

**The fatty acid amide hydrolase inhibitor URB597 modulates serotonin-dependent emotional behaviour, and serotonin<sub>1A</sub> and serotonin<sub>2A/C</sub> activity in the hippocampus**

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## ABSTRACT

The fatty acid amide hydrolase (FAAH) inhibitor URB597 increases anandamide, resulting in antidepressant/anxiolytic-like activity, likely via CB<sub>1</sub> receptor-mediated modulation of serotonin (5-HT) and norepinephrine (NE) neurotransmission. However, the relative importance of the 5-HT and NE systems in these effects and on effects of URB597 on postsynaptic 5-HT receptors remain to be determined. Using behavioural and electrophysiological approaches, we assessed the effects of single and repeated URB597 treatment on responses predicting antidepressant/anxiolytic activity, and on hippocampal 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptor sensitization. Acute single or serial URB597 treatment, compared to vehicle, reduced immobility in the forced swim test (FST), increased open arm visits in the elevated plus maze and shortened feeding latency in the novelty-suppressed feeding test (NSFT). Repeated URB597 treatment yielded more profound behavioural effects, which were associated with an increase in hippocampal brain-derived neurotrophic factor (BDNF). The 5-HT synthesis inhibitor parachlorophenylalanine (pCPA), but not the NE neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP4) prevented URB597-mediated antidepressant/anxiolytic-like response in the FST and NSFT, while DSP4 did not further affect URB597-mediated increase in raphe 5-HT neuron firing. Repeated URB597 administration decreased hippocampal pyramidal firing in response to 5-HT<sub>2A/C</sub> and 5-HT<sub>1A</sub> stimulation with 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) and 8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT), respectively, suggesting plastic adaptation of these receptors. The effects of single and repeated URB597 administration on hippocampal cell firing in response to DOI or 8-OH-DPAT were similar in magnitude and intensity to the positive control citalopram. These data indicate that URB597 acts, either directly or indirectly, on the 5-HT system, increase hippocampal BDNF expression, and modify 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> function.

**Keywords:** *5-HT<sub>2A/C</sub>, 5-HT<sub>1A</sub>, endocannabinoids, electrophysiology, fatty acid amide hydrolase*

## INTRODUCTION

Endocannabinoids are lipid molecules ubiquitously present in the brain. Of these, the arachidonic acid derivatives *N*-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol are well characterized. Endocannabinoid signalling with the CB<sub>1</sub> receptor subtype (CB<sub>1</sub>R) is involved in a myriad of physiological functions. Human genetic polymorphisms of the *CNR1* gene encoding for CB<sub>1</sub>R influences vulnerability to depressive disorders. In preclinical studies, pharmacological or genetic blockade of endocannabinoid and CB<sub>1</sub>R signalling confers neurobehavioural phenotypes mimicking depression (for review, Gorzalka and Hill, 2011; Hillard and Liu, 2014; Moreira and Lutz, 2008). CB<sub>1</sub>R activation by cannabinoid compounds, such as (–)-trans- $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), affects mood and emotion, as well as memory, nociception, motor function and energy homeostasis (Onaivi et al., 1995; Terranova et al., 1996; for review, Chaperon and Thiébot, 1999; Gorzalka and Hill, 2011; Moreira and Lutz, 2008). CB<sub>1</sub>R agonists elicit thymoleptic-like activity in a dose-dependent manner (Ashton et al., 1981; Bambico et al., 2007; El-Alfy et al., 2010). Likewise, amplifying the intrinsic level of anandamide by pharmacological (Kathuria et al., 2003, Gobbi et al., 2005, Realini et al., 2011; for review, Hillard and Liu, 2014; Gorzalka and Hill, 2011) or genetic (Bambico et al., 2010a, Cassano et al., 2010; for review, Hillard and Liu, 2014; Gorzalka and Hill, 2011) deactivation of its catabolic enzyme called fatty acid amide hydrolase (FAAH) leads to antidepressant/anxiolytic-type behaviour and reverses chronic stress-induced behavioral abnormalities (Bortolato et al., 2007). These effects are associated with a modulation of central monoamine activity, mediated by enhanced CB<sub>1</sub>R signaling, and blocked by CB<sub>1</sub>R antagonists. The exact mechanism by which anandamide-CB<sub>1</sub>R transmission conveys its action remains to be explored. Furthermore, it is not clear whether CB<sub>1</sub>R-mediated therapeutic-like effects may involve serotonin (5-HT) or norepinephrine (NE).

Chronic treatment with different classes of conventional antidepressants produces variable effects on 5-HT and NE, monoamine molecules implicated in depressive-type behavior and antidepressant response. Antidepressants that rapidly enhance synaptic 5-HT, such as selective serotonin reuptake inhibitors (SSRIs), gradually desensitize

auto-inhibitory 5-HT<sub>1A</sub> receptors located on the somatodendritic domain of 5-HT-releasing neurons in the brainstem raphe nuclei. Antidepressants that directly increase norepinephrine (NE) levels, such as the tricyclics and norepinephrine reuptake inhibitors (NRIs), gradually sensitize postsynaptic 5-HT<sub>1A</sub> receptors in some brain regions [for reviews, see Blier (2001) and Bambico et al. (2009)]. Both direct 5-HT-acting and NE-acting antidepressants increase the tonic activity of hippocampal 5-HT<sub>1A</sub> receptors (Haddjeri et al., 1998, Besson et al., 2000) and downregulate postsynaptic 5-HT<sub>2A/C</sub> receptors (Stahl, 1994). These adaptations typically ensue after at least two weeks, coinciding with the onset of therapeutic action (Blier, 2001; Bambico et al., 2009).

The hippocampus receives dense afferents from 5-HT neurons in the dorsal raphe (DR) and NE neurons in the locus coeruleus (LC) nucleus, and is an important locus where functional receptor modifications are associated with the antidepressant response. The activity of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and its neurogenic effects in the hippocampus are highly sensitive to stress and is upregulated by chronic antidepressant treatment (Nibuya et al., 1995). Antidepressant-linked enhancement of BDNF production and neurogenesis has been reported to be modulated by 5-HT receptors such as the 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> subtypes (Klempin et al., 2010). Several lines of evidence also suggest a role for the hippocampal endocannabinoid system in depressive disorders (for review, Hillard and Liu, 2014; Gorzalka and Hill, 2011). Hippocampal CB<sub>1</sub>R density, endocannabinoid content and FAAH activity are altered by chronic stress (Hill et al., 2008) and corticosterone exposure (Bowles et al., 2012), similarly observed in genetic animal models (Vinod et al., 2012).

Because the relative contributions of the 5-HT and NE systems to URB597-mediated antidepressant/anxiolytic-like response were not clear from our previous studies, we first sought to determine whether URB597 would elicit both antidepressant-like activity in the forced swim test (FST) and anxiolytic-like activity in the novelty-suppressed feeding test (NSFT), as well as in the elevated plus maze test (EPMT) and social interaction test (SIT). We then tested if these were dependent on 5-HT or NE transmission by scrutinizing the impact of 5-HT synthesis inhibition and NE-selective lesion on coping behaviour in the FST and NSFT. We further examined whether these

behavioural were associated with increased BDNF levels and modifications in the function of postsynaptic 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptors (via the differential response of pyramidal neurons to local, iontophoretic application of 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> agonists and/or antagonists) in the hippocampus.

## EXPERIMENTAL PROCEDURES

**Animals.** Adult male Sprague-Dawley rats (Charles-River Saint-Constant, PQ, Canada), weighing 300-320 grams, were pair-housed under standard conditions as previously described (Bambico et al., 2007; Bambico et al., 2010b; Bambico et al., 2012). Experiments were conducted between 10:00 and 22:00. All procedures were in accordance to the ethical guidelines by the local institutional animal care and use committee and the Canadian Institutes of Health Research.

**Drugs.** The carbamate FAAH inhibitor cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester (URB597), the CB<sub>1</sub>R/CB<sub>2</sub>R agonists (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN55,212-2) and the CB<sub>1</sub>R antagonist rimonabant were dissolved in a vehicle consisting of 5% Tween 80, 5% polyethylene glycol, and 90% saline (0.9% NaCl solution) to a final concentration of 0.1, 0.2 and 1.0 mg ml<sup>-1</sup>, respectively. The tricyclic antidepressant desipramine and the SSRI citalopram and paroxetine were dissolved in 0.9% saline to a final concentration of 10 mg ml<sup>-1</sup>. The NE neuron-selective toxin *N*-(2-chloroethyl)-*N*-2-bromobenzylamine (DSP4) and the 5-HT synthesis inhibitor *para*-chlorophenylalanine (*p*CPA) were dissolved in 0.9% saline to a final concentration of 50 and 350 mg ml<sup>-1</sup>, respectively. For iontophoresis, the following drugs were used: the 5-HT<sub>1A</sub> receptor agonist 8-hydroxy-2-dipropylaminotetralin HBr (8-OH-DPAT, 50 mM in 200 mM NaCl, pH 4.0 – 4.5), the 5-HT<sub>2A/C</sub> receptor agonist 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI, 10 mM in 200 mM NaCl, pH 4.0–4.5), and quisqualic acid (1.5 mM in 400 mM NaCl, pH 8.0). Ethylurethane (1.8 g kg<sup>-1</sup>) was used as anesthesia for electrophysiology (Labonte et al., 2009). Drugs were purchased from

Sigma-Aldrich Canada Ltd. except for URB597, which was synthesized at the University of Urbino following a procedure previously described (Mor et al., 2004).

**Drug Treatment.** Distinct cohorts of animals were used for each behavioral and electrophysiological test, and for each treatment schedule, unless stated otherwise. Drugs were administered intraperitoneally (ip) in all behavioural tests, and intravenously (iv) or iontophoretically in electrophysiological experiments.

*Single or Serial Treatment.* First, we tested the effect of a single URB597 administration in the social interaction test (SIT), elevated plus maze test (EPMT) and novelty-suppressed feeding test (NSFT) (n=6-8/group). URB597 was injected 30 min prior to tests at a dose of 0.1 mg·kg<sup>-1</sup> (0.3 mg·kg<sup>-1</sup> was also tested in EPMT). In the forced swim test (FST) (5=7/group), drugs were administered serially (23, 5, and 0.75 h prior to test, as previously described, Bambico et al., 2007) at a dose of 0.03-0.3 mg·kg<sup>-1</sup> for URB597, 0.2 mg·kg<sup>-1</sup> for WIN55,212-2 and 10 mg·kg<sup>-1</sup> for desipramine and citalopram.

*Repeated Treatment.* Second, we tested the effect of repeated URB597 (0.1 mg·kg<sup>-1</sup>) treatment with or without rimonabant (1 mg·kg<sup>-1</sup>) in the FST and NSFT, as well as on brain BDNF content (n=6-8/group). ~~For repeated treatment, in the FST and NSFT,~~ URB597 was administered once daily for 7 days. Animals used for an open field test (OFT) were taken from the previous EPMT cohort, as the EPMT has a low stress load. Animals were injected serially before the FST or OFT on the 7<sup>th</sup> day or once, 30 min before the NSFT on the 7<sup>th</sup> day. Rimonabant (or vehicle) was administered 15 min prior to each URB597 (or vehicle) injection.

*5-HT and NE Lesions.* Third, we examined the effects of pCPA (350 mg·kg<sup>-1</sup>, 72 h and 48 h prior to test) or DSP4 (50 mg·kg<sup>-1</sup>, 7 days prior to test) on serially administered URB597 (0.1 mg·kg<sup>-1</sup>) and desipramine (10 mg·kg<sup>-1</sup>) in the FST and after single administration of URB597 (0.1 mg·kg<sup>-1</sup>) in the NSFT (6-9/group). All animals received the same number of injections. Across injections days, animals received either the drugs or the vehicle. For example, naive controls received the vehicle on the 1<sup>st</sup>, 4<sup>th</sup> and 5<sup>th</sup> days, followed by testing on the 7<sup>th</sup> day. Our lab has previously employed these protocols. We used DSP4, which preferentially lesions neurons of the dorsal

noradrenergic bundle (LC nucleus) because URB597 and CB<sub>1</sub>R/CB<sub>2</sub>R agonists, like NE-acting antidepressants, have previously been shown to activate these neurons (Muntoni et al., 2006). Although DSP4 may yield residual NE content in the LC nucleus in mice (Cassano et al., 2009); in rats, a significant >50% decrease was detected in frontal cortex and hippocampus (Hamani et al., 2010). We depleted endogenous 5-HT using pCPA (Chaput et al., 1990), which markedly decreased 5-HT content by >30% in the frontal cortex and by >50% in the hippocampus, and blocked antidepressant-like behaviour (Chaput et al., 1990; Bambico et al., 2007; Hamani et al., 2010).

*Electrophysiology and Microiontophoresis.* A total of 60 animals were used for electrophysiology. For basal recordings, the first group was administered with DSP4 (50 mg·kg<sup>-1</sup>, 7 days prior to test), followed by recordings after acute URB597 (0.1 mg·kg<sup>-1</sup>) or WIN55,212-2 (0.05-0.2 mg·kg<sup>-1</sup>) treatment. In a second group, URB597 (0.1 mg·kg<sup>-1</sup>) was administered once daily for 7 days with or without rimonabant (1 mg·kg<sup>-1</sup>) or DSP4 pretreatment (50 mg·kg<sup>-1</sup>, 7 days prior to test). Recordings were done 30 min after the last injection. In a third group, URB597 (0.1 mg·kg<sup>-1</sup>) was administered with or without rimonabant (0.1 mg·kg<sup>-1</sup>) once daily for 21 days prior to recording 30 min after the last injection. For electrophysiology with microiontophoresis, DOI or 8-OH-DPAT was iontophoresed 30 min after a single injection or 30 min after the last of 7 once-daily injections of URB597 (0.1 mg·kg<sup>-1</sup>), citalopram (10 mg·kg<sup>-1</sup>) or vehicle.

### **Behavioral assay.**

*OFT.* Ambulatory activity (total distance traveled in cm) in an OFT arena (80 × 80 × 15 cm) was recorded for 5 min under low lighting (20 lux) (Bambico et al., 2007).

*FST.* Passive (immobility) coping behaviour was examined in the FST, as previously described (Bambico et al., 2007). Twenty-four hours after a 15-min pre-exposure to an inescapable water-filled bin, rats were re-exposed for 5 min after having received drug injections prior to testing. The behavioural endpoints encoded in the FST and in subsequent behavioural tests were analyzed by an automated tracking system (Videotrack, Viewpoint Life Science, Inc., Montreal, Canada). Custom-made plates arrayed with infrared light-emitting diodes were suspended above the bins. Infrared

light-sensitive CCD cameras were used to capture and store images. The FST was conducted toward the end of the light phase/beginning of the dark phase, in a dark room with red lighting.

*EPMT.* In a cross-shaped, elevated (80 cm) maze, exploration time in the two open (50×10 cm) and two walled (closed) (50×10×40 cm) arms was recorded for 5 min, under white light (350 lux) (Bambico et al., 2010b).

*NSFT.* Anxiety-induced inhibition (latency) in the consumption of 12 chow pellets spread across the central area of an unfamiliar arena (80 × 80 × 30 cm) after 48-h food-deprivation was tested as previously described (Bambico et al., 2010b). Before the test, feeding latency was observed in the familiar home cage. Cut-off time was 600 s.

*SIT.* As described by Cassano et al. (2011), each test animal was placed together with an unfamiliar conspecific partner in an arena (80 × 80 × 30 cm). Social behaviour was recorded for 5 min and assessed offline by a rater blind to the grouping. Endpoints included the total time of social contact (investigating, sniffing, following, mounting and crawling under or over the partner) and total time spent in aggressive/antagonistic behaviour. An increase in social interaction is indicative of an anxiolytic effect, whereas a specific decrease in social interaction indicates an anxiogenic effect.

**BDNF measurements.** BDNF protein in the hippocampus (HPC), prefrontal cortex (PFC) and cerebellum (CBL) was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison WI), according to manufacturer's instructions. Brains were dissected from frozen tissue and lysed in 1:100 volumes of modified lysis buffer (100 mM PIPES, pH 7, 500 mM NaCl, 0.2% Triton X-100, 0.1% NaN<sub>3</sub>, 2% BSA, 2 mM EDTA·Na<sub>2</sub>·2H<sub>2</sub>O, 200 mM PMSF frozen in isopropanol, 10 mM leupeptin frozen separately in deionized water, 0.3 M aprotinin frozen separately in 0.01 M HEPES pH 8 and 1 mM pepstatin frozen separately in DMSO (Szapacs et al., 2004). Plates were read with an automated reader using the SPF software.

**Electrophysiology.** *In vivo* single-unit extracellular recordings and microiontophoresis



(Npi electronic GmbH microiontophoresis system, Tamm, Germany) on 5-HT neurons of the DR (1.0 mm anterior to the interaural line) and on pyramidal neurons of the dorsal hippocampus CA<sub>3</sub> region (4.2 mm lateral and 4.2 mm anterior to lambda) (Paxinos and Watson, 2005) were carried out after intraperitoneal single (30-45 min prior) or repeated (once daily for 7 days, recordings 45-60 min after the 7<sup>th</sup> injection) drug administrations. The procedures were as previously described (Bambico et al., 2007; Bambico et al., 2010; Bambico et al., 2012). We used single-barreled or multi-barreled glass micropipettes (Harvard Applied Scientific Instrumentation, OR, USA). The side barrels had impedances ranging from 50 to 150 MΩ, and contained NaCl solution (2 M) for automatic current balancing, and the following drug solutions: (1) DOI was used to assess the sensitivity of 5-HT<sub>2A/2C</sub> receptors and was ejected as a cation (+10 to +100 nA, 1–2 min-currents) and retained with a current of –11 nA. (2) 8-OH-DPAT was used to assess the sensitivity of 5-HT<sub>1A</sub> receptors and was ejected as a cation (+1 to +10 nA, 1–2 min currents) and retained with a current of –11 nA. 5-HT neurons were identified according to standard criteria, as previously described (Bambico et al., 2007). These include a slow (0.1–4 Hz) and prominently regular firing rate and a broad positive action potential (0.8–3.5 ms; 1.4 ms first positive and negative deflections). Pyramidal neurons were identified by their steady response to standard short pulses of quisqualate and by large amplitudes (0.5-1.2 mV), long durations (0.8-1.2 ms), and simple spike patterns alternating with complex spike discharges (Bambico et al., 2010a). Low current ejection of quisqualate was used to activate very low spontaneous firing rate to at least 2 Hz (Bambico et al., 2010a). Recording sites were marked by iontophoretic ejection (1–10 mA, negative current for 10 min) of Pontamine Sky Blue for later histological verification. Groups were counterbalanced across recording times (Domingues-Lopez et al., 2012; Bambico et al., 2010a).

**Data analysis.** Data are presented as mean ± standard error of the mean (SEM). Behavioural data were analyzed using general linear model, mixed design analysis of variance (ANOVA) or with one-way ANOVA. ELISA data on BDNF were normalized to the mean control level [100%] before effect of URB597 was compared to vehicle

treatment (control). Electrophysiological data on firing rate were presented either as integrated firing rate histograms (bar = rate/10 sec) or mean population firing rate in Hz  $\pm$  SEM. To obtain mean firing, neurons were continuously sampled across 4-5 electrode descents within 1.5 hours of recording. For recordings involving microiontophoresis, neural response to each drug current ejection was expressed as percentage increase/decrease from pre-drug (baseline/zero current) activity, before they were plotted as data points (mean % excitation or inhibition  $\pm$  SEM) on line graphs. These data were submitted to a two-way mixed design ANOVA (drug  $\times$  current). In all data sets, the Student-Newman-Keuls (SNK) test was used for multiple post hoc comparisons. Probability value of  $p \leq 0.05$  was considered to be statistically significant.

## RESULTS

### **Behavioural and neurobiological effects of single and repeated treatment with URB597**

One-way ANOVA ( $F_{6,40}=3.942$ ,  $p=0.003$ ), followed by post-hoc comparisons (SNK test), revealed that when compared to vehicle (control, CTR), an attenuation in immobility duration ensued after serial treatment with URB597 at a dose of 0.1 ( $p=0.028$ ) and 0.3  $\text{mg}\cdot\text{kg}^{-1}$  ( $p=0.016$ ), WIN55,212-2 at 0.1  $\text{mg}\cdot\text{kg}^{-1}$  ( $p=0.019$ ), desipramine at 10  $\text{mg}\cdot\text{kg}^{-1}$  ( $p=0.002$ ) and citalopram at 10  $\text{mg}\cdot\text{kg}^{-1}$  ( $p=0.017$ ) ( $n=7$  in each of these groups; Fig. 1a). This effect was dose-dependent for URB597 since the lowest dose (0.03  $\text{mg}\cdot\text{kg}^{-1}$ ;  $n=5$ ) failed to achieve a significant difference (Fig. 1a).

Unpaired t-test for social investigation and aggressive behaviour showed no difference between CTRs and URB597-treated animals (0.1  $\text{mg}\cdot\text{kg}^{-1}$ ,  $n=8$ )/group; Fig. 1b). In the EPMT, one-way ANOVA on the duration of area visits showed a significant effect in the closed arm ( $F_{2,19}=3.455$ ,  $p=0.05$ ) and in the open arm ( $F_{2,19}=3.55$ ,  $p=0.049$ ; Fig. 1c). Post-hoc comparisons determined that compared to CTR ( $n=8$ ), URB597 at 0.3  $\text{mg}\cdot\text{kg}^{-1}$  ( $n=7$ ) yielded significantly longer open arm visits ( $p<0.05$ ) and shorter closed arm visits ( $p<0.05$ ). This decrease in closed arm visits did not achieve significance ( $p=0.066$ ) with 0.1  $\text{mg}\cdot\text{kg}^{-1}$  ( $n=7$ ) of URB597. In the NSFT, unpaired t-tests revealed that URB597 compared to CTR markedly shortened feeding latency in the novel environment

( $t_{11}=3.031$ ,  $p=0.011$ ) but not in the home cage (Fig. 1d). This suggests that URB597 specifically acted on reversing anxiety-like feeding suppression induced by novelty, and did not seem to affect normal feeding in a familiar environment. Thus, URB597 produces significant effects in the EPMT and NSFT, but not in the SIT. We initially validated the aforementioned tests in chronic mild stress studies and anxiolytic (benzodiazepine) treatment that decreased and increased, respectively, the onset of approach and feeding in the NSFT, open arm duration in the EPMT and social investigation in the SIT.

Because single or serial administration is not predictive of onset or time course of behavioural and neurobiological changes, we then employed a repeated schedule. Seven once-daily treatment with URB597 ( $0.1 \text{ mg}\cdot\text{kg}^{-1}$ ) similarly decreased immobility in the FST (One-way ANOVA,  $F_{2,21}=6.732$ ,  $p=0.006$ ; SNK test: vs. CTR,  $p<0.01$ ;  $n=8/\text{group}$ ; Fig. 2a). In the NSFT, unpaired t-tests showed that URB597 compared to CTR shortened feeding latency in the novel environment ( $t_{14}=2.708$ ,  $p=0.017$ ) but not in the home cage environment ( $n=8/\text{group}$ ; Fig. 2b). These observed behaviours were prevented by daily co-administration with the CB<sub>1</sub>R antagonist rimonabant ( $1.0 \text{ mg}\cdot\text{kg}^{-1}$  intraperitoneal; URB 0.1 vs. URB 0.1+Rim 1.0: FST  $p<0.01$ , Fig. 2a; NSFT  $p<0.05$ ; Fig. 2b), and were accompanied by changes in BDNF protein levels, suggesting that long-term neuroplastic changes may contribute to the observed behavioural effects. Unpaired t-tests showed that URB597, compared to CTR, led to greater BDNF level (normalized,  $\geq$  CTR levels = 100%) in hippocampal ( $t_{12}=-2.61$ ,  $p=0.02$ ;  $n=7/\text{group}$ ) and prefrontocortical ( $t_{10}=-2.327$ ,  $p=0.04$ ;  $n=6/\text{group}$ ) but not in cerebellar homogenates ( $n=7/\text{group}$ ; Fig. 2c).

### **Effect of *p*CPA and DSP4 on the behavioural action of URB597 in the FST and NSFT**

To determine the differential contribution of 5-HT and NE in the behavioural activity of URB597 ( $0.1 \text{ mg}\cdot\text{kg}^{-1}$ ) in the FST and NSFT, we compared pretreatment with saline to that of the 5-HT synthesis inhibitor *p*CPA and the NE-selective toxin DSP4. One-way ANOVA revealed a significant effect of treatment in the FST ( $F_{5,39}=3.573$ ,  $p=0.009$ ; Fig. 3a) and NSFT ( $F_{5,42}=3.912$ ,  $p=0.005$ ; Fig. 3b). Post-hoc comparisons showed that URB597 decreased immobility ( $p<0.05$ ) and feeding latency ( $p<0.05$ ). *p*CPA had no

effect on these behaviours alone but prevented URB597-mediated effects partially in the FST and completely in the NSFT. DSP4 did not significantly affect immobility nor feeding latency, and did not reverse URB597-mediated effects (n=6-9/group; Fig. 3a-3b).

In the FST, DSP4 also prevented the immobility-attenuating effect of serial treatment with desipramine (one-way ANOVA,  $F_{2,18} = 3.692$ ,  $p = 0.045$ ; vehicle+vehicle [CTR] vs. vehicle+desipramine(DMI),  $p < 0.05$ ; vehicle+vehicle vs. DSP4+DMI, not significant[n.s.]; vehicle+DMI vs. DSP4+DMI,  $p < 0.05$ ; n=7/group; Fig. 3a, right panel). In the OFT, there were no significant differences in locomotor activity (distance travelled, cm) among the treatment groups [CTR (n=7) =  $2886.30 \pm 555.21$ , URB597 0.1 mg·kg<sup>-1</sup> (n=6) =  $2678.80 \pm 633.26$ ; URB597 0.3 mg·kg<sup>-1</sup> (n=6) =  $2568.60 \pm 301.95$ ) indicating that the behavioural changes observed in the FST were not confounded by nonspecific locomotor changes.

#### **Serotonergic action of URB597 and the effect of DSP4**

To further verify the non-involvement of NE in the observed behavioural property of repeated URB597 treatment, we examined 5-HT neuron activity in the DR in DSP4-treated animals. Consistent with our previous findings (Gobbi et al., 2005; Bambico et al., 2007), single administration of URB597 (0.1 mg·kg<sup>-1</sup>, intravenous) gradually increased 5-HT neuron firing rate and WIN55,212-2 (cumulative 0.05-0.2 mg·kg<sup>-1</sup>, intravenous) rapidly increased it (n=3 neurons/drug treatment; Fig. 4a). This time course difference is likely due to slow anandamide accumulation and subsequent CB<sub>1</sub>R activation following FAAH inactivation, as previously described (Gobbi et al., 2005; Bambico et al., 2009).

Repeated treatment with URB597 (0.1 mg·kg<sup>-1</sup>), compared to that of vehicle, enhanced the spontaneous firing rate of DR 5-HT neurons (One-way ANOVA,  $F_{3,56} = 6.732$ ,  $p < 0.01$ ; CTR, n=16 vs. URB, n=17,  $p = 0.019$ ; Fig. 4b). This effect of URB597 was reversed by the CB<sub>1</sub>R antagonist rimonabant (1 mg·kg<sup>-1</sup>) (URB+Rim, n=13 vs. CTR, n=16, n.s.; URB+Rim, n=13 vs. URB, n=17,  $p = 0.01$ ) but not by DSP4 (URB vs. DSP4+URB, n=7, n.s.) (Fig. 4b). Chronic URB597 (0.1 mg·kg<sup>-1</sup>, once daily for 21 days) maintained an enhanced 5-HT neuron firing, which was blocked by daily co-treatment

with rimonabant (two-way ANOVA, URB x rimonabant interaction,  $p < 0.01$ ; vehicle+vehicle[CTR],  $n=23$  vs. URB+vehicle,  $n=34$ ,  $p < 0.05$ ; CTR vs. vehicle+Rim,  $n=27$ , n.s.; CTR vs. URB+Rim,  $n=15$ , n.s.; URB+vehicle vs. URB+Rim,  $p < 0.01$ ; Fig. 4c)

### **Effect of repeated treatment with URB597 on hippocampal 5-HT<sub>2A/C</sub> receptors sensitivity**

We next measured neurophysiological changes associated with repeated treatment. Hippocampal plasticity, such as changes in BDNF expression, has been linked to augmentation of 5-HT input in the hippocampus by 5-HT-acting antidepressants. We assessed the status of 5-HT activity in the hippocampus by measuring modifications in 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptor subtype sensitivity. Microiontophoretic delivery of DOI in the CA<sub>3</sub> subfield of the dorsal hippocampus elicited an 85% inhibitory responses and 15% excitatory response from all recorded pyramidal neurons. The predominant inhibitory responses were consistent with reports that activation of 5-HT<sub>2A/C</sub> receptors decreases the somatodendritic excitability of pyramidal neurons (Carr et al., 2002; Gobbi and Janiri, 1999). It is also conceivable that inhibitory responses may have been produced by activation of 5-HT<sub>2A/C</sub> receptors on interneuron terminals synapsing with pyramidal cell bodies, while excitatory responses are mediated by 5-HT<sub>2A/C</sub> receptors activation located on pyramidal cell bodies (Jakab and Goldman-Rakic, 1998). The degree of excitations or inhibitions was apparently proportional to the DOI current, with the effect of the lowest current being markedly different from that of the highest current. Pilot experiments showed that both inhibitions and excitations were prevented by intravenous pre-treatment with the 5-HT<sub>2A/C</sub> antagonist ritanserin (also, see Labonte et al., 2009). Two-way mixed-design ANOVA on inhibitory responses, with DOI current as within-groups factor and drug treatment as between-groups factor, showed a significant main effect of single treatment ( $F_{2,94}=148.613$ ,  $p < 0.01$ ). Compared to vehicle, both URB597 and citalopram decreased DOI-induced inhibitions ( $p < 0.01$ ;  $N=3-7$ /data point; Fig. 5a-b). After repeated administrations of either URB597 or citalopram, inhibitory responses were somewhat milder but significantly lower than vehicle treatment (main treatment effect,  $F_{2,98}=8.770$ ,  $p < 0.01$ ; vehicle vs. URB or citalopram,  $p < 0.01$ ; Fig. 5c). Two-way mixed-design ANOVA on the DOI-induced excitatory responses revealed no effect of URB597, neither by single (Fig. 6a) nor repeated

treatment (Fig. 6b). Citalopram also did not reveal any significant effects (Fig. 6a-6b) (n=3-6/data point). These data indicate that 5-HT<sub>2A/C</sub> receptors were significantly desensitized by URB597 treatment, a possible consequence of increased 5-HT input.

### **Effect of repeated treatments with URB597 and citalopram on hippocampal 5-HT<sub>1A</sub> receptor function**

Microiontophoretic delivery of increasing 8-OH-DPAT currents in the CA<sub>3</sub> subfield of the dorsal hippocampus elicited increases in pyramidal responses that were predominantly inhibitory (Fig. 7a and 7b). There was only null to slight current-dependent response to 8-OH-DPAT with the range of current used. However, pilot experiments showed that these inhibitions are reversed by intravenous administration of the 5-HT<sub>1A</sub> antagonist WAY100,635, suggesting that these responses were likely mediated by 5-HT<sub>1A</sub> receptors activation. Two-way mixed-design ANOVA with drug treatment as between-group factor and 8-OH-DPAT current as within-group factor revealed a significant main effect of repeated ( $F_{3,131}=14.708$ ,  $p<0.01$ ; Fig. 7b) but not of single treatment (Fig. 7a). Both URB597 and citalopram significantly attenuated these inhibitory responses when compared to their respective controls ( $p<0.01$ ; n=5-10/data point; Fig. 7b). These observations suggest that hippocampal 5-HT<sub>1A</sub> receptors desensitize following repeated URB597 treatment.

## **DISCUSSION**

We present two key findings in this study. First, we have determined a key role for the 5-HT system, but not the NE system, in URB597-mediated effects predicting antidepressant/anxiolytic activity, as demonstrated by lesion studies. Second, in the hippocampus, considered an important locus of downstream neurobiological effects linked to antidepressant/anxiolytic action, repeated URB597 administration modified the sensitivity of 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptors to their respective agonists, which likely resulted from prolonged enhancement of 5-HT input. These changes in behaviour were associated with increased levels of BDNF.

In the FST, serial treatment with URB597 led to a significant reduction in immobility without affecting locomotor activity. This paradigm does not recapitulate the clinical time

course of antidepressants. Nevertheless, these observed effects are highly predictive of the efficacy of monoamine-acting antidepressants (Bambico et al., 2009). We have previously established that such is mediated by indirect activation of CB<sub>1</sub>R signaling conferred by increasing cerebral anandamide. It has been demonstrated that URB597 can also increase other non- CB<sub>1</sub>R active fatty acid ethanolamides (Kathuria et al., 2002), and further investigations need to address their role in emotional behaviour. However, here we show that behavioural and neurobiological effects are abrogated by the co-administration of CB<sub>1</sub>R antagonists, consistent with our previous reports (Gobbi et al., 2005), and similar to that observed with direct CB<sub>1</sub>R agonists (Bambico et al., 2007) and endocannabinoid reuptake blockers (Bortolato et al., 2007). Therefore, these are likely mediated mainly by anandamide-CB<sub>1</sub>R signaling. The precise neurobiological mechanisms are far from being completely elucidated although there are several lines of evidence that point to the role of the central 5-HT system. First, the behavioral profile of direct and indirect CB<sub>1</sub>R agonists in different tests for antidepressant-related activity consistently assumes those displayed by 5-HT-modulating antidepressants such as SSRIs (for review, Bambico et al., 2009; Hillard and Liu, 2014; Gorzalka:2011gz). In the FST, this was marked by a selective increase in swimming without affecting climbing behavior (Gobbi et al., 2005, Bambico et al., 2007). Second, CB<sub>1</sub>R agonists and FAAH inhibitors have been shown to increase 5-HT neural activity (Gobbi et al., 2005; Bambico et al., 2007; Page et al., 2008, Mendiguren and Pineda, 2009, Bambico et al., 2010a) and 5-HT efflux in limbic areas, including the medial prefrontal cortex and the hippocampus (Gobbi et al., 2005; for review, Hillard and Liu, 2014; Gorzalka:2011gz). Third, the antidepressant-like activity of many CB<sub>1</sub>R agonists including  $\Delta^9$ -THC (Haring et al., 2013) and WIN55,212-2 (Bambico et al., 2007) is abolished by pre-treatment with the 5-HT synthesis inhibitor pCPA. On the other hand, some evidence also suggests the involvement of the NE system. For instance, the decrease in FST immobility observed after prolonged treatment with the CB<sub>1</sub>R agonist HU-210 was completely prevented by the administration of adrenergic receptor antagonists (Morrish et al., 2009). Furthermore, along its effects on 5-HT, CB<sub>1</sub>R agonists modulate the NE neural activity (Mendiguren and Pineda, 2006, Muntoni et al., 2006) and synaptic release (Page et al., 2008). Also, prolonged treatment of URB597 unlike direct 5-HT-acting antidepressants,

does not desensitize 5-HT<sub>1A</sub> autoreceptors in the DR (Gobbi et al., 2005). In adolescent rats, it increased CB<sub>1</sub>R binding in the LC (Marco et al., 2009), implicating the involvement of the dorsal adrenergic bundle in some of URB597's behavioural effects. Remarkably, we show here that URB597-induced reductions in immobility in the FST and in feeding latency in the NSFT are nullified by pCPA but not by DSP4. These are convergent with our data demonstrating that DSP4 pre-treatment failed to completely prevent the increase in spontaneous 5-HT neuron firing induced by single and/or repeated systemic administrations of URB597 or WIN55,212-2. On the other hand, enhancement in 5-HT firing by bupropion (Dong and Blier, 2001) and NK1 antagonists (Gobbi et al., 2007) is abolished by DSP4. These together confirm that the therapeutic-like activity of both URB597 and CB<sub>1</sub>R agonists, unlike those of bupropion and NK1 antagonists, are mainly dependent upon the integrity of the 5-HT system and less of the NE system. Behavioural data on anxious reactivity showed that single and repeated URB597 treatment, compared to vehicle treatment, was anxiolytic in the NSFT indicated by a shorter feeding latency in a novel environment. No effect was seen in the home cage suggesting absence of hyperphagic activity typically associated with cannabinoids (for review, Bambico et al., 2009; Hill and Liu, 2014; Gorzalka and Hill, 2011). A similar anxiolytic effect was seen in the EPMT where single URB597 treatment increased open arm visits and decreased closed arm visits, evident at a dose of 0.3 mg·kg<sup>-1</sup> but not at 0.1 mg·kg<sup>-1</sup>. These data are consistent with those reported by others (Patel and Hillard, 2006, Moreira et al., 2008). By contrast, in the SIT, there was no difference in social investigation time and aggressive episodes between URB597-treated and vehicle-treated animals. The anxiolytic-like response to cannabinoids and URB597 has not always been replicated across different social behaviour and conflict paradigms (EPMT and NSFT), owing to the variable strain, age and handling procedures employed by different investigators, as well as to the sensitivity of the paradigm to environmental conditions (Naidu et al., 2007, Haller et al., 2009). Because endocannabinoid synthesis and release occur *on demand*, URB597 can only increase anandamide levels and CB<sub>1</sub>R signaling when neurons are stimulated by specific conditions (Bambico et al., 2009, Haring et al., 2013), hence, reports of profound anxiolytic activity under aversive test conditions and equivocal effects under milder conditions (Haller et al., 2009). It is quite



conceivable that the observed lack of behavioural effect of URB597 in the SIT is due to the milder stress load of the SIT paradigm, compared to the EPMT and NSFT paradigms.

Electrophysiological recordings revealed that URB597 and citalopram directly modified the functional states of hippocampal 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptors, which are likely mediated by an increase in 5-HT outflow, as treatments also increased 5-HT neural activity. This lends further credence to the 5-HT-dependence of URB597's behavioural effects. In particular, we found that postsynaptic 5-HT<sub>2A/C</sub> receptor response is downregulated by both repeated URB597 and citalopram treatment. This is not surprising since we also found that cortical postsynaptic 5-HT<sub>2A/C</sub> receptors of FAAH null-mutant mice are desensitized, a possible correlate of their anxiety/depression-resistant phenotype (Bambico et al., 2010a). It is known that prolonged antidepressant treatment could downregulate postsynaptic 5-HT<sub>2A/C</sub> receptors (Quesseveur et al., 1997, Quesseveur et al., 2012). This effect is associated with the anxiolytic efficacy. Indeed, 5-HT<sub>2A/C</sub> antagonists yield potent anxiolytic activity (Kennett et al., 1994, Adamec et al., 2004) or augment the therapeutic activity of antidepressants (Quesseveur et al., 2012). In contrast, upregulation of 5-HT<sub>2A/C</sub> function is anxiogenic in humans (Germine et al., 1994) and animal models, including chronic corticosterone exposure (Zahorodna and Hess, 2006). Interestingly, we found that a single administration of URB597 already led to a decrease in 5-HT<sub>2A/C</sub> response that was much more profound than after citalopram treatment. This appears to agree with the anxiolytic response to a single URB597 administration in the NSFT and EPMT. In this event, URB597's acute effect may have been instigated by an enhanced anandamide-CB<sub>1</sub>R tonus on GABAergic terminals synapsing with pyramidal neurons. This hyperpolarizes the GABAergic terminal thereby increasing the threshold for 5-HT<sub>2A/C</sub>-mediated excitatory release of GABA, and disinhibiting the postsynaptic pyramidal neuron.

The attenuated responses observed after microiontophoretic applications of 8-OH-DPAT suggests a desensitization of hippocampal 5-HT<sub>1A</sub> receptors by both repeated URB597 and citalopram treatment, indicating a plastic change in 5-HT<sub>1A</sub> receptor function. An increase in the tonic activity of hippocampal 5-HT<sub>1A</sub> receptors, assessed with intravenous injection of the 5-HT<sub>1A</sub> antagonist WAY100635, has also been reported

after chronic treatment with several classes of antidepressant drugs (Haddjeri et al., 1998). Indeed, we have previously shown an increase of 5-HT<sub>1A</sub> hippocampal tonic activity in FAAH knockout mice and after prolonged CB<sub>1</sub>R agonist or URB597 treatment in rats and mice (Bambico et al., 2010; Bambico et al., 2012). These changes were likely due to increased 5-HT output. Indeed, we found that repeated and chronic URB597 treatment resulted in an increase in 5-HT neural firing activity. However, these do not seem to depend on 5-HT<sub>1A</sub> autoreceptor desensitization, which is linked to the therapeutic onset of chronic SSRI administration (Gobbi et al., 2005). This somewhat divergent but parallel serotonergic mechanisms of URB597 and SSRIs may be consistent with the fact that these drugs have been shown to potentiate each other's therapeutic-like activity (Umathe et al., 2011), indicating that URB597 may potentially be used as an adjunctive therapy to SSRIs. The proposed mechanisms for URB597's action are depicted in Fig. 8.

We also detected a significant enhancement in hippocampal BDNF in parallel with URB597-induced functional changes in 5-HT receptors. It has been shown that enhanced 5-HT transmission, such as after chronic SSRI treatment, leads to increased hippocampal plasticity that may be mediated by increased BDNF activity (Nibuya et al., 1995). Stimulation of 5-HT<sub>2A/C</sub> but not of 5-HT<sub>1A</sub> receptors also decreases hippocampal BDNF mRNA levels (Vaidya et al., 1997). This suggests a negative modulation of BDNF-mediated plasticity by 5-HT<sub>2A/C</sub> activity, which seems consistent with our data. We cannot rule out the contribution of other brain regions in the behavioural effects of URB597 since we also noted that it potently increased prefrontocortical BDNF. Studies examining the outcome of monoamine depletion on URB597 or endocannabinoid-mediated effects on hippocampal BDNF and monoamine receptor function are under way.

Altogether, these data further build upon the notion that the endocannabinoid system may represent an alternative target for the development of novel antidepressant/anxiolytic therapeutics. Amplifying the endocannabinoid-CB<sub>1</sub>R signaling via FAAH inhibitors is a favorable strategy as these agents produce therapeutic benefits devoid of the cannabimimetic side effects of direct CB<sub>1</sub>R agonists. The downstream mechanisms of FAAH inhibitors seem to converge with those of 5-HT-acting

antidepressants, including modifications of hippocampal 5-HT<sub>1A</sub> and 5-HT<sub>2A/C</sub> receptor function, and enhancement in BDNF production. Such a pharmacological approach provides potential for safe monotherapy or as adjuvant with respect to conventional antidepressant treatment.

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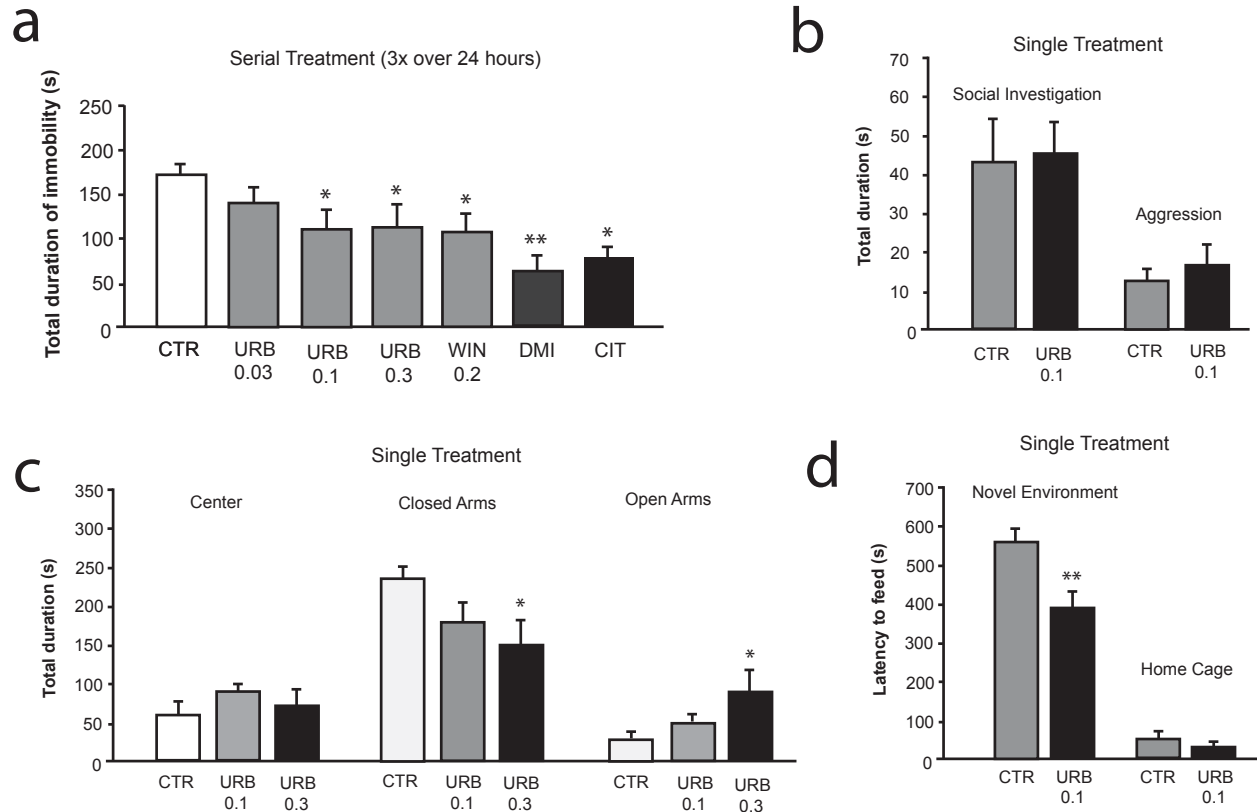
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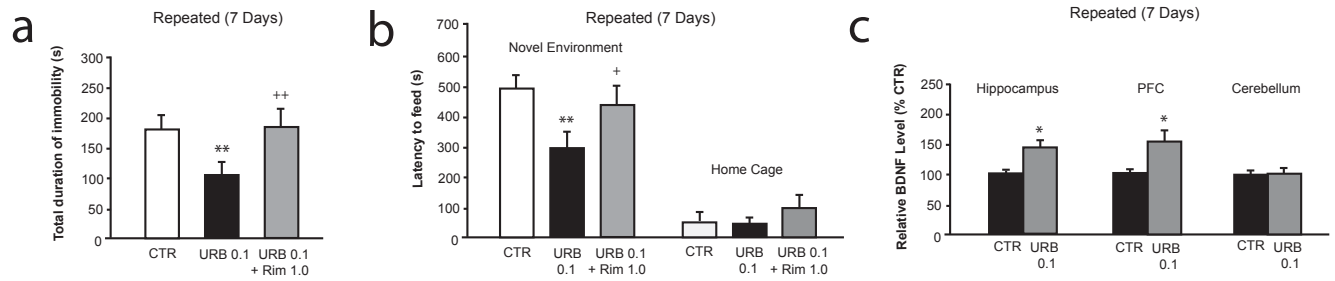
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## FIGURES AND LEGENDS



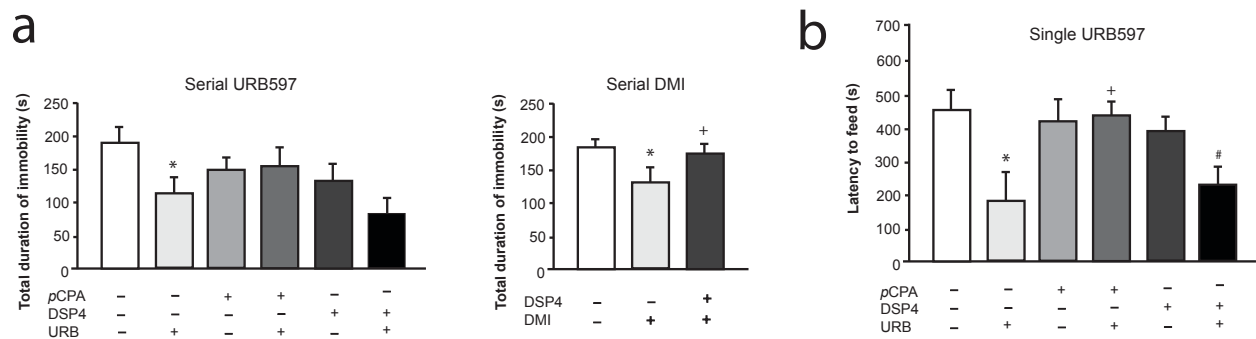
**Figure 1.** In the FST (a), URB597 (URB, 0.1, 0.3 mg·kg<sup>-1</sup>,ip) significantly decreased immobility. Citalopram (CIT, 10 mg·kg<sup>-1</sup>,ip), desipramine (DMI, 10 mg·kg<sup>-1</sup>,ip) and the cannabinoid receptor agonist WIN55,212-2 (WIN, 0.2 mg·kg<sup>-1</sup>,ip) also decreased immobility. URB597 (0.1 mg·kg<sup>-1</sup>,ip) had no effect on (b) social investigation nor aggressive behaviour, but decreased (c) anxiety-like behaviour in the EPMT at a dose of 0.3 mg·kg<sup>-1</sup>. In the NSFT (d), URB597 (0.1 mg/kg) significantly decreased feeding latency in a novel environment but not in the home cage. \*p<0.05 , \*\*p<0.01 vs. CTR. N=5-8/group.

Figure 2



**Figure 2.** Repeated URB597 treatment (URB 0.1 mg·kg<sup>-1</sup>,ip) ~~once daily for 7 days~~ significantly decreased immobility duration in the FST (a) and feeding latency in the NSFT (b). These effects were blocked by rimonabant (Rim, 1.0 mg·kg<sup>-1</sup>,ip) (a-b), and were associated with increased BDNF (pg gram<sup>-1</sup> of tissue, normalized to the mean control level [100%]) in the hippocampus (HPC) and prefrontal cortex (PFC), but not in the cerebellum (CBL) (c). \*p<0.05, \*\*p<0.01 vs. CTR; +p<0.05, ++p<0.01 vs. URB. N=6-8/group.

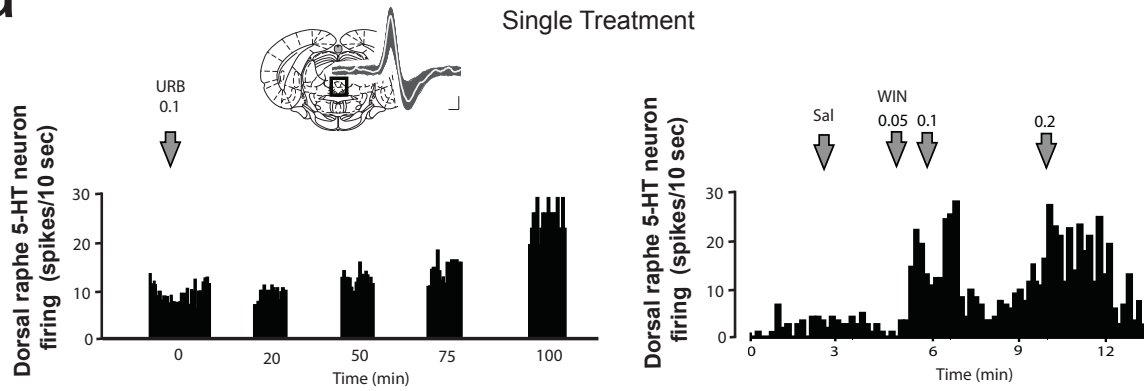
Figure 3



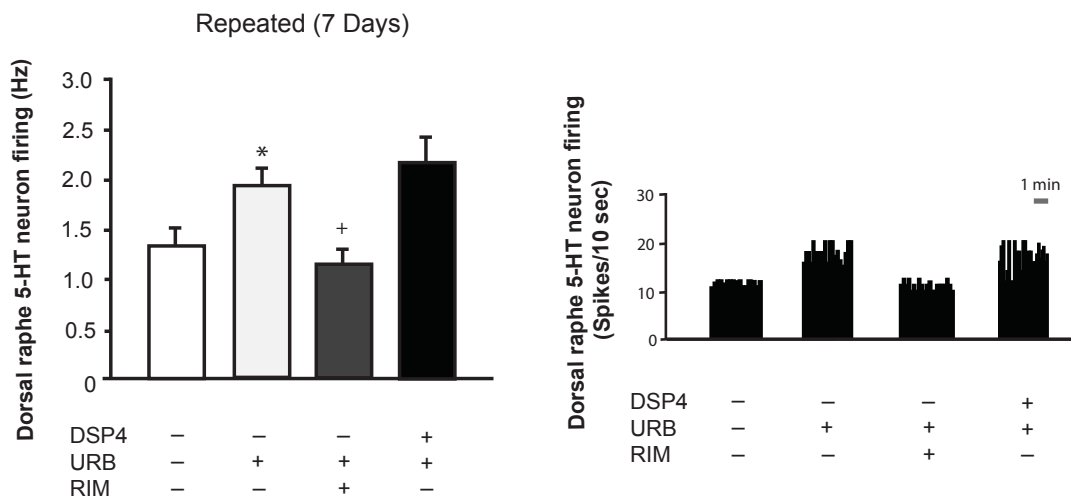
**Figure 3.** pCPA (350 mg·kg<sup>-1</sup>,ip; 72 h and 48 h prior to test) but not DSP4 (50 mg·kg<sup>-1</sup>,ip; 7 days prior to test) significantly attenuated URB597-induced (URB, 0.1 mg·kg<sup>-1</sup>,ip) reduction in (a, left panel) immobility in the FST and (b) latency to feed in the NSFT. DSP4 prevented the immobility-attenuating effect of desipramine (DMI, 10 mg·kg<sup>-1</sup>,ip) in the FST (a, right panel). Matrices below bar graphs indicate presence (+) or absence (-) of drug administrations. \*p<0.05 vs. CTR; +p<0.05 vs. DMI (a) or URB (b); #p<0.05 vs. DSP4+vehicle.. N=6-9/group.

Figure 4

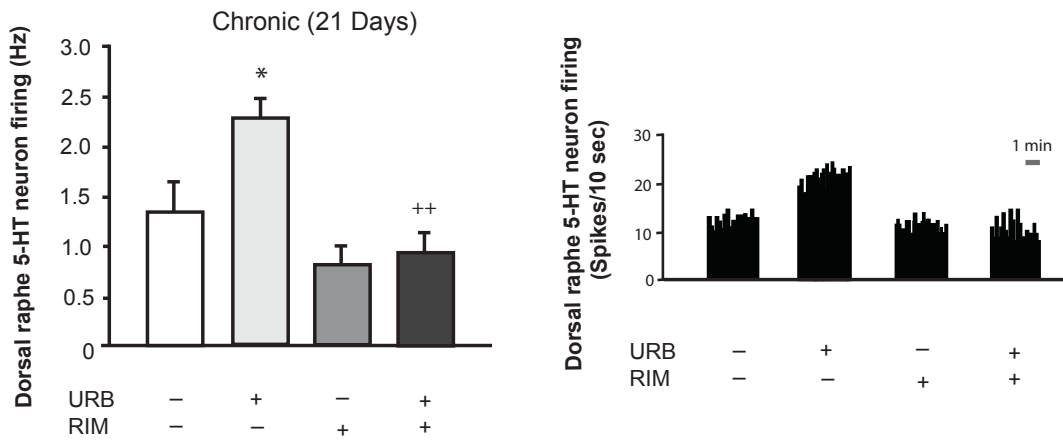
a



b

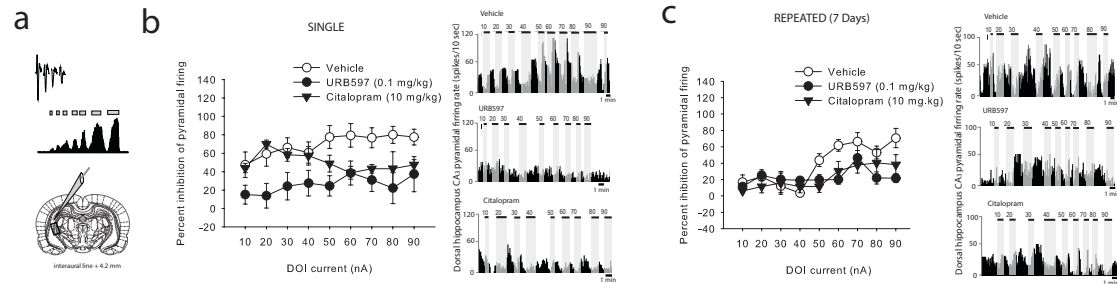


c



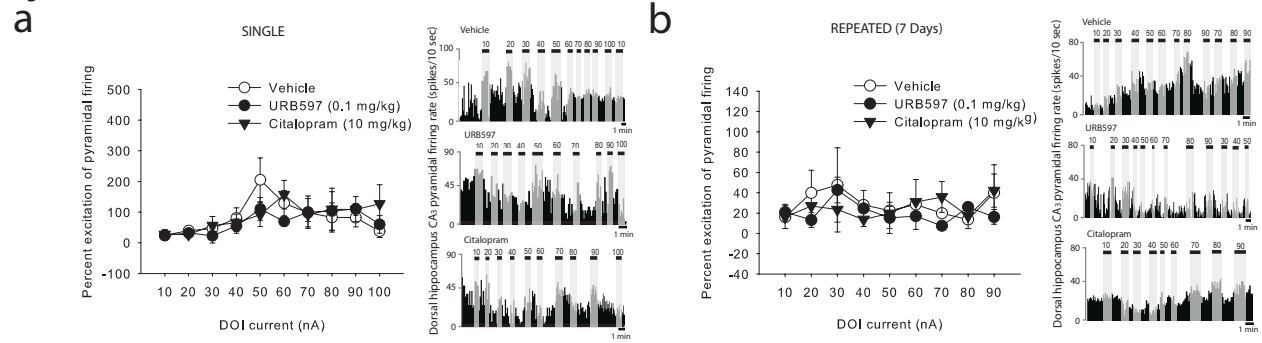
**Figure 4.** In DSP4-treated animals (50 mg·kg<sup>-1</sup>,ip, 7 days before recording), single injection of URB597 (URB 0.1 mg·kg<sup>-1</sup>,iv) (a) gradually increased 5-HT neuron activity while WIN55-212,2 (WIN 0.05, 0.1, 0.2 mg·kg<sup>-1</sup>,iv, cumulative) but not saline (Sal,iv) rapidly increased it (n=3 neurons/drug treatment). Repeated (once daily for 7 days) (b) and chronic (once daily for 21 days) (c) treatment with URB597 (URB 0.1 mg·kg<sup>-1</sup>,ip) increased DR 5-HT neuron firing rate. Rimonabant (Rim, 1 mg·kg<sup>-1</sup>,ip, daily co-administration, 15 min before URB597 treatment) but not DSP4 pretreatment blocked URB597-mediated increase in 5-HT neuron firing. Right panels show integrated firing rate histograms for each treatment group. Matrices below bar graphs indicate presence (+) or absence (-) of drug administrations (-,- or -,-,- = CTR). \*p<0.05 vs. CTR; + p<0.05 vs. URB+vehicle; ++p<0.01 vs. URB+vehicle. N=7-17 neurons/group for repeated treatment. N=15-34 neurons/group for chronic treatment.

Figure 5



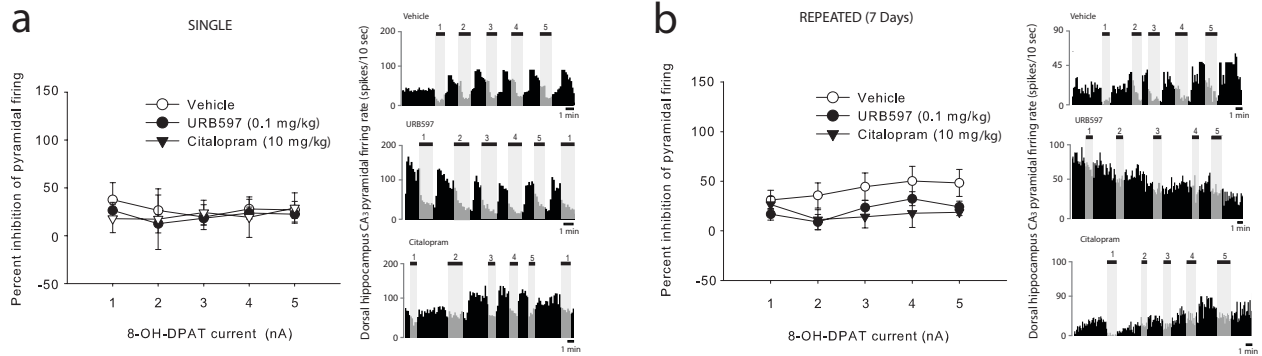
**Figure 5.** Leftmost panel (a) shows a pyramidal neuron spike waveform, and an integrated firing rate histogram showing pyramidal neuron response to quisqualate ejections. The plate at the bottom is a rat brain coronal section showing the location of recordings. Single (b) and repeated (once daily for 7 days) (c) administration of URB597 (URB 0.1 mg·kg<sup>-1</sup>,ip) or citalopram (CIT 10 mg·kg<sup>-1</sup>,ip) decreased inhibition of hippocampal pyramidal neurons in response to microiontophoretic application of DOI (10–90 nA ejection current). Data points across DOI currents (abscissa) represent mean ( $\pm$  SEM) of percent inhibition (ordinate) in pyramidal activity from pre-drug (zero current) activity preceding each ejection (unshaded region).  $p < 0.05$ , significant main effect of treatment, URB vs. CTR, CIT vs. CTR.  $N = 3-7$  neural responses/data point. The firing rate histograms show inhibitory response (ordinate, spikes per 10 seconds) of representative pyramidal neurons across currents (numbers on top) after single or repeated drug treatments.

Figure 6



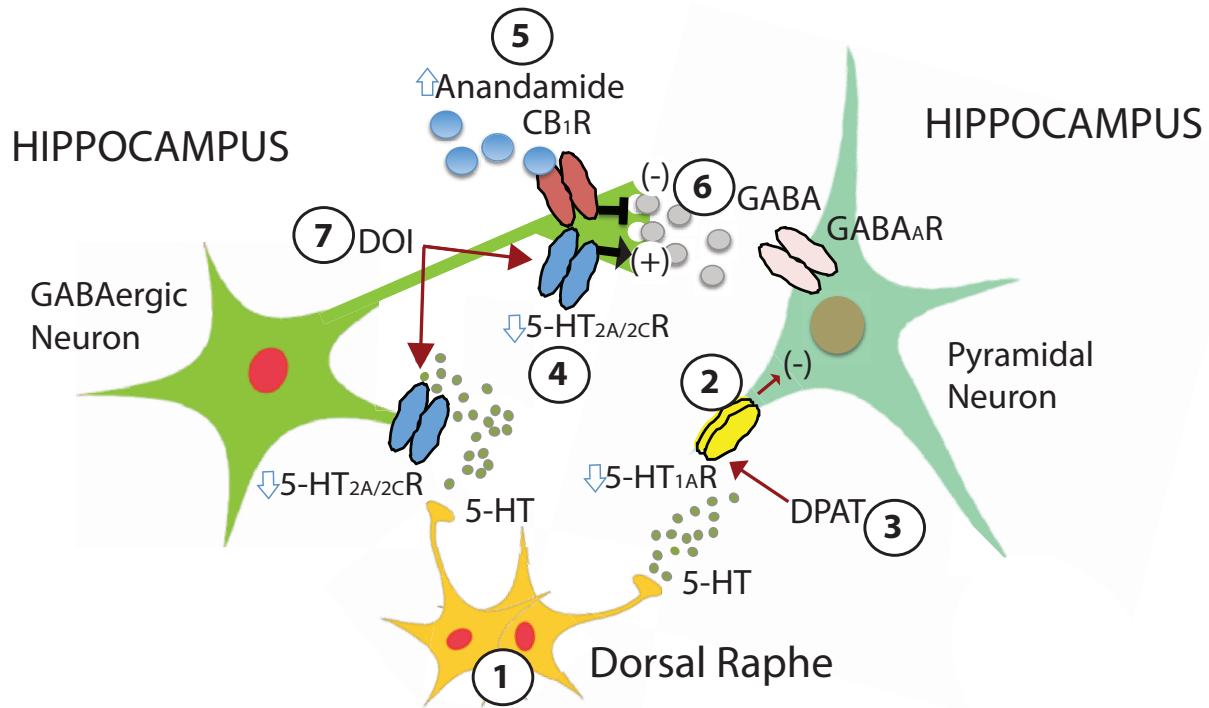
**Figure 6.** Neither single (a) nor repeated (7 days) (b) administration of URB597 (URB 0.1 mg·kg<sup>-1</sup>,ip) or citalopram (CIT 10 mg·kg<sup>-1</sup>,ip) significantly altered hippocampal pyramidal excitation in response to microiontophoretic application of DOI (10–100 nA ejection current). Data points across DOI currents (abscissa) represent mean ( $\pm$  SEM) of percent excitation (ordinate) in pyramidal activity from pre-drug (zero current) activity preceding each ejection (unshaded region). N=3-6 neural responses/data point. The firing rate histograms show excitatory response (ordinate, spikes per 10 seconds) of representative pyramidal neurons to different DOI currents (numbers on top) after single or repeated drug treatments.

Figure 7



**Figure 7.** Repeated (once daily for 7 days) (b) but not single (a) administration of URB597 (URB 0.1 mg·kg<sup>-1</sup>,ip) or citalopram (CIT 10 mg·kg<sup>-1</sup>,ip) decreased inhibition of pyramidal neurons in response to microiontophoretic application of 8-OH-DPAT (1-5 nA ejection current). Data points across 8-OH-DPAT currents (abscissa) represent mean ( $\pm$  SEM) of percent decrease (ordinate) in pyramidal activity from pre-drug (zero current) activity preceding each ejection (unshaded region) (ordinate)  $\pm$  SEM, across 8-OH-DPAT current (abscissa).  $p < 0.05$ , significant main effect of treatment, URB vs. CTR, CIT vs. CTR.  $N = 5-10$  neural responses/data point. The firing rate histograms show the response (ordinate, spikes per 10 seconds) of representative pyramidal neurons to different 8-OH-DPAT currents (numbers on top) after single or repeated drug treatments.





**Figure 8.** Proposed mechanisms of action of URB597 in the hippocampus. Repeated URB597 enhances 5-HT neural firing and release (1), desensitizing (downward arrow) 5-HT<sub>1A</sub> receptors (Rs) (2), and blunting inhibition by the 5-HT<sub>1A</sub>R agonist 8-OH-DPAT on pyramidal neurons (3). Single or repeated URB597 treatment enhances 5-HT neural firing and release (1), desensitizing presynaptic 5-HT<sub>2A/C</sub>Rs (downward arrow) (4). Anandamide and CB<sub>1</sub>R signaling is increased (upward arrow) (5), hyperpolarizing GABAergic terminals and decreasing (minus) GABA release and receptor activation in pyramidal neurons (6). Consequently, the GABA-releasing action (plus) and GABA-mediated inhibitory response of pyramidal neurons to the 5-HT<sub>2A/C</sub>R agonist DOI is markedly attenuated (7).

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