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Suppression of sex behavior by kappa opiates and stress steroids occurs via independent neuroendocrine pathways

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ABSTRACT

Endocannabinoids and their receptors are found throughout the brain of all vertebrates. By virtue of their wide distribution, endocannabinoids have the potential to affect many behaviors. Prior research has shown that cannabinoids inhibit courtship-clasping and mediate behavioral responses to stress in male rough-skinned newts, *Taricha granulosa*, and cannabinoid signaling is initiated by rapid actions of the steroid corticosterone (CORT) at its specific membrane receptor (mCR). This same mCR also recognizes κ-opioid receptor agonists and antagonists. Prior behavioral studies show that κ-opioid agonists suppress clasping behavior in a dose dependent manner. Combined, these studies suggest that κ-opioid agonists might suppress clasping behavior via the same pathway initiated by CORT: up-regulation of endocannabinoid signaling. We examined whether pretreatment with a CB_1 antagonist, AM281, would block κ-opioid-mediated suppression of clasping. We found that the CB_1 antagonist did not reverse κ-opioid-induced suppression of clasping, revealing that while endocannabinoids mediate CORT-induced suppression of clasping, endocannabinoids do not mediate the κ-opioid-induced suppression of clasping.

1. Introduction

The ability of an animal to respond appropriately when faced with an immediate threat is critical to survival. Behavioral responses to acute stressors have been identified in all major groups of vertebrates (Breuner et al., 1998; Burmeister et al., 2001; Lumley et al., 1999; Mitra and Sapolsky, 2008; Sandi et al., 1996). While glucocorticoids (stress-steroids) are instrumental in the chronic disease states developed after experiencing prolonged stress, they also initiate rapid behavioral responses to direct threats or acutely stressful situations (Breuner et al., 1998; Coddington et al., 2007; Moore and Miller, 1984). A majority of the neuroendocrine research into stress-steroid effects has focused on long-term genomic effects (Akama and McEwen, 2005; Carrasco and Van de Kar, 2003; Ferguson et al., 2008; Ferguson and Sapolsky, 2007; Kaufer et al., 2004; Sapolsky, 2000). However, the neuroendocrine regulation of rapid behavioral/neuronal responses to immediate threats is not yet well understood. Examining neural mechanisms of hormone regulation of behavior in the rough-skinned newt, *Taricha granulosa*, provides a unique perspective for three main reasons. First, *Taricha* offers the opportunity to examine the effects of acute stress on a well-characterized behavior, male courtship clasping (see below) (Propper, 1991; Rose and Moore, 1999). Second, the functional properties of the key medullary neurons that regulate clasping behavior have been well characterized using single-unit extracellular recordings (Rose et al., 1993, 1995, 1998; Rose and Moore, 1999). Third, the stress-induced neuroendocrine cascade and behavioral impact of corticosterone (CORT) on clasping; (Moore and Miller, 1984; Moore and Orchinik, 1994; Orchinik et al., 1991, 1994), κ-opiates (Deviche and Moore, 1987; Evans et al., 2000), and endocannabinoids (Coddington et al., 2007) have been well characterized in this species.

The behavior we have focused on in this study is courtship clasping, a robust behavior that is rapidly suppressed by acute stress. The courtship clasping behavior engaged by male *Taricha* is both consistent and predictable: male *Taricha* grasp sexually attractive females with their hind and forelimbs in an ardent amplectic clasp (Arnold, 1977; Propper, 1991). The essential element of clasping is a synchronized tightening of male hind legs, which is initiated and maintained in response to somatosensory stimulation of the male's cloacal region on the ventrum between the hind limbs. Previous studies have shown that when male *Taricha* are first subjected to an acute stressor and then presented with a female, they consistently fail to clasp (Coddington et al., 2007; Coddington and Moore, 2003; Moore and Miller, 1984).

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In *Taricha*, three neuromodulators have been shown to rapidly suppress clasping: CORT (Moore and Miller, 1984), κ-opioid receptor agonists (Deviche and Moore, 1987), and cannabinoids (Coddington et al., 2007; Soderstrom et al., 2000). Corticosterone administration, or exposure of males to stressors that naturally stimulate CORT secretion, rapidly suppresses clasping without affecting locomotor behavior (Moore and Miller, 1984). The behavioral response to CORT occurs within minutes of experiencing an acute stressor or administration of CORT, and is too rapid to be mediated by traditional genomic mechanisms; instead, ligand-binding assays using *Taricha* neuronal membranes revealed that CORT most likely binds to a membrane-associated G-protein coupled receptor (mCR) (Orchinik et al., 1991). This membrane-bound receptor gives an animal the advantage of rapid responses to hormonal changes.

Evidence from *in vitro* studies suggests that κ-opioid agonists might suppress clasping by binding to the same site on the same membrane receptor used by CORT, the mCR (Evans et al., 2000). U50488 binds to both the mCR and it's own native κOR in *in vitro* preparations of *Taricha* brains; binding to the mCR with the same affinity as CORT (K_i = 250 nM; (Evans et al., 2000)), and binding its own receptor (κOR) with a higher affinity (K_i = 3.4 nM; (Bradford et al., 2005). Furthermore, the mCR has a single CORT binding site that has very high specificity and affinity for the CORT and κ-opiate ligands, and very low affinity for mineralocorticoids or classical intracellular glucocorticoid receptor antagonists (Orchinik et al., 1991; Evans et al., 2000). Given that the κ-opioid agonist U50488 binds to the mCR *in vitro*, it is reasonable to hypothesize that κ-opioids might suppress clasping via the same mCR and neuroendocrine pathway used by CORT (H_0 in Fig. 1). Because the molecular identity of the mCR has yet to be determined, an arsenal of specific/selective antagonists and agonists is not yet available. We do have the capacity to test this hypothesis using behavioral pharmacology. If CORT and κ-opioids share the same mCR, then κ-opioids, like CORT, should also suppress courtship behaviors in *Taricha*. Indeed, studies examining courtship behavior of *Taricha* (Deviche and Moore, 1987) and other animals (Agmo et al., 1994) supports the notion that κ-opioids suppress courtship behaviors, but the mechanism remains unknown.

κ-opioid agonists also have discrete behavioral effects via their own receptors, such as suppression of locomotor activity (Deviche et al., 1989). Studies in *Taricha* (Deviche et al., 1989) and mice (Ukai and Kameyama, 1985) have revealed that high doses of κ-opioid agonists will depress locomotor activity, and that this effect is reversed by the general opiate antagonist naloxone. Therefore, it is also possible that κ-opioids might suppress clasping indirectly through binding to κ-opioid receptors.

Behavioral, molecular, and binding data suggest that CORT and κ-opioids might share a common mechanism, binding the mCR, by which they suppress courtship behaviors and mediate stress-induced behavioral responses (Fig. 1). Behavioral and electrophysiological data show that CORT inhibits clasping by up-regulating the signaling of endogenous cannabinoids (Coddington et al., 2007). This is likely to be a conserved mechanism by which CORT exerts its rapid effects in the central nervous system of vertebrates, as this neuroendocrine cascade has also been demonstrated in the hypothalamic magnocellular neurons of rodents (Di et al., 2003, 2005a,b). If CORT and κ-opioid agonists are working through the mCR, then κ-opioid agonists should elicit the same phenotype as CORT, which includes both CB signaling and specific suppressive effects on clasping. If so, the κ-opioid agonist U50488 would suppress clasping without suppressing locomotor activity, and this effect would be blocked by pretreatment with a cannabinoid antagonist as we have seen with CORT. Alternatively, if κ-opioid effects are elicited through its own receptor, then blocking cannabinoid signaling should have no effect on κ-opioid-mediated suppression of clasping, and doses of κ-opioid agonist that suppress clasping would also suppress locomotor activity.

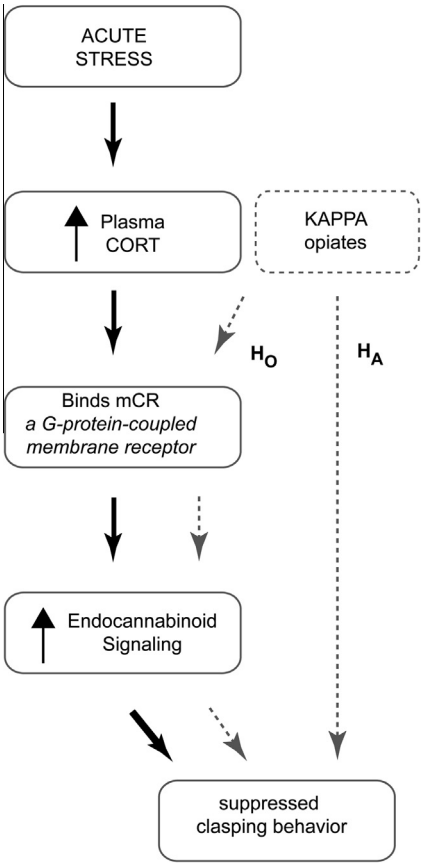

Fig. 1. Schematic outline of hypotheses. The neuroendocrine pathway by which acute stress leads to suppression of clasping behavior has been well established (indicated by solid boxes and arrows). Dotted boxes and lines outline the hypotheses tested in the current study (null hypothesis = H_o, alternative hypothesis = H_a). Based on several lines of evidence from behavioral, molecular, and binding studies we predicted that κ-opioid agonists suppress clasping by their action on membrane CORT receptors, mCR. If this is correct, then blocking endocannabinoid signaling should block κ-opioid-mediated suppression of clasping and blocking κ-opioid receptors should not block κ-opioid-mediated suppression of clasping.

2. Methods

2.1. Animals

Animals were collected from January Pond in Lincoln County, OR (Latitude 44.6, Longitude 123.6). Sexually mature and active adult male rough skinned newts (*T. granulosa*, 15 ± 1 g) were collected during the active breeding season (March 2007 and 2010). Sexually mature and active males are identified by the following criteria: smooth epidermis, thickened neck and dermis, enlarged tail height, mate-seeking behavior, and >13 g. Males were held in community tanks (10 Gallon glass aquaria: 20 × 10 × 12 inches) maintained with running, dechlorinated water (20 °C, *depth* 30 cm). Each tank held up to 30 male newts and had Styrofoam floats for the animals to rest on. Four chopped earthworms were fed to each community tank daily. Sexually mature and receptive females were captured en route to their natural breeding ponds and were housed in 10-gallon aquaria complete with mosses, ferns and dechlorinated water (20 °C). Females were injected intraperitoneally (i.p.) every 48 h with 5 I.U./ml prolactin (Sigma–Aldrich, USA) to maintain their sexual receptivity and attractiveness to

males. No more than 8 females occupied the same aquarium. Females were misted daily, tanks were cleaned bi-weekly, and each tank was given one chopped earthworm daily. The diet of males and females was augmented weekly with calcium-fortified crickets. All animals were in a climate controlled room under a light:dark regimen of 10.5 L:13.5 D.

2.2. Experiment 1 – dose response effect of a κ-opioid agonist U50488

In order to verify whether U50488 does suppress clasping in a dose-dependent manner *in vivo*, and to determine the behaviorally effective dose of U50488 to use in subsequent experiments we performed a dose–response experiment. Male newts received a single 0.1 ml intraperitoneal (i.p.) injection of one of the following doses of the κ-opioid agonist U50488: 0, 0.07, 0.7, 7.0, 70 mg/kg. A total of six trials were run with ten males randomly assigned among the five treatments for each trial, ensuring 2 males per treatment group were examined in each trial ($N = 12$ per treatment group). The males were placed individually in their testing arenas for 30 min prior to receiving i.p injections of U50488. Females were added 30 min after injections and incidence of clasping was recorded during the following 30 min period. Details about the drug preparations and testing arenas are outlined in Sections 2.4 and 2.5, respectively.

2.3. Experiment 2 – Do κ-opioid agonists suppress clasping via endocannabinoid signaling?

A time-dependent dual injection protocol was used to determine whether κ-opioid agonists suppress clasping via mCR-mediated cannabinoid signaling. The intention of this design is to allow time for cannabinoid antagonists to act prior to introducing κ-opioids. Male newts were randomly allocated to one of seven treatment groups: (a) vehicle injection followed by a second vehicle injection (no-drug control) ($N = 28$); (b) CB_1 antagonist AM281 (5 μg/0.1 ml) followed by vehicle injection of amphibian Ringers ($N = 28$); (c) AM281 followed by κ-opioid agonist U50488 (7 mg/kg) + κ-opioid antagonist nor-Binaltorphimine dihydrochloride (nBNI; 50 μg/kg) ($N = 28$); (d) vehicle injection followed by U50488+nBNI ($N = 28$); (e) vehicle injection followed by nBNI ($N = 28$); (f) AM281 followed by vehicle ($N = 28$); (g) AM281 followed by U50488 ($N = 30$). The first injection (AM281 or vehicle – 1% DMSO, 1% cyclodextrin, and 98% amphibian Ringer) was administered intraperitoneally (i.p.) 30 min after males were placed in their testing arenas. The second injection (κ-opioid agonist and/or nBNI, or vehicle) was administered i.p. 9 min later, allowing the CB1 antagonist to take effect prior to the administration of κ-opioids. After males had been in testing arenas for 60 min (30 min after CB antagonist injection and 20 min after κ-opioid injection), two gravid females were placed into each arena and behavioral measures for assessed for the following 30 min. All injections were administered by a person blind to the treatments and performed between 1:00 pm and 3:00 pm to minimize potential confounds due to circadian changes in endogenous hormones.

Male clasping behavior was observed and recorded from 0 to 30 min after females were added to the chamber. We recorded incidence of clasping by counting the number of males observed clasping females in the 30 min period. The criteria for a clasp include the male must be observed clasping a female for longer than 1 min. For display purposes, the incidence of clasping is expressed as proportion of males that successfully clasped during the 30 min observation period. Latency of males to clasp a female was measured using a stopwatch, and we assessed locomotor activity pre- and post-drug administrations. We report locomotor activity as the difference in the number of lines crossed by males in 3 min immediately before the first injection and 16 min after the second injection but prior to female introduction. Our criterion for crossing a line was defined as the newt's entire head passing over a line. Observers were blind to the treatments.

2.4. Drug preparation

A CB_1 antagonist, AM281 (Tocris, USA) was solubilized in 1% dimethyl sulfoxide (DMSO), 1% cyclodextrin (Sigma–Aldrich, USA), and 98% amphibian Ringer (6.5 g NaCl, 0.14 g KCl, 0.12 g $CaCl_2$, 0.2 g $NaHCO_3$/liter) to a final concentration of 0.3 mg/kg (5 μg/0.1 ml injection) as in previous behavioral studies (Coddington et al., 2007). Control injections consisted of the vehicle alone (i.e., the same solution as above without the addition of AM281). A κ-opioid agonist, U50488 (5 μg/0.1 ml), and κ-opioid antagonist, nBNI (50 μg/kg), (Tocris, USA) were solubilized using amphibian Ringer's solution. Prolactin (Sigma–Aldrich, USA) was solubilized in amphibian Ringer's solution to 5 I.U./ml. All newts were injected intraperitoneally (i.p.) with 0.1 ml of each reagent as described below.

2.5. Testing arenas

The testing arenas were plastic circular containers (h 17 cm, D 26 cm) containing dechlorinated water measuring 6 cm deep (20 °C). On the floor of each testing arena 8 lines were drawn, radiating out from the center, dividing the base of the arena into 8 equal regions. These lines allowed locomotor activity to be quantified as the number of lines crossed. Two males were tested together in each arena to minimize experimental stress. The 12 testing arenas were organized into three equal rows, and activity of newts was recorded from above with a CCD camera under low light.

2.6. Statistical analysis

The incidence of clasping was analyzed using a 1-way Analysis of Variance (ANOVA) followed by a post hoc analysis using Bonferroni-corrected t-tests. IC_{50} for the κ-opioid agonist U50488 was determined using a non-linear regression analysis [model: log(inhibitor) vs. response (three parameters)]. Latency and Activity data were independently analyzed using non-parametric ANOVA's (Kruskal–wallis test) followed by a post hoc analysis with Dunn's Multiple Comparison's test because these data were skewed in their distribution, and the treatment groups had inconsistent variability. All data analyses were performed using Graphpad Prism 5 software.

3. Results

3.1. Experiment 1 – dose response effect of κ-opioid agonist U50488

Suppression of clasping by the κ-opioid agonist U50488 was dose-dependent (Kruskal–Wallis = 37.01, $P > 0.0001$). Doses 7 and 70 mg/kg of the κ-opioid agonist significantly suppressed clasping with respect to controls and lower κ-opioid agonist doses (Dunn's $P < 0.01$; Fig. 2). We therefore used 7 mg/kg for all subsequent experiments. IC_{50} for κ-opioid agonist U50488 was 0.6 mg/kg with 95% confidence intervals 0.38–0.96 mg/kg ($R^2 = 0.959$, d.f. = 17).

3.2. Experiment 2 – Do κ-opioid agonists suppress clasping via endocannabinoid signaling?

The incidence of clasping differed significantly overall among treatment groups ($F_{6,28} = 11.80$, $P < 0.0001$; Fig. 3A). However, direct comparisons of individual groups revealed no significant

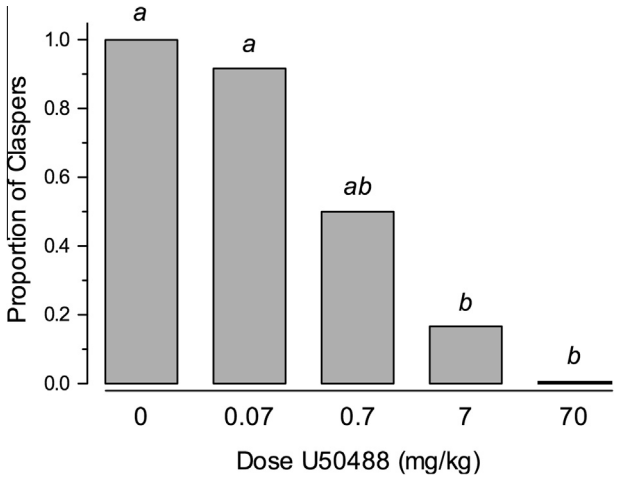

Fig. 2. Incidence of clasping was suppressed in a dose-dependent manner by κ-opioid agonist. Behavior was observed for a 30 min period, 30 min after 0.1 ml i.p. injection of U50488 ($N = 12$ per treatment group). Columns that do not share a letter are significantly different from each other ($P < 0.05$).

difference between males who received κ-opioid agonist alone ($n = 28$) and those receiving CB_1 antagonist prior to κ-opioid agonist administration ($n = 30$). There was, however, a significant difference between cannabinoid antagonist and control conditions (AM281+Ringer and vehicle+Ringer) versus both κ-opioid agonist conditions (AM281:U50488 and vehicle:U50488) (Dunn's, $P < 0.01$). Most significantly, the U50488-induced (veh:U50488) suppression of clasping suppression of clasping was blocked by co-administration of the κ-opioid antagonist nBNI (veh:U50488+nBNI) (Dunn's $P < 0.01$), but not by pre-treatment with CB_1 antagonist AM281 (AM:U50488). There were no differences in latency among groups (KW = 8.714, $P = 0.19$; Fig. 3B), however, there was a significant difference in the mean change in the number of lines crossed among treatment groups (KW = 65.12, $P < 0.0001$; Fig. 3C). Post-hoc Bonferroni-corrected t-tests revealed no difference among control conditions (AM281:Ringer, vehicle:Ringer, AM281:U50488+nBNI, vehicle:U50488+nBNI, vehicle:nBNI) and no significant difference between experimental conditions (vehicle:U50488 and AM281:U50488). However, control conditions differed significantly from experimental conditions (Dunn's $P < 0.05$).

4. Discussion

We hypothesized that κ-opioid agonists and CORT act through a shared mCR to suppress male courtship-clasping without affecting locomotion. This notion was supported by significant evidence: both κ-opioid agonists and CORT suppress clasping behavior (Deviche and Moore, 1987), CORT suppresses clasping by acting at a membrane receptor mCR (Orchinik et al., 1991) and rapidly up-regulating endogenous cannabinoid signaling (Coddington et al., 2007), mCR has κ-like properties (Bradford et al., 2005), and specific κ-opioid agonists bind and compete with CORT for the mCR with high affinity (Evans et al., 2000). Consistent with past research, this study found that the κ-opioid agonist U50488 significantly suppressed clasping in a dose-dependent manner. However, we found that pretreatment with the CB_1 antagonist AM281 did not block κ-opioid-mediated suppression of clasping. Furthermore, we found that although κ-opioid agonists suppressed clasping, they also suppressed locomotion, as has been shown in an earlier study (Deviche et al., 1989). This κ-opioid-mediated reduction in activity appears to be mediated via a κ-opioid receptor

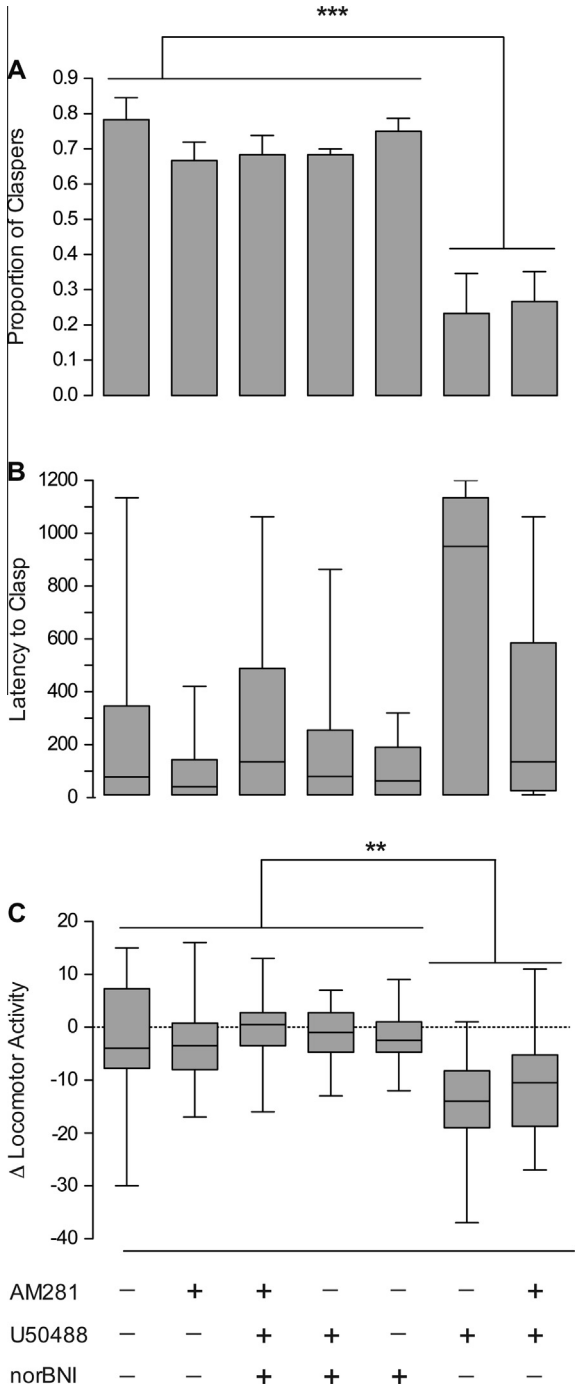

Fig. 3. The cannabinoid antagonist AM281 did not block κ-opioid-mediated suppression of clasping, but the κ-opioid antagonist nBNI did block κ-opioid-mediated suppression of clasping. (A) Proportion of males observed clasping during 50 min post-treatment monitoring period. The κ-opioid agonist U50488 (7 mg/kg) suppressed the incidence of clasping. (B) Latency to clasp is represented as median and interquartile range, and was not significantly affected by the κ-opioid agonist or a cannabinoid antagonist (AM281; 0.3 mg/kg). (C) Locomotor activity was significantly increased by 7 mg/kg dose of κ-opioid agonist U50488, and this effect was blocked by co-administration of κ-opioid antagonist nBNI. Locomotor activity was measured as the difference in the number of lines crossed in a 3 min period pre-1st injection and post-2nd injection just prior to females being introduced. Activity is represented here as median and interquartile range. We monitored a total of 30 male newts in experimental condition AM281+ κ-opioid and 28 in each of the six other conditions. All males were tested once only. Statistically significant differences among treatment groups are indicated by asterisks ($^{**}P < 0.01$, $^{***}P < 0.001$); lines drawn across groups indicate statistical similarity in median ± range or mean ± SD.

(κOR) as κ-opioid-mediated decrease in clasping and activity was alleviated by co-administration with a κOR antagonist nBNI. Thus, our results suggest that suppression of clasping by κ-opioid agonists is likely via a mechanism independent of the mCR and endocannabinoid signaling.

We found that administration of the κ-opioid agonist U50488 suppressed clasping behavior of male *Taricha*. We observed a dose-dependent decrease in the incidence of clasping by males receiving κ-opioid agonist, reaching significance at doses of 7 mg/kg. These results are consistent with previous studies that indicate κ-opioid agonists, and not μ-opioids, suppress male sexual behaviors of male *Taricha* (Deviche and Moore, 1987) and mammals (Agmo et al., 1994).

The key piece of evidence that suggests that κ-opioid agonists suppress clasping via a route other than mCR is that the potent CB_1 antagonist AM281 failed to block the κ-opioid agonist-induced suppression of clasping. Prior studies have established that CORT suppresses clasping by binding its specific mCR (Orchinik et al., 1991), and blockade of CB_1 receptors reverses the suppressive effects of CORT on clasping (Coddington et al., 2007); essentially, acute effects of CORT require endocannabinoid signaling to mediate appropriate responses to acute stress. This up-regulation of endocannabinoid signaling in response to glucocorticoids binding their membrane receptor site seems to be a neuroendocrine cascade that is conserved across taxonomic species and brain regions (Evanson et al., 2010). In amphibians, CORT-mediated suppression of *Taricha* clasping occurs at clasp-controlling neurons located in the rostromedial medulla of the hindbrain (Coddington et al., 2007). In mammals, glucocorticoids provide negative feedback to hypothalamic neurons, mediated by a membrane receptor and the subsequent up-regulation of endocannabinoid signaling (Di et al., 2003, 2005a,b; Evanson et al., 2010; Tasker and Herman, 2011). Administration of a cannabinoid antagonist at doses equivalent to or lower that those used in this study in mammalian hypothalamic slices and amphibian behavioral and single-unit electrophysiology preparations resulted in the blockade of rapid CORT effects. Given that the cannabinoid antagonist AM281 did not block the suppressive effects of the κ-opioid agonist U50488 on clasping, our data strongly suggest that κ-opioids are not acting through the mCR.

κ-opioid-mediated suppression of clasping is most likely occurring via its own native receptor, κOR. U50488 binds to two different receptors in *in vitro* preparations of *Taricha* brains: the mCR and κOR. The κ-opioid agonist U50488 binds mCR with the same affinity as CORT (K_i = 250 nM; (Evans et al., 2000)), and binds its own receptor (κOR) with a higher affinity (K_i = 3.4 nM; (Bradford et al., 2005). These *in vitro* studies suggest that if presented with equivalent abundance of both mCRs and κORs in one preparation, κ-opioid agonists would bind preferentially to their own κOR. Therefore, the intraperitoneal administration of a κ-opioid agonist is likely to result in binding κOR before binding to mCRs. The present behavioral study may illustrate κ-opioid agonist specificity for its own specific receptor.

Although *in vitro* binding data demonstrate that κ-opioid agonists bind specifically to the mCR, *in vivo* data from this and other (Deviche et al., 1989) studies are inconsistent with this observation. Several alternative mechanisms may explain the apparent disagreement between the *in vitro* binding and *in vivo* behavioral data. First, the κ-opioid agonist U50488 binds both receptors *in vivo*; binding κOR to affect locomotor activity and mCR to affect clasping, but the behavioral manifestation of κ-opioid agonist binding its κOR overshadowing that of the mCR. If this were the case, then we would expect the animals treated with U50488+nBNI to have diminished clasping (κ-opioid agonist binding mCR) but normal locomotor activity (κ-opioid agonist binding to κOR blocked by nBNI antagonist). However, we do not observe this; animals treated with U50488+nBNI have normal locomotor activity and exhibit normally high levels of clasping. This is the most compelling evidence suggesting that while κ-opioid agonists may bind mCR *in vitro*, *in vivo* we observe that κ-opioid agonists affect courtship behavior indirectly and most profoundly by suppressing locomotor activity. Our data are also consistent with other studies demonstrating that κ-opioid agonists diminish locomotor activity in *Taricha* (Deviche et al., 1989; Lowry et al., 1990) and mammals (Kamerling et al., 1988; Narita et al., 1993; Ukai and Kameyama, 1985). Furthermore, the evidence from Deviche et al. (1989) strongly suggest that κ-opioid-mediated suppression of locomotion occurs via κOR, based on the observation that naloxone (a non-specific opiate antagonist) blocks κ-opioid-mediated suppression of locomotion. Another alternative hypothesis is that the κ-opioid agonist U50488 is suppressing clasping by binding the mCR, but initiating an alternative cellular response that does not involve endocannabinoids. While data from heterologous expression systems indicate that opiates can initiate alternative cellular mechanisms, this is thought to be due to heterodimerization with alternative opiate receptors. We are unaware of precedence for a single receptor initiating alternative cellular responses in response to alternative ligands binding.

It is possible that the dual injection protocol used in our study resulted in a generalized stress response mediated by κ-opioid agonists. However, this scenario seems unlikely as male newts in control conditions received dual injections and were capable of clasping females, indicating the dual injection protocol alone was not significantly stressful to the animal.

It is also possible that the time between drug injections exceeded the effective half-life of cannabinoid antagonist AM281. In order to ensure drug access to the brain, females were presented 20 min after U50488 injection, and 30 min after CB_1 antagonist treatment. There may be a critical interval of time during which complete reversal by CB_1 antagonist may be observable. While the effective half-life and timing of dual injections is a potential confound in any study, we suggest that this is unlikely to be a problem in our current study as behavioral analysis was performed within an hour of the administration of both AM281 and U50488.

In summary, *in vitro* data strongly suggested that κ-opioid agonists bind mCR receptors with a high affinity, which implied that κ-opioid agonists might suppress clasping via the same cellular signaling route as the glucocorticoid CORT, by employing endocannabinoid signaling at the CB_1 receptor. Consistent with this notion, the ability of male *Taricha* to clasp females was suppressed by the κ-opioid agonist U50488 in a dose-dependent manner. However, this κ-opioid-mediated suppression of clasping was reversed by a κOR antagonist nBNI, but not reversed by a CB_1 antagonist. These data suggest that κ-opioid agonists are not acting *in vivo* at the mCR receptor and that endocannabinoids do not mediate the κ-opioid agonist effects of courtship clasping. Thus, we propose that κ-opioid-mediated suppression of courtship-clasping in male newts and other vertebrates is likely to be an indirect effect of κ-opioid agonists suppressing overall activity, unlike CORT which acts specifically to suppress the clasp itself (Coddington and Moore, 2003).

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