesic support. Catheter patency was verified daily with post-session flushing of catheters. The resistance to flow was used as the first indicator of possible catheter occlusion. If catheter occlusion was suspected, 0.2 mL methohexital (10 mg/mL) was administered (i.v.) and loss of muscle tone within 5 sec indicated a patent catheter. If catheter patency was lost, the day's data point was excluded and the rat was excluded from self-administration studies until a left jugular catheter was implanted or was removed from the study if stability was not met after the second surgery.

Apparatus

Sessions were run in modular operant chambers, equipped with two retractable levers, a stimulus light above each lever, and a house light located on the opposite wall (Med Associates, St.Albans VT). Chambers were located in sound-attenuating cabinets equipped with fans to provide ventilation and mask external noise. For the self-administration sessions, a spring-leash tether connected to the vascular access buttons on the rat housed the infusion line through which drug injections were delivered via an infusion pump (Razel Model A) outside of the experimental chamber.

Self-administration procedures

Briefly, sessions started with extension of two levers, a priming infusion of the dose of drug available for that session and illumination of a houselight; all indicating the availability of a drug.

Designations of the “active” and “inactive” lever were counter-balanced across rats. Responding on the active lever resulted in intravenous drug infusions; responding on the inactive lever had no scheduled consequence. Initially, a single response (fixed ratio 1; FR1) on the active lever resulted in the retraction of both levers, illumination of the stimulus light above the active lever, and infusion of 3 ug/kg/infusion remifentanil 3 µg/kg remifentanil (n=30) or 56 µg/kg oxycodone (n=40) over the course of 4-6 seconds. The stimulus light remained on for 10 sec following completion of each FR (10 sec timeout; TO) after which the light turned off and levers were extended again. Once the rat received >10 infusions for 2 consecutive days, the response requirement was gradually increased to FR10 (remifentanil) or FR 3 (oxycodone). Sessions lasted 1 h (remifentanil) or 2 h (oxycodone) in duration or until 100 ratios were completed. Following completion of each daily session, catheters were flushed with 0.1 mLs of heparinized saline (30 IU/mL) to maintain catheter patency. Acquisition of stable self-administration was defined as >10 infusions/day for 3 consecutive days and <25% variability in number of infusions with no upward or downward trends evident.

Remifentanil self-administration under a fixed ratio 10 schedule of reinforcement

Rats were initially trained to respond under a FR10 schedule of reinforcement using a training dose of 3 µg/kg/infusion remifentanil. The effects of 30 mg/kg ML375 were determined across a full remifentanil dose-response curve by substituting 1-10 µg/kg/infusion remifentanil or saline (dose order randomized per animal) for the maintenance dose for three consecutive days followed by a single session in which animals were pretreated 15 min before the start of the session with ML375 30 mg/kg. A follow-up session at the same dose of remifentanil was assessed to evaluate any long-term effects on behavior. Animals were then returned to the maintenance dose until reaching the stability criteria at which point another dose of remifentanil was substituted. Only rats that completed all conditions of the study were included for a within-subject assessment.

Remifentanil or oxycodone self-administration under a progressive ratio schedule of reinforcement

Rats were initially trained to respond under a FR3 schedule of reinforcement (training doses of 3 µg/kg/infusion remifentanil or 56 µg/kg/infusion oxycodone) similar to the methods described above. Once rats responded reliably, a progressive ratio schedule of reinforcement was initiated. The response requirement for each subsequent reinforcer was determined by the exponential equation described by Richardson and Roberts (1996), ratio = [5 × e(R × 0.2)] – 5, in which the first response requirement (1 response) corresponds to the 1st value given by this equation (1) and was followed by 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, etc. Sessions ended when 30 min had elapsed without a completed ratio; the last completed ratio was determined as the break point (BP). Rats then self-administered 1-10 µg/kg/infusion remifentanil or 10-100 µg/kg/infusion oxycodone. The effects of vehicle, 3, 10 and 30 mg/kg ML375 was determined on each dose of remifentanil or oxycodone. The self-administration doses for each rats were quasi-randomized to account for behavioral history, repeated dosing, etc., with each rat self-administered the maintenance dose (3 µg/kg/infusion remifentanil or 56 µg/kg/infusion oxycodone) for at least 1 session such that altering opioid doses for self-administration was always occurring from a similar baseline rate of responding. Self-administration of each dose was allowed to stabilize (as noted above) before testing the effects of ML375 or naltrexone on opioid self-administration. Only rats that completed all conditions of the study were included for a within-subject assessment.

Oxycodone-induced antinociception

The effects of ML375 on oxycodone-induced anti-nociception were assessed in the hot plate and tail flick tests. In the hot plate assay, pain responses were determined by placing a rat on a hot plate maintained at 55 °C (Analgesia Meter model HP/fj, Omnitech Electronics, Columbus, OH) and measuring the latency to jump or lick a hindpaw. If an animal failed to respond within 60 s (cutoff time), it was removed from the hot plate and was assigned a score of 60 s.

In the tail flick assay, the terminal 5 cm of a rat's tail were immersed in water heated to 55 °C using a water bath (need manufacturer) and the latency to withdraw (flick) the tail before and after treatment was determined. If a rat failed to respond within 10 s (cutoff time), it was removed from the test and assigned a score of 10 s.

Spontaneous locomotor exploration

The impact of ML375 on spontaneous locomotor exploration of an unfamiliar open field was tested using a SmartFrame Open Field System (Kinder Scientific, San Diego, CA) with a 16 × 16 array of infrared photo beams located 2.5 cm above the floor of the chamber (see Bubser et al. 2014). Fifteen minutes after i.p. administration of vehicle or ML375 (10 or 30 mg/kg) animals were placed in the open field and both the time course of ambulation (beam breaks per 5-min bins) and total ambulations (beam breaks in the 60-min session) were examined.

Bioanalysis for determining potential drug-drug interaction

Bioanalysis of *in vivo* plasma and brain samples for quantitation of ML375 (VU0483253) was performed essentially as described previously [52], except calibration curves for plasma and brain samples were constructed independently in blank samples of each matrix. Quantitation of oxycodone was performed using the same sample preparation techniques, LC-MS/MS instrumentation, and analysis methods used for ML375, with the following exceptions – the HPLC gradient started at 5% mobile phase B after a 0.2 min hold and was linearly increased to 90% B over 1.2 min, held at 90% B for an additional 0.1 min, and then returned to 5% B over 0.2 min, which was followed by a 0.3 min re-equilibration period.

M_5 mRNA is expressed in dopaminergic neurons in the substantia nigra

In the SNc, approximately 90% of M_5 cells co-expressed TH (see Supplemental Figure S2A). About 80% of TH cells in the SNc expressed M_5 (see Supplemental Figure S2B). Cells expressing vGAT in the SN for the most part did not express M_5 (see Supplemental Figure S2C).

Supplementary Table 1. Brain and plasma concentrations after single or combined administration of ML375 (56.6 mg/kg, i.p.; 0.75 h) and oxycodone (5.6 mg/kg, s.c.; 0.5 h).

	N	*ML375*		*Oxycodone*	
		Plasma [ng/mL]	*Brain [ng/g]*	*Plasma [ng/mL]*	*Brain [ng/g]*
ML375 + Vehicle	*5*	*668* ± 147	1261 ± 338		
Vehicle + Oxycodone	*5*			1017 ± 251	2915 ± 420
ML375 + Oxycodone	*7*	565 ± 127	1062 ± 281	761 ± 157	2265 ± 299
% Compound alone [#]		85	84	75	78

[#] *% Compound alone* = 100 x [concentration after combined administration] / [concentration after single administration]; Data are means ± S.E.

Two-tailed t-tests confirmed a lack of effect of co-administration of ML375 and oxycodone concentrations

ML375 levels
Plasma: ML375+Vehicle vs. ML375 + OXY t_{10}*=0.52, p = 0.64*
Brain ML375+Vehicle vs. ML375 + OXY t_{10}*=0.45, p = 0.66*

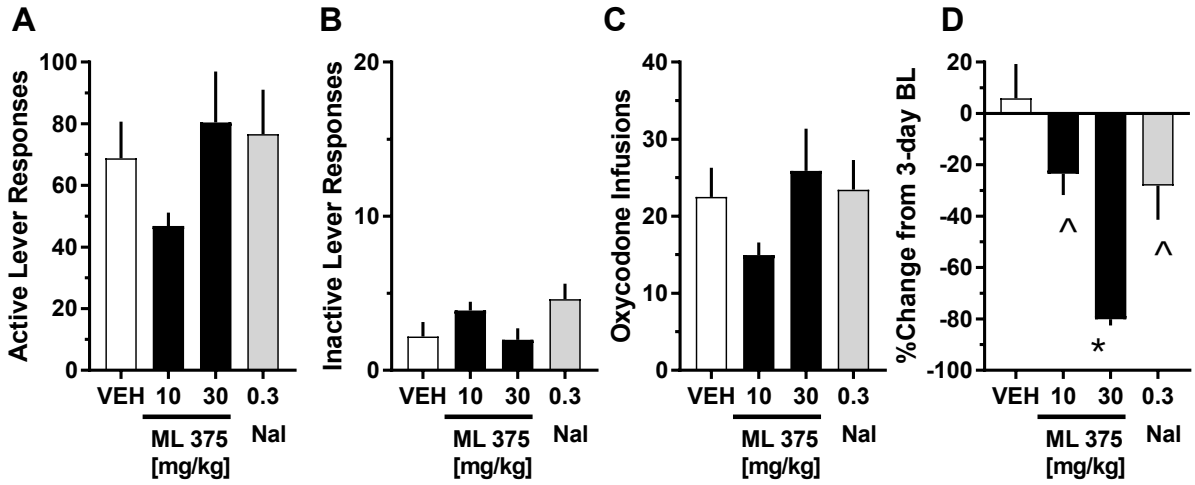
Oxycodone levels
Plasma: Vehicle + OXY vs. ML375 + OXY t_{10}*=0.91, p = 0.38*
Brain Vehicle + OXY vs. ML375 + OXY t_{10}*=1.30, p = 0.22*

Supplementary Table 2. Density of TH-, vGAT-, and M_5- mRNA expressing cells in the ventral tegmental area and substantia nigra

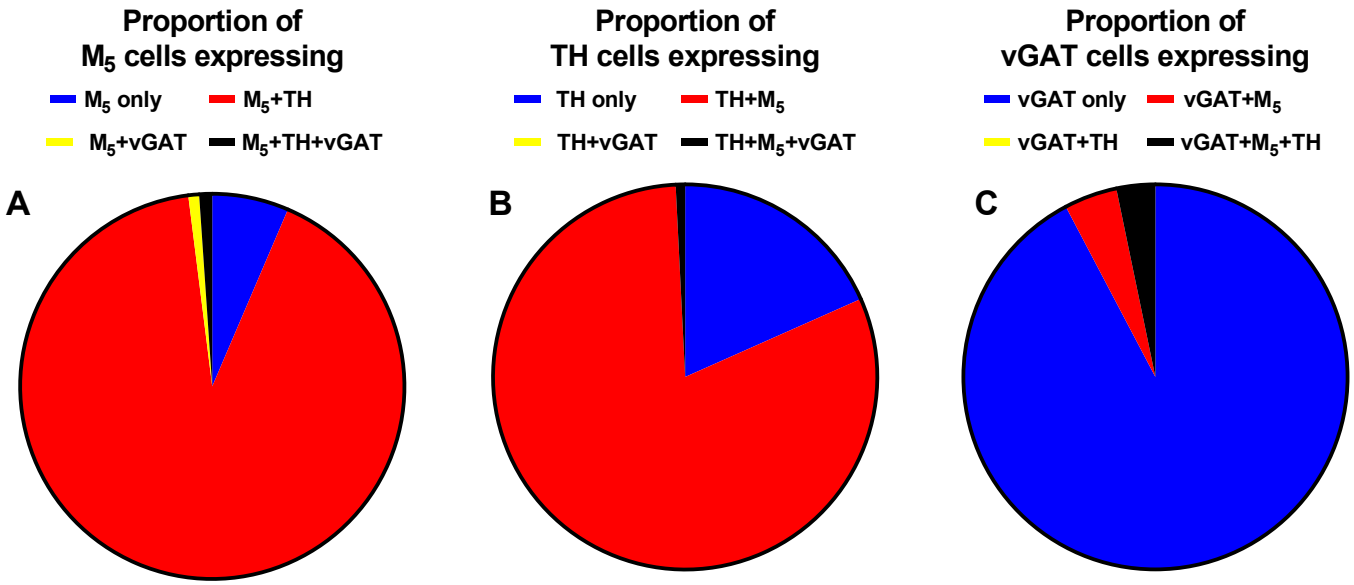
	VTA	*SNc*
	number of cells/mm^2	
TH	104.5 ± 34.6	66.0 ± 21.6
vGAT	80.0 ± 17.8	85.7 ± 30.8
M_5	15.5 ± 4.3	20.1 ± 7.5
TH + M_5	112.3 ± 29.7	297.7 ± 39.9
vGAT + M_5	14.4 ± 6.0	3.9 ± 3.9
TH + vGAT	37.9 ± 16.5	0.0 ± 0.0
TH + vGAT + M_5	8.4 ± 4.5	2.8 ± 2.0

M_5, M_5 muscarinic acetylcholine receptor; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; vGAT, vesicular GABA transporter; VTA, ventral tegmental area. Cell density (number of cells/mm^2) is expressed as mean ± S.E.M. of 4 animals.

Supplementary Figure S1. Oxycodone self-administration is not different prior to cue-reactivity test. On the last three days of oxycodone (56 µg/kg/infusion) self-administration the mean number of responses was higher on the active than on the inactive lever (68.2±6.4 vs. 3.1±0.5, t_{25}=9.95, p<0.001). There was no difference in oxycodone self-administration parameters, such as active lever presses ($F_{(3,22)}$=1.31, n.s. [Figure S1A]), inactive lever presses ($F_{(3,22)}$=2.15, n.s. [Figure S1B]), and number of oxycodone infusions ($F_{(3,22)}$=1.32, n.s. [Figure S1C]) between the different treatment groups. When data were expressed as % change from 3-day baseline, drug treatment still altered responding ($F_{(3,22)}$=10.6, p<0.001). In the 30 mg/kg ML375 group, active lever presses were reduced compared to vehicle (*q*=7.96, p<0.001), but also relative to the 10 mg/kg ML375 (*q*=4.90, p<0.05) and the naltrexone (*q*=4.50, p<0.05) groups (Figure S1D). Values represent the mean ± S.E.M. (n=6-8/ groups). * p<0.05, *vs.* vehicle, ^ p<0.05 vs. 30 mg/kg ML375.



Supplementary Figure S2. Co-expression of M_5, TH, or vGAT transcripts in the substantia nigra. Proportion of M_5, (**A**) TH **(B)**, or vGAT **(C)** cells in the substantia nigra that are single labeled or double labeled for TH or vGAT (**A**), M5 or vGAT (**B**), or M5 or TH (**C**) as well as cells triple labeled for these mRNA species (**A – C**). Data are means of 4 animals per group.



Supplementary Figure S3. ML375 does not affect food-maintained responding on a PR schedule or spontaneous locomotor exploration. (A) Administration of 30 mg/kg ML375 does not alter the number of reinforcers (sucrose pellets) earned in rats with an oxycodone self-administration history. **(B)** ML375 (10 and 30 mg/kg, i.p.) does not affect locomotor exploration in an open field. Values represent the mean ± S.E.M of 7 animals/group (A) or 4 animals/group (B).