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Species differences in the effects of the κ -opioid receptor antagonist zyklophin

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Abstract

We have shown that dysregulation of the dynorphin/kappa-opioid receptor (DYN/KOR) system contributes to escalated alcohol self-administration in alcohol dependence and that KOR antagonists with extended durations of action selectively reduce escalated alcohol consumption in alcohol-dependent animals. As KOR antagonism has gained widespread attention as a potential therapeutic target to treat alcoholism and multiple neuropsychiatric disorders, we tested the effect of zyklophin (a short-acting KOR antagonist) on escalated alcohol self-administration in rats made alcohol-dependent using intermittent alcohol vapor exposure. Following dependence induction, zyklophin was infused centrally prior to alcohol self-administration sessions and locomotor activity tests during acute withdrawal. Zyklophin did not impact alcohol self-administration or locomotor activity in either exposure condition. To investigate the neurobiological basis of this atypical effect for a KOR antagonist, we utilized a κ -, μ -, and δ -opioid receptor agonist-stimulated GTPyS coupling assay to examine the opioid receptor specificity of zyklophin in the rat brain and mouse brain. In rats, zyklophin did not affect U50488-, DAMGO-, or DADLE-stimulated GTPyS coupling, whereas the prototypical KOR antagonist nor-binaltorphimine (norBNI) attenuated U50488-induced stimulation in the rat brain tissue at concentrations that did not impact μ - and δ -receptor function. To reconcile the discrepancy between the present rat data and published mouse data, comparable GTPyS assays were conducted using mouse brain tissue; zyklophin effects were consistent with KOR antagonism in mice. Moreover, at higher concentrations, zyklophin exhibited agonist properties in rat and mouse brains. These results identify species differences in zyklophin

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efficacy that, given the rising interest in the development of short-duration KOR antagonists, should provide valuable information for therapeutic development efforts.

Keywords

alcohol dependence and withdrawal; GTPyS coupling; kappa-opioid receptor; alcohol self-administration; zyklophol

Introduction

Alcoholism, a chronic relapsing disorder characterized by continued alcohol use despite numerous adverse consequences, has a prevalence of ~5.3% in the United States, with ~4.4% of the population diagnosed as alcohol-dependent (Lee et al., 2010). An impaired physiological state accompanied by negative affective states and impaired cognitive control are devastating symptoms of alcoholism that promote excessive alcohol consumption and can adversely affect treatment outcome (Dvorak, Lamis, & Malone, 2013; Leeman, Fenton, & Volpicelli, 2007; Walker & Koob, 2008). However, none of the FDA-approved drugs target these symptoms of alcohol dependence and as such there is a pressing need for better therapeutics that could increase treatment compliance and reduce relapse episodes.

Kappa-opioid receptors (KORs) and their endogenous ligand, dynorphin (DYN) (Chavkin, James, & Goldstein, 1982), are present and positioned to modulate numerous neurotransmitters in motivational and emotional neurocircuitry (Sirohi, Bakalkin, & Walker, 2012; Tejada et al., 2013; Tejada, Shippenberg, & Henriksson, 2011). Alterations in the DYN/KOR system in motivational, emotional, and decision-making circuitry have been posited to contribute to multiple neuropsychiatric disorders, including alcohol dependence (Barg et al., 1993; Bazov et al., 2013; Hiller, Itzhak, & Simon, 1987; Risser et al., 1996). Recently, it was shown that dysregulation of the DYN/KOR system contributes to behavioral deficits that drive an organism to excessively seek and use alcohol in alcohol dependence (Walker & Koob, 2008; Walker, Valdez, McLaughlin, & Bakalkin, 2012), due to the fact that site-specific blockade of the DYN/KOR system alleviates alcohol dependence-induced negative affective states and escalated alcohol consumption (Berger, Williams, McGinnis, & Walker, 2013; Kissler et al., 2014; Nealey, Smith, Davis, Smith, & Walker, 2011; Valdez & Harshberger, 2012; Walker & Koob, 2008).

As a result of such evidence, the KOR has been proposed as a potential therapeutic target to treat addictive and neuropsychiatric disorders such as alcohol dependence and depression (Aldrich & McLaughlin, 2009; Knoll & Carlezon, 2010; Shippenberg, Zapata, & Chefer, 2007; Tejada et al., 2011; Walker et al., 2012). An extended duration of action, lasting from weeks to months, characterizes classical KOR antagonists (Chartoff et al., 2012; Melief et al., 2011; Metcalf & Coop, 2005; Schlosburg et al., 2013; Walker, Zorrilla, & Koob, 2011; Whitfield et al., 2015), and general opioid-receptor antagonists with an extended duration of action are currently approved for the treatment of alcohol dependence in the USA (Mannelli, Peindl, Masand, & Patkar, 2007). However, an alternate strategy for the treatment of alcohol dependence using a short-acting mixed mu-opioid receptor antagonist/partial KOR agonist (nalmefene; prescribed for use on an as-needed basis) was recently approved for use in the

European Union (Mann, Bladström, Torup, Gual, & van den Brink, 2013). However, all currently approved opioid-receptor ligands for the treatment of alcohol dependence modulate the function of multiple opioid receptors. Given the wealth of preclinical data implicating modification of aberrant signaling through KORs as a potential therapeutic target, there have been considerable efforts to develop novel KOR-selective antagonists with a short duration of action.

Zyklophin ([N-benzyl-Tyr¹-cyclo(d-Asp⁵,Dap⁸)]dynorphin A(1–11)NH₂), a cyclic peptide with a short duration (<12 h) of action, has recently been characterized as a novel KOR antagonist in mouse models (Aldrich, Patkar, & McLaughlin, 2009). In the present study, we evaluated zyklophin efficacy for attenuating excessive alcohol consumption during withdrawal in alcohol-dependent rats. We have previously shown that the long-acting KOR antagonist norBNI selectively attenuated withdrawal-induced escalation of alcohol self-administration in alcohol-dependent rats (Kissler et al., 2014; Kissler & Walker, 2015; Nealey et al., 2011; Walker & Koob, 2008), and it was hypothesized that zyklophin would show selective efficacy in dependent animals for reducing excessive alcohol self-administration during acute withdrawal.

Materials and methods

Animals

Male Wistar rats or C57BL/6J mice ~70 days of age were used in the experiments. Upon arrival, animals were housed in an environmentally controlled vivarium with food and water available *ad libitum* and were gently handled on a daily basis. All work adhered to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and followed Institutional Animal Care and Use Committee guidelines. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

Operant alcohol self-administration

Rats were trained to self-administer a 10% alcohol (w/v) solution using a sweetener-fade method (Kissler et al., 2014; Nealey et al., 2011; Samson, 1986; Walker & Koob, 2008). In standard operant chambers (Med Associates, St. Albans, VT), rats pressed a single lever and received 0.1 mL of solution. Following stability (<10% deviation over 3 sessions), the animals were divided into two groups (n = 8/group) that were matched for baseline alcohol self-administration. Half of these animals underwent alcohol self-administration studies and the other half were tested for locomotor activity.

Surgical procedure

Bilateral guide cannulae were implanted in the lateral ventricles using stereotaxic coordinates (from bregma: DV: −3.7; AP: −0.8; and ML: ±1.5; Paxinos & Watson, 2005) under isoflurane gas anesthesia (~2%) and secured to the skull with four jeweler screws and dental acrylic. To preserve patency and reduce risk of infection, obturators were inserted into each guide cannulae. Following surgery, rats were allowed to recover for one week and post-operative care (saline, Flunixin, Baytril, subcutaneous injection) was provided during that

time. Following the conclusion of the experiments, cannulae placements were confirmed by injecting 1 μ L 0.6% cresyl violet over 1 min, extracting the brain, and confirming intraventricular dye penetration.

Intermittent alcohol-vapor exposure

Following recovery, rats were subjected to alcohol vapor according to an intermittent schedule (14 h on, 10 h off), with controls exposed to air. This procedure reliably induces alcohol dependence-like phenotypes (e.g., escalated self-administration and negative affective-like behavior) as shown previously (Kissler et al., 2014; Walker & Koob, 2008). Blood ethanol concentrations (BECs) were analyzed from samples collected prior to daily vapor termination using the Analox AM1 (Analox Instruments Ltd., Lunenburg, MA). BECs were also assessed prior to any behavioral testing. Target BECs of 175–225 mg% were maintained throughout the experiments.

Drugs

Zyklophih-HCl (J. Aldrich, University of Kansas) was dissolved in artificial cerebral spinal fluid (aCSF) (pH 7.2–7.4), composed of 145-mM NaCl, 2.8-mM KCl, 1.2-mM MgCl₂, 1.2-mM CaCl₂, 5.4-mM d-glucose, and 0.25-mM ascorbic acid, or assay buffer. NorBNI and U50488 were purchased from Tocris Bioscience (Ellisville, MO). DAMGO and DADLE were purchased from Sigma Chemical Co. (St. Louis, MO). All drugs for GTPyS coupling assay were dissolved in the assay buffer.

Infusions

Following air or intermittent alcohol-vapor exposure, rats self-administered 10% alcohol (w/v) for 30 min twice per week during acute withdrawal (6–8 h after vapor termination) in operant chambers. Upon stability (<10% deviation over 3 days), rats received sham intracerebroventricular (ICV) infusions via insertion of internal cannulae into the guide cannulae for 2 min, followed by a 1.0-h waiting period before self-administration testing. Once stability was again achieved (<10% over 2 sessions), animals were infused with 1.0 μ L of aCSF on each side over 1 min with the internal cannulae remaining in place for 1 min to allow for vehicle diffusion. Infusions of aCSF were repeated until stability was achieved again (<10% deviation over 2 sessions). Sham and aCSF infusions were performed to habituate the animal to the infusion process.

Pharmacology

After stable alcohol self-administration was demonstrated following aCSF infusions, rats received ICV infusions of zyklophih (0.0–30.0 nmol in 1 μ L aCSF over 60 sec). To control for drug-order and carry-over effects, zyklophih was administered according to a within-subject Latin-square design counterbalanced for drug dose with a vehicle (aCSF) dose included. One hour following infusions, rats were allowed to selfadminister 10% alcohol (w/v) for 30 min.

Locomotor activity testing

Separate groups (n = 4–5/group) of rats were tested for locomotor activity following ICV infusion of aCSF or the highest zyklophin dose (30 nmol). As explained above, rats were subjected to intermittent alcohol-vapor exposure and controls were airexposed. Following dependence induction, during acute withdrawal rats received sham and aCSF infusions to habituate the animals to the infusion process. Finally, all rats received ICV infusions of aCSF or zyklophin (30 nmol) and 1 h later were placed in a clean cage identical to their home cages. The animals were allowed to explore for 10 min and AnyMaze video tracking software (Stoelting Co., Wood Dale, IL) was used to measure distance traveled.

[³⁵S] GTPγS assay

Assays were conducted as described previously (Kissler et al., 2014; Mizoguchi et al., 2004), with some modifications. Rat accumbens or mouse whole brain tissue was examined. Briefly, tissue was homogenized (40–45 strokes; glass homogenizer with Teflon™ plunger; on ice) in 1.5 mL (rat tissue) or 15 mL (mouse whole brain) of membrane buffer (pH 7.4, 50.0-mM Tris-HCl, 3.0-mM MgCl₂, and 1.0-mM EGTA). Homogenates were centrifuged (15,000 rpm, 4 °C for 30 min), re-suspended in 1.5 mL (rat brain tissue) or 30 mL (mouse whole brain) membrane buffer, homogenized (12–15 strokes; on ice) again and finally centrifuged. Pellets were homogenized (12–15 strokes; on ice) in 1.5 mL (rat brain tissue) or 20 mL (mouse whole brain) assay buffer (pH 7.4, 50.0-mM Tris-HCl, 3.0-mM MgCl₂, 0.2-mM EGTA, 100.0-mM NaCl). Protein estimation was conducted using BCA protein assay (Pierce).

Homogenized (12–15 strokes; on ice) before addition, protein homogenate (3.0 µg) was incubated with U50488 (a KOR agonist; 5.0 µM for rat tissue or 10 µM for mouse tissue), DADLE (a delta-opioid receptor agonist; 0.5 µM) or DAMGO (a mu-opioid receptor agonist; 0.5 µM) in the presence of norBNI (0–0.02 µM for rat tissue or 0–0.05 µM for mouse tissue) or zyklophin (0.0–5.0 µM) in triplicate for 90 min at 25 °C, with 50- µM GDP and 0.05-nM (rat tissue) or 0.1-nM (mouse tissue) [³⁵S] GTPγS in a total volume of 1.0 mL. Unlabeled GTPγS (10.0 µM) was used to assess nonspecific binding. Specific binding was obtained by subtracting nonspecific binding from total binding. The reaction was quickly terminated by filtration through Whatman GF/B glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD), followed by 3–5 washes with ice-cold phosphate buffer (pH 7.4). Bound radioactivity on the filters was counted by liquid scintillation spectrophotometry on the following day.

Data analysis

A mixed-model two-way analysis of variance (ANOVA) compared the baseline and post-dependence ethanol self-administration for the vapor- and air-exposed animals. The within-subject variable was session (the average of the final two stable self-administration sessions prior to and following the dependence induction period) and the between-groups variable was level of vapor exposure (air or vapor exposure). Subsequently, univariate analysis for alcohol self-administration prior to and following the dependence induction period was individually conducted for the air- and vapor-exposed groups. In air- and vapor-exposed animals, zyklophin dose-response data for operant alcohol self-administration (g/kg) was

analyzed using a mixed-model two-way ANOVA with exposure condition as the between-groups factor and dose as the within-subject factor. Univariate ANOVAs were used to individually compare locomotion (distance traveled) following aCSF or zyklophin [30 nmol] in air- or vapor-exposed animals.

A two-way repeated-measures ANOVA was used to compare the effect of the agonists and/or antagonists on basal signaling state in the GTP γ S assay. Individual concentration-response curves were analyzed using a one-way ANOVA. *Post hoc* Least Significant Difference (LSD) tests were conducted if a main effect for dose was found.

Results

Chronic intermittent alcohol exposure resulted in significant escalation of alcohol self-administration in alcohol-dependent rats compared to air-exposed controls (Fig. 1). A mixed-model ANOVA revealed a significant main effect of alcohol exposure ($F[1,14] = 29.015$, $p < 0.001$, power = 0.999) and a significant interaction ($F[1,14] = 15.850$, $p = 0.001$, power = 0.959) indicating successful induction of alcohol dependence. Fig. 2 displays the effect of zyklophin (0–30 nmol) on alcohol dependence-induced escalated alcohol self-administration. The mixed-model ANOVA identified a main effect of vapor ($F[1,6] = 18.244$, $p = 0.005$, power = 0.942), whereas no significant differences were found for zyklophin dose ($p > 0.05$, power = 0.352). Zyklophin (30 nmol) did not alter locomotion in either condition ($p > 0.05$, power = 0.066; Fig. 3).

Next, we proceeded to assess zyklophin efficacy, in parallel with the KOR antagonist norBNI, for the ability to functionally antagonize opioid agonist-stimulated GTP γ S coupling in rat and mouse brain tissue. Two-way repeated-measures ANOVAs were used to examine the effect of norBNI and zyklophin on basal- (~12.0 fmol/mg), U50488-, DAMGO-, or DADLE-stimulated GTP γ S coupling in the rat brain tissue (Fig. 4A & 4B, respectively). Fig. 4A illustrates the main effect of agonist exposure ($F[2,113,19.014] = 144.874$, $p < 0.001$, power = 1.0), norBNI concentration ($F[2,18] = 10.459$, $p = 0.001$, power = 0.971) and a significant agonist $\times$ norBNI interaction ($F[6,54] = 3.236$, $p = 0.009$, power = 0.898). NorBNI did not affect basal signaling itself but blocked U50488-stimulated GTP γ S coupling ($F[2,18] = 22.558$, $p < 0.001$, power = 1.0) in a concentration-dependent manner. A KOR-specific effect of norBNI was evident at the lower concentration tested by a selective blockade of U50488-stimulated GTP γ S coupling without an impact on DAMGO- or DADLE-stimulated GTP γ S coupling. At the higher concentration, norBNI completely abolished U50488-stimulated GTP γ S coupling (106% reduction) and also partially, but significantly, blocked DADLE-stimulated GTP γ S coupling (34% reduction). The two-way repeated-measures ANOVA conducted on the effect of zyklophin on basal-, U50488-, DAMGO-, or DADLE-stimulated GTP γ S coupling in rat brain tissue revealed a main effect of agonist exposure ($F[3,15] = 172.172$, $p < 0.001$, power = 0.972). Interestingly, at the highest concentration (5.0 μ M) tested, zyklophin did not affect U50488-, DAMGO-, or DADLE-stimulated GTP γ S coupling (Fig. 4B), but significantly stimulated GTP γ S coupling ($F[2,10] = 12.572$, $p = 0.002$, power = 0.975) compared to the assay buffer (control) in the rat brain tissue, indicating agonist activity.

In mouse brain tissue, the effect of norBNI and zyklophin on basal-(~12.0 fmol/mg), U50488-, DAMGO-, or DADLE-stimulated GTPyS coupling was analyzed using a two-way repeated-measure ANOVA (Fig. 5A & 5B, respectively). Fig. 5A shows the coupling data in which there was a main effect of agonist exposure ($F_{[3,21]} = 37.184$, $p = 0.000$, power = 1.0), norBNI concentration ($F_{[2,14]} = 24.352$, $p = 0.000$, power = 1.0) and significant agonist $\times$ norBNI interaction ($F_{[6,42]} = 3.518$, $p = 0.007$, power = 0.914) (Fig. 5A). NorBNI alone did not affect basal signaling, but blocked U50488-stimulated GTPyS coupling in a concentration-dependent manner ($F_{[2,14]} = 12.103$, $p = 0.001$, power = 0.982). At the highest concentration tested, norBNI completely blocked U50488-stimulated GTPyS coupling, whereas DADLE-stimulated GTPyS coupling was partially, yet significantly, blocked. At the highest concentration, norBNI significantly blocked all agonist-stimulated GTPyS coupling. The two-way repeated-measures ANOVA conducted on the effect of zyklophin on basal-, U50488-, DAMGO-, or DADLE-stimulated GTPyS coupling in the mouse brain tissue (Fig. 5B) identified a main effect of agonist exposure ($F_{[3,21]} = 65.887$, $p = 0.000$, power = 1.0), zyklophin concentration ($F_{[3,21]} = 3.4$, $p = 0.037$, power = 0.682), and a significant agonist $\times$ zyklophin dose interaction ($F_{[9,63]} = 4.47$, $p < 0.001$, power = 0.996). Interestingly, at higher concentrations, zyklophin significantly stimulated GTPyS coupling in the mouse brain tissue ($F_{[3,21]} = 6.075$, $p = 0.004$, power = 0.918) compared to the assay buffer (control). Zyklophin at 0.5 μ M concentration significantly blocked both U50488- and DADLE-stimulated GTPyS coupling, and this effect was abolished at a higher zyklophin concentration (5.0 μ M). When assessed alone, zyklophin significantly stimulated GTPyS signaling in rat ($F_{[2,10]} = 12.572$, $p = 0.002$, power = 0.975) and, under higher concentrations, in mouse ($F_{[3,21]} = 6.075$, $p = 0.004$, power = 0.918) brain tissue.

Discussion

The primary objective of the present study was to evaluate zyklophin efficacy for attenuating alcohol dependence-induced escalated alcohol self-administration. Following one month of intermittent alcohol vapor exposure, the alcohol-dependent group displayed escalated alcohol self-administration (Fig. 1) when tested 6–8 h into withdrawal, a dependence-like phenotype that is consistent with previous reports (Kissler et al., 2014; O'Dell, Roberts, Smith, & Koob, 2004; Walker & Koob, 2008). Zyklophin (0–30 nmol) was administered ICV, and its effect on alcohol self-administration was examined in alcohol vapor- and air-exposed rats. One hour pretreatment with ICV zyklophin has been shown to significantly block KOR-induced effects (Aldrich & McLaughlin, 2009); therefore, in the present experiment zyklophin was administered 1 h prior to behavioral testing. However, zyklophin (20 or 30 nmol) did not affect alcohol self-administration in either group (Fig. 2).

We have previously shown that selective KOR antagonism with norBNI attenuates escalated alcohol self-administration during acute withdrawal and protracted abstinence in alcohol-dependent Wistar rats (Kissler et al., 2014; Kissler & Walker, 2015; Nealey et al., 2011; Walker & Koob, 2008; Walker et al., 2011). Zyklophin has been reported to have KOR-selective effects in mice and was developed as a short-acting KOR antagonist (Aldrich, Patkar, et al., 2009), but did not show a prototypical KOR antagonist profile in the present study. Namely, zyklophin did not alter escalated alcohol self-administration in alcohol-dependent rats (Fig. 2). This lack of effect could be related to differences in short- or long-

acting KOR antagonist-induced activation of c-Jun N-terminal kinase (JNK) signaling (Bruchas et al., 2007), although it is currently unknown whether altered JNK signaling contributes to the therapeutic efficacy of KOR antagonists in alcohol dependence. Differences in the functional potency of opioid ligands has been attributed to species differences in mu- and delta-opioid receptor binding capacity and affinity (Yoburn, Lutfy, & Candido, 1991), and it follows that putative genetic differences in KOR expression contribute to the observed differences in zyklophin potency. However, zyklophin did not attenuate alcohol self-administration in a KOR antagonist-predicted manner in rats, even when tested at 10× the effective dose in mice (Aldrich, Patkar, et al., 2009). At the highest zyklophin dose tested (30 nmol), there was a slight, non-significant decrease in the alcohol self-administration in both groups. In order to examine if there is any non-specific central depressant effect following a higher dose of zyklophin, locomotor activity was examined in alcohol-dependent and non-dependent rats following 1 h of zyklophin (30 nmol) administration. Locomotor data suggested no non-specific effects as zyklophin administration did not alter locomotor activity when compared to aCSF-infused controls (Fig. 3). Pharmacokinetics and metabolism play a critical role in defining the time course of drug action, and it may be that the species differences in the zyklophin efficacy observed in the present study could be a consequence of pharmacokinetics and metabolism differences between rats and mice (Lin & Lu, 1997; Martignoni, Groothuis, & de Kanter, 2006; Stamou, Wu, Kania-Korwel, Lehmler, & Lein, 2014). In the present experiment, zyklophin was administered 1 h prior to behavioral testing, based on previous studies in mice (Aldrich, Patkar, et al., 2009). Therefore, it could be that zyklophin has a different time course of action in rats compared to mice, and evaluating zyklophin at various pretreatment times in the current paradigm seems necessary. However, before any further *in vivo* behavioral testing could occur, it was of primary importance to investigate possible species differences in the zyklophin KOR antagonist profile at the receptor level.

We utilized a GTPyS-coupling assay in order to examine zyklophin specificity at opioid receptors in the rat brain. The GTPyS-coupling assay was carried out as described previously (Kissler et al., 2014), and the effect of norBNI or zyklophin on U50488-, DAMGO-, and DADLE-stimulated GTPyS coupling was examined. U50488, DAMGO, and DADLE significantly stimulated GTPyS coupling in the rat brain tissue compared to the assay buffer (control) (Fig. 4). NorBNI did not affect basal signaling but blocked U50488-stimulated GTPyS coupling in a concentration-dependent manner. At higher concentration, norBNI also blocked DADLE-stimulated GTPyS coupling (Fig. 4A). The order of potency for norBNI was $\kappa > \delta > \mu$, although it must be noted that there was no effect of norBNI on DAMGO-stimulated GTPyS coupling, with these data consistent with previous reports (Black, Jales, Brandt, Lewis, & Husbands, 2003; Takemori, Ho, Naeseth, & Portoghesi, 1988). Conversely, zyklophin (5.0 μ M) significantly stimulated GTPyS coupling in rat brain tissue (Fig. 4B), compared to control assay buffer without modifying U50488-, DAMGO-, or DADLE-stimulated GTPyS coupling (Fig. 4B). The GTPyS coupling data for zyklophin revealed that zyklophin does not act as a KOR antagonist in rat brain tissue and supports our behavioral data. Instead, zyklophin appeared to display positive efficacy, as at higher concentrations it significantly stimulated GTPyS coupling. On the other hand, norBNI at lower concentrations selectively blocked U50488-stimulated GTPyS coupling, suggesting a

KOR-selective antagonism which is also seen in behavioral testing in which norBNI has been shown to selectively impact dependence-induced escalated alcohol self-administration in rats (Kissler et al., 2014; Walker et al., 2011).

Given the lack of effect in rats and considering that the initial characterization of zyklophin occurred exclusively in mice (Aldrich, Patkar, et al., 2009), it was important to functionally confirm KOR antagonism and compare the effects of zyklophin and norBNI on agonist-stimulated opioid receptor function in rat and mouse. Therefore, we next examined the effect of norBNI or zyklophin on U50488-, DAMGO-, and DADLE-stimulated GTPyS coupling in rat and mouse brain tissue. U50488, DAMGO, and DADLE significantly stimulated GTPyS coupling in mouse brain tissue compared to control assay buffer (Fig. 5A & 5B). NorBNI did not affect basal signaling but blocked U50488-stimulated GTPyS coupling in a concentration-dependent manner. At higher concentrations, norBNI also blocked DADLE- and DAMGO-stimulated GTPyS coupling (Fig. 5A). On the other hand, zyklophin significantly stimulated GTPyS coupling in the mouse brain tissue compared to the assay buffer (control; Fig. 5B). In addition, zyklophin blocked U50488- and DADLE-stimulated GTPyS coupling at a moderate concentration (Fig. 5B). At the highest concentration, zyklophin antagonist effects were indiscernible, putatively due to occlusion by zyklophin-stimulated GTPyS coupling. Based on the mouse GTPyS coupling data, zyklophin behaved similarly to norBNI at lower concentration; however, KOR antagonism was not discernible at higher concentrations of zyklophin.

An important consideration is that efficacy is proportional to the magnitude and direction of response produced by a ligand. However, the actual response is dependent on the state and physiologic environment of the receptor (Kenakin, 2009, 2011; Sirohi, Dighe, Madia, & Yoburn, 2009; Sirohi, Kumar, & Yoburn, 2007). Therefore, it is possible that zyklophin positive efficacy, as seen in our data, could be attributed to assay-specific parameters used in the present study. However, in both tissues (rat or mouse), the norBNI efficacy profile was very similar and consistent with the previous reports, supporting the specificity of the assay. Moreover, zyklophin did not alter U50488-, DAMGO-, or DADLE-stimulated signaling in the rat tissue preparation, while demonstrating positive efficacy that suggests agonist effects. However, efficacy is not only a ligand-dependent entity and ligands can display multiple efficacies based on the system tested (Kenakin, 2002, 2011; Kenakin & Beek, 1980; Yoburn et al., 1991). Interestingly, a recent study reported strain-dependent off-target effects (KOR-independent) following zyklophin administration in mice (Dimattio, Yakovleva, Aldrich, Cowan, & Liu-Chen, 2014). Similar KOR-independent effects have been reported for 5'-GNTI (a long-acting KOR antagonist) that produced effects in KOR-KO mice (Inan, 2010). Taken together, there appear to be additional molecular targets of zyklophin action and further evaluation is needed to determine the precise receptor population(s) mediating zyklophin's effects.

In summary, in mouse brain tissue, zyklophin showed selective KOR-antagonist effects through attenuation of U50488-stimulated coupling at lower concentrations, which supports previously published data (Aldrich, Patkar, et al., 2009) in reference to zyklophin's KOR-antagonist profile. Conversely, in the rat brain, zyklophin did not affect U50, 488-stimulated signaling at any concentration tested, which corresponds well with the lack of a KOR

antagonist-like effect on escalated alcohol self-administration during acute withdrawal in alcohol-dependent animals (Kissler et al., 2014; Nealey et al., 2011; Walker & Koob, 2008; Walker et al., 2011). To our surprise, in rat brain and mouse brain tissue at higher concentrations, zyklophin alone showed an agonist profile by significantly stimulating GTPyS coupling. Taken together, there are clear species differences in zyklophin efficacy, with these results providing valuable information for those using rodent species to evaluate KOR involvement in addictive and neuropsychiatric disorders. Given the rising interest in the development of KOR antagonists (e.g., Carlezon & Miczek, 2010), these data should provide valuable information for therapeutic development efforts.

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Highlights

- Zyklophin did not impact alcohol dependence-induced escalated alcohol consumption
- Zyklophin did not antagonize κ , μ or δ agonist-stimulated effects in the rat brain
- In the mouse brain, Zyklophin displayed KOR antagonism at lower concentration
- At higher concentration, Zyklophin behaved as an agonist in rat and mouse brains

Fig. 1. Chronic alcohol vapor exposure increases self-administration in alcohol-dependent rats
Mean ($\pm$ SEM) baseline alcohol consumption during 30-min operant self-administration sessions when tested at a time point corresponding to acute withdrawal. Alcohol-dependent rats ($n = 8/\text{group}$) consumed significantly more alcohol than non-dependent control animals. AW = acute withdrawal (6 h after vapor termination); *** $p < 0.01$, compared to air-exposed controls.

Fig. 2. Zyklophin did not alter alcohol self-administration in vapor- and air-exposed rats
Alcohol-dependent rats ($n = 4/\text{group}$) displayed escalated alcohol consumption compared to control animals treated with aCSF (artificial cerebrospinal fluid). ICV zyklophin did not alter alcohol consumption in either exposure condition. $*p < 0.05$, compared to air-exposed controls. Mean ($\pm$ SEM) alcohol consumption during aCSF or drug infusion is presented.

Fig. 3. The effect of zyklophin on locomotion in air- and vapor-exposed rats
Zyklophin (30 nmol, ICV) did not alter the mean ($\pm$ SEM) distance traveled compared to aCSF-infused controls in air- or vapor-exposed rats ($n = 4/\text{group}$).

Fig. 4. Effect of norBNI and zyklophin on U50488-, DAMGO-, and DADLE-stimulated GTPyS coupling in rat brain tissue

U50488, DAMGO, and DADLE significantly stimulated GTPyS coupling in the rat brain tissue compared to the assay buffer (control). *** $p < 0.001$, ** $p < 0.01$ compared to the basal signaling. **A)** NorBNI did not affect basal signaling but concentration-dependently blocked U50488-stimulated GTPyS coupling. At higher concentration, norBNI also reduced DADLE-stimulated GTPyS coupling (^λ $p < 0.05$; ^{λλλ} $p < 0.001$ compared to agonist-stimulated GTPyS coupling). **B)** Zyklophin significantly stimulated GTPyS coupling in the rat brain tissue compared to control assay buffer (^{λλ} $p < 0.01$ compared to the baseline signaling) without affecting U50488-, DAMGO-, or DADLE-stimulated GTPyS coupling. Data from 3–5 independent experiments conducted in duplicate are presented as mean $\pm$ SEM.

Fig. 5. Effect of norBNI and zyklophin on U50488-, DAMGO-, and DADLE-stimulated GTP γ S coupling in the mouse brain tissue

U50488, DAMGO, and DADLE significantly stimulated GTP γ S coupling in the mouse brain tissue compared to the assay buffer (control). *** $p < 0.001$ compared to the basal signaling. **A)** NorBNI did not affect basal signaling but concentration-dependently blocked U50488-stimulated GTP γ S coupling. At higher concentration, norBNI also blocked DADLE- and DAMGO-stimulated GTP γ S coupling. ^{λλ} $p < 0.01$, ^λ $p < 0.05$ compared to the agonist-stimulated GTP γ S coupling. **B)** Zyklophin significantly stimulated GTP γ S coupling in mouse brain tissue compared to the assay buffer (control). * $p < 0.05$ compared to the baseline signaling. Zyklophin blocked U50488- and DADLE-stimulated GTP γ S coupling at 0.5 μ M concentration while leaving DAMGO-stimulated GTP γ S coupling intact. At higher concentration, zyklophin antagonist effect was not discernable. ^{λλ} $p < 0.01$ compared to the agonist-stimulated GTP γ S coupling. Data represent mean ($\pm$ SEM) from four experiments conducted in duplicate, using two mouse brains.