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Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action.

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ABSTRACT

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug (NSAID) used for the topical treatment of inflammations of the oral and vaginal mucosa. Virtually nothing is known about the central pharmacological actions of BZY. Yet, there are reports of voluntary systemic overdosage of BZY in drug addicts, resulting in a euphoric, hallucinatory state. In the present study, we investigated the reinforcing properties of BZY in a rat self-administration paradigm. We found that BZY has a powerful reinforcing effect and that this effect is greatly facilitated in animals that already had substance experience, having previously self-administered heroin and cocaine, indicating cross sensitization between BZY and other common drugs of abuse. We then assessed the effect of BZY on Prelimbic Cortex-to-Nucleus Accumbens (PLCx-NAcc) glutamatergic transmission, using field recordings in rat parasagittal brain slices. BZY dose-dependently reduced both field excitatory post synaptic potential (fEPSP) amplitude and Paired Pulse Ratio (PPR), suggesting a presynaptic mechanism of action. Similarly to the *in vivo* paradigm, also the electrophysiological effects of BZY were potentiated in slices from animals that had undergone cocaine and heroin self-administration. Furthermore, BZY-induced LTD-like responses in the PLCx-NAcc circuitry were significantly reduced in the presence of the CB1 receptor antagonist AM251. These findings provide firm evidence of the abuse liability of BZY and suggest a possible cannabinoidergic mechanism of action. Further research is needed in order to give insights into the molecular mechanism underlying BZY psychoactive and reinforcing effects, to better understand its abuse potential.

Keywords: Benzydamine, hallucinogen, psychostimulant, cannabinoid receptor type 1, CB1 receptor, fEPSP, antinflammatory, NSAID, drug abuse, self-administration

INTRODUCTION

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug that was synthetized by Angelini's laboratories in 1964 and made commercially available since 1966 for the symptomatic treatment of acute inflammatory states of the oral and vaginal mucosae. A number of pharmaceuticals preparations for topical use are available (e.g., TANTUM® Verde and GineTANTUM® in Italy, Difflam® in the UK, Tanflex® and Benzidan® in Turkey, Flogo-Rosa® in Brazil). Benzydamine is also contained in medications for systemic use (with concentrations of BZY ten times lower than those for topical use), as in the case of Benflogin® in Brazil, which, however, is no longer available on the market.

The mechanisms of action of BZY are complex. The anti-inflammatory effect mainly depends on the inhibition of prostaglandin synthesis, probably due to disruption of calcium mobilization/sequestration at the level the plasma membrane of inflammatory cells (Jeremy et al., 1991). Benzydamine also exhibits a less specific mechanism of action termed 'membrane stabilization', resulting in the inhibition of granule release by neutrophils and lysosomes (Quane et al., 1998). In addition to the anti-inflammatory/analgesic effect, BZY has local anesthetic effects (Sato and Maehara, 1967; Silvestrini et al., 1966a, 1966b).

Relatively little is known about the effects of BZY on the central nervous system. Symptoms such as hallucinations, dizziness, and anxiety have been reported as a consequence of unintended systemic ingestion (Gómez-López et al., 1999; Ballesteros et al., 2009; Settimi et al., 2012; Acar et al., 2014). Most important, there is evidence of recreational use of BZY in various countries, including Brazil, Italy, Poland, and Turkey (Anand et al., 2007; Opaleye et al., 2009, 2011; Settimi et al., 2012; Balaban et al., 2013; Barwina et al., 2014). In Brazil, for example, BZY has been abused by street youth since at

least the early 1990s, probably due to its low price and ready availability (Opaleye et al., 2009). In the following two decades its use spread to Brazil club scene, with the blossoming of theme rave parties (Benflogin® parties) and pop songs titled 'Benzydamine'. Informal self-reports, hosted by internet drug forums and social networks, BZY and provide information about route of administration, dosage, and substance preparation from commercial preparations, as well as advice about other psychotropic substances to be assumed with BZY to enhance its pleasurable effects and dampen the undesired ones (Souza et al., 2008). Benzydamine seems to be particularly popular among poly-drug users (Anand et al., 2007; Opaleye et al., 2009). We have recently reported BZY use among the outpatients of an addiction clinic (Villa Maraini in Rome, Italy) with a history of heroin and cocaine co-abuse (Malavasi et al., 2012).

The major aim of the present study was to assess the reinforcing effect of BZY in animal model of drug abuse. In particular, we investigated the ability of this drug to sustain intravenous self-administration in drug-naïve rats as well as in rats that had been previously trained to self-administer cocaine and heroin, to mimic the drug history of polydrug abusers.

The second aim of our study was to begin characterizing the neurobiological actions of BZY, as virtually nothing is known in this regard. We investigated the electrophysiological effects of BZY on PLCx-NAcc synapses, a circuitry that is thought to play an important role in drug reward and drug seeking (Ikemoto and Panksepp, 1999; Kalivas and Volkow, 2005; Kalivas et al., 2009) and that has been shown to undergo neuroplastic changes following repeated drug administration (Van den Oever et al., 2012; Quintero, 2013; Kalivas et al., 2009). Interestingly, other drugs of abuse with hallucinogenic properties similar to those of BZY, such as ketamine and PCP, have been shown to reduce glutamatergic transmission

(Anis et al., 1983). We also explored the possible involvement of CB1 receptors in the electrophysiological effects of BZY, as the chemical structure of BZY shares some features (e.g., the benzoyl indole structure) with CB1 synthetic agonists, which also have hallucinogenic properties (Forrester, 2012; Harris and Brown, 2013).

MATERIALS AND METHODS

Experiment 1: BZY self-administration

Animals and surgery: All the experimental procedures were carried out according the guidelines established by the European legislation (Directive 2010/63/EU) and the Italian legislation (L.D. 26/2014).

A total of 23 male Sprague-Dawley rats (Harlan Laboratories) weighting 250-300 g upon arrival were used. The rats were housed in pairs in transparent plastic cages (40 cm in length, 24.5 cm in width, and 18 cm in height) with stainless steel grid tops and flat bottoms covered with ground corncob bedding, in a temperature (21±1 °C) and humidity (70%) controlled room with a 14-h dark/10-h light cycle (lights off at 0700 hours). The rats were gently handled twice a week for two weeks before undergoing surgical catheterization, following procedures described previously in detail (Caprioli et al., 2008). On the day of surgery, the rats received an intraperitoneal injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). The catheter consisted of 10.5 cm of silicone tubing (0.37-mm inner diameter and 0.94-mm outer diameter) sheathed at 3.4 cm from its proximal end by a 5-mm piece of heat-shrink tubing. The catheter was

inserted into the right jugular vein and secured to the surrounding soft tissues with silk thread. Catheter distal end was externalized through a small incision at the nape of the neck and connected to an L-shaped 22-gauge cannula, which was secured to rat's skull using dental cement and stainless steel screws. After surgery, the rats were given 15 mg i.v. enrofloxacin (Baytril®, KVP Pharma + Veterinär Produkte Gmbh, Kiel, Germany). The catheters were flushed daily (1800 hours) with 0.1 ml of sterile saline solution containing 0.4 mg of enrofloxacin and 25 IU heparin (Marvecs Services, Agrate Brianza, Italy). The rats had ad libitum access to food and water throughout the experiment, except during the self-administration sessions. The rats were allowed to recover from the surgery for 7-10 days and were then assigned to either the drug-naïve group (n=12) or the drug-experienced group (n=11) before the start of drug self-administration.

Apparatus: The apparatus (ESATEL S.r.l.; Rome, Italy) consisted of self-administration chambers (28.5-cm length, 27-cm width, and 32-cm height) made of transparent plastic (front and rear walls), aluminum (sidewalls and ceiling), and stainless steel (grid floor). Plastic trays covered with pinewood shaving were placed under the cage floors. Each cage was equipped with a counterbalanced arm holding a liquid swivel and two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor. Cue lights consisting of either a set of triple (green, red, and yellow) LED lights (in the case of heroin/cocaine self-administration) or a single white light (for BZY self-administration) were positioned above each the two levers. The self-administration cages were placed within sound- and light-attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable

logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software developed by Aries Sistemi S.r.l. (Rome, Italy).

Self-administration procedures in the drug-naïve group: The rats in the drug-naïve group underwent fourteen 3-h daily sessions of BZY self-administration. Independent groups (n=4) self-administered one of three infusion doses of BZY (Sigma Aldrich): 250, 500, and 1000 µg/kg (dissolved in 40 µl of sterile saline solution). The sessions took place during the dark phase between 0900 and 1700 hours. During the session both levers were extended, but only one of them (counterbalanced across animals) was 'active' whereas pressing on the other lever had no scheduled consequences except resetting the counter for the active lever. The number of consecutive presses (on the active lever) required to obtain a single drug infusion (fixed ratio, FR) was progressively increased during training: FR1 on sessions 1-7, FR2 on session 8, FR3 on session 9, FR4 on session 10, and finally FR5 on sessions 11-14. Once the appropriate FR had been reached, a drug infusion was delivered over a period of 3 s and at the same time both levers retracted for a time-out period of 40 s. A white cue light above the active lever was on throughout the session except during the time-out period. Rats that did not spontaneously self-administer at least one infusion within the first 5 min of the session were placed with their forepaws on the active lever, so as to trigger a priming infusion. Priming infusions were administered again at times 60 and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 5-60 and 60-120 min. On FR5 sessions (days 11-14) priming infusions were given only to rats that failed to self-administer at least one infusion within the 0-5 min period. These experimenter-induced lever presses were opportunely subtracted from the total. The rats were allowed to self-administer a maximum of 50 infusions within a single session.

Self-administration procedures in the drug-experienced group: The rats in the drug-experienced group were first trained to self-administer cocaine and heroin on alternate days for 12 consecutive sessions (as previously described in detail by Caprioli et al., 2009 and Montanari et al., 2015). Briefly, during this phase both levers were paired with cocaine and heroin in a counterbalanced manner. At the start of each session, only the lever associated with the drug to be self-administered on that session was extended, and the appropriate set of cue lights was turned on. Thus, during this phase pressing on both levers was reinforced and there was no 'inactive' lever. After the completion of this procedure, the rats were transferred to a different experimental room and given a 5-day period of rest. The rats were then trained to self-administer one of three infusion doses of BZY (n=3-4), as described above for drug-naïve rats. Notice that the self-administration procedures for BZY differed from those for heroin and cocaine in three major ways: i) only one lever (either the left or the right lever, on alternate days) was extended during heroin and cocaine self-administration whereas both levers (one 'active' and one 'inactive') were available during BZY self-administration sessions; ii) the cue lights signaling drug availability consisted of a set of triple (green, red, and yellow) LED lights in the case of heroin/cocaine self-administration versus a single white light in the case of BZY self-administration; iii) two distinct testing rooms (and related contextual cues) were used for heroin/cocaine versus BZY self-administration.

Experiment 2: Slice electrophysiology

Animals: Acute brain slices were obtained from 18 drug-naïve and 5 drug-experienced male Sprague-Dawley rats of the same age of those used in the BZY self-administration

experiment (P60-90 for drug-naïve rats and P80-100 for drug-experienced rats). Drug-experienced animals underwent alternate cocaine and heroin self-administration training as detailed in the previous experiment and were then given 5-7 days of rest in their home cage before being anesthetized for brain slice collection. Notice that the rats used in the electrophysiology experiments did not receive BZY self-administration training.

Slice collection: Animals were deeply anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Sigma-Aldrich) and decapitated. The brain was rapidly removed and 300 µm thick, parasagittal slices were obtained using a Leica 1200T vibratome, and immersed in a cutting solution containing (in mM): KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 10, $CaCl_2$ 0.5, Choline 120, $NaHCO_3$ 26, Glucose 10. The slices were then incubated in artificial cerebrospinal fluid (aCSF) in a holding chamber at 32°C for the first 60 min and at room temperature thereafter. Each slice was transferred to a recording chamber constantly perfused with aCSF (31-32°C, 3-4 ml/min) with the following composition (in mM): NaCl 126, KCl 1.25, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, Glucose 10. All solutions were saturated with a 95% O_2 – 5% CO_2 gas mixture.

Stimulation and recordings: Field excitatory post synaptic potentials (fEPSPs) were recorded by placing a glass recording electrode, filled with aCSF, in the Core of the Nucleus Accumbens (NAcc-Core). Prelimbic cortical afferents were stimulated by a 25 µs square wave current delivered at 0.03 Hz (a sweep every 30 s) through a bipolar stainless steel microelectrode placed at the border between PLCx and NAcc. The $GABA_AR$ antagonist picrotoxin (100 µM) was added to the perfusion aCSF due to presence of strong GABAergic inhibition in the NAcc-Core. Signals were fed into a Multiclamp 700b amplifier (Axon

Instruments), filtered at 1 kHz, converted by a Digidata 1322A (Axon Instruments), acquired and analyzed using Clampex 9.2 software.

The stimulus intensity that evoked a fEPSP of 50-60% of the maximal response was chosen to acquire the baseline response (15 min) for all experiments. For short term plasticity experiments, a fEPSP of ~50% of the maximum was obtained and two stimuli were delivered with a 120 ms inter-stimulus interval (ISI). Paired Pulse Ratio was calculated as fEPSP2/fEPSP1 (3 sweeps average) right before and after BZY bath-perfusion. For all experiments, BZY was added at the final concentration to the perfusion aCSF. Preliminar experiments showed that at the concentration of 30 µM BZY was effective in decreasing the PLCx-NAcc fEPSP by about 50% after 30 min of exposure, whereas at the concentration of 100 µM fEPSP was virtually abolished. Thus, whereas in the acute perfusion experiments the exposure time was 10 minutes (Fig. 2), in the long term plasticity study the exposure time to 100 µM BZY was reduced to 8 min (Fig. 4).

Data analysis and statistics

Data were analyzed using IBM SPSS 20 statistical software. *In vivo* data were analyzed using a 4-way mixed ANOVA with between-subject factors drug experience (drug naïve vs. drug-experienced) and dose (3 levels) and with repeated measures on the factors lever (active vs. inactive) and session (14 levels). Electrophysiological data were analyzed using ANOVAs with repeated measures on the factor time. Data from PPR experiments were analyzed using paired samples t-test.

RESULTS

BZY self-administration

Figure 1 illustrates the reinforcing effects of BZY on lever pressing as a function of session and drug experience. The acquisition of BZY self-administration was greatly facilitated in drug-experienced rats relative to drug naïve rats. Notice that drug-experienced rats readily learned to discriminate the BZY-paired lever from the 'inactive' lever even though during the cocaine/heroin training phase the rate of lever pressing on the two levers was virtually identical (see Supplementary Information, Fig. S1). A 4-way ANOVA indicated a significant main effect of lever [$F_{1,17}$=30.008; p<0.001], session [$F_{13,221}$=8.646; p=0.001], and drug experience [$F_{1,21}$=7.721; p=0.013]. There were also lever*session [$F_{13,221}$=10.511; p<0.001] and lever*drug experience [$F_{1,21}$=5.629; p=0.03] interactions. Furthermore, a 3-way ANOVA of the number of infusions yielded a main effect of session [$F_{13,221}$=2.672; p=0.014] and of drug experience [$F_{1,17}$=14.564; p=0.001], as well as session*drug experience [$F_{13,221}$=3.183; p=0.004] and session*training dose [$F_{26,221}$=2.119; p=0.016] interactions.

Electrophysiological recordings

Bath applications of BZY at concentrations of 30 µM for 30 min or 100 mM BZY for 10 min decreased PLCx-NAcc fEPSP amplitude by 36.1±6.8% (from 0.77±0.06 mV to 0.51±0.09 mV, n=7) and 64.8±5.7% (from 0.75±0.07 mV to 0.27±0.05 mV, n=7), respectively (Fig. 2). Repeated measures ANOVA run separately for 30 µM and 100 mM experimental groups on the fEPSP amplitude from the time period 15-45 showed a significant effect of time for both the 30 µM [$F_{60,360}$=7.954; p<0.001] and the 100 mM concentration[$F_{60,360}$=18.774; p<0.001].

Furthermore, 2-way repeated measures mixed ANOVA conducted on data from the time period 15-25 min yielded significant main effects of time [$F_{20,240}$=9.763; p<0.001], concentration [$F_{1,12}$=25.583; p<0.001], and a time*concentration interaction [$F_{20,240}$=5.253; p<0.001]. To exclude that this severe depression in synaptic transmission could be due to non-specific toxic effects of BZY, pulses of higher current intensity were delivered at the end of each experiment. Under these experimental conditions the depressed fEPSPs recovered to the baseline amplitude values (data not shown).

We also performed short-term plasticity experiments by measuring PPRs to assess if BZY-mediated effects are due to alterations of neurotransmitter release probability. In control conditions, the second synaptic response (fEPSP2) was smaller than the first (fEPSP1) (Fig. 2d), a phenomenon termed paired pulse depression. Benzydamine perfusion (30 μM) further decreased PPR (from 0.85±0.05 baseline to 0.59±0.03 after 30 min perfusion; n=7; t_6=4.978; p=0.003), suggesting a presynaptic mechanism of drug action (Creager et al., 1980; Wu and Saggau, 1994). Due to the deep depression of the fEPSP induced by 100 μM BZY (Fig. 2f), PPR data collected from these experiments were not included in the analyses.

The fEPSP depression induced by BZY was significantly enhanced in drug-experienced animals compared to naïve rats (Fig. 3). Two-way mixed ANOVA for repeated measures unveiled a significant main effect of experience [$F_{1,21}$=9.237; p=0.006] and experience*time interaction [$F_{59,1239}$=3.573; p=0.01], indicating sensitization of PLCx-NAcc synapses to acute effects of BZY perfusion, in drug-experienced rats. Interestingly, baseline glutamatergic transmission was lower in drug-experienced rats (Fig. 3c), as indicated by main effect of stimulus intensity [$F_{4,84}$=44.224, p<0.001] and experience [$F_{1,21}$=11.570; p=0.009].

As shown in Fig. 4a, bath application of 100 µM BZY for 8 min induced long term depression of fEPSP amplitude with a peak of -60.1±5.0% at min 25-35 (from 0.55±0.04 mV to 0.22±0.03 mV; n=9; t_8=9.984; p<0.001), indicating that BZY can cause long-term changes of PLCx-NAcc synaptic connectivity. Longer time of BZY perfusion often resulted in the complete suppression of the fEPSP (Fig. 2). The fEPSP amplitude did not fully recover during the wash out (from 0.55±0.04 mV baseline to 0.36±0.03 mV at time 70-80 min; t_8=6.347; p<0.001) and was still reduced (-33.4±4.5%) at the end of recordings. This stable LTD-like response was consistently reduced by the co-application of the CB1 receptor antagonist AM251 ($F_{1,19}$=18.635; p<0.001; Fig. 4a). In the presence of AM251 2 µM, BZY-induced fEPSP depression was smaller than in the BZY group both at the depression peak (-35.8±5.7% vs. -60.1±5.0%; n=12; t_{19}=3.071; p=0.006) and at the end of recordings (-13.9±3.5% vs. -33.4±4.5%; t_{19}=3.420; p=0.003). Repeated measures mixed ANOVA on PPR data showed a significant time point*treatment interaction [$F_{2,40}$=5.548; p=0.007]. The reduction of PPR by BZY (from 0.85±0.03 baseline to 0.73±0.04 in the BZY group; t_8=2.664; p=0.012) was fully counteracted by AM251 co-perfusion (from 0.86±0.05 baseline to 0.93±0.07 in the BZY+AM251 group; t_{11}=1.577; p=0.143; Fig 4b). AM251 did not affect PPR *per se* (0.93±0.06, p=0.175 vs baseline PPR, data not shown). Consistent with these results during the perfusion of the CB1 agonist WIN55,212-2 (1 µM) the amount of fEPSP depression by BZY was smaller by 62.8±4.7% than BZY applied alone (n=10, Fig c), thus suggesting that BZY and WIN55,212-2 share a common pathway. Taken together these data indicate that the effects of BZY on glutamatergic neurotransmission are likely mediated by cannabinoid mechanisms.

DISCUSSION

We report here three major novel findings. First, we found that BZY, a non-steroidal anti-inflammatory drug, is self-administered intravenously by rats and that the reinforcing effects of BZY are greatly enhanced by a history of heroin and cocaine self-administration. Second, we found that BZY induces LTD-like plasticity at PLCx-NAcc synapses. Third, we found that this latter effect was significantly reduced by the CB1 receptor antagonist AM251, suggesting a cannabinoidergic mechanism of action.

Benzydamine abuse is well documented in Brazil (Opaleye et al., 2009, 2011) and other countries, including Italy (Malavasi et al., 2012; Settimi et al., 2012), Poland (Anand et al., 2007), and Turkey (Balaban et al., 2013). The finding that BZY is reinforcing in the rat sheds a light on two aspects of BZY abuse in humans. The first aspect concerns its addictive potential. There is little information on the subjective effects of BZY in the scientific literature, except for its hallucinogenic effects (Opaleye et al., 2009). Yet, hallucinogenic substances such as LSD, mescaline, and DOM, have relatively little addictive potential in humans (Nutt et al., 2007) and there is little or no evidence of self-administration of in rodents (Deneau et al., 1969; Yanagita, 1986). However, informal subjective reports posted in drug forums, indicate that BZY has both stimulant and hallucinogenic properties. Substances with mixed hallucinogenic and psychostimulant effects, such as MDMA or ketamine, have greater abuse potential than purely hallucinogenic drugs and are self-administered by monkeys (Lamb and Griffiths, 1987; Fantegrossi et al., 2002) and rats (Ratzenboeck et al., 2001; Schenk et al., 2003; De Luca et al., 2011; De Luca and Badiani,

2012). In summary, the reinforcing effects of BZY described here in the rat are consistent with the stimulant and hallucinogenic effects reported in drug forums.

The second important aspect of BZY abuse is that most cases concern individuals with a history of drug addiction and polydrug use (Anand et al., 2007; Opaleye et al., 2009; Malavasi et al., 2012), suggesting that the reinforcing effects of BZY are facilitated by previous drug exposure. Indeed, in the present study we found that although BZY acted as a positive reinforcer in both naïve rats and in rats with a history of cocaine and heroin self-administration, the latters acquired BZY self-administration at a very low unit dose, which was not effective in naïve rats.

The mechanism underlying the reinforcing effects of BZY is unknown. Actually, to the best of our knowledge, this is the first study reporting on the neurobiological effects of BZY. We have shown that BZY can produce short- and long-term changes in excitatory synaptic transmission in the PLCx-NAcc circuitry. In particular, acute BZY reduced PLCx-NAcc glutamatergic transmission via a presynaptic mechanism. The depressive effect of BZY on glutamatergic transmission was greater, similar to its reinforcing effect, in rats that had previously self-administered cocaine and heroin than in naïve rats. This phenomenon might be related to differences in baseline PLCx-NAcc glutamatergic transmission as indicated by the input-output curves for the two groups (see Fig. 3) or to alterations of cannabinoid signalling between drug-experienced and drug-naïve animals (Rivera et al., 2013). Interestingly, previous studies have shown that cocaine and d-amphetamine can depress excitatory neurotransmission at PLCx-NAcc synapses via dopamine DA1 receptors (Nicola et al, 1996). However, BZY-induced LTD does not appear to depend on dopaminergic

mechanisms since it was not affected by the selective D1 antagonist SCH23390 (data not shown).

In contrast, the BZY-induced LTD of glutamatergic transmission seems to depend at least in part on the presence of CB1 receptors on glutamatergic presynaptic terminals. The CB1R antagonist AM251 reduced in fact BZY-induced LTD as well as BZY-induced changes in PPR. Interestingly amphetamine has been found to induce CB1-dependent LTD in the rat amygdala (Huang et al., 2003). The mechanism by which BZY interacts with CB1 receptors remains unclear. Benzydamine might act as a direct agonist at CB1 receptors. Activation of CB1 receptors by WIN 55,212,2 has been shown in fact to induce persistent and long lasting fEPSP LTD in the NAcc (Robbe et al., 2002). Consistent with this hypothesis, occlusion experiments the CB1 receptor agonist WIN corroborated that the partial inhibition of BZY by AM251 was due to the involvement of CB1 receptors (Fig. 5). Alternatively, BZY might influence endocannabinoid synthesis and release (Löffler et al., 1987), which in turn regulates glutamatergic transmission (Domenici et al., 2006). Of course, given that AM251 dampened but not abolished BZY-induced LTD it is not possible to exclude the contribution of other neurotransmitter systems.

The critical role of CB1R in modulating BZY-induced synaptic plasticity provides a potential mechanism for the abuse potential of BZY. Interestingly, the chemical structure of BZY shares some features (like the presence of a benzoyl indole) with several CB1 synthetic agonists (i.e. the JWH series compounds), which have been shown to possess hallucinogenic proprieties (Forrester, 2012; Harris and Brown, 2013). Future self-administration studies utilizing CB1 antagonists will be necessary to fully elucidate the degree to which CB1 receptors are involved in the reinforcing effects of BZY.

In conclusion, we provide here the first empirical evidence of the reinforcing effects of BZY and of its ability to produce short- and long-term changes in glutamatergic transmission in PLCx-NAcc synapses (a circuitry implicated in drug reward), possibly mediated by a cannabinoidergic mechanism of action. Further research is needed in order to better characterize these neurophysiological effects of BZY. Our findings also suggest that individuals with a history of substance abuse might be more prone to develop BZY abuse not only as a consequence of a general predisposition to experiment with psychoactive substances, but also because of a specific pharmacological cross-sensitization to its reinforcing effects. This could be of particular relevance for the management of detention facilities or rehabilitation clinics, considering that an addict might consume BZY (o similar substances) as a substitute drug without even being noticed by supervisors, thus unpredictably compromising health conditions along with therapy efficacy.

Funding and Disclosure

Author contribution

The study was designed by AB, SM, MM, RA, EM, and ES. RA, MM, and ES conducted the experiments under the supervision of AB and SM. Data analysis was conducted by RA, AB, and SM. RA and AB drafted the manuscript, which was critically reviewed by SM. All authors approved the final version for publication.

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Figure Legends

Figure 1. Lever pressing behavior during 3-h daily sessions of BZY self-administration. FR is progressively increased throughout training. Drug experienced animals readily acquire the self-administration behavior for all dosages, while naïve animals do so only for higher dosages. Values are displayed as means ± SEM.

Figure 2. Effect of BZY perfusion on PLCx-NAcc glutamatergic fEPSP. **a)** Mean normalized fEPSP amplitude during perfusion of BZY 30 µM (n=7 slices/3 animals, 30 min) and 100 µM (n=7 slices/3 animals, 10 min). **b)** Schematic of electrode placement with mediolateral coordinates from bregma. **c-e)** Representative time-course of an experiment for BZY 30 µM and 100 µM perfusion with sample traces from different time points. Traces were obtained by averaging 3-4 sweeps. **d-f)** Paired Pulse Ratio (PPR) values measured right before and right after the 30 minutes perfusion of BZY 30 µM or the 10 minutes perfusion of BZY 100 µM. BZY induced a significant reduction in PPR at PLCx-NAcc synapses. Representative traces are displayed above the histograms. * indicates significantly different ($p<0.05$) from preBZY PPR. Values are displayed as means ± SEM.

Figure 3. fEPSP depression induced by 30 µM BZY is enhanced in drug experienced rats' brain slices. **a)** BZY-induced fEPSP depression in brain slices from naïve (n=13 slices/7 animals) and drug experienced rats (n=10/5). **b)** Representative traces from experiments on brain slices collected from naïve or drug experienced rats. Traces were obtained averaging 3-4 sweeps at baseline and the end of the 30 min BZY perfusion. **c)** Input/output curve in baseline PLCx-NAcc glutamatergic transmission in naïve versus drug experienced animals. **d)** Paired Pulse Ratio (PPR) values measured right before and right after the 30 minutes

perfusion of BZY 30 µM. * indicates significant differences ($p<0.05$) between drug naïve and drug experienced rats. Values are displayed as means ± SEM.

Figure 4. BZY induces stable LTD at the PLCx-NAcc synapses that is partially related to cannabinoid signaling. For the sake of clarity, the effects of AM251 and WIN55,212 are shown separately even thought the controls are the same. **a)** Normalized fEPSP amplitude in response to 100 µM BZY perfusion for 8 min (n=10 slices/6 animals). AM251 (2 µM) co-perfusion significantly reduces the BZY-induced LTD (n=12 slices/8 animals). **b)** Paired Pulse Ratio values measured at different time points (as depicted by numbers) show that BZY-mediated alteration of glutamate release was significantly inhibited by the CB1 antagonist AM251. **c)** WIN55,212 (1 µM) reduces fEPSP amplitude by 14.5±3.2%. In the continuous presence of the CB1 agonist, fEPSP depression by 100 µM BZY is by 14±4.7% (n=10 slices /6 animals). Arrows indicate onset and offset of BZY perfusion. **d)** Co-perfusion of WIN55,212 masks BZY effects on Paired Pulse Ratio. * indicates significantly different ($p<0.05$) from BZY group, same time point (2); # indicates significantly different ($p<0.05$) from BZY baseline (1). Values are displayed as means ± SEM.

Figure 1

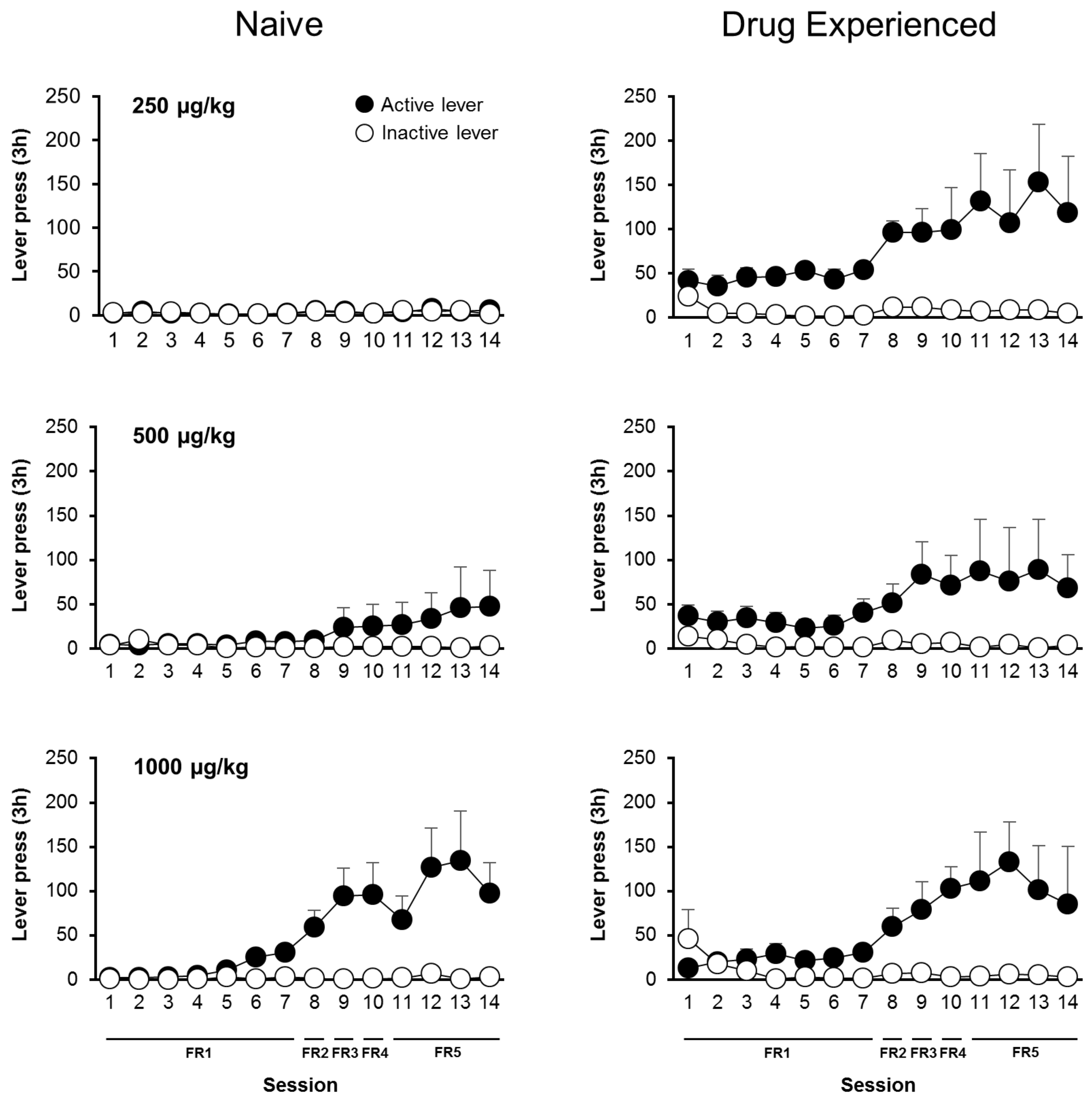

Figure 2

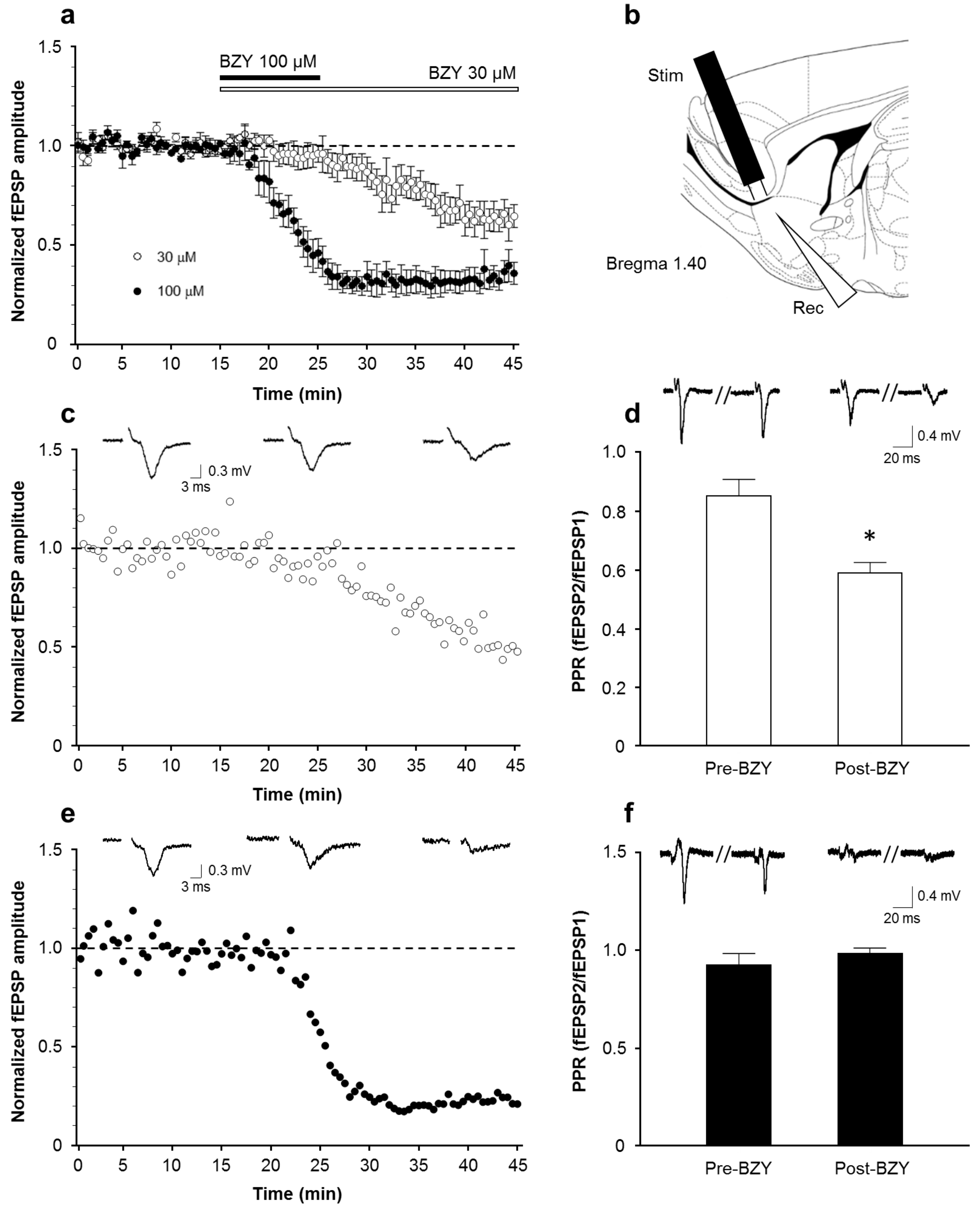

Figure 3

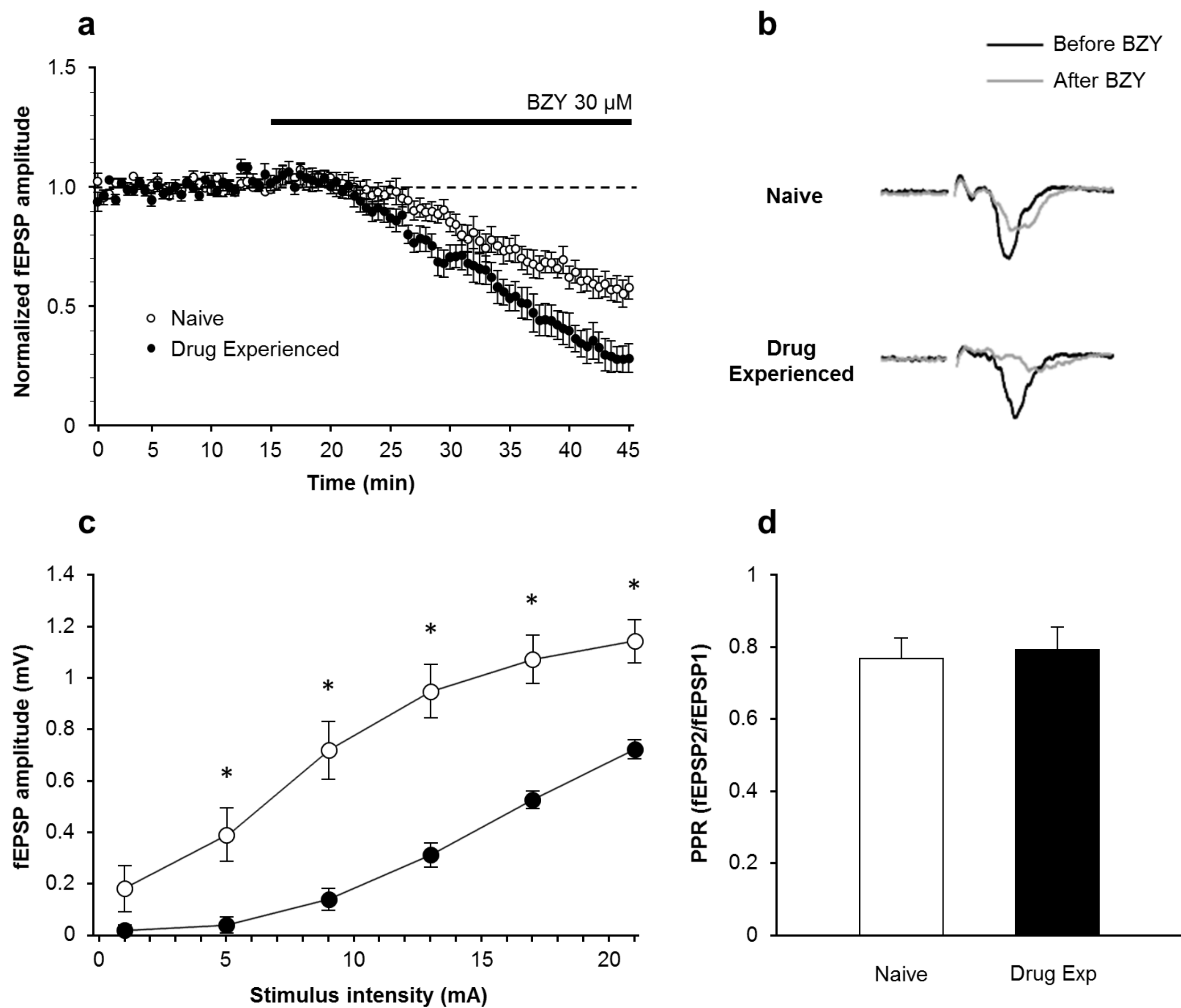

Figure 4