Psychopharmacology (2016) 233:3449–3459
DOI 10.1007/s00213-016-4384-9

ORIGINAL INVESTIGATION

Metabolic shift of the kynurenine pathway impairs alcohol and cocaine seeking and relapse

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Received: 16 February 2016 / Accepted: 16 July 2016 / Published online: 30 July 2016

Abstract
Rationale The glutamatergic system plays a key role in the maintenance of drug use and development of drug-related conditioned behaviours. In particular, hyper-glutamatergic activity and N-methyl-D-aspartate receptor (NMDAR) activation may drive drug craving and relapse. Inhibition of kynurenine-3-monooxygenase (KMO) shifts the metabolic kynurenine pathway towards production of kynurenic acid, which leads to a reduction of glutamatergic/NMDAR activity via different mechanisms.
Objectives In this study, we investigated whether drug-seeking and relapse behaviour could be modified by the metabolic shift of endogenous kynurenine pathway.
Methods An inhibitor of kynurenine-3-monooxygenase (KMO) Ro61-8048 (4 and 40 mg/kg) and its prodrug JM6 (100 and 200 mg/kg) were tested in two behavioural rat models for drug seeking and relapse—the alcohol deprivation effect (ADE) model in long-term alcohol-drinking rats and the model of cue-induced reinstatement of alcohol- and cocaine-seeking behaviour.
Results Our results show that relapse-like alcohol drinking during the ADE was abolished by repeated intraperitoneal administration of Ro61-8048 and significantly reduced by its oral prodrug JM6. Cue-induced reinstatement of both alcohol- and cocaine-seeking behaviour was also abolished by administration of Ro61-8048.
Conclusions Pharmacological enhancement of endogenous kynurenic acid levels provides a novel treatment strategy to interfere with glutamatergic/NMDAR activity as well as with craving and relapse in alcohol-dependent patients and drug addicts.

Keywords Kynurenine-3-monooxygenase · Kynurenic acid · Ethanol · Cocaine · Craving · Relapse

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Introduction

The glutamatergic system has probably received the greatest attention during the last few years with respect to its role in addictive behaviours. This system was shown to play a key role in the maintenance of drug use and development of drug-related conditioned behaviours. In particular, it has been demonstrated that administration of N-methyl-D-aspartate receptor (NMDAR) antagonists can disrupt the consolidation or reconsolidation of drug-cue associations (von der Goltz et al. 2009; Alaghband and Marshall 2013; Vengeliene et al. 2015a), can reduce cue-induced drug-seeking (Bäckström and Hyytiä 2004, 2006; but see also Eisenhardt et al. 2015a), and can reduce relapse-like behaviour (Vengeliene et al. 2005; Spanagel 2009). Based on these, and numerous further publications, NMDAR antagonists have been suggested as a treatment for drug addiction and for alcohol addiction in particular (Gass and Olive 2008; Spanagel 2009; Kalivas and Volkow 2011; Holmes et al. 2013). However, given the psychotomimetic side effect profile of NMDAR antagonists that includes hallucinations, paranoid delusions, confusion, learning and memory deficits (Holmes et al. 2013), alternative approaches are warranted.

There are several approaches one can take in order to maintain therapeutic efficacy while eliminating the adverse effects of NMDAR antagonists. One possible strategy is to identify intracellular signalling cascades coupled to NMDAR activation that are differentially involved in therapeutic vs. adverse effects of NMDAR antagonists (Vengeliene et al. 2015b). Another innovative approach is to modulate NMDAR activation via the endogenous kynurenine pathway.

In this study, we investigated whether drug-seeking and relapse-like behaviour could be modified by the metabolic shift of the endogenous kynurenine pathway. Several products of this pathway are biologically active and especially kynurenic acid (KYNA) acts as an antagonist of NMDAR (Birch et al. 1988; Parsons et al. 1997), as well as a negative allosteric modulator of the α7nACh receptor (Hilmas et al. 2001) and thus exerts a neuroprotective effect on the brain (Schwarcz et al. 2012; Vécsei et al. 2013). In our study, we used an inhibitor of kynurenine-3-monooxygenase (KMO) Ro61-8048 (Röver et al. 1997) and its prodrug JM6 (Zwilling et al. 2011). Both compounds are known to not effectively cross the blood-brain barrier (Zwilling et al. 2011); however, it was shown that oral Ro61-8048 administration in gerbils led to 85 % inhibition of the cerebral KMO (Röver et al. 1997), suggesting that brain penetration of this compound is sufficient to produce central effects. In addition, peripheral KMO inhibition was shown to increase formation of brain penetrant L-kynurenine (Chiarugi and Moroni 1999), which in turn elevated brain levels of KYNA and reduced striatal levels of glutamate (Moroni et al. 2005; Justinova et al. 2013). In order to examine the effect of KMO inhibition on drug-seeking and relapse-like behaviour, we used two behavioural models—the alcohol deprivation model and the cue-induced reinstatement model of both alcohol- and cocaine-seeking behaviour.

In the alcohol deprivation model, relapse-like drinking is measured as a robust, although temporary, increase in voluntary alcohol intake after a period of deprivation, the alcohol deprivation effect (ADE). Following repeated deprivation phases, the ADE is characterized by an increased demand for alcohol that is dissociated from normal behaviour involving eating or drinking (Sanchis-Segura and Spanagel 2006; Vengeliene et al. 2013, 2014; Eisenhardt et al. 2015b) and therefore resembles a typical relapse situation in alcoholic patients. In previous studies in rats, the expression of ADE was attenuated by repeated sub-acute treatment with several NMDA receptor antagonists (Hölter et al. 2000; Vengeliene et al. 2005, 2008; Spanagel 2009).

The reinstatement model (de Wit and Stewart 1981) examines the resumption of responding in an operant task of a previously extinguished drug-reinforced behaviour in response to non-contingent drug delivery (i.e. priming), environmental stimuli previously associated to it or stressful stimuli (Sanchis-Segura and Spanagel 2006; Bossert et al. 2013). A cue-induced increase in alcohol-seeking is attenuated by some NMDAR antagonists (Bäckström and Hyytiä 2004; Spanagel 2009), but these effects are usually accompanied by a generalized impairment in motor behaviour (Bachteler et al. 2005; Spanagel 2009; Holmes et al. 2013; Eisenhardt et al. 2015a), and therefore the specific contribution of NMDARs to alcohol-seeking responses is unclear. However, a series of cue-induced reinstatement studies with other drugs of abuse, especially with cocaine, support a critical role of NMDARs in mediating drug-seeking responses (Bäckström and Hyytiä 2006, 2007; Feltenstein and See 2007; Engblom et al. 2008; Mameli et al. 2009).

Here, we studied the effects of Ro61-8048 and its prodrug JM6 in the ADE model and reinstatement model.

Materials and methods

Animals

Sixty-five 2-month-old male Wistar rats (from our own breeding colony at the CIMH, Mannheim, Germany) were used for the ADE experiments and blood L-kynurenine measurements, and thirty 2-month-old male Wistar rats (Harlan Laboratories, Venray, Netherlands) were used for ethanol cue-induced reinstatement experiments. All animals were housed individually in standard rat cages (Ehret, Emmendingen, Germany) under a 12 h artificial light–dark cycle (lights on at 7:00 a.m.). Fourteen 2-month-old male Sprague-Dawley rats (Charles River, Germany), were used for cocaine cue-induced reinstatement experiments. Standard laboratory rat food (Ssniff, Soest, Germany) and tap water were provided ad libitum throughout the experimental period (unless stated otherwise). Body weights were measured weekly. All experimental procedures are approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, Germany) and were carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Alcohol drinking solutions were prepared from 96 % ethanol (Sigma-Aldrich, Taufkirchen, Germany) and then diluted with tap water. Cocaine-HCl (Sigma-Aldrich) was dissolved in sterile saline. Ro61-8048 (generously provided by AbbVie, Ludwigshafen, Germany) was dissolved in sterile water (pH = 8) and injected intraperitoneally (i.p.) in a volume of 5 ml/kg. JM6 (synthetized at Gladstone Institute of Neurological Disease, Zwilling et al. 2011) was suspended in 0.1 % of tween 80 and administered orally (p.o.) in a volume of 3 ml/kg.

Long-term voluntary alcohol consumption with repeated deprivation phases

After 2 weeks of habituation to the animal room, rats were given ad libitum access to tap water and to 5, 10 and 20 % ethanol solutions (*v*/*v*) as well. Drinking of alcohol and water was monitored daily/weekly by weighing bottles. From these data, water consumption (in milligramme per kilogramme of body weight per day, ml/kg/day) and alcohol consumption (calculated in gramme of pure alcohol per kilogramme of body weight per day, g/kg/day) was calculated. The first 2-week deprivation period was introduced after 8 weeks of continuous alcohol availability. After the deprivation period, rats were given access to alcohol again and eight more deprivation periods were introduced in a random manner. The long-term voluntary alcohol drinking procedure including all deprivation phases lasted for approximately 1 year.

Relapse-like drinking (pharmacological studies I)

In order to study the effects of drug treatment on the expression of the ADE, rats were divided into groups (n = 7–8 per group) in such way that the mean baseline total alcohol intake was approximately the same in each group (i.e., 2.7 g/kg/day). Baseline drinking was monitored daily for 1 week. After the last day of baseline measurement, the ethanol bottles were removed from the cages leaving the animals with free access to food and water for 2 weeks. Thereafter, the first three groups of animals were subjected to a total of five i.p. injections (starting at 7 p.m. with 12-h intervals) of either vehicle or Ro61-8048 (4 and 40 mg/kg). The other three groups received 3 p.o. administrations (once daily at 9 a.m.) of either vehicle or JM6 (100 and 200 mg/kg). The alcohol bottles were reintroduced after the second drug administration and the occurrence of an ADE was determined. Total ethanol (in gramme per kilogramme of body weight per day) and water intake (in millilitre per kilogramme of body weight per day) were measured daily for a subsequent week. Body weights were recorded 24 h before the first and 12 h after the last drug administration.

Home-cage locomotor activity measurements by the E-motion system

In order to test for any sedative effects resulting from the drug treatment, home-cage locomotor activity was monitored by use of an infrared sensor connected to a recording and data storing system (Mouse-E-Motion by Infra-e-motion, Henstedt-Ulzburg, Germany). A Mouse-E-Motion device was placed above each cage (30 cm from the bottom) so that the rat could be detected at any position inside the cage. The device was sampling every second whether the rat was moving or not. The sensor could detect body movement of the rat of at least 1.5 cm from one sample point to the successive one. The data measured by each Mouse-E-Motion device were downloaded into a personal computer and processed with Microsoft Excel. Monitoring of locomotor activity started 4 days before drug treatment procedure and was continued for four more post-treatment days. The percentage of each rat's locomotor activity during and after treatment days was calculated by using the "before treatment" activity data as a reference.

Blood L-kynurenine measurements (pharmacological studies II)

In order to see whether chronic alcohol drinking interferes with peripheral KMO activity, we measured basal blood L-kynurenine levels in a separate group of chronically drinking rats deprived from ethanol for 2 weeks (n = 14) as well as in the group of age-matched naïve rats (n = 6). Chronic alcohol drinking rats were derived from the long-term voluntary alcohol consumption procedure with repeated deprivation phases as described in the previous paragraph. Ethanol deprived rats were further divided into two groups, and one group was administered with vehicle (n = 6), whereas another one was given 40 mg/kg of Ro61-8048 (n = 8). Blood L-kynurenine levels were measured 30, 120 and 180 min following drug administration. In order to see whether ethanol acutely interferes with KMO activity and Ro61-8048 induced L-kynurenine formation, 0.5 g/kg of 10 % ethanol was administered i.p. 1 h before the last L-kynurenine measurements.

L-kynurenine levels were measured in serum using Biotrend ELISA kit (Biotrend Chemikalien GmbH, Köln, Germany). For this purpose, blood was collected from the tail vein and centrifuged at 5,000×*g* for 15 min, at 4 °C (Centrifuge 5430R, Eppendorf® Microcentrifuges, Hamburg, Germany). Serum was quickly separated and stored at −20 °C until the analysis. ELISA was performed according to the manufacturer's instructions and absorbance was read at 450 nm in a Biotek PowerWave XS Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Standard curve and sample analysis were done using GraphPad Prism software.

Cue-induced reinstatement of alcohol seeking

Operant alcohol self-administration apparatus

Cue-induced reinstatement of alcohol seeking was carried out in operant chambers (MED Associates Inc., St. Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with a response lever on each side panel of the chamber. Responses at the active lever activated a syringe pump that delivered a ~30-μl drop of fluid into a liquid receptacle next to it. Responses at the inactive lever were recorded but had no programmed consequences. A light stimulus (house light) was mounted above both response

levers of the self-administration chamber. An IBM compatible computer controlled the delivery of fluids, presentation of stimuli and data recording.

Alcohol self-administration conditioning and extinction phase

All animal training and testing sessions were performed during the dark phase of their light/dark cycle. Animals were trained to self-administer either 10 % (*v*/*v*) ethanol or water in daily 30-min sessions using a fixed-ratio 1 (FR 1) schedule. The purpose of the conditioning phase was to train the animals to discriminate between the availability of ethanol and water. Discriminative stimuli predicting ethanol or water availability were presented during each ethanol or water self-administration session (one 30-min session/day). An orange flavour extract served as the contextual stimulus (S+) for ethanol, whereas water availability was signalled by a lemon grass extract (S−). These olfactory stimuli were generated by depositing few drops of the respective extract into the bedding of the operant chamber before each session. In addition, each lever press resulting in ethanol delivery was accompanied by a 5-s blinking-light conditioned stimulus (CS+), whereas a 5-s constant-light stimulus (CS−) was presented with water delivery. The 5-s period served as a "time-out", during which responses were recorded but not reinforced. At the end of each session, the bedding of the chamber was changed and trays were thoroughly cleaned. During the first 2 days of conditioning, animals were kept fluid deprived for 20 h/day. Subsequently, alcohol and water sessions were conducted without fluid deprivation in a random manner until the animals received a total of 10 alcohol and 10 water sessions.

After completing the conditioning phase, rats were subjected to daily 30-min extinction sessions for five consecutive days, which in total was sufficient to reach reduced response rates approximating the extinction criterion of 20 % of the last conditioning sessions. Extinction sessions began by extending the levers without presenting olfactory discriminative stimuli. Responses at the previously active lever activated the syringe pump, without resulting in the delivery of either alcohol or water or the presentation of response-contingent cues (stimulus blinking-light or constant-light).

Ethanol cue-induced reinstatement (pharmacological studies III)

Reinstatement testing began 3 days after the final extinction session. In these tests, rats were exposed to the same conditions as during the conditioning phase, except that the liquids (ethanol or water) were not made available. Sessions were initiated by the extension of both levers and the presentation of either the ethanol- (S+) or water- (S−) associated discriminative stimuli. Responses at the active lever were followed by the activation of the syringe pump and the presentation of the CS+ (blinking-light) in the S+ condition or the CS− (constant-light) in the S− condition. One-half of the animals were tested under the S+/CS+ condition on day 1 and under the S−/CS− condition on day 2. For the second half of the animals, conditions were reversed. The number of responses on both the active (i.e. ethanol-associated lever for S+/CS+ condition and water-associated lever for S−/CS− condition) and inactive lever (i.e. water-associated lever for S+/CS+ condition and ethanol-associated lever for S−/CS− condition) was recorded throughout the experiment.

To test the effect of Ro61-8048 on the cue-induced reinstatement of alcohol seeking, animals were divided into two groups on the basis of their performance during the last conditioning and extinction sessions (n = 10 per group). For the test, one group of animals was injected with the vehicle, while the other two groups received either 4 or 40 mg/kg of Ro61-8048. Drug administration was performed 2 h before the reinstatement test procedure to ensure the maximal inhibition of the KMO during the reinstatement test (Röver et al. 1997). Please note that 4 mg/kg of Ro61-8048 had no effect on either ADE or ethanol cue-induced reinstatement and was excluded from the further study.

Cue-induced reinstatement of cocaine seeking

Catheter implantation

Rats were anesthetised with 4 % isoflurane and maintained anesthetised with ~2 % isoflurane during the entire surgery. A catheter composed of a Micro-Renathane® tube (internal diameter, 0.58 mm; external diameter, 0.94 mm; Bilaney Consultants, Düsseldorf, Germany) was implanted into the right jugular vein, and a compatible back-mount (Bilaney Consultants) was passed under the skin and protruded in the mid-scapular region. Rats were given 9–14 days recovery before cocaine self-administration (CSA) sessions began. Catheters were flushed daily with a heparinized solution (100 I.U./ml) containing 1 mg/ml of enrofloxacin (Baytril®).

Self-administration apparatus

Cue-induced reinstatement of cocaine seeking was carried out in operant chambers (Imetronic, Pessac, France) enclosed in ventilated sound-attenuating cubicles. Two nose-poke holes were located on the opposite walls of the chambers, 5 cm above the grid floor. Nose-poke responses were recorded by the interruption of a photo-beam projected across the hole. Poking in one (active) hole resulted in the delivery of 40 μl (0.26 mg) of cocaine over a period of 2 s. Poking in the other (inactive) hole was recorded but had no programmed consequences. A white cue light was located 9.5 cm above the active hole, and a blue cue light was on the opposite wall 33 cm above the grid floor. A sound generator ("beep", 3 kHz, 60 dB) was located on the back wall 40 cm from the grid floor.

Experiments were controlled and data collected with Windows-compatible SK_AA software.

Cocaine self-administration conditioning and extinction phase

All animal training and testing sessions were performed during the dark phase of their light/dark cycle. Rats were trained to self-administer cocaine in 2-h daily sessions until stable responding was established. During the first three sessions, animals were trained under a fixed-ratio 3 (FR3) schedule of reinforcement. FR5 schedule was used for the reminder of conditioning phase. Cocaine availability was signalled by the blue cue light, which was constantly on during the session. Following the required number of nose pokes in the active hole, the white cue light was illuminated and a beep-sound was generated, after 1 s the infusion pump was activated. The white cue light and beep-sound was maintained for 4 s in total. Cocaine infusion was followed by a 40-s time-out period during which nose-poking at either hole had no scheduled consequences.

After completing the conditioning phase, rats entered extinction training, during which nose poking did not result in either the administration cocaine or the presentation of response-contingent cues. Extinction lasted for 17 sessions, which in total was sufficient to reach reduced response rates approximating the extinction criterion of 20 % of the last conditioning sessions.

Cocaine cue-induced reinstatement (pharmacological studies IV)

Reinstatement testing was performed on the next day after the final extinction session. In this test, rats were exposed to the same conditions as during the conditioning phase, except that the cocaine was not made available. Operant chamber was constantly illuminated by the blue cue light which served as a contextual stimulus for cocaine availability. Following the required number of nose pokes in the active hole, the white cue light was illuminated and a beep-sound was generated, after 1 s the infusion pump was activated. The number of nose-poking in both the active and inactive holes was recorded throughout the test.

To test the effect of Ro61-8048 on the cue-induced reinstatement of cocaine seeking, animals were divided into two groups on the basis of their performance during the last conditioning and extinction sessions (n = 7 per group). For the test, one group of animals was injected with the vehicle, while the other one received 40 mg/kg of Ro61-8048. Drug administration was performed 2 h before the reinstatement test. All animals were habituated to the treatment procedure during the last three extinction sessions.

Statistical analysis

Data obtained from ADE measurements (total alcohol intake, water intake) and locomotor activity was analysed using a two-way ANOVA with repeated measures [factors were: treatment group and day]. Data obtained from basal L-kynurenine measurements was analysed by use of the unpaired t test (factor: group). Effect of Ro61-8048 on L-kynurenine levels was analysed by a two-way ANOVA with repeated measures [factors were: treatment group and time]. Data analysis regarding the effects of treatment on the change in the rat body weight was performed using a one-way ANOVA [factor was: treatment group]. Data obtained from the cue-induced alcohol- and cocaine-seeking experiments was analysed by use of a three-way ANOVA with repeated measures [factors were: treatment group, lever-responses/nose pokes (active vs. inactive) and session (extinction vs. reinstatement)]. Whenever significant differences were found, Student Newman–Keuls post hoc tests were performed. The chosen level of significance was $p < 0.05$.

Results

Effect of the administration of Ro61-8048 on L-kynurenine levels and relapse-like drinking

Following the re-introduction of alcohol solutions after a period of abstinence, the vehicle-treated groups showed a typical increase in alcohol consumption, indicating the occurrence of an ADE. This increase was not different from that observed during the first deprivation periods (data not shown). Hence, a two-way ANOVA for repeated measures revealed a general increase in alcohol intake after a deprivation phase as compared to basal drinking [factor day: $F(6,108) = 37.4$, $p < 0.001$ and $F(6,126) = 55.9$, $p < 0.0001$ for Ro61-8048 and JM6 treatment groups, respectively]. Analysis of data also showed that both Ro61-8048 and JM6 treatments significantly reduced alcohol intake during post-abstinence days when compared to intake by vehicle-treated animals [factor treatment group × day interaction effect: $F(12,108) = 5.8$, $p < 0.001$ and $F(12,126) = 1.9$, $p < 0.05$ for Ro61-8048 and JM6 treatment groups, respectively]. The subsequent post hoc tests demonstrated that treatment of rats with 4 mg/kg of Ro61-8048 did not affect post-abstinence drinking, whereas twice daily administration of 40 mg/kg of Ro61-8048 completely abolished relapse-like drinking (Fig. 1a). Once daily administration of 200 mg/kg of JM6 reduced post-abstinence drinking; however, the alcohol intake was still significantly increased during the first post-deprivation day as compared to the baseline consumption (Fig. 1b). Water intake in Ro61-8048 and JM6 treated rats was either unchanged or tended to be increased during treatment days when compared to water

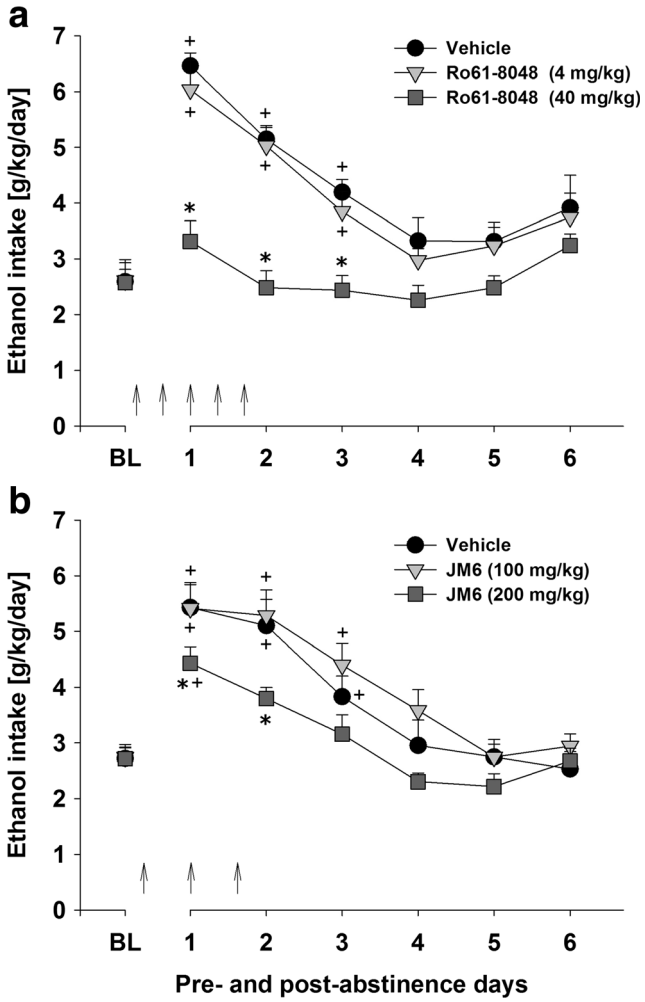

Fig. 1 Intake of total ethanol (calculated in gramme of pure alcohol per kilogramme of body weight per day) in (**a**) vehicle, 4 mg/kg of Ro61-8048 and 40 mg/kg of Ro61-8048 ($n = 7$ per treatment condition) and (**b**) vehicle, 100 mg/kg of JM6 and 200 mg/kg of JM6 ($n = 8$ per treatment condition) treated rats before and after a deprivation period of 2 weeks. The last week measurements of ethanol intake is given as baseline drinking—BL. Animals received a total of five, once every 12 h, injections of Ro61-8048 and three, once daily, oral administrations of JM6 (*arrows*). The ethanol bottles were reintroduced after the second drug administration. Data are presented as means ± S.E.M. *Plus sign* (+) indicates significant differences to baseline drinking, *asterisk* (*) indicates significant differences from the vehicle control group, $p < 0.05$

intake by vehicle-treated animals [factor treatment group × day interaction effect: $p = 0.12$ and $p = 0.15$ for Ro61-8048 and JM6 treatment groups, respectively] (data not shown), demonstrating that treatment selectively affected alcohol consumption.

Locomotor activity data was analysed using recordings of 12-h post-injection intervals that corresponded to the animals' active phase. Overall, there was a general reduction in home-cage activity seen in all animal groups, which was likely caused by alcohol intoxication during post-abstinence drinking days [factor day: $F(6,108) = 29.3$, $p < 0.001$ and $F(6,126) = 30.5$, $p < 0.001$ for Ro61-8048 and JM6 treatment groups, respectively] (Fig. 2). However, a two-way ANOVA revealed that treatment with 40 mg/kg of Ro61-8048 had an additional, although small, sedative effect on animal home-cage activity [factor treatment group: $F(2,18) = 5.0$, $p < 0.05$] (Fig. 2a). This activity change was temporary and recovered to basal levels immediately after treatment stopped.

Treatment with either Ro61-8048 or JM6 did not lead to significant changes in the animals' body weight, demonstrating that food intake and/or metabolism was not altered during the treatment days [factor treatment group: $p = 0.44$ and $p = 0.47$ for Ro61-8048 and JM6 treatment groups, respectively].

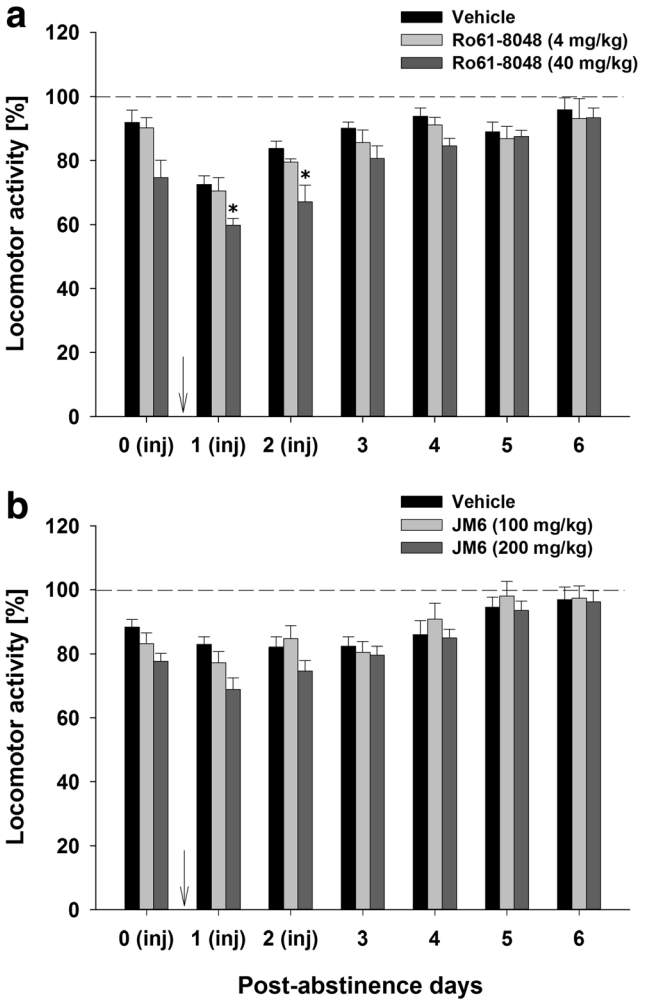

Fig. 2 Locomotor activity in (**a**) vehicle, 4 mg/kg of Ro61-8048 and 40 mg/kg of Ro61-8048 ($n = 7$ per treatment condition) and (**b**) vehicle, 100 mg/kg of JM6 and 200 mg/kg of JM6 ($n = 8$ per treatment condition) treated rats. Locomotor activity is shown as 12-h post-injection intervals of animals' active phase. The percentage of each rat's locomotor activity during and after treatment days was calculated with respect to basal activity prior to treatment (*dashed line*). Injection days are marked as "inj". The *arrow* indicates re-exposure of animals to alcohol solutions. Data are presented as means ± S.E.M. *Asterisk* (*) indicates significant differences from the vehicle control group, $p < 0.05$

Blood L-kynurenine measurements showed that chronic ethanol consumption had no effect on L-kynurenine production. In fact, blood L-kynurenine levels in chronically drinking rats deprived from ethanol for 2 weeks were not different from that in age-matched ethanol naïve rats ($p = 0.89$) (Fig. 3a). Administration of 40 mg/kg of Ro61-8048 significantly elevated L-kynurenine levels when compared to vehicle-treated animals [factor treatment group: F(1,12) = 58.8, $p < 0.001$ and factor treatment group × time interaction effect: F(2,24) = 26.1, $p < 0.001$] (Fig. 3b). Further post hoc analysis showed that ethanol alone had no effect on L-kynurenine production. Thus, L-kynurenine levels remained unchanged in the vehicle-treated group after ethanol adminstration (Fig. 3b).

Effects of the administration of Ro61-8048 on cue-induced reinstatement of alcohol-seeking behaviour

At the end of the conditioning phase, rats exhibited 97 ± 8 ethanol-associated lever presses (S+/CS+ condition) and 21 ± 2 water-associated lever presses (S−/CS− condition). The number of operant responses progressively faded away across five extinction sessions. Thus, during the last extinction sessions, lever presses dropped down to 8 ± 1 and 7 ± 1 for the previously ethanol-reinforced and water-reinforced lever, respectively.

Three-way ANOVA revealed that the number of responses during the ethanol cue-induced reinstatement test increased significantly when compared to the last extinction sessions [factor session: $F(1,36) = 71.6$, $p < 0.001$]. This increase was mainly caused by the higher responses on the active lever in the vehicle-treated group [factor treatment group × lever × session interaction: $F(1,36) = 20.1$, $p < 0.001$] (Fig. 4). Treatment with 40 mg/kg of Ro61-8048 significantly reduced lever responses during the reinstatement test under S+/CS+ condition [factor treatment group: $F(1,36) = 38.9$, $p < 0.001$ and treatment group × session interaction: $F(1,36) = 39.5$, $p < 0.001$] (note that 4 mg/kg dose is not shown in the Fig. 4). Further post hoc analysis showed that responding on the active lever during the reinstatement test by the Ro61-8048-treated group was not different from that during the last extinction sessions. Responding on the inactive lever was not considerably lower in Ro61-8048-treated animals as compared to that of the vehicle-treated group (Fig. 4b).

The number of responses during the S−/CS− session was also found to be higher than that observed during the last extinction sessions [factor session: $F(1,36) = 8.6$, $p < 0.01$]. This increase was not selective to the treatment condition and lever suggesting that it was probably driven by general arousal of an animal [factor treatment group × lever × session interaction: $p = 0.23$] (data not shown).

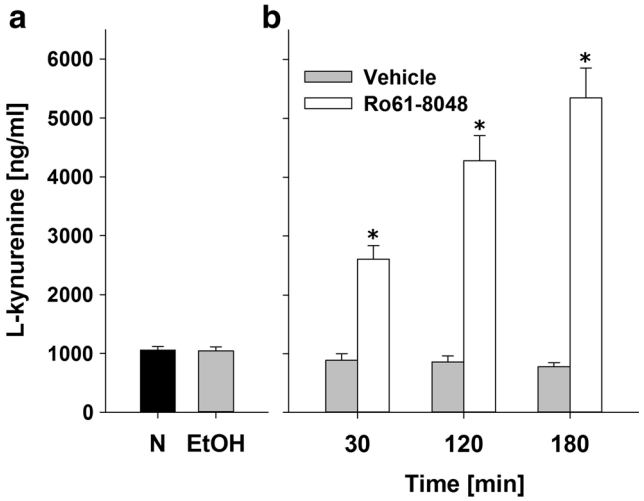

Fig. 3 Blood content of L-kynurenine in (**a**) naïve (*N*) and chronically drinking rats after a deprivation period of 2 weeks (abstinent, EtOH), and in (**b**) abstinent rats following administration of either vehicle or 40 mg/kg of Ro61-8048 ($n = 6$–8). Measurements were performed 30, 120 and 180 min after vehicle and Ro61-8048 administration. Both vehicle and Ro61-8048-treated groups were administered with 0.5 g/kg of ethanol 1 h before the last L-kynurenine measurement. Data are presented as means ± S.E.M. *Asterisk* (*) indicates significant differences from the vehicle control group, $p < 0.05$

Effects of the administration of Ro61-8048 on cue-induced reinstatement of cocaine-seeking behaviour

At the end of the conditioning phase, rats exhibited 1,046 ± 352 cocaine-associated nose pokes and 51 ± 15 inactive nose pokes. The number of operant responses progressively faded away across 17 extinction sessions. Thus, during the last extinction sessions, active nose pokes dropped down to 72 ± 16, and inactive pokes were 33 ± 4.

During the cocaine cue-induced reinstatement test, the number of nose pokes significantly increased compared to

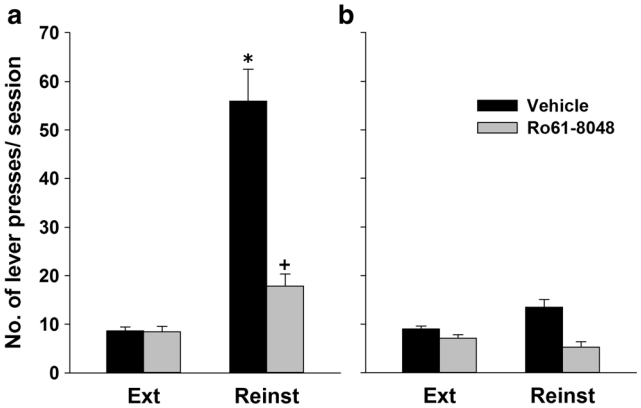

Fig. 4 The effect of vehicle and 40 mg/kg of Ro61-8048 ($n = 10$ per treatment condition) on ethanol cue-induced reinstatement. Data are shown as the number of responses on the active (**a**) and inactive (**b**) levers during the last extinction sessions (Ext) and during the reinstatement test (Reinst). Data are presented as means ± S.E.M. *Asterisk* (*) indicates significant differences to the extinction lever responses; *plus sign* (+) indicates significant differences from the vehicle control group, $p < 0.05$

the last extinction sessions, which was confirmed by three-way ANOVA [factor session: F(1,24) = 17.2, $p < 0.001$]. This was caused by an increase in active nose pokes in the vehicle-treated group [factor treatment group × nose pokes × session interaction: $F(1,24) = 8.8$, $p < 0.01$] (Fig. 5). Administration of 40 mg/kg of Ro61-8048 completely abolished cocaine seeking; thus, in these animals the number of active nose pokes during the reinstatement testing was not different from that during the extinction phase [factor treatment group: $F(1,24) = 11.7$, $p < 0.01$ and treatment group × session interaction: $F(1,24) = 15.2$, $p < 0.001$]. Further post-hoc analysis showed that inactive nose pokes were not significantly different in the Ro61-8048-treated group as compared to that in the vehicle-treated group (Fig. 5b).

Discussion

The present study demonstrates that KMO inhibition causes a significant reduction of relapse-like excessive alcohol intake during the post-abstinence drinking days in a four-bottle free choice paradigm in male rats. Relapse-like drinking during these days was abolished by repeated intraperitoneal administration of Ro61-8048 and significantly reduced by its oral prodrug JM6. Although Ro61-8048 treatment was slightly sedative, neither water intake, nor body weight was affected by either Ro61-8048 or JM6 demonstrating that therapeutic safety of these compounds is within acceptable limits. Importantly, repeated oral once daily administration of the pro-drug JM6 did not have significant effect on home-cage activity. JM6 provides long-lasting inhibition of KMO, because it slowly produces Ro61-8048 in the gut of the animal. In this way, more stable concentration of Ro61-8048 can be achieved, which lowers the probability of developing side effects. Cue-induced reinstatement of both alcohol- and cocaine-seeking behaviour was also abolished by administration of Ro61-8048. The latter finding is in line with a recent report showing that Ro61-8048 abolished delta-9-tetrahydrocannabinol (THC)-seeking behaviour (Justinova et al. 2013) pointing to a more general mechanism involved in drug-seeking responses.

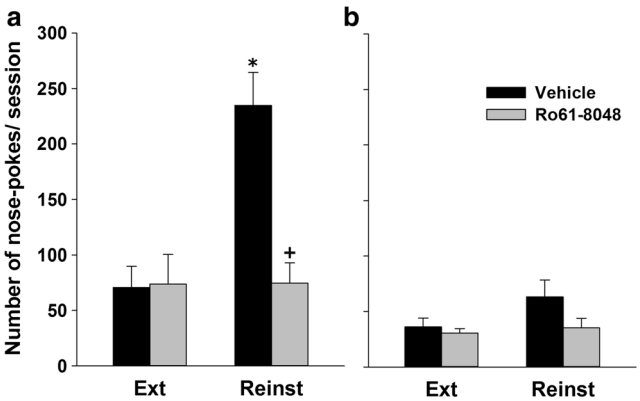

Fig. 5 The effect of vehicle and 40 mg/kg of Ro61-8048 ($n = 7$ per treatment condition) on cocaine cue-induced reinstatement. Data are shown as the number of active (**a**) and inactive (**b**) nose pokes during the last extinction sessions (Ext) and during the reinstatement test (Reinst). Data are presented as means ± S.E.M. *Asterisk* (*) indicates significant differences to the extinction nose pokes; *plus sign* (+) indicates significant differences from the vehicle control group, $p < 0.05$

Despite the attractiveness of targeting the NMDAR with the aim to reduce drug relapse related behaviours, there are a few setbacks related to this target. Even though the NMDAR plays a crucial role in the occurrence of drug-cue associations, antagonists of this receptor are surprisingly not very efficient in reducing cue-induced drug seeking (Vengeliene et al. 2008; Eisenhardt et al. 2015a). For instance, the competitive NMDAR antagonist CGP39551 was shown to have no effect on either alcohol or cocaine cue-induced reinstatement (Bäckström and Hyytiä 2004, 2006). Blocking of NMDAR channel also failed to reduce drug-seeking responses in rats (Bespalov et al. 2000; Bäckström and Hyytiä 2004; Bachteler et al. 2005). On the other hand, the NMDAR glycine binding site antagonist L-701.324 reduced both alcohol as well as cocaine-seeking behaviour (Bäckström and Hyytiä 2004, 2006). In case of alcohol consumption during the post-abstinence phase, NMDAR antagonists were efficient in reducing drinking independently on which binding site of the receptor they were acting (Vengeliene et al. 2005). The other problem with targeting the NMDAR is poor tolerability of antagonist treatment due to easily occurring side-effects. For instance, repeated administration of different NMDAR antagonists during the ADE test in rats caused a significant loss of body weight, demonstrating that food intake and/or metabolism was altered during the treatment days (Vengeliene et al. 2005). In addition, the psychotomimetic side effect profile of NMDAR antagonists that includes hallucinations, paranoid delusions, confusion, learning and memory deficits is of great concern (Holmes et al. 2013). Therefore, any alternative approach to maintain treatment efficacy while eliminating the adverse effects of NMDAR antagonists is of great therapeutic interest. One innovative strategy is to modulate NMDAR activation via the endogenous kynurenine pathway, which might ultimately produce fewer side effects compared to targeting NMDAR directly. Both JM6 and Ro61-8048 do not effectively cross the blood-brain barrier (Zwilling et al. 2011), which may also contribute to a better safety profile of these compounds. Indeed, NMDAR antagonist MK-801 was shown to disrupt memory formation in the passive avoidance task in rats, whereas Ro61-8048 had no effect on performance in this test (Moroni et al. 2005).

Our study showed that blood L-kynurenine levels in deprived rats after long-term drinking experience were similar to

that in age-matched alcohol naive rats. Furthermore, comparison of gene-expression profiles in the nucleus accumbens of these rats showed that neither kynurenine 3-monooxygenase nor kynurenine aminotransferase gene expression was changed after chronic alcohol consumption (Oliver Ståhlin and Valentina Vengeliene, unpublished results). Hence, it is unlikely that our long-term alcohol drinking procedure had irreversible effect on KYNA synthesis in the brain and interfered with Ro61-8048 treatment. KMO inhibitor Ro61-8048 dramatically increased blood KYNA levels and completely abolished both relapse-like alcohol drinking and cue-induced reinstatement of alcohol- and cocaine-seeking behaviour, showing that this target is superior to a direct blockade of NMDAR activity. The effect of KMO inhibition is in part similar to that of NMDAR antagonist treatment; however, there are also a few differences. Inhibition of KMO shifts the metabolic kynurenine pathway towards production of KYNA, which is an endogenous NMDAR antagonist acting at a glycine binding site of this receptor (Birch et al. 1988; Parsons et al. 1997). KYNA can also antagonize non-NMDAR (Rózsa et al. 2008), however, only at the higher doses, which were unlikely reached in our study (Röver et al. 1997). And finally, KYNA is known to have an inhibitory effect on α7nACh receptor function (Hilmas et al. 2001), which may contribute to a well-known neuroprotective effect of KYNA due to suppression of glutamate release (Schwarcz et al. 2012; Vécsei et al. 2013).

Metabolic shift of the kynurenine pathway caused by KMO inhibition may be advantageous at several levels. First of all, blocking the co-agonist glycine binding side of NMDAR seems to be the only effective way of targeting this receptor in order to reduce cue-induced drug-seeking behaviour (Bäckström and Hyytiä 2004, 2006). Hence, elevation of KYNA levels is beneficial in this respect. And second, both drugs, JM6 and Ro61-8048, beside their main effect to increase KYNA production, were shown to reduce extracellular glutamate concentration in the brain (Moroni et al. 2005; Zwilling et al. 2011). This effect seems to be caused by the effect of KYNA on the α7nACh receptors (Carpenedo et al. 2001). Although antagonizing the α7nACh receptor alone does not seem to be sufficient to reduce relapse-like behaviour (Kuzmin et al. 2009), reduction of glutamatergic activity seen after administration of KMO inhibitors may have an additional beneficial anti-relapse effect. Increased glutamatergic transmission was found during alcohol withdrawal (Spanagel and Kiefer 2008; Spanagel 2009; Hermann et al. 2012; Spanagel and Vengeliene 2013), as well as during alcohol-associated cue responding in a reinstatement test (Gass et al. 2011). Reducing glutamatergic activity during acute or conditioned withdrawal may help to lower glutamate-induced neurotoxicity and alleviate withdrawal symptoms. Interestingly, it was also shown that glutamate release was increased during acute cocaine withdrawal (Gabriele et al. 2012), suggesting that reduction of glutamatergic activity may also be advantageous in cocaine relapse prevention. As for the conditioned withdrawal, increased glutamate release was measured during the reinstatement of drug-seeking behaviour independently on whether animals were trained to self-administer alcohol (Gass et al. 2011), cocaine (McFarland et al. 2003) or heroin (LaLumiere and Kalivas 2008). Hence, reduction of overall glutamatergic activity during drug or cue re-exposure may have an additional effect to that of NMDAR antagonism on lowering drug craving and seeking. Interestingly, KMO inhibition had no effect on cocaine self-administration (Justinova et al. 2013), suggesting that this treatment may be specific to relapse-like situations.

In conclusion, our results show, for the first time, that metabolic shift of the kynurenine pathway towards production of KYNA, impaired alcohol and cocaine-seeking and relapse-like behaviour in rats. These findings complement those derived from the experiments on THC seeking (Justinova et al. 2013). We suggest that KMO could be used as a pharmacological target for developing novel relapse-preventing drugs, which may have similar profile to NMDAR antagonists. However, higher efficiency, due to reduction of glutamatergic activity, and better side-effect profile could be expected from KMO inhibitors.

Acknowledgements We would like to thank Sabrina Koch for the excellent technical assistance and Paul Muchowski for supplying JM6. Financial support for this work was provided by the Bundesministerium für Bildung und Forschung (e:Med program, FKZ: 01ZX1311A (Spanagel et al. 2013)). TT was funded by CAPES Foundation, Ministry of Education of Brazil, Brasília—DF 70040-020, Brazil.

Compliance with ethical standards

Conflict of interest All authors declare no financial interests or potential conflicts of interest.

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