Title: Orexin-1 receptor signaling in ventral pallidum mediates demand for the opioid remifentanil

Running title: Orx-1 receptor signaling in VP mediates remifentanil demand

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Abstract:

Signaling at the orexin-1 receptor (Ox1R) is important for motivation for various drugs of abuse. Recently, our laboratory showed that systemic blockade of Ox1Rs decreased motivation for the potent and short-acting opioid remifentanil (Porter-Stransky *et al*, 2017). However, the central sites through which orexin acts to mediate motivation for opioids are not known. Here, we investigated ventral pallidum (VP) as a potential site of orexin action, as VP is a known mediator of opioid reward and is densely innervated by orexin-immunoreactive fibers. We used a within-session behavioral economics (BE) paradigm in which remifentanil price (responses/µg iv remifentanil) was sequentially increased throughout the session. Rats were implanted with bilateral cannulae into VP, through which microinjections of SB334867 (SB), and orexin 1 receptor (Ox1R) antagonist, were given prior to BE testing. Rats were then extinguished and subjected to cue-induced reinstatement following intra-VP SB microinjection. We found that inhibition of Ox1R signaling in VP reduced both motivation (increased demand elasticity) for remifentanil and cued reinstatement of extinguished remifentanil-seeking without affecting baseline consumption or general locomotor activity. These effects were specific to the VP, as control injections of SB immediately dorsal to VP did not affect remifentanil-seeking. Together, these findings indicate a selective role of Ox1R signaling in VP in motivation and relapse for the opioid remifentanil.

Introduction:

In recent years, abuse of prescription opioids has risen rapidly and continues to be a major health issue (Compton and Volkow, 2006; Dobbs and Fogger, 2018). There is significant urgency to understand the neurobiological and behavioral mechanisms underlying opioid addiction to facilitate development of new treatments for this disorder. The hypothalamic orexin (hypocretin) system has emerged as a key modulator of drug seeking behaviors across a variety of drugs of abuse, including opioids (Baimel *et al*, 2015; Harris *et al*, 2005; James *et al*, 2017; Mahler *et al*, 2014a). Orexin-containing neurons are restricted to dorsal hypothalamus and project widely throughout the brain, targeting two G-protein-coupled receptors (orexin receptors 1 and 2; Ox1R and Ox2R, respectively). Signaling at Ox1R modulates motivational properties of opioids; systemic blockade of Ox1R signaling reduces breakpoints for heroin on a progressive ratio task (Smith and Aston-Jones, 2012) and attenuates demand (motivation) for remifentanil on a behavioral economics (BE) paradigm (Porter-Stransky *et al*, 2017). Systemic Ox1R blockade also reduces low-effort self-administration of heroin and remifentanil, indicating an additional role for the orexin system in mediating the hedonic properties of opiate reward. Despite these data, little is known about where in the reward circuitry orexin acts to contribute to opioid reward behavior.

One region of interest is ventral pallidum (VP), as this brain structure is innervated by orexin fibers (Baldo *et al*, 2003; Peyron *et al*, 1998) and VP neurons express both Ox1Rs and Ox2Rs (Marcus *et al*, 2001). Human studies indicate that drug-related cues robustly activate VP (Childress *et al*, 2008), and inactivation of VP in rat prevents various drug behaviors, including acquisition and expression of learned preferences for environments paired with morphine reward (Dallimore *et al*, 2006). Moreover, VP inactivation decreases fixed-ratio 5 heroin self-administration (Hubner and Koob, 1990). Interestingly, the posterior VP contains the greatest density of orexin fibers in the VP (Baldo *et al*, 2003), and previous studies have shown that orexin microinjections into this region enhances the hedonic impact of sucrose (Ho and Berridge, 2013). Taken together, these studies indicate a role for VP in opioid reward behavior and point to a role for orexin signaling in VP in mediating these processes.

To test this directly, we utilized a within-session BE paradigm that provides a quantitative approach to measuring both the motivation for, and the hedonic value of, remifentanil in the

same animal. We focused on remifentanil based on our recent report implicating Ox1R signaling in remifentanil-seeking behavior, and because of evidence that remifentanil may contribute to prescription opioid addiction (Levine and Bryson, 2010). We report that the selective Ox1R antagonist SB334867 (SB) infused directly into the posterior portion of VP reduced motivation (increased demand elasticity) for remifentanil, but not its hedonic properties. Blockade of VP Ox1R signaling also blocked cued reinstatement of remifentanil seeking. Together, our findings point to VP as a critical site of Ox1R signaling in driving particularly the motivation for remifentanil reward, and thus highlight a novel target for therapeutics designed to treat opioid addiction.

Materials and Methods

Subjects

Male Sprague Dawley rats (n=24; initial weight 275–300 g; Charles River, Raleigh, USA) were single-housed and maintained under a 12h reverse light/dark cycle (lights off at 08:00h) in a temperature and humidity-controlled animal facility at Rutgers University. Food and water were available ad libitum. All experimental procedures were approved by the Rutgers Institutional Animal Care and Use Committee and were conducted according to the Guide for the Care and Use of Laboratory Animals. Rats were handled daily after a 3-day acclimation period at the facility; all experiments were performed in the rats' active (dark) phase.

Drugs

Remifentanil (obtained from the National Institute of Drug Abuse, Research Triangle Park, USA) was dissolved in 0.9% sterile saline for intravenous (iv) self-administration. SB334867 (SB), a selective antagonist for the Ox1R (National Institute of Drug Abuse) was dissolved in sterile artificial cerebrospinal fluid (aCSF) at a concentration of 1mM for VP microinjections. For systemic administration, SB was suspended in 10% 2-hydroxypropyl-b-cyclodextrin in sterile water and 2% DMSO (Sigma-Aldrich, USA). SB (30 mg/kg) or vehicle was injected at a volume of 4.0ml/kg/i.p., 30min prior to testing. We chose this dose of SB as it increases demand elasticity and attenuates free consumption and reinstatement responding for remifentanil (Porter-

Stransky *et al*, 2017). A within-subjects design was used whereby each rat received both SB and vehicle; order was counterbalanced across subjects.

Intravenous catheter and stereotaxic surgery

Rats were prepared with an indwelling catheter into the jugular vein as described previously (Mahler *et al*, 2014b; McGlinchey *et al*, 2016). Animals were then placed in a stereotaxic frame (Kopf, Tujunga, USA) and implanted with bilateral stainless steel guide cannulae (22G , 11mm, Plastics One) 1mm dorsal to VP (−0.8mm posterior, ±2.6mm medial–lateral, −7.5mm ventral, relative to bregma; (Paxinos and Watson, 1998). Cannulae were secured to the skull using jeweler's screws and dental acrylic; stylets were placed into the guide cannula to prevent occlusion.

VP microinfusions

For VP microinjection experiments, rats were given bilateral microinjections over a 70-sec period and injectors were left in place for 1min. To acclimate rats to the infusion procedure, the day prior to the first microinfusion test injection cannulae (28G) were bilaterally inserted into the guide cannula for 1 min (no infusions). Rats received microinfusions (0.3µl/side) of either SB (1 mM) or aCSF vehicle, 5min prior to being tested on the BE paradigm, cue-induced reinstatement or locomotor activity. SB and vehicle microinfusions were counterbalanced in a within-subjects design and administered via polyethylene tubing connected to gastight 10µl Hamilton syringes (Hamilton, USA) set in an infusion pump (Model 975, Harvard Apparatus, USA). Each rat received a maximum of 6 VP microinfusions.

Control micoinjections of SB were carried out dorsal to VP, along the cannula tract, to confirm that our observed changes in behavior were not due to diffusion of SB along the path of least resistance to fluid flow. This within-subject control was performed using injectors that projected 0.2mm below the tip of the guide cannula. Dorsal control microinjections were performed in a session prior to VP injection sessions.

Following the final behavioral test, rats were deeply anesthetized with ketamine/xylazine (56.6⁄8.7mg⁄kg) and received bilateral microinfusions of pontamine sky blue (0.3µl); brains were flash-frozen in 2-methylbutane and stored at −80°C, sectioned into 40µm-thick sections,

mounted, Nissl-stained with neutral red, and cover slipped to localize cannula tract damage and verify injection sites.

Behavioral-economics threshold procedure

Rats were trained to self-administer remifentanil as described in Supplementary Materials and Methods before being trained on the BE task as described previously (Porter-Stransky *et al*, 2017). Briefly, the cost of drug was altered by changing the amount of drug infused every 10min; therefore, each 110min session tested 11 doses of remifentanil (2, 1, 0.6, 0.3, 0.2, 0.1 0.06, 0.03, 0.02, 0.01 and 0.006 μg/infusion). Each infusion was accompanied by presentation of a light-tone compound cue; responses on the inactive lever had no consequence. During BE training, there were no limits on how much drug rats could take in a session, nor where there time-out periods in which drug was unavailable. Subjects were trained daily on the BE procedure until stable responding, (≤20% variation in α and Q_0 over 3 consecutive days). Rats were tested on the BE procedure for a minimum of 3d and until behavior re-stabilized between drug treatments.

Extinction and reinstatement tests

After BE testing, rats were subjected to extinction training and locomotor activity; the order of extinction training and locomotion tests was counterbalanced. During each 2h extinction session, lever presses yielded neither remifentanil nor cues. Rats received extinction training for at least 7d and until they met the criteria of ≤25 active lever presses for at least two consecutive sessions. Rats were given two 2h cue-induced reinstatement tests with either SB or vehicle pretreatment (counterbalanced), in which pressing the active lever yielded the cues previously paired with remifentanil infusions, but no remifentanil. Rats received at least 2d of extinction (≤25 active lever presses) between tests (Mahler and Aston-Jones, 2012a; Smith *et al*, 2009c).

Locomotor testing

One day after completion of BE or cue-reinstatement testimg, rats were tested for locomotor activity as described previously (McGlinchey *et al*, 2016) using Digipro Software (Omnitech Electronics). Rats were placed in a chamber for 2h to acclimate to the environment. The next day, rats received a microinjection of either SB or aCSF into VP 5min before being tested in the

locomotor chamber for 2h. A within-subjects design was utilized, and drug order was counterbalanced across rats.

Data analysis

Demand curves were generated for each BE session as previously described (Bentzley *et al*, 2013; Porter-Stransky *et al*, 2017). An exponential demand equation (Hursh and Silberberg, 2008) was applied to these curves to generate estimates of preferred remifentanil intake at zero cost (Q_0) and demand elasticity (α, the slope of the demand curve). Larger α values indicate greater demand elasticity and are characterized by a greater reduction in responding as drug price increases; this is interpreted as decreased motivation as described in our previous work (Bentzley et al., 2013). Smaller α values indicated less demand elasticity and are symptomatic of continued responding for drug despite increases in the cost to obtain drug (increased motivation). The curve fitting was performed using a least sum of squares approach, as in our previous reports (Bentzley *et al*, 2013; Bentzley *et al*, 2014; Porter-Stransky *et al*, 2017).

Analyses were performed in GraphpadPrism V6, except for multiple linear regression analyses that were performed using SPSS Statistics (V19). Acquisition of self-administration, individual differences in BE parameters and the effects of SB on locomotor activity were analyzed using repeated measures ANOVA and paired t-tests. The effects of SB on active/inactive lever responding during self-administration tests were assessed using separate 2-way repeated measures ANOVAs with 'treatment' (vehicle, SB) and 'lever type' (active, inactive) as the variables. Corrections (Dunnett) were applied to post hoc tests to reduce the risk of type 1 errors. Linear regression was used to correlate individual Q_0 and α values. For multiple linear regression analyses, Q_0 and α values were set as the independent variables, with the dependent variables being active lever responses during cued reinstatement, or the difference in responding between SB and vehicle treatments.

Results:

Experiment1:

Blockade of Ox1Rs reduces economic demand for remifentanil

During FR1 training, rats made more presses on the active lever versus the inactive lever (main effect, $F_{(11, 131)}=3.501$, $p<0.001$) beginning on the third session (Bonferroni post hoc test, $p<0.05$) and throughout the remainder of the training sessions ($p<0.01$; Fig. 1b). This was associated with a steady increase in remifentanil infusions across training (one-way repeated measures ANOVA; $F_{(5,65)}=11.64$, $p=0.0003$; Fig. 1c). Next, rats were trained on a BE threshold procedure during which demand for drug under varying costs was evaluated in a within-session design (Fig. 1d). There was a significant inverse correlation between baseline Q_0 and α in these BE tests (Fig. 1e; $r=-0.6783$, $p=0.0311$), such that individuals with high Q_0 values exhibited more persistent drug taking as the cost for remifentanil increased than individuals with low Q_0. Together, these data demonstrate that individuals who exhibit high free consumption (Q_0) for remifentanil also exhibit higher motivation for drug (more inelastic behavior, lower α). Blockade of Ox1Rs with SB significantly increased demand elasticity (decreased motivation for remifentanil, Fig. 1f; 30 mg/kg SB versus vehicle, $t_9=2.551$, $p=0.0312$). Additionally, this dose of SB reduced remifentanil consumption at low effort (free consumption) compared with vehicle (decreased Q_0, Fig. 1g; $t_9=2.382$, $p=0.0411$).

Ox1R signaling is involved in cue-induced reinstatement of remifentanil seeking

Following extinction training (Fig. 2a), animals were tested for cue-induced reinstatement remifentanil-seeking following either SB (30 mg/kg i.p.) or aCSF (counterbalanced) injections. A 2×3 repeated measures ANOVA revealed a significant main effect of treatment ($F_{(2, 32)}=7.720$, $p=0.0018$), and lever ($F_{(2,32)}=34.56$, $p<0.0001$), and a significant treatment × lever interaction ($F_{(2,32)}=8.672$, $p<0.0010$). Post-hoc analyses showed that presentation of remifentanil-associated cues with active lever presses prompted a significant reinstatement of drug seeking after vehicle pretreatment measured by increased responding on the active lever (vehicle vs. extinction, $p<0.001$). SB significantly reduced reinstatement of responding on the active lever (SB vs. vehicle, $p<0.01$). There was no effect of SB or vehicle pretreatment on inactive lever responding ($p>0.05$; Fig. 2b).

Experiment 2:

Antagonism of Ox1Rs in VP decreases motivation but not free consumption for remifentanil

To determine the role of orexin 1 receptor signaling in VP on demand for remifentanil, a separate group of animals with bilateral cannula in VP were trained to self-administer remifentanil. Rats acquired self-administration (Fig. 3b,c) and exhibited stable demand for remifentanil (that was correlated with Q_0 values; Fig. 3d) as in Experiment 1. Microinfusion of SB into VP significantly increased demand elasticity (α; i.e., decreased motivation for remifentanil) compared to injections of aCSF vehicle or to the dorsal infusions of SB (Fig. 3e; $F_{(2, 4)}$=15.02, p=0.0012). In contrast, SB microinfusions into VP did not alter remifentanil free consumption (Q_0; Fig.3f; $F_{(2,4)}$=0.8576, p=0.4358).

Blockade of Ox1Rs in VP attenuates cue-induced reinstatement of remifentanil seeking

Lever pressing was extinguished as in Experiment 1 (Fig. 4a) before. rats were microinjected with either SB or aCSF (counterbalanced) bilaterally into VP 5min prior to reinstatement test sessions. A 2×3 factor repeated measures ANOVA revealed a significant main effect of treatment ($F_{(2, 52)}$=8.117, p=0.0009), and lever ($F_{(2,52)}$=61.38, p<0.0001), and a significant treatment×lever interaction ($F_{(2,52)}$=8.280, p<0.0010). Post-hoc analyses showed that presentation of remifentanil-associated cues with active lever presses prompted a significant reinstatement of drug seeking after vehicle pretreatment measured by increased responding on the active lever (vehicle vs. extinction, p<0.001). SB significantly reduced reinstatement of responding on the active lever (SB vs. vehicle, p<0.05; Fig 4b). There was no effect of SB or aCSF pretreatment in VP on inactive lever responding (p>0.05; Fig. 4b).

Blocking Ox1Rs in VP does not affect locomotor activity

Pretreatment with SB did not alter the total distance traveled in the chambers (Fig. 4c; t_{13}=0.9848), nor was there an effect of bilateral microinfusion of SB on either horizontal (p=0.0754; Fig 4d) or vertical (p=0.1747; Fig. 4e) activity over the 2h test session. These results indicate that effects of blocking VP Ox1Rs on demand elasticity and reinstatement is more likely motivational than motoric in nature.

Blockade of Ox1R signaling in VP attenuates cue-induced reinstatement of cocaine seeking in proportion to baseline demand

Multiple linear regression analysis revealed that the magnitude of responding during cued reinstatement after vehicle microinfusion into VP was predicted by baseline demand elasticity ($p=0.008$) but not by baseline free consumption ($p=0.118$; Fig. 5a), with animals with the lowest α (highest motivation) responding the most during reinstatement testing. In addition, the reduction in responding during cue-induced reinstatement following SB microinfusion into VP was predicted by the animals' baseline remifentanil demand elasticity ($P=0.002$) but not baseline Q_0 values ($p=0.068$; Fig. 5b). The reduction in responding during cue-induced reinstatement following SB was also predicted by animals' baseline reinstatement (responding during cued reinstatement after aCSF microinjection into VP; $p=0.000$; Fig. 5c), such that animals with higher reinstatement responding exhibited greater reductions in responding following SB microinfusions into VP.

Discussion

The ventral pallidum has drawn attention for its role in reward and incentive motivation (Root *et al*, 2015; Smith *et al*, 2009a). Because VP receives input from hypothalamic orexin neurons, which are known to regulate motivated behavior, here we investigated the effect of both systemic and intra-VP Ox1R antagonism on demand for the short-acting opioid remifentanil. We confirm previous findings that systemic blockade of Ox1Rs with SB increased the elasticity of demand for remifentanil (α; an inverse measure of motivation), decreased free remifentanil consumption (Q_0) and attenuated cue-induced reinstatement of drug seeking (Porter-Stransky *et al*, 2017). We extend these findings to show a role for VP Ox1Rs in remifentanil demand. We report that intra-VP SB microinfusions recapitulated the effect of systemic SB injections on demand elasticity for remifentanil (decreased motivation). However, unlike systemic injections, intra-VP treatment with SB did not affect free consumption (Q_0). Further, we show that signaling at Ox1Rs in VP is critical for the expression of cue-induced reinstatement of extinguished remifentanil seeking. Finally, we showed that the rats' baseline remifentanil demand elasticity (but not free consumption), measured before any testing, predicted the later cue-induced reinstatement responding as well as the ability of Ox1R antagonism to reduce such reinstated responding, which is analogous to what has been shown previously with cocaine (Bentzley and Aston-Jones, 2015). Together, these results indicate a specific role for orexin signaling in VP in the control of motivated responding for the opioid remifentanil.

Orexin signaling in VP and motivated opioid seeking

A clear role has been established for orexin signaling in drug seeking behavior (Harris *et al*, 2005; James *et al*, 2017; Mahler *et al*, 2012b). In the case of psychostimulants and alcohol, signaling at the Ox1R appears to uniquely mediate drug seeking under circumstances where high levels of effort are required to obtain the drug, or when drug motivation is augmented by external stimuli, such as drug-associated stimuli or stressors (Bentzley *et al*, 2015; Borgland *et al*, 2009; James *et al*, 2017; Moorman and Aston-Jones, 2009; Moorman *et al*, 2017; Smith *et al*, 2009b). We have previously shown that this role extends to opioids; systemic treatment with SB reduces motivated responding for both heroin (Smith *et al*, 2012) and remifentanil (Porter-Stransky *et al*, 2017), and activation of LH orexin neurons is strongly correlated with the degree of morphine seeking in a conditioned place preference paradigm (Harris *et al*, 2005). However, unlike with other drugs of abuse, these studies also highlighted a role for orexin signaling in mediating the hedonic properties of opioids, as systemic SB treatment reduced FR1 responding for heroin and low-cost consumption (Q_0) for remifentanil. Here we confirm a role for Ox1R signaling in both the motivational and hedonic properties of remifentanil reward by showing that systemic SB treatment both increased demand elasticity (reduced motivation) and decreased Q_0 values in the same BE test session. Changes in remifentanil free consumption are unlikely due to motor deficits, as SB treatment does did affect general locomotor activity. Together, these findings indicate that orexin-based therapies may be effective at reducing both opioid use (by blocking their reinforcing properties) and craving during abstinence (when motivation is augmented), without affecting the pursuit of other reinforcers such as food.

Our findings raise the question of whether orexin mediates the motivational and hedonic properties of opioid reward via distinct or overlapping circuitries. VP has been identified as a key structure mediating motivation for food (Kalivas and Nakamura, 1999; Kelley and Berridge, 2002; Smith and Berridge, 2005), drugs and other rewards (Hubner *et al*, 1990; Mahler *et al*, 2014b; Perry and McNally, 2013). In particular, VP appears to be a component of the brain circuitry regulating effort-related choice behavior, as VP inactivation reduces willingness to work on an instrumental task to obtain sucrose reward (Farrar *et al*, 2008). The caudal portion of VP receives moderate input from orexin fibers and expresses a relatively high density of Ox1Rs, indicating that this region may be an important site for orexin modulation of reward behavior.

Indeed, we show here that intra-VP microinfusions of SB mimic the effect of systemic SB in terms of increasing α (demand elasticity) for remifentanil. Intriguingly, VP microinfusions of SB did not affect Q_0, indicating that VP represents a site through which orexin mediates motivation for remifentanil, but not its hedonic properties. This finding is interesting in light of a recent study showing that microinjections of orexin-A into posterior VP enhanced the hedonic impact ('liking') of sucrose (Ho *et al*, 2013). This raises the important question of why orexin signaling in this portion of the VP might modulate the hedonic properties of food but not opioid reward. Importantly, the study by Ho and Berridge (2013) did not investigate the effect of intra-VP infusions of orexin-A on motivation for sucrose, and thus it will be interesting in future studies to determine if orexin signaling in this area mediates motivation for sucrose as we observed for remifentanil.

Orexin signaling in VP and cued reinstatement behavior

It is notable that across all drugs of abuse tested to date, systemic SB is highly effective at reducing cued reinstatement of extinguished drug seeking (Moorman *et al*, 2017; Plaza-Zabala *et al*, 2013; Smith *et al*, 2012; Smith *et al*, 2009c). Here, we confirm that SB is also effective at reducing cued reinstatement of remifentanil seeking. Previous work has identified the ventral tegmental area (VTA) as a key site for orexin signaling in mediating cued reinstatement of cocaine seeking (James *et al*, 2011b; Mahler *et al*, 2013). To date, however, the central sites of orexin action in mediating cued reinstatement for opioids have been unclear. Here, we show that intra-VP infusion of SB blocks cued reinstatement of remifentanil seeking, consistent with a role for this site as a key mediator of orexin's effects on motivated behavior. Orexin may also act at other sites to contribute to cued reinstatement of remifentanil seeking behavior; the nucleus accumbens shell may be a candidate given that orexin signaling in this region mediates stress-induced reinstatement of morphine-seeking (Qi *et al*, 2013), as might the paraventricular thalamus given evidence that this region is highly responsive to drug cues (Dayas *et al*, 2008; James *et al*, 2011a) and blockade of orexin signaling in this region reduce cued drug seeking behaviors (Matzeu *et al*, 2016; Matzeu *et al*, 2014). It will be important that future studies seek to identify whether the role for orexin signaling in VP (and other regions) in cued reinstatement extends to other opioids, including heroin and fentanyl.

Considerations regarding VP and locomotor activity

VP has been shown to be critical for the modulation of locomotor activation (Swerdlow *et al*, 1984; Williams and Herberg, 1987). As a result, impairment of motoric function could be a potential confound in our finding that blockade of orexin signaling in this region decreases motivation and cued reinstatement of remifentanil seeking. Locomotor or arousal effects do not appear, however, to account for our results. First, animals did not appear ataxic at the time of testing. Further, in locomotor activity tests there were no differences between rats microinjected with SB vs. its vehicle, indicating that blockade of Ox1R signaling in VP does not produce a generalized disruption of locomotor activity. Moreover, SB in VP did not attenuate inactive presses on either the BE task or cued reinstatement tests. Similarly, other studies have shown that SB injections, either systemically or locally in other reward-related regions, has little or no effect on general locomotor activity or responding for other rewards (Borgland *et al*, 2009; James *et al*, 2011b; Smith *et al*, 2009b). Thus, we believe that the effects reported here following SB microinjections into VP are not due to a general impairment in arousal or motor behavior.

Conclusion:

We found that systemic Ox1R antagonism with SB reduces motivation for remifentanil (increased demand elasticity, α), free remifentanil consumption (Q_0) and cued reinstatement of remifentanil seeking. We report that the motivational and hedonic effects of orexin signaling are mediated via distinct circuitries, by showing that intra-VP infusions of SB preferentially block motivated behavior without affecting the hedonic/reinforcing efficacy of remifentanil. The present results, along with previous work from our lab and others', indicate that Ox1R antagonism has potential as a pharmacological strategy for the treatment of opioid abuse, and provide new insights into the corresponding neural substrates. Importantly, the current study focused on the posterior VP, but VP is a heterogeneous structure with differences in cell morphology and connectivity patterns along the rostral–caudal axis, as well as different functional roles in reward-related behavior (Chan *et al*, 2016; Mahler *et al*, 2014b; Root *et al*, 2015; Smith *et al*, 2005). Future research should analyze roles for rostral VP in opiate addiction, as well as whether specific orexin neuronal populations or their targets regulate different aspects of opioid motivation. Finally, our data supports our previous observations (Porter-Stransky *et al*, 2017) that BE offers a highly predictive behavioral marker of remifentanil addiction behaviors,

including cued reinstatement, suggesting a potential utility for the BE paradigm as a behavioral marker for opioid addiction liability in a clinical setting.

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Figure Legends.

Figure 1. Effects of Ox1R blockade on demand for remifentanil. (a) Timeline for behavioral procedure. Rats were trained to self-administer remifentanil and then underwent BE training. They extinguished this behavior, and then tested for 2 h in cue-induced reinstatement sessions. Arrows in timeline indicate 30 mg/kg SB or vehicle treatments (counterbalanced) 30 min prior to each test. (b-c) Mean behavioral performance during remifentanil self-administration training. Data reflect the minimum 6 days of self-administration training. (d) A representative example of a demand curve for a single animal during a single remifentanil BE session. (e) Consumption of remifentanil at null cost (Q_0) and demand elasticity (α) are negatively correlated. Each dot represents data from a single animal. (f-g) SB (30 mg/kg, ip) increased α (decreased motivation; n=10) and reduced Q_0 (free consumption) for remifentanil during BE sessions. Bar graphs represent mean ± standard error of mean (SEM). *p<0.05 compared with vehicle; **p<0.01.

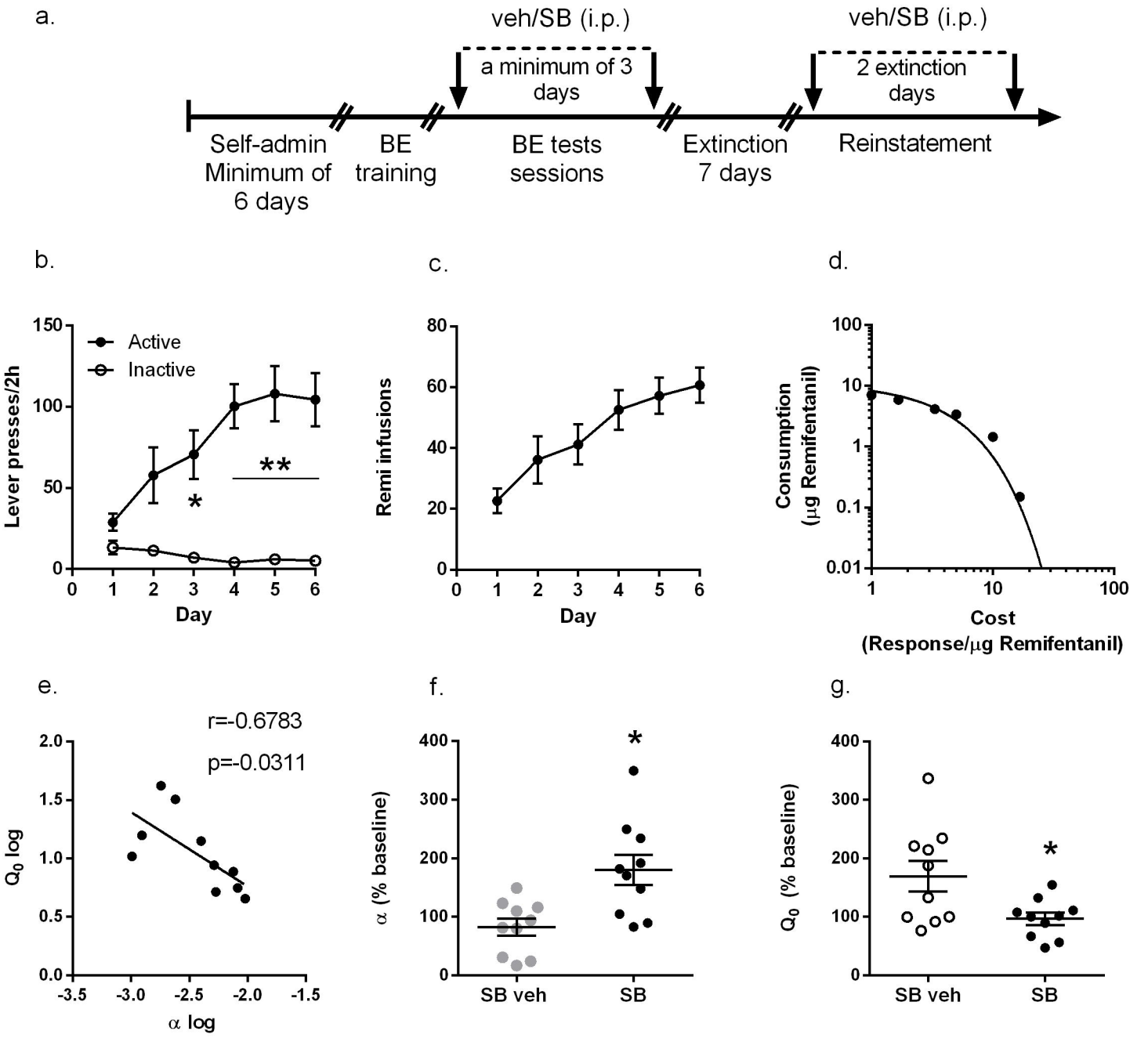
Figure 2. Ox1R antagonism attenuates cue-induced reinstatement of remifentanil seeking. (a) Mean number of active and inactive lever presses during 7 days of extinction training (n=10). (b) Average active lever presses during the last 3 days of extinction training and 2 h cue-induced reinstatement testing 30 min after pretreatment with 30 kg/mg SB or vehicle. SB had no effect on inactive lever presses. Bar graphs represent mean ± standard error of mean (SEM). *p<0.05; **p<0.01; ***p<0.001 compared with vehicle.

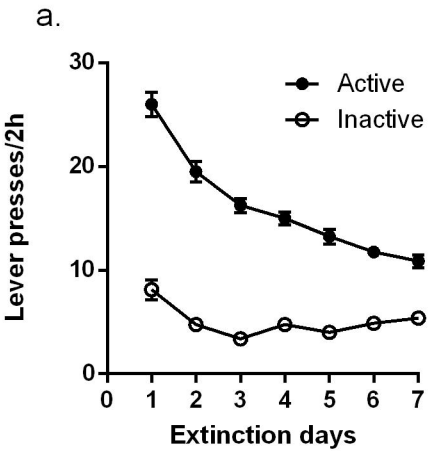
Figure 3. Ox1R antagonism in VP decreases motivation for remifentanil. (a) Experimental design of behavioral training and testing indicating the order and number of injections in VP. (b-c) Mean behavioral performance of all rats in Experiment 2 during remifentanil self-administration (n=14). Data show average number of infusion, and active and inactive lever presses during a minimum of 6 days of FR1 sessions. Again, rats performed significantly more lever presses on the active (drug associated) lever compared to the inactive lever (one-way repeated measures ANOVA; $F_{(11,\ 167)}$=13.29, p<0.0001) beginning on the second session (Bonferroni post hoc test, p<0.05) and throughout the remainder of the training sessions (p<0.01; Fig. 3b). This was associated with an increase in remifentanil intake ($F_{(5,83)}$= 23.91, p<0.0001; Fig. 3c). (d) There was a significant correlation between α and Q_0 (Pearson, r=-0.8405, p=0.0002) such that high takers at null effort (high Q_0) displayed significantly higher motivation for remifentanil than low takers. (e) Demand elasticity (α) was significantly increased

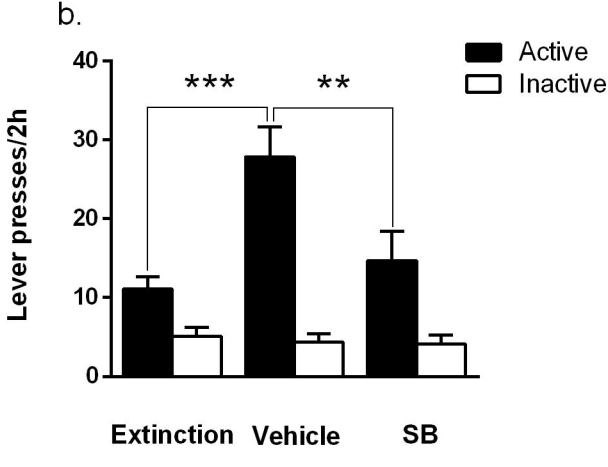
(motivation was decreased) following intra-VP delivery of SB (0.3μL) compared to microinfusion of aCSF or microinfusion of SB just dorsal to VP (n=14, repeated ANOVA, $F_{(2, 41)}$=15.02, p=0.0012). (f) Consumption of remifentanil at null cost (Q_0) was not altered by intra-VP SB microinfusion (n=14, repeated ANOVA, $F_{(2, 41)}$=0.8576, p=0.4358), indicating that OxR1 blockade in VP does not alter the hedonic properties of remifentanil. (g) Representative photomicrograph of cannula injection sites in VP (neutral red Nissl stain, frontal section, midline at center). Injections sites are depicted by blue dye microinfused from cannulae at end of experiments. Brain atlas inset shows bilateral injection sites across all rats (black circles; n=14). Scale bar, 40 μm. Bar graphs represent mean ± standard error of mean (SEM). *p<0.05; **p<0.01 compared with dorsal SB; n.s.=not statistically different.

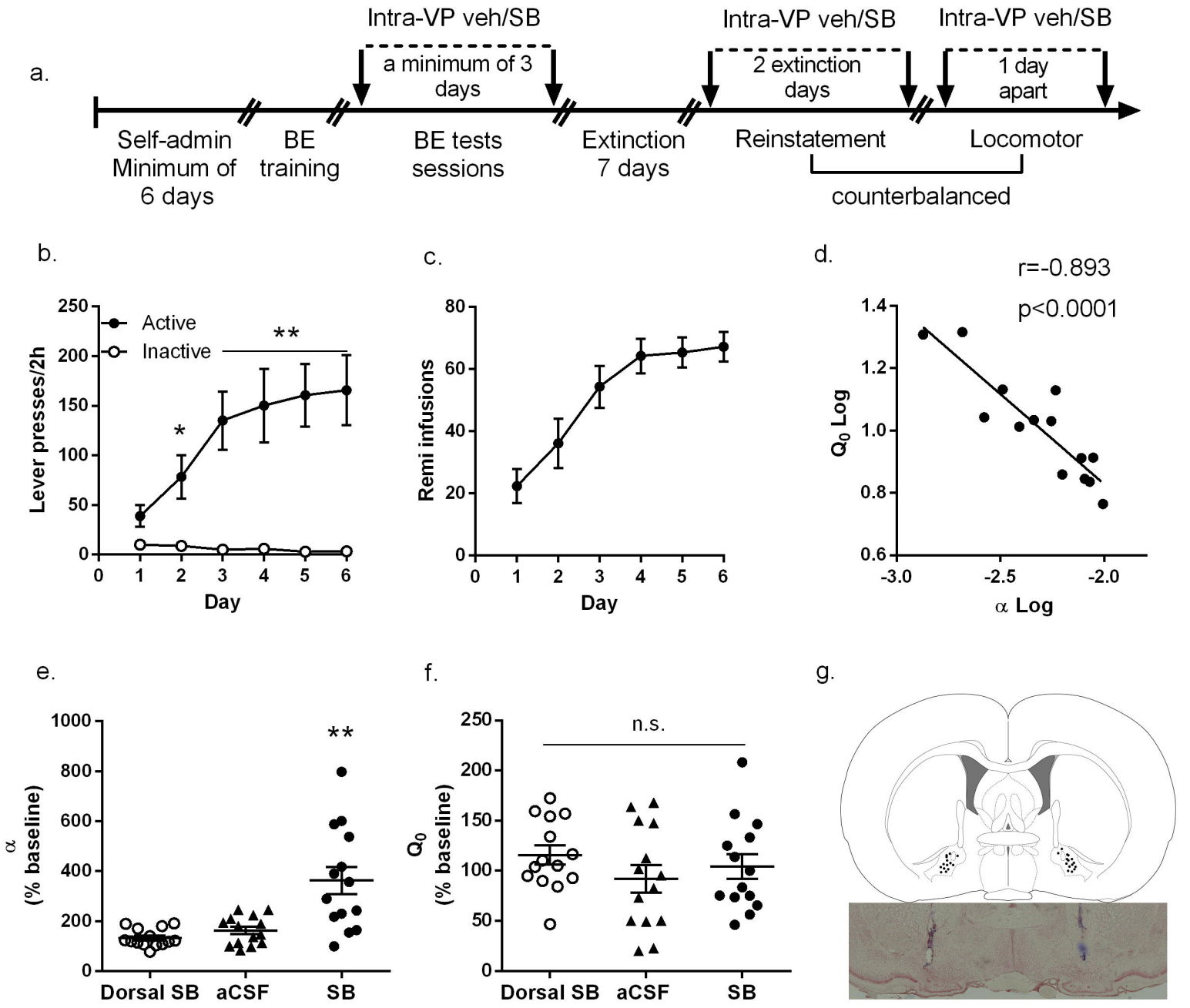
Figure 4. Blockade of Ox1R signaling in VP attenuates cue-induced reinstatement of remifentanil seeking. (a) Mean number of active and inactive lever presses during 7 days of extinction training after BE training and testing (n=14). (b) Microinfusion of SB into VP reduced active lever responding during cue-induced reinstatement of remifentanil seeking compared to the same rats that received aCSF microinjections (n=14; one-way ANOVA, p=0.0079). There was no effect of SB vs. aCSF microinjections on inactive lever presses. (c) Rats displayed no differences in total distance traveled in locomotor chambers after SB microinjection into VP compared to vehicle (n=14). Microinjection of SB into VP had no effect on (d) horizontal or (e) vertical activity. Bar graphs represent mean ± standard error of mean (SEM). *p< 0.05; ***p<0.001; n.s.=not statistically different.

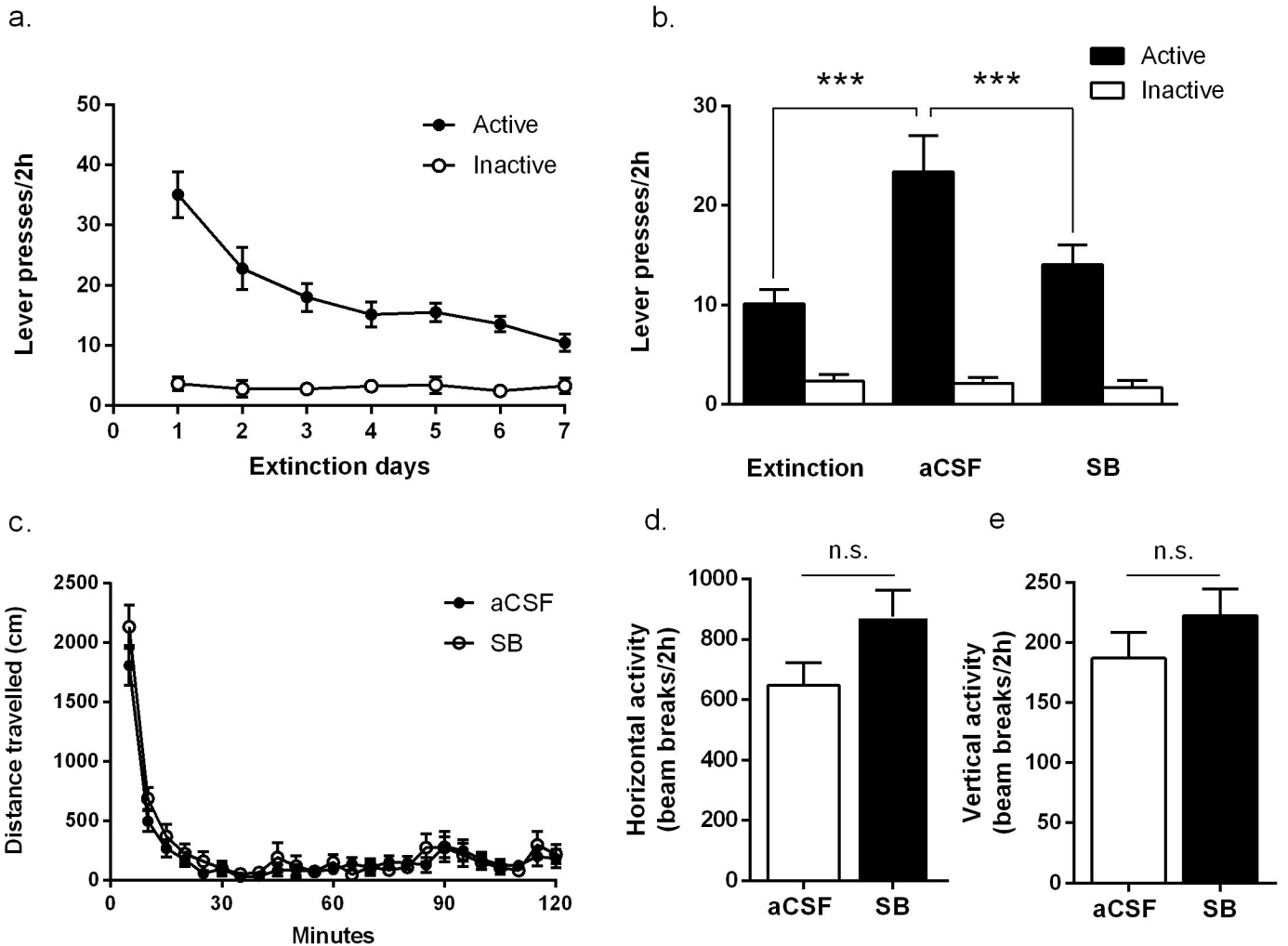
Figure 5. Baseline demand predicted reinstatement propensity and efficacy of SB microinfusion into VP. (a) Baseline demand elasticity (α), but not free cocaine consumption (Q_0), significantly correlated with the magnitude of cue-induced reinstatement with aCSF microinjection (α; β =-0.674, p=0.008), (Q_0; β=0.438, p=0.118). (b) Baseline demand elasticity (α) (β=-0.751, P=0.002), but not free cocaine consumption (Q_0) (β=0.502, P=0.068), correlated with the magnitude of the reduction of cue-induced reinstatement with SB microinjection into VP. (c) Baseline reinstatement (active lever presses after aCSF microinjection) predicted the magnitude of reduction of cue-induced reinstatement with SB microinjection into VP (β=0.855, p=0.000).

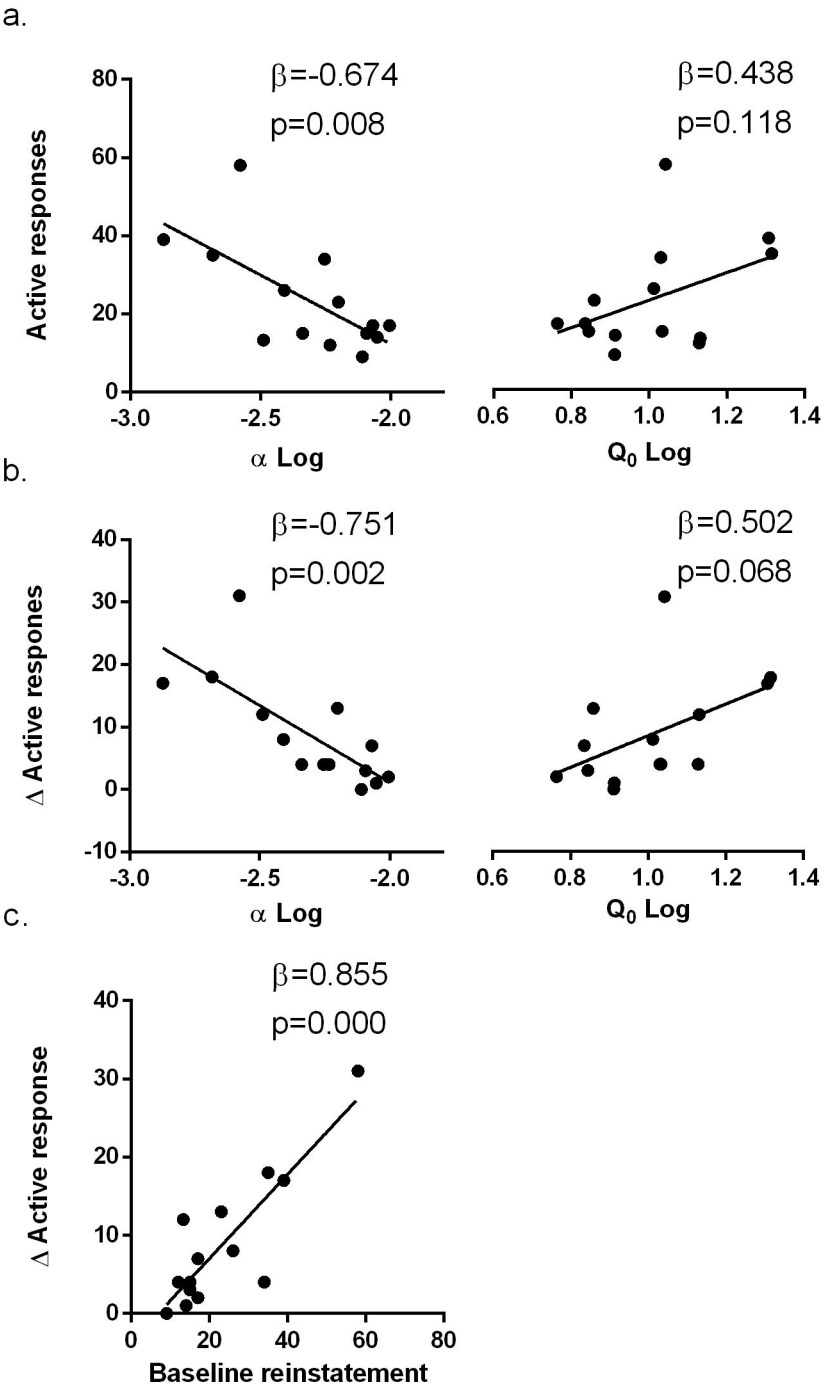

a.
veh/SB (i.p.)
a minimum of 3 days
veh/SB (i.p.)
2 extinction days
Self-admin Minimum of 6 days
BE training
BE tests sessions
Extinction 7 days
Reinstatement
b.
Active
Inactive
Lever presses/2h
Day
c.
Remi infusions
Day
d.
Consumption (μg Remifentanil)
Cost (Response/μg Remifentanil)
e.
r=-0.6783
p=-0.0311
Q0 log
α log
f.
α (% baseline)
SB veh
SB
g.
Q0 (% baseline)
SB veh
SB


a.
Active
Inactive
Lever presses/2h
30
20
10
0
0
1
2
3
4
5
6
7
Extinction days


b.
Active
Inactive
***
**
Lever presses/2h
40
30
20
10
0
Extinction
Vehicle
SB


a.
Intra-VP veh/SB
a minimum of 3 days
Intra-VP veh/SB
2 extinction days
Intra-VP veh/SB
1 day apart
Self-admin Minimum of 6 days
BE training
BE tests sessions
Extinction 7 days
Reinstatement
Locomotor
counterbalanced
b.
Active
Inactive
Lever presses/2h
Day
c.
Remi infusions
Day
d.
r=-0.893
p<0.0001
Q0 Log
α Log
e.
α (% baseline)
Dorsal SB
aCSF
SB
f.
n.s.
Q0 (% baseline)
Dorsal SB
aCSF
SB
g.


a.
Lever presses/2h
Extinction days
Active
Inactive
b.
Lever presses/2h
***
***
Active
Inactive
Extinction
aCSF
SB
c.
Distance travelled (cm)
Minutes
aCSF
SB
d.
Horizontal activity (beam breaks/2h)
n.s.
aCSF
SB
e
Vertical activity (beam breaks/2h)
n.s.
aCSF
SB


a.
β=-0.674
p=0.008
β=0.438
p=0.118
Active responses
α Log
Q0 Log
b.
β=-0.751
p=0.002
β=0.502
p=0.068
Δ Active respones
α Log
Q0 Log
c.
β=0.855
p=0.000
Δ Active response
Baseline reinstatement