Acute and chronic modulation of striatal endocannabinoid-mediated plasticity by nicotine

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ABSTRACT

The endocannabinoid (eCB) system modulates several phenomena related to addictive behaviors, and drug-induced changes in eCB signaling have been postulated to be important mediators of physiological and pathological reward-related synaptic plasticity. Here, we studied eCB-mediated long-term depression (eCB-LTD) in the dorsolateral striatum, a brain region critical for acquisition of habitual and automatic behavior. We report that nicotine differentially affects *ex vivo* eCB signaling depending on previous exposure *in vivo*. In the nicotine-naïve brain, nicotine facilitates eCB-signaling and LTD, whereas tolerance develops to this facilitating effect after subchronic exposure *in vivo*. In the end, a progressive impairment of eCB-induced LTD is established after protracted withdrawal from nicotine. Endocannabinoid-LTD is reinstated 6 months after the last drug injection, but a brief period of nicotine re-exposure is sufficient to yet again impair eCB-signaling. LTD induced by the cannabinoid 1 receptor agonist WIN55,212-2 is not affected, suggesting that nicotine modulates eCB production or release. Nicotine-induced facilitation of eCB-LTD is occluded by the dopamine D2 receptor agonist quinpirole, and by the muscarinic acetylcholine receptor antagonist scopolamine. In addition, the same compounds restore eCB-LTD during protracted withdrawal. Nicotine may thus modulate eCB-signaling by affecting dopaminergic and cholinergic neurotransmission in a long-lasting manner. Overall, the data presented here suggest that nicotine facilitates eCB-LTD in the initial phase, which putatively could promote neurophysiological and behavioral adaptations to the drug. Protracted withdrawal, however, impairs eCB-LTD, which may influence or affect the ability to maintain cessation.

Keywords dopamine, endocannabinoid, synaptic plasticity.

INTRODUCTION

The ability for neuronal circuits to adapt is necessary for integrative and higher functions of the brain, and long-term modification of synaptic activity is considered the cellular basis for learning and memory (Nabavi *et al.* 2014). However, drugs of abuse may also induce remodeling of neural networks, and drug-induced synaptic plasticity has been postulated as a neurobiological underpinning of addiction (Gerdeman *et al.* 2003; Jones & Bonci 2005). Drug-induced synaptic plasticity contributes to the reorganization of neural circuits as addiction forms, but whether these changes induce addictive behaviors or simply reflects compensations in neural function to drug effects are not known (Ungless *et al.* 2001).

Postsynaptically synthesized endocannabinoids (eCBs), which mediate synaptic depression through activation of cannabinoid 1 receptors (CB1R) at excitatory and inhibitory synapses, have been implicated in both drug addiction and addictive behaviors (Arnold 2005; Adermark & Lovinger 2007b, 2009; Fattore *et al.* 2010). The eCB system appears to modulate several phenomena related to addiction, including drug-induced reward, the motivation to procure the drug and behavioral sensitization (Vinod *et al.* 2008). CB1Rs are highly expressed in the dorsolateral striatum (DLS), a

critical brain region for the gradual acquisition of habitual and automatic behaviors (Gremel & Costa 2013). Striatal CB1Rs may thus be crucial for establishing behavioral patterns that are directed compulsively toward drug usage (Gerdeman et al. 2003). Interestingly, studies from several laboratories have demonstrated disruption of striatal eCB-LTD following acute and protracted exposure to drugs of abuse (Fourgeaud et al. 2004; Clarke & Adermark 2010; Adermark et al. 2011; Atwood, Kupferschmidt, & Lovinger 2014). Therapeutic approaches aimed at restoring eCB-mediated synaptic plasticity might thus potentially have impact on the treatment of addiction (Zlebnik & Cheer 2016).

Associations between behavioral responses to nicotine and the eCB system have previously been shown with respect to reward, conditioning and drug reinstatement (Castane et al. 2002; Cohen et al. 2002; Merritt et al. 2008; Gamaleddin et al. 2013). Inhibition of eCB signaling attenuates nicotine-induced activation of the mesolimbic dopamine system, reduces self-administration and inhibits acquisition and expression of nicotine-induced place preference (Castane et al. 2002; Cohen et al. 2002; Cheer et al. 2007; Hadjiconstantinou & Neff 2011). On the opposite, activation of CB1Rs or inhibition of eCB metabolism enhances nicotine self-administration and expression of nicotine-induced place preference (Merritt et al. 2008; Gamaleddin et al. 2013). Interestingly, eCB levels are altered by acute and repeated nicotine exposure (Gonzalez et al. 2002; Buczynski, Polis, & Parsons 2013), and the endogenous ligand for the nicotinic acetylcholine receptor (nAChR), acetylcholine, appears to regulate eCB-mediated plasticity in the striatum (eCB-LTD) (Partridge et al. 2002; Adermark 2011). The striatal eCB system has repeatedly been linked to neuronal adaptations that form persistent drug-related habits and addictive behaviors. Nicotine-induced modulation of striatal eCB-signaling could thus play a key role in initiating the neurophysiological and behavioral adaptations elicited by this drug. Therefore, this study aimed to define acute and long-term effects produced by nicotine on eCB-LTD in the DLS of Wistar rats.

MATERIALS AND METHODS

Drugs

Pharmacological agents were dissolved in appropriate solvent and diluted in artificial cerebrospinal fluid (aCSF) to final concentration shortly before use. (−)-Nicotine hydrogen tartrate salt was dissolved to final concentration in aCSF shortly before use, while the non-selective nAChR antagonist mecamylamine hydrochloride was dissolved in H_2O (50 mM) and applied at 10 µM. The dopamine D1 receptor antagonist SCH23390 hydrochloride was dissolved

in H_2O (20 mM) and used at 0.5 µM. The dopamine D2 receptor antagonist sulpiride was dissolved in ethanol to 10 mM and used at 5 µM, while the agonist quinpirole was dissolved in aCSF to final concentration (5 µM) shortly before use. CB1R agonist WIN55,212-2 and antagonist AM251 were both dissolved in DMSO to 20 mM and used at 2 µM. The fatty acid amide hydrolase (FAAH) inhibitor TC-F 2 was dissolved in DMSO (20 mM) and further diluted to 5 µM. Scopolamine was dissolved in aCSF to final concentration shortly before use (5 µM). The mGluR2/3 agonist LY354740 was dissolved in DMSO (1 mM) and further diluted in aCSF to 200 nM, while the mGluR1/5 agonist (S)-3,5-DHPG (DHPG) was dissolved in DMSO (20 mM) and further diluted in aCSF to 100 µM. Substances were purchased from Sigma-Aldrich (Stockholm, Sweden) and Tocris Bioscience (Bristol, UK).

Nicotine treatment

Male Wistar rats (Taconic, Ejby, Denmark) (280–350 g) were group-housed with a 12-hour light/dark cycle with food and water ad libitum. One week after arrival, the rats received daily injections of nicotine (0.36 mg/kg subcutaneous, dissolved in 0.9 percent NaCl with pH adjusted to 7.2–7.4 with $NaHCO_3$) or vehicle (0.9 percent NaCl) on weekdays during 3 weeks, resulting in a total of 15 injections. One subgroup of animals only received five nicotine injections before being sacrificed. Behavioral sensitization to repeated nicotine treatment was confirmed by monitoring locomotor activity as previously described (Morud et al. 2016). A subset of animals was maintained drug-free for 28 weeks (6 months) following the initial nicotine treatment after which they received six additional doses of nicotine (for schematic overview of protocol see Fig. 2). The experimental protocols were approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden.

Brain slice preparation

Brain slice preparation and field potential recordings were performed as previously described (Clarke & Adermark 2010). In brief, the animals were deeply anesthetized with isoflurane (Forene, Baxter, Kista, Sweden) and decapitated. The brains were placed in ice-cold modified aCSF containing (in mM) 194 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-glucose, saturated with oxygen. The brain slices were sectioned coronally (300 µm) by using a Leica VT 1200S Vibratome (Leica Microsystems AB, Bromma, Sweden) and were allowed to equilibrate for 15 minutes at 30°C, and then for at least 1 hour at room temperature in normal aCSF containing (in mM) 124 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-glucose, with osmolarity adjusted to approximately

300 mosmol/kg with sucrose, continuously bubbled with a mixture of 95 percent O_2/5% CO_2 gas.

Field potential recordings

One hemisphere of a slice was transferred to a recording chamber and perfused with pre-warmed aCSF kept at 30°C. A stimulating electrode (monopolar tungsten electrode, type TM33B, World Precision Instruments, FL, USA) was placed at the border of the subcortical white matter and the striatum, and postsynaptic spikes (PSs) were evoked in the DLS (Fig. 1) by paired-pulse stimulation (50-ms interpulse interval), evoked at a frequency of 0.05 Hz. Stimulus intensity (0.01–0.035 mA) was adjusted so that the PS amplitude was approximately half maximum the size of the maximal response. Signals were amplified by a custom-made amplifier, filtered at 3 kHz and digitized at 8 kHz. High-frequency stimulation (HFS)

of cortical afferents synapsing on dopamine D1 and D2 receptors expressing medium spiny neurons in the DLS has previously been established to induce postsynaptic eCB production and release, resulting in eCB-LTD (Adermark & Lovinger 2009). After monitoring a stable baseline for at least 10 minutes, eCB-mediated LTD was induced by HFS (4 trains of 50 pulses delivered at 100 Hz with a 4-s intertrain interval) of afferent fibers as previously described (Adermark & Lovinger 2009). In selected experiments, the brain slices from nicotine-treated or vehicle-treated rats were pre-treated with agonists/antagonists targeting CB1R signaling (WIN55,212-2, AM251, and TC-F 2), nAChRs (nicotine and mecamylamine), muscarinic acetylcholine receptor (mAChRs; scopolamine), dopamine receptors (quinpirole, sulpiride, and SCH23390), mGluRs (DHPG and LY354740) or $GABA_A$ receptors (bicuculline). All compounds were perfused for at least 20 minutes prior to HFS and remained in the bath
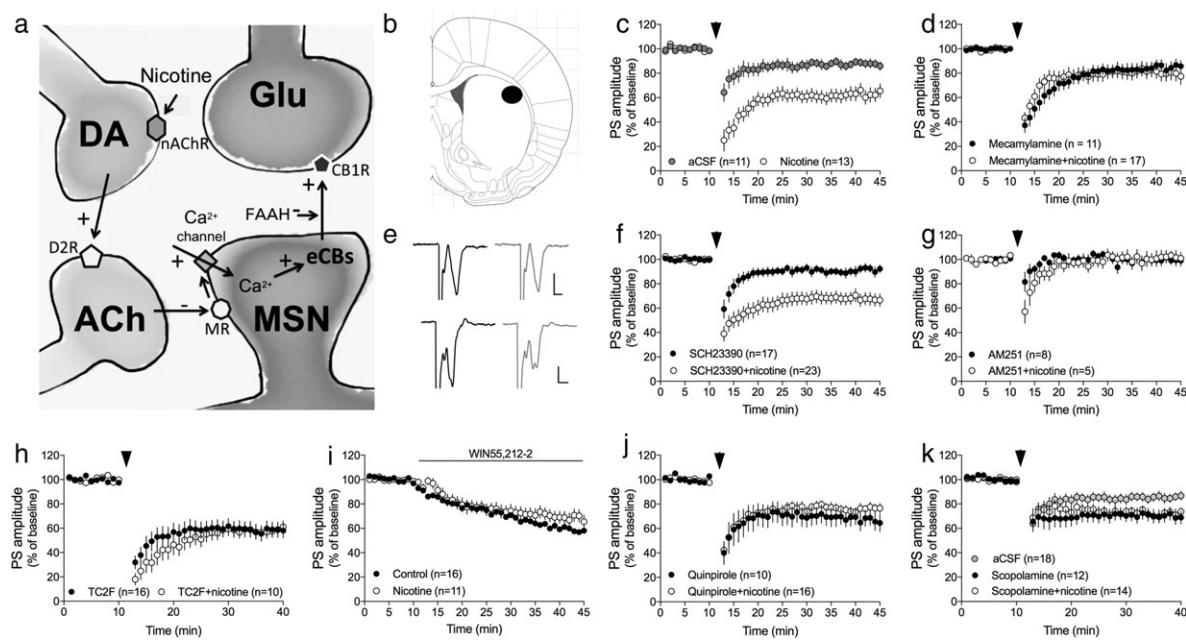

Figure 1 Nicotine facilitates endocannabinoid-mediated long-term depression (eCB-LTD).

throughout the experiment. In all experiments, slices from nicotine-treated rats were run in parallel to vehicle-treated controls.

Statistical analysis

Data were analyzed with Clampex 10.1 (Molecular Devices, Foster City, CA) and assembled in GRAPHPAD PRISM. Gaussian distribution was tested with D'Agostino and Pearson omnibus normality test. All data are presented as mean values ± SEM, and the level of significance was set to $P < 0.05$. Group effects and treatment effects were analyzed by using two-way ANOVA and *t*-test when applicable. Mann Whitney *U*-test was used for nonparametric data.

RESULTS

Nicotine exposure *ex vivo* facilitates eCB-LTD in slices from nicotine naïve rats

Continuous bath perfusion of nicotine (1 μM, starting ≥20 min prior to HFS) significantly enhanced LTD in a manner that was inhibited by pre-treatment with the non-competitive nAChR antagonist mecamylamine (10 μM) (two-way ANOVA $_{t\ =\ 15-45min}$: aCSF versus nicotine: $F_{(1,\ 24)} = 20$, $P < 0.001$; mecamylamine versus mecamylamine + nicotine: $F_{(1,\ 27)} = 0.02$, $P > 0.05$) (Fig. 1). The net outcome of synaptic plasticity in the striatum is balanced by long-term potentiation (LTP) and LTD at both excitatory and inhibitory synapses (Adermark & Lovinger 2009). The decrease in PS amplitude induced by HFS could thus be influenced by changes in LTP formation. However, inhibition of dopamine D1 receptors (SCH23390, 0.5 μM), which prevents striatal LTP (Lovinger 2010), did not modulate LTD or the effect displayed by nicotine ($F_{(1,\ 35)} = 12$, $P < 0.01$) (Fig. 1f). On the other hand, HFS-induced LTD was completely blocked by the CB1Rs antagonist AM251 (2 μM), establishing a role for eCBs in mediating this form of LTD (eCB-LTD) (Fig. 1g). Importantly, nicotine did not promote LTD formation in slices pre-treated with AM251 (AM251: $F_{(1,\ 11)} = 2.01$, $P > 0.05$) (Fig. 1g). Previous research has put forward anandamide as the primary eCB mediating HFS-LTD (Lerner & Kreitzer 2012). Reducing the breakdown of anandamide by inhibiting FAAH (TC-2F, 5 μM) significantly enhanced eCB-LTD ($F_{(1,\ 32)} = 8.37$, $P < 0.01$), further supporting a role for anandamide in eCB-LTD mediated by HFS in this brain region (Lerner & Kreitzer 2012). Nicotine did not further promote eCB-LTD in TC-2F-treated slices ($F_{(1,\ 24)} = 0.31$, $P > 0.05$) (Fig. 1h).

To assess if nicotine facilitates HFS-LTD by enhancing postsynaptic eCB signaling or by presynaptic mechanisms, a subset of experiments were performed where LTD was induced by the CB1R agonist WIN55,212-2 (2 μM). WIN55,212-2-induced LTD was not enhanced in slices pretreated with nicotine, indicating that nicotine facilitates eCB production or release ($F_{(1,\ 14)} = 0.26$, $P > 0.05$) (Fig. 1i). nAChRs are located on dopaminergic terminals and could exert its influence over eCB production by altering dopamine-release (Fig. 1a) (Yan, Song, & Surmeier 1997; Partridge *et al*. 2002). Supporting this theory, the dopamine D2 receptor agonist quinpirole occluded nicotine-induced facilitation of LTD ($F_{(1,\ 23)} = 1.62$, $P > 0.05$) (Fig. 1j). Dopamine D2 receptor activation has been postulated to be upstream from mAChRs, which exert a strong control over calcium influx in rat striatal neurons (Howe & Surmeier 1995; Wang *et al*. 2006). Pre-treatment with the unselective mAChR antagonist scopolamine (5 μM) also occluded nicotine-induced facilitation of eCB-LTD ($F_{(1,\ 29)} = 1.32$, $P > 0.05$) (Fig. 1k). Nicotine could thus act by facilitating postsynaptic calcium influx, thereby reducing the threshold for eCB production and release (Fig. 1a).

Repeated nicotine treatment *in vivo* does not affect *ex vivo* eCB-LTD

In the next set of experiments, eCB-LTD was studied in brain slices from adult rats (280–350 g) receiving 15 doses of nicotine over a period of 3 weeks. This treatment paradigm, where treatment is interrupted to promote incubation effects, has repeatedly been shown to produce long-lasting behavioral sensitization, which might even be lifelong (Vezina, McGehee, & Green 2007; Morud *et al*. 2016). Locomotor activity was recorded to confirm that a sensitized response to the locomotor-stimulatory properties of nicotine was established after 15 days of nicotine administration (paired *t*-test, 1st versus 15th injection: $t = 6.30$, df = 12, $P < 0.001$) (Fig. 2b). To avoid acute withdrawal effects, eCB-LTD was studied 1 week after the last nicotine exposure. eCB-Mediated LTD induced by HFS was not significantly modulated in brain slices from animals receiving intermittent nicotine-treatment as compared with vehicle-treated controls (two-way ANOVA: $F_{(1,22)} = 0.59$, $P > 0.05$) (Fig. 2), neither was synaptic depression induced by the CB1R agonist WIN55,212-2 ($F_{(1,17)} = 0.05$, $P > 0.05$) (data not shown). However, *ex vivo* exposure to nicotine (1 μM) did not facilitate eCB-LTD in brain slices from animals treated repeatedly with nicotine *in vivo* (slices from nicotine-treated rats with or without nicotine-administration *ex vivo*; $F_{(1,17)} = 0.09$, $P > 0.05$) (Fig. 2c). To further define the influence of repeated nicotine on eCB-LTD, one set of rats was treated with nicotine for 5 days after which slice recordings were performed 2–5 hours after the last drug injection. Similar to the data retrieved following 15 days of treatment, synaptic depression induced by HFS was not significantly modulated in this group of rats ($F_{(1,15)} = 0.00$, $P > 0.05$) (data not shown).
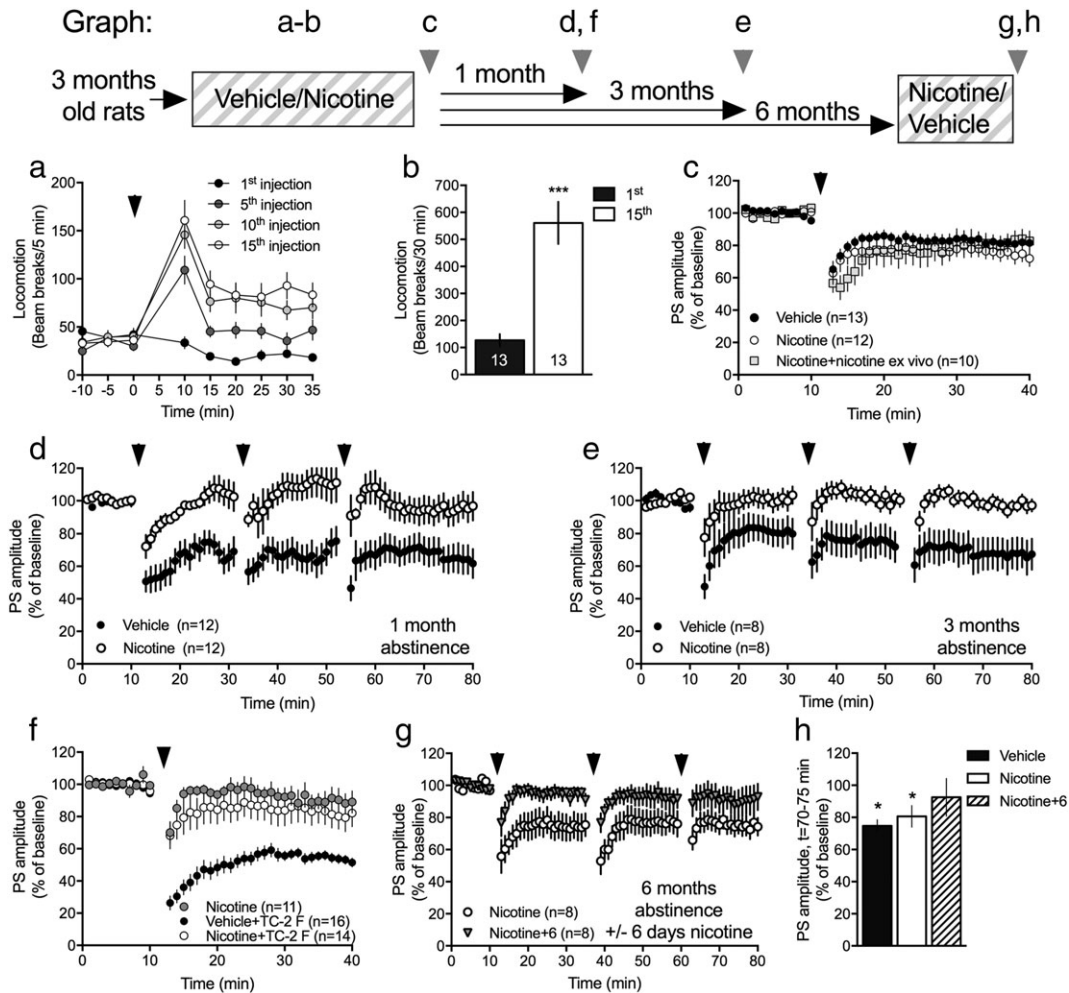
Figure 2 Impaired endocannabinoid-mediated long-term depression (eCB-LTD) following protracted abstinence. (a) Time course graph showing locomotor activity during the progress of behavioral sensitization. (b) Bar graph showing behavioral sensitization toward the locomotor-stimulatory properties of nicotine when comparing the first with the last session. (c) Repeated nicotine-treatment terminated 1 week prior to recordings did not alter high-frequency stimulation (HFS)-induced eCB-LTD. Facilitation of eCB-LTD by *ex vivo* nicotine, however, was not present in brain slices from rats previously exposed to nicotine. (d and e) Endocannabinoid-LTD was significantly impaired in brain slices from rats treated with nicotine 1 or 3 months earlier. (f) The fatty acid amide hydrolase (FAAH) inhibitor TC-2F did not restore eCB-LTD in brain slices from nicotine treated rats. (g) Following 6 months of withdrawal from nicotine eCB-LTD was reinstated (nicotine) but impaired after brief re-administration of nicotine (nicotine + 6). (h) The bar graph shows mean PS amplitude ± SEM after 70–75 minutes in brain slices from vehicle, from rats treated with nicotine 6 months earlier (nicotine), and previously treated rats receiving six additional exposures to nicotine (nicotine + 6). Time course figures show mean amplitude as compared with baseline ± SEM. The arrows mark time points for HFS. Significance compared with baseline *$P < 0.05$. The number of brain slices (*n*), obtained from at least three different animals, is indicated in each figure

Impaired eCB-LTD following protracted withdrawal

Recent research suggests that nicotine withdrawal produces progressive neuroadaptations in striatal subregions (Adermark *et al.* 2016). In a way to define temporal changes in eCB signaling, HFS-induced LTD was assessed after 1, 3 and 6 months of nicotine withdrawal. Slice electrophysiology revealed that eCB-LTD was impaired after both 1 and 3 months of nicotine withdrawal (two-way ANOVA, vehicle versus nicotine-treated: 1-month withdrawal: $F_{(1,20)} = 12$, $P < 0.01$; 3-month withdrawal: $F_{(1,14)} = 7.87$, $P < 0.05$) (Fig. 2d and e). Inhibiting the breakdown of anandamide by pre-treating the slices with

the FAAH-inhibitor TC-2F (5 μM) was not sufficient to restore eCB-LTD in brain slices from rats receiving nicotine 1 month earlier (nicotine-treated: with versus without TC-2F: $F_{(1,22)} = 0.72$, $P > 0.05$; TC-2F-treated slices: vehicle versus nicotine: $F_{(1,27)} = 12.8$, $P < 0.01$) (Fig. 2f). Following three additional months of withdrawal eCB-LTD was reinstated (two-way ANOVA, vehicle versus nicotine-treated: 6 months withdrawal: $F_{(1,12)} = 0.73$, $P > 0.05$) (Fig. 2g). A brief period of re-administration of nicotine *in vivo* (nicotine + 6), however, was sufficient to once again impair eCB-LTD (vehicle: $t = 6.69$, $df = 7$, $P < 0.001$; nicotine: $t = 2.88$, $df = 5$, $P < 0.05$; nicotine + 6: $t = 0.64$, $df = 5$,

$P > 0.05$; two-way ANOVA: nicotine versus nicotine + 6; $F_{(1,14)} = 4.74$, $P < 0.05$) (Fig. 2g and h).

Dopamine D2 receptor activation restores eCB-LTD in nicotine-treated rats

To define the locus of impaired LTD, another batch of rats was treated with nicotine for 3 weeks and underwent 3 months of withdrawal before electrophysiological recordings were performed. Supporting data from the first batch of animals, eCB-LTD was impaired in brain slices from animals receiving nicotine 3 months earlier (two-way ANOVA: $F_{(1, 17)} = 11.34$, $P < 0.01$) (Fig. 3a). To determine if changes in presynaptic properties could underlie impaired eCB-LTD, presynaptic depression was induced by the mGluR2/3 receptor agonist LY354740 (20 nM). LY354740, which primarily activates autoreceptors located on glutamatergic terminals, suppressed PS amplitude to a similar extent in both treatment groups ($F_{(1, 20)} = 0.63$, $P > 0.05$) (Fig. 3b). LTD

induced by the CB1R agonist WIN55,212-2 (2 μM) was also comparable between the two groups, suggesting that presynaptic eCB signaling remains intact (two-way ANOVA, vehicle versus nicotine: aCSF pre-treated: $F_{(1, 31)} = 0.00$, $P > 0.05$; bicuculline pre-treated (20 μM): $F_{(1, 20)} = 0.00$, $P > 0.05$) (Fig. 3c). Administration of DHPG (100 μM), which previously has been shown to induce eCB-LTD by activating postsynaptic group I mGluRs (Kreitzer & Malenka 2005), only suppressed PS amplitude in vehicle treated rats, supporting the hypothesis that impaired eCB-LTD is linked to reduced postsynaptic eCB production or release (two-way ANOVA, vehicle versus nicotine: $F_{(1, 21)} = 6.16$, $P < 0.05$) (Fig. 3d).

In an attempt to restore eCB-LTD following 3 months of withdrawal, the slices were perfused with nicotine for 20 minutes prior to HFS and continuous throughout the experiment. eCB-LTD was robustly induced in brain slices from vehicle-treated rats (unpaired *t*-test $t = 35–40$ min, $t = 6.82$, df = 14, $P < 0.001$), but not in
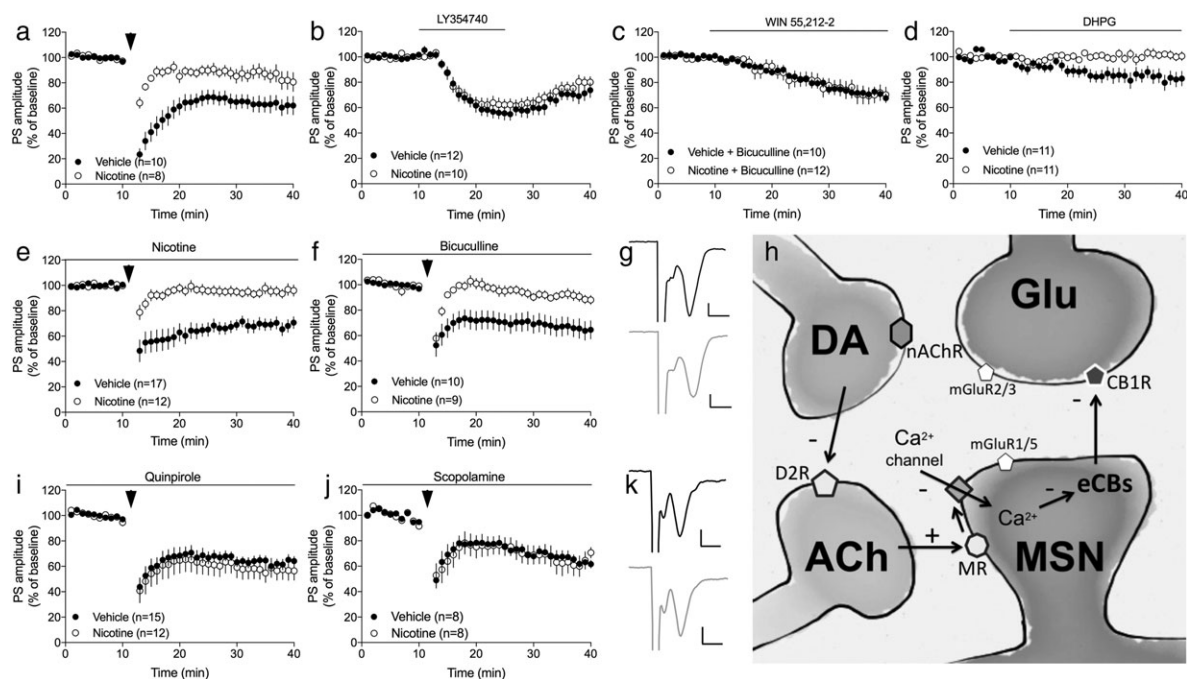

Figure 3 Dopamine D2 receptor activation rescues impaired endocannabinoid-mediated long-term depression (eCB-LTD) after 3 months of nicotine abstinence. (a) Endocannabinoid-LTD was impaired in brain slices from rats treated 3 months earlier with nicotine. (b) Activation of presynaptic mGluR2/3s with LY354740-induced LTD in both treatment groups. (c) WIN55,212-2-induced LTD independent of treatment suggesting that presynaptic eCB-signaling remains intact during nicotine withdrawal. (d) Group I mGluR agonist DHPG selectively suppressed post-synaptic spike (PS) amplitude in brain slices from vehicle-treated rats. (e and f) Neither nicotine nor bicuculline restored eCB-LTD in brain slices from nicotine-treated rats. (g) Example traces show evoked PS at baseline (black) and following high-frequency stimulation (HFS; gray) in a slice from a vehicle-treated rat. (h) Simplified schematic drawing showing postulated signaling pathways underlying impaired eCB-LTD. Dopamine D2 receptor tone is reduced following protracted abstinence from nicotine, leading to enhanced cholinergic activity and muscarinic acetylcholine receptor (mAChR) activation. This, in turn, reduces L-type calcium channel activation and decreases the chance for postsynaptic calcium to reach the level required for eCB production. (i) Activation of dopamine D2 receptors prior to HFS reinstated eCB-LTD in slices from nicotine-treated rats, (j) as did the mAChR antagonist scopolamine. (k) Example traces show evoked PS at baseline (black) and following HFS (gray) in a slice from a nicotine-treated rat. Calibration is 2 ms and 0.1 mV. Time course figures show mean amplitude as compared with baseline ± SEM. The arrows mark time points for HFS. The number of brain slices (*n*) is indicated in each figure, and taken from at least three different animals

slices from rats treated with nicotine 3 months earlier ($t = 1.04$, df = 14, $P > 0.05$; two-way ANOVA, vehicle versus nicotine: $F_{(1, 28)} = 23$, $P < 0.001$) (Fig. 3e). Pre-treatment with the GABA$_A$ receptor antagonist bicuculline was also ineffective in restoring LTD ($F_{(1, 17)} = 10$, $P < 0.01$) (Fig. 3f). Administration of the dopamine D2 receptor agonist quinpirole (5 μM) for 20 minutes prior to HFS, and continuous throughout the experiment, reinstated eCB-LTD (two-way ANOVA $t = 15–40$ min vehicle versus nicotine: $F_{(1, 25)} = 0.58$, $P < 0.01$) (Fig. 3i), as did the unselective mAChR antagonist scopolamine (5 μM) ($F_{(1, 14)} = 0.03$, $P > 0.05$) (Fig. 3j).

DISCUSSION

The data presented here suggest that nicotine differentially affects eCB signaling *ex vivo* depending on previous exposure *in vivo*. In the nicotine-naïve brain, nicotine facilitates eCB-signaling and plasticity, whereas after subchronic exposure, tolerance develops to this facilitating effect, and, finally, a progressive impairment of eCB-induced LTD is established after protracted withdrawal from nicotine. We also show that even though plasticity is restored after 6 months of withdrawal, brief *in vivo* re-exposure is sufficient to disrupt eCB signaling again. Even though no causality can be established here, it is possible that nicotine-induced facilitation of eCB signaling and synaptic plasticity contributes to the addictive properties of nicotine and habitual nicotine use.

Perfusion of nicotine to drug-naïve slices potentiated eCB-LTD induced by HFS but not LTD induced by the CB1R agonist WIN55,212-2, indicating that nicotine facilitates eCB production or release, or reduces eCB metabolism. This is in line with previous findings from the ventral tegmental area showing enhanced levels of the eCBs arachidonoylethanolamide and 2-arachidonoyl-glycerol after nicotine self-administration (Buczynski *et al.* 2013). It is also in agreement with previous studies showing that antagonists targeting nAChRs on dopaminergic terminals impair eCB-signaling (Partridge *et al.* 2002). Inhibition of nAChRs on dopaminergic terminals has been postulated to reduce the activation of dopamine D2 receptors located on cholinergic interneurons, thereby increasing acetylcholine-release and mAChR activation, resulting in reduced postsynaptic calcium influx (Yan *et al.* 1997; Partridge *et al.* 2002). Elevated postsynaptic calcium is a prerequisite for eCB production (Adermark & Lovinger 2007a), and mAChRs exert a strong control over calcium influx in rat striatal neurons (Howe & Surmeier 1995; Wang *et al.* 2006) (Fig. 1i). Changes in cholinergic activity, putatively connected to changes in dopaminergic tone, may thus regulate calcium influx and thereby the threshold for eCB

signaling via mAChRs. This hypothesis is also supported by the data presented here showing that nicotine exposure was insufficient to facilitate eCB-LTD in slices pre-treated with dopamine D2 agonist or mAChR antagonist. Importantly, the firing frequency of striatal cholinergic interneurons has been shown to be reduced following nicotine administration *ex vivo* (Storey *et al.* 2016). Nicotine-induced suppression of cholinergic activity could thus reduce mAChR activation, thereby relieving the break on L-type calcium channels to allow postsynaptic calcium to reach the threshold required for eCB production (Howe & Surmeier 1995; Wang *et al.* 2006; Adermark & Lovinger 2007a). It should be noted, however, that there is still some debate as to where the dopamine D2 receptors required for eCB-LTD are located and whether they exert a direct or indirect control over medium spiny neuron excitability (Lerner & Kreitzer 2012). It is thus possible that the influence from cholinergic neurons lies upstream, rather than downstream from dopamine D2 receptor activation. Nevertheless, nicotine did not facilitate eCB-LTD in rats receiving repeated nicotine administration *in vivo*, indicating that tolerance to this effect develops over time. This finding could be related to long-lasting desensitization of nAChR and a blunted dopamine elevation in response to repeated nicotine administration, which has previously been reported in self-administrating rats using *in vivo* microdialysis (Rahman *et al.* 2004).

Following protracted withdrawal from nicotine, eCB-LTD was completely blocked for up to 3 months. This finding is partially supported by previous studies, showing impaired eCB-LTD and reduced levels of the eCB arachidonoylethanolamide in the dorsal striatum following repeated administration of nicotine (Gonzalez *et al.* 2002; Abburi *et al.* 2016). Impaired eCB-LTD was not restored by TC-2F suggesting that this finding is not caused by enhanced FAAH activity, but rather linked to a reduced postsynaptic release of eCBs. The progressive impairment of eCB signaling could be linked to elevated extracellular levels of acetylcholine, which has previously been shown in the ventral striatum after continuous nicotine-administration followed by withdrawal (Carcoba *et al.* 2014). In fact, a rise in extracellular levels of acetylcholine, coupled with a decrease in dopamine, has been reported during withdrawal from several different drugs of abuse, including nicotine (Avena & Rada 2012). Considering that eCB-LTD requires a tight temporal and spatial linkage between dopamine release with other events involved in the induction of plasticity (Calabresi *et al.* 2007), transformations in dopaminergic and cholinergic systems could thus underlie impaired eCB-LTD, not only after nicotine exposure but also for other drugs of abuse.

Drug addiction is a chronic relapsing disorder that involves progressive adaptations of cortico-striatal

networks (Yin, Ostlund, & Balleine 2008; Belin *et al.* 2009; Belin *et al.* 2013; Adermark *et al.* 2016), and the data presented here suggest that the eCB system might play a role in mediating these transformations. Even though no causal relationship with behavioral transformations was assessed in this study, it is possible that nicotine-induced facilitation of eCB signaling could promote neuroadaptations and contribute to the initial conditioning and motivational effects elicited by nicotine. In addition, impaired eCB signaling, as detected following protracted withdrawal, might lead to impaired memory consolidation and compromised behavioral adaptations, possibly influencing or affecting the recovery from addiction and re-learning during smoking cessation (Subramaniyan & Dani 2015). This hypothesis is partially supported by preclinical studies showing impaired learning and memory following prenatal or postnatal nicotine exposure (Han *et al.* 2014). Taken together, the eCB-system has been linked to several neurobiological processes related to nicotine dependence (Scherma *et al.* 2008), and our data show that nicotine produces long-lasting effects on eCB-LTD that could be important for the addictive properties of nicotine and for habitual nicotine use (Gerdeman *et al.* 2003). In addition, our data suggest that the signaling pathways that cause maladaptive plasticity are rapidly recruited during re-exposure. Future studies will define the behavioral adaptations associated with impaired eCB-LTD and show if restoring striatal eCB-signaling could have a significant impact on the treatment of nicotine addiction.

Author Contribution

L.A. was responsible for the concept of the study. L.A. performed the field potential recordings and wrote the manuscript. J.M. performed the *in vivo* drug treatment, behavioral experiments and analysis of this part of the data. A.L. assisted during electrophysiological and behavioral recordings. B.S. and M.E. contributed with scientific expertise, interpretation of results and revisions of the paper. All authors critically reviewed content and approved the final version for publication.

Acknowledgements

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