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Long-term application of cannabinoids leads to dissociation between changes in cAMP and modulation of $GABA_A$ receptors of mouse trigeminal sensory neurons

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ABSTRACT

Antinociception caused by cannabinoids may have a partial peripheral origin in addition to its central site of action. In fact, we have observed that anandamide selectively and reversibly inhibits $GABA_A$ receptors of putative nociceptive neurons of mouse trigeminal sensory ganglia via CB1 receptor activation to inhibit adenylyl cyclase and decrease cAMP with downstream posttranslational alterations. Since cannabinoids are often used chronically, we studied changes in cAMP levels and GABA-mediated currents of trigeminal neurons following 24 h application of anandamide (0.5 μM) or the synthetic cannabinoid WIN 55,212-2 (5 μM). With this protocol GABA responses were similar to control despite persistent fall in cAMP levels. Inhibition by WIN 55,212-2 of GABA effects recovered after 30 min washout and was not associated with changes in CB1 receptor expression, indicating lack of CB1 receptor inactivation and transient loss of negative coupling between CB1 receptors and $GABA_A$ receptors. The phosphodiesterase inhibitor rolipram (100 μM; 24 h) enhanced cAMP levels and GABA-mediated currents, suggesting $GABA_A$ receptors were sensitive to persistent upregulation via cAMP. While the adenylyl cyclase activator forskolin (1–20 μM) facilitated cAMP levels and GABA currents following 30 min application, this action was lost after 24 h in line with the drug limited lifespan. The PKA inhibitor PKI 14–22 (10 μM) increased cAMP without changing GABA currents. These data indicate that modulation of $GABA_A$ receptors by intracellular cAMP could be lost following persistent application of cannabinoids. Thus, these observations provide an insight into the waning antinociceptive effects of these compounds.

1. Introduction

Cannabinoid neurobiology is undergoing major growth as discussion on the therapeutic use of such compounds heralds important developments for a variety of clinical ailments (Cheer and Hurd, 2017). Endocannabinoids like anandamide (AEA) and synthetic derivatives induce their antinociceptive effects by binding G protein-coupled receptors (GPCRs) type 1 and 2 (CB1 and CB2) at multiple levels along pain pathways (Cravatt and Lichtman, 2004; Guindon and Hohmann, 2009), including inhibition of cAMP synthesis and subsequent activation of protein kinase A (PKA; Howlett et al., 2010). While CB1 is extensively present in the central nervous system to mediate pain-suppression and behavioral effects (Woodhams et al., 2017), such receptors are also found in sensory ganglia (Ahluwalia et al., 2000; Bridges et al., 2003; Hohmann and Herkenham, 1999; Mitrirattanakul et al., 2006; Price et al., 2003; Sañudo-Peña et al., 1999). Indeed, the endocannabinoid system inhibits the initiation of the nociceptive signaling in primary sensory afferents (Agarwal et al., 2007), and spinal processing (Strangman and Walker, 1999). Conversely, CB2 is mainly located to the immune system (Galiègue et al., 1995), and its expression in the nervous system, especially during inflammation and in neuropathic pain, is debated (Anand et al., 2008; Van Sickle et al., 2005; Wotherspoon et al., 2005).

We recently found that the acute activation of CB1 selectively

Abbreviations: AC, adenylyl cyclase; AEA, N-arachidonoylethanolamine or anandamide; CB1, type 1 cannabinoid receptor; CB2, type 2 cannabinoid receptor; GS, glutamine synthetase; DRG, dorsal root ganglion; FSK, forskolin; GABA, γ-aminobutyric acid; PKA, protein kinase A; PKI, PKA inhibitor 14–22; RLP, rolipram; SGCs, satellite glial cells; TG, trigeminal ganglion

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depresses gabazine-sensitive $GABA_A$ receptor mediated currents in mouse cultured trigeminal ganglion (TG) neurons (Eroli et al., 2018). Hayasaki and colleagues proposed that GABA mediates a cross-talk between TG neurons and the surrounding satellite glial cells (SGCs) (Hayasaki et al., 2006), which form a functional unit in the TG (Ceruti et al., 2011). Activation of $GABA_A$ receptors on sensory neurons, therefore, produces a depolarizing response that can be excitatory and pro-nociceptive *in vitro* (Ault and Hildebrand, 1994; Waite et al., 2000) and *in vivo* (Jang et al., 2017). As TG neurons are immunopositive for various $GABA_A$ subunits (Hayasaki et al., 2006), which contain consensus sites for phosphorylation mediated by PKA (McDonald et al., 1998; Moss and Smart, 1996), thus identifying a molecular target for CB1 receptor modulation of $GABA_A$ receptors.

Since CB1 are expressed by GABAergic neurons (Kano et al., 2009), the present study aimed at expanding our former observations on the early effects of CB1 on GABA currents by investigating whether CB1 (and CB2) might affect GABAergic signaling over a longer timespan. Our goal arose from the realization that chronic activation of CB1 receptors produces pharmacological tolerance in humans and animals (Martin et al., 2004; Sim-Selley, 2003), a phenomenon that *in vitro* is reported to develop after 5–30 min (Coutts et al., 2001; Hsieh et al., 1999; Rinaldi-Carmona et al., 1998) and can last *in vivo* for up to 12 days (Bass and Martin, 2000). Observational studies indicate that, should endocannabinoids be made readily available, their human or animal assumption persists for many hours (Lee et al., 2015). Little is, however, known whether habituation to the pain-killing effects develops within just one day and whether the peripheral (sensory ganglion) component remains unabated. At a cellular and molecular level, CB1 tolerance may be due to multiple mechanisms including desensitization, downregulation, and downstream signaling adaptation (Falenski et al., 2010; Martin et al., 2004; Sim-Selley, 2003). While most studies focused on CB1 turn-over and adaptation in the central nervous system, fewer examined the longer term effects of the spinal cord and the peripheral nervous system (Falenski et al., 2010; Fan et al., 1996; Rubino et al., 2000a; Sim-Selley, 2003). *In vitro* studies used a variety of different cell lines and cultures, leading to controversial results (Coutts et al., 2001; Grimsey et al., 2010; Hsieh et al., 1999; Keren and Sarne, 2003; Laprairie et al., 2014; Rinaldi-Carmona et al., 1998). We employed a model system of TG cultures to find out if 24 h application of CB1 agonists can change CB1 expression and function, and alter their cellular targets. It is well established that activated CB1 (and CB2) can inhibit the activity of adenylyl cyclase (AC) to decrease cAMP levels and the associated downstream signaling mediated by PKA (Howlett, 1985; Howlett et al., 2002; Howlett and Fleming, 1984). Thus, we assessed if the acute or sustained application of endocannabinoid agonists and antagonists impacted on the expression of CB1, their ability to inhibit cAMP concentration, and to negatively modulate $GABA_A$ receptors on nociceptive neurons. This approach offered also the opportunity to investigate whether raising cAMP concentrations had the opposite effects of endocannabinoid administration.

2. Materials and methods

2.1. Animals

Post-natal 14–15 day old (P14-15) or adult (≥30 days old) C57BL/6J mice were used in the present study. All procedures were conducted in agreement with the Italian Welfare Act and the European Communities Council Directive (2010/63/EU), and were approved by the ethical committee of the Scuola Superiore di Studi Avanzati (SISSA). All the efforts were made to minimize animal suffering.

2.2. Primary TG cultures

Standard primary cell cultures of mouse TG were prepared as described previously (Fabbretti et al., 2006; Simonetti et al., 2006). Terminally-anesthetized (i.p. urethane, 10% solution) mice were decapitated and their brain was removed to expose the trigeminal ganglia. Excised TG were mechanically dissected, and then underwent enzymatic digestion in F12 medium (Invitrogen), supplemented with 100U/ml penicillin, and 100 g/ml streptomycin, containing 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNase (Sigma) for 12 min at 37 °C. Dissociated ganglia were centrifuged for 3 min at 1400 rpm and the pellet was suspended in F12 medium, plus streptomycin/penicillin and 5% fetal bovine serum (FBS). Finally, cells were plated on poly-L-lysine (Sigma) coated Petri dishes, glass coverslips or multiwells. Cultured cells were kept in a controlled atmosphere of 5% CO_2 and 37 °C and always used 24 h after plating.

2.3. Drug treatments

All drug treatments were performed on 24 h TG cultures with parallel untreated controls (sham). For short or long treatments, drugs were diluted from stock solutions to their final concentrations in the culture medium and incubated at 37 °C as indicated in the Results. For acute application during electrophysiological tests, drugs were dissolved in physiological solution. We used either the CB1 endogenous agonist AEA or the synthetic cannabinoid agonist WIN 55,212-2 ((R)-4,5-dihydro-2-methyl-4(4-morphoinylmethyl)-1(1-naphthalenylcarbonyl)-6H-pyrrolo[3,2,1-ij]quinolin-6-one); Devane et al. (1992) at the concentration of 0.5 and 5 μM respectively, which were calculated as the IC_{50} values from former dose/response experiments on GABA currents and were found to target selectively $GABA_A$ receptors with no influence on TRPV1 receptors (Eroli et al., 2018). The CB1 antagonist AM251 (Gatley et al., 1996, 1997) was used at the concentration of 0.1 μM, which in previous studies resulted adequate to inhibit the action by AEA on GABA currents without changes in control responses (Baur et al., 2012; Lenkey et al., 2015; Eroli et al., 2018). Forskolin (FSK), a standard activator of AC (Dessauer et al., 2017; Seamon and Daly, 1981), was applied to TG cultures at 1 μM (D'Arco et al., 2009; Veeraraghavan and Nistri, 2015) or 20 μM (Eroli et al., 2018) concentrations for 30 min (Florio et al., 1999). In a separate set of experiments, TG cultures were incubated for 24 h with FSK to evaluate the persistence of its effects on cAMP levels and any associated cytotoxicity. Table 1 shows significant increase in cAMP induced by 30 min application of 1 μM or 20 μM FSK.

Rolipram (RLP; 100 μM), a selective phosphodiesterase inhibitor (Mourlevat et al., 2003), was applied to TG cultures at different time points (10, 30, 45, 60 min and 24 h) to evaluate possible uncoupling of $GABA_A$ function from the modulation by the cAMP system. Twentyfour h incubation with 100 μM RLP was also used to assess possible cytotoxicity. To evaluate the involvement of PKA in $GABA_A$ receptor modulation, we used the PKA inhibitor fragment 14–22 amide, myristoylated (PKI; P9115 Sigma) at the concentration of 10 μM (Rathee et al., 2002) for 30 min, alone or in combination with FSK, to investigate a potential negative feedback mechanism through which activated PKA inhibits AC (Chen et al., 1997; Defer et al., 2000; Iwami et al., 1995).

Table 1
FSK-mediated effects on cAMP levels.

	cAMP (pmol/μg)
SHAM	0.305 ± 0.0227
1 μM FSK	1.922 ± 0.254 ($p \leq 0.001$)
20 μM FSK	10.662 ± 2.336 ($p \leq 0.01$)

For each condition data are the mean from 3 wells run in duplicate. Paired *t*-test was used for analysis.

2.4. Immunostaining

Immunocytochemistry was performed as previously described (Vilotti et al., 2013) to assess the expression of CB1 and CB2. Cells were blocked in 5% bovine serum albumin (BSA), 5% horse serum or normal goat serum, 0.1% Triton X-100 in PBS 1x to eliminate unspecific binding. Samples were incubated with the following primary antibodies diluted in the blocking solution: anti-β-tubulin III (T2200, 1:1000, Sigma, rabbit), anti-glutamine synthase (MAB302, 1:500 (culture), 1:300 (slices), Millipore, mouse), anti-CB1 (K-15, 1:100, Santa Cruz, goat), anti-CB2 (AB5642P, 1:500, Millipore, rabbit). Immunoreactivity was detected using Alexa 594 or Alexa 488 (1:400, Molecular Probes Invitrogen) secondary antibodies. Samples were mounted on a microscope slide with a Vectashield Antifade Mounting Medium with DAPI (Vector Labs) to counterstain the nuclei. The specificity of anti-CB1 antibody has been previously validated in our lab (Veeraraghavan and Nistri, 2015). To validate the specificity of the CB1 primary antibody, we used its blocking peptide (supplied by the CB1 (K-15) antibody manufacturer) in 1:10 dilution at room temperature as instructed by the supplier. TG sections were then incubated in the neutralized mixture and processed as mentioned above. No CB1 staining was subsequently observed (Supplemental Fig. 1 A). Anti-CB2 antibody was validated by us by western blotting (Supplemental Fig. 1 B). Mouse spleen was used as positive control tissue (Galiègue et al., 1995), whereas HEK293 and mouse brain lysates were used as negative controls (Callén et al., 2012; Galiègue et al., 1995). CB2 protein was detected in the spleen, but not in the HEK293 or brain homogenates, neither it was found in TG lysates. Immunostaining of TG cultures or slices further confirmed lack of CB2 expression: indeed, β-tubulin III positive neurons and glutamine synthetase (GS) stained satellite glial cells (SGCs) were CB2 immunonegative, either in culture or *in situ* (Supplemental Fig. 1C and D).

Immunohistochemistry was performed on slices from adult mice to investigate the *in situ* expression of CB1 and CB2 as previously described (Vilotti et al., 2013). The same blocking solution used for immunocytochemistry was employed to block unspecific binding and dilute the antibodies. After 1 h blocking, slices were incubated overnight with primary antibodies: anti-β-tubulin III (1:1000), anti-glutamine synthetase (1:300), anti-CB1 (1:300), anti-CB2 (1:500). One h incubation in Alexa 594 or Alexa 488 secondary antibodies (1:400) followed. Finally, slices were stained with DAPI to counterstain nuclei. Images were collected with a Zeiss fluorescence microscope or with a Nikon confocal laser-scanning microscope, and processed using ImageJ software (National Institutes of Health, U.S.A; open source).

2.5. Immunoprecipitation, SDS-PAGE and western blot

Standard SDS-polyacrilamide gel electrophoresis (SDS-PAGE) and Western blot were performed as previously described (D'Arco et al., 2009; Fabbretti et al., 2004; Vilotti et al., 2013) to detect the expression of cannabinoid receptors in the entire TG, or in primary TG cultures. Samples were lysed in ice cold 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS, Sigma) lysis buffer (0.5% CHAPS, 50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol) supplemented with protease inhibitors (Sigma). Total protein content of TG primary cultures or intact ganglia was measured with the Bicinchoninic Acid kit for protein determination (BCA, Sigma) as indicated by the manufacturer's protocol. In each gel lane, 30 μg of protein extracts were loaded. Cell culture lysates were immunoprecipitated by incubation with 20 μL of Protein A/G magnetic beads (Pierce), plus 40 μL of CHAPS supplemented with protease inhibitors, plus 1 μL of anti-CB1 receptor antibody (ab23703, Abcam, rabbit) overnight at 4 °C. Non-immunoprecipitated controls of culture lysates were processed in parallel. We used the following antibodies: primary anti-CB1 antibody (1:500), horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:1000) or, for immunoprecipitated samples, anti-rabbit Jackson secondary antibody (1:10000, Jackson ImmunoResearch). The chemoluminescent signal was detected by incubating the immunoblots with LiteAblot Plus Enhanced Chemiluminescent Substrate (EuroClone, Milan) or ECL (Amersham, Little Chalfont, UK), and quantified using ImageJ. Signal intensity was normalized to the β-actin control detected with mouse anti-β-actin-HRP conjugated antibody (1:5000, Sigma). For initial CB1R or CB2 receptor expression tested with Western blotting, three independent experiments were done: each one included one petri dish with HEK293T cells, one with a brain lysate, one with a spleen lysate, and one obtained from two ganglia. Drug tests were based on n = 5 independent experiments, in each one of them six ganglia were pooled from three mice. Thus, pooled tissues were plated in three petri dishes that were used for control (sham), AEA- or AM251-treated.

2.6. Biotinylation of surface expressed receptors

For biotinylation experiments, cells were incubated with 1 mg/ml EZ-Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) as previously described in detail (Fabbretti et al., 2006). Pull-down of biotinylated proteins was obtained with Streptavidin agarose resin (Pierce) for 2 h at 4 °C according to the manufacturer's instructions. Samples were processed for Western blotting using the same CB1 antibody (1:1000) as detailed before. Positive control for biotinylation assay was obtained by checking the surface expression of the transferrin receptor detected with an antibody purchased from Santa Cruz Biotechnology (1:1000, Heidelberg, Germany). For control of correct gel loading, the expression of β-actin in the intracellular fraction was always checked. Tests were n = 5 independent experiments in each one of them six ganglia were pooled from three mice. Thus, pooled tissues were plated in three petri dishes that were used for control (sham), AEA- or AM251-treated.

2.7. cAMP ELISA assay

ELISA assay was performed to investigate the changes in the concentration of basal and FSK-induced intracellular cAMP in TG cultures. Each well contained lysates from two TGs and duplicate samples were run throughout. The assay was performed following the manufacturers' instructions (Abnova; http://www.abnova.com/products/products_detail.asp?catalog_id=KA0886) as previously reported (Veeraraghavan and Nistri, 2015; Vilotti et al., 2016). cAMP levels were calculated from the best fit curve obtained from serially diluted cAMP standard concentrations, and then normalized to the total protein concentration assayed with a DS-11 spectrophotometer (DeNovix) expressed as pmol/μg of protein. Data were usually obtained by averaging signals from 8 wells (harvested from 8 ganglia from 4 mice) for each experimental condition. Different concentrations of AEA (0.1, 0.5, 1, 5, 10 μM) were applied to TG cultures for 30 min, to build a dose/response curve of the effects exerted on basal cAMP levels (Fig. 1). The estimated IC_{50} of AEA to inhibit cAMP production was 0.3 μM, a concentration near 0.5 μM, that is the AEA IC_{50} responsible to depress GABA-evoked responses previously recorded in our lab (Eroli et al., 2018). Thus, we decided to use 0.5 μM AEA for all subsequent ELISA treatments. Supplemental Fig. 2 summarizes the protocols used for ELISA experiments. AEA (0.5 μM) was applied to cultured cells either alone for 30 min (acute application) or 24 h (sustained application), or, in certain experiments, together with 30 min FSK. Additionally, AEA was co-applied with 0.1 μM AM251 for 24 h and, for certain experiments, this application was followed by 1 μM FSK for 30 min. WIN-55,212-2 (5 μM) was applied either alone for 30 min or 24 h and, in certain experiments, together with 1 μM FSK (30 min). AM251 (0.1 μM) was applied alone for 24 h to evaluate the possible existence of a constitutive CB1 activity (Pertwee, 2005) in TG cultures or together with the other agents. Finally, Supplemental Fig. 2 indicates also the protocols for sustained application of FSK, the scheme for PKI application alone or together with FSK and the scheme for RLP application.

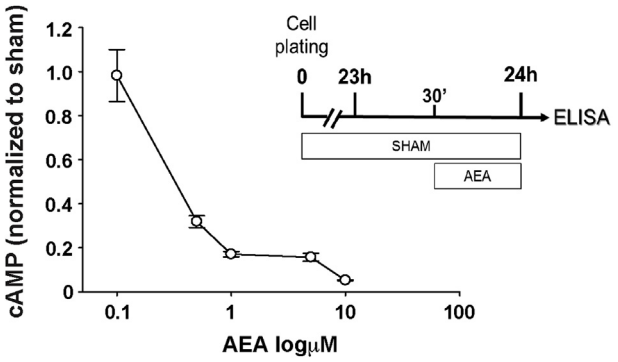

Fig. 1. AEA dose/response curve. The plot shows average cAMP levels quantified by ELISA assay and normalized to sham values. AEA (0.1, 0.5, 1, 5, 10 μM) was applied for 30 min. For each concentration, each datapoint is the mean (± SEM) from 8 wells. When not visible, error bars are within the symbol size.

2.8. Electrophysiology

Patch clamp experiments were performed as previously described in detail on small to medium somatic diameter (15–20 μm) trigeminal neurons believed to represent the main population of nociceptors (Hullugundi et al., 2013; Nair et al., 2010). These cells were superfused continuously (2 ml/min) with physiological solution containing (in mM): 152 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES; pH adjusted to 7.4 with NaOH), and were patch-clamped in the whole-cell configuration under voltage-clamp conditions. Their responses were filtered at 1 kHz using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), and acquired by means of a DigiData 1200 Interface and pClamp 8.2 software (Molecular Devices, Sunnyvale, CA, USA). The pipette resistance was 3–4 MΩ when filled with the following solution (in mM): 140 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 2 Mg_2ATP_3, 2 GTP, 10 HEPES, and 10 EGTA (pH adjusted to 7.2 with KOH). Cells were held at −65 mV holding potential after correcting for 3.5 mV liquid junction potential, as calculated with JP calculator of pClamp software. Recordings were performed on small- and medium-size neurons with a capacitance less than 22 pF: the large majority of these cells (about 90%) expressed functional $GABA_A$ receptors (Hullugundi et al., 2013) and correspond to P2X and TRPV1 sensitive nociceptors (Simonetti et al., 2006). To induce $GABA_A$ receptor currents, GABA (Tocris) was applied with 2 s pulses at the concentration of 10 μM to evoke half maximal responses (Eroli et al., 2018; Fabbro and Nistri, 2004; Vilotti et al., 2013) using a fast superfusion system (Rapid Solution Changer RSC-200; BioLogic Science Instruments, Claix, France). To investigate the effect of sustained endocannabinoid application on GABA-mediated currents, TG cultures were pre-incubated for 24 h with AEA or WIN 55, 212-2, then drugs were washed out with physiological solution and recording commenced with standard applications of GABA. Further tests involved applying WIN 55,212-2 (5 μM), RLP (100 μM) or PKI (10 μM) after about 30 min in physiological solution. FSK (30 min) was tested at 1 or 20 μM; only the higher concentration significantly enhanced GABA-mediated currents (29.16 ± 2.75 vs 38.36 ± 3.39 pA/pF, n = 8, p = 0.0001) while 1 μM was ineffective (22.6 ± 2.1 vs 22.4 ± 2.5 pA/pF; p = 0.8783; n = 6 cells). Further tests were, therefore, performed with 20 μM FSK. In each experiment drug-treated cultures were always compared with their sister cultures prepared on the same day, processed with identical procedure and taken as control (sham). Primary cultures display inherent variability in cell responses because the ganglion cell population is heterogeneous even when recording only from cells with a relatively narrow range of somatic diameter. Furthermore, intrinsic membrane resistance as well as intensity of receptor expression are not homogeneous, yielding different control values when applying exogenous agonists. Furthermore, the value of electrode resistance (albeit always small) conditions access resistance and series resistance, all factors that impact on response amplitude. Thus, it is not unexpected that absolute values of electrophysiological responses have a 10% standard error (see Results). For these reasons, we systematically run, in each set of experiments (and then for each treatment), their own internal controls for data comparison.

2.9. Chemicals

GABA, AEA, RLP and AM251 were purchased from Tocris (Bristol, UK); FSK and PKA inhibitor fragment 14–22 (P9115) were purchased from Sigma (Milan, Italy); WIN 55,212-2 mesylate was purchased from MedChemExpress (Sollentuna, Sweden). The stock solution of AEA was prepared with ethanol (final concentration less than 0.05%). Stock solutions of WIN 55,212-2, AM251, FSK and RLP were prepared by dissolving them in DMSO (final solvent 0.5% or less). GABA and PKA inhibitor stocks were prepared in distilled water.

2.10. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM), with n indicating the number of cells, animals, or independent experiments, as indicated in figure legends. Statistical analysis was performed using the Student's t-test or the Mann–Whitney rank sum test for parametric and non-parametric data respectively, according to the choice directed by the software (SigmaPlot and Systat Software Inc., San Jose, CA, USA). Paired *t*-test or Wilcoxon signed rank test were used for the analysis of parametric and non-parametric, paired sample data, respectively. GraphPad Prism 6 (San Diego, California, USA) software was used to calculate the best fit-curve to extrapolate the unknown cAMP concentrations in the ELISA assay. A p value ≤ 0.05 was accepted as an index of statistical significance.

3. Results

3.1. Effect of sustained application of AEA or WIN 55,212-2 on $GABA_A$-mediated currents

We have previously reported that transient (10–30 min) exposure to CB1 agonists significantly depressed GABA currents by approximately 50% (Eroli et al., 2018). The present voltage-clamp experiments investigated whether sustained (24 h) pretreatment with CB1 agonists remained effective to inhibit $GABA_A$-mediated responses (Eroli et al., 2018). Fig. 2 A and B show that, when compared with sham responses from parallel cell cultures, the size of GABA-evoked currents after 24 h exposure to AEA (0.5 μM) or WIN 55,212-2 (5 μM) was similar. Nevertheless, after 30 min washout of WIN 55,212-2, GABA currents were again inhibited by acute application of this agent (Fig. 2 C), suggesting that functional recovery of CB1 activity had occurred.

3.2. CB1 expression and stability under culture condition and drug application

The electrophysiological data raised a number of questions concerning the (reversible) loss of modulation of $GABA_A$ receptor-mediated responses when CB1 agonists were applied for 24 h. We first addressed the issue of any CB1 change after prolonged exposure to cannabinoids. As depicted in Fig. 3 A, in control condition TG neurons (labeled with the neuronal marker β-tubulin III, red) expressed the CB1 receptor (green) throughout dendrites, soma and axons: on average 83 ± 3% neurons were CB1 immunoreactive. No CB1 signal was detected in non-neuronal cells. We further confirmed the presence of the CB1 protein with western blotting of TG lysates (Fig. 3 B). Mouse brain tissue and human embryonic kidney 293 (HEK293) cells (Callén et al., 2012) were used as positive and negative controls, respectively, to confirm antibody specificity. Western blot analysis of CB1 expression revealed a

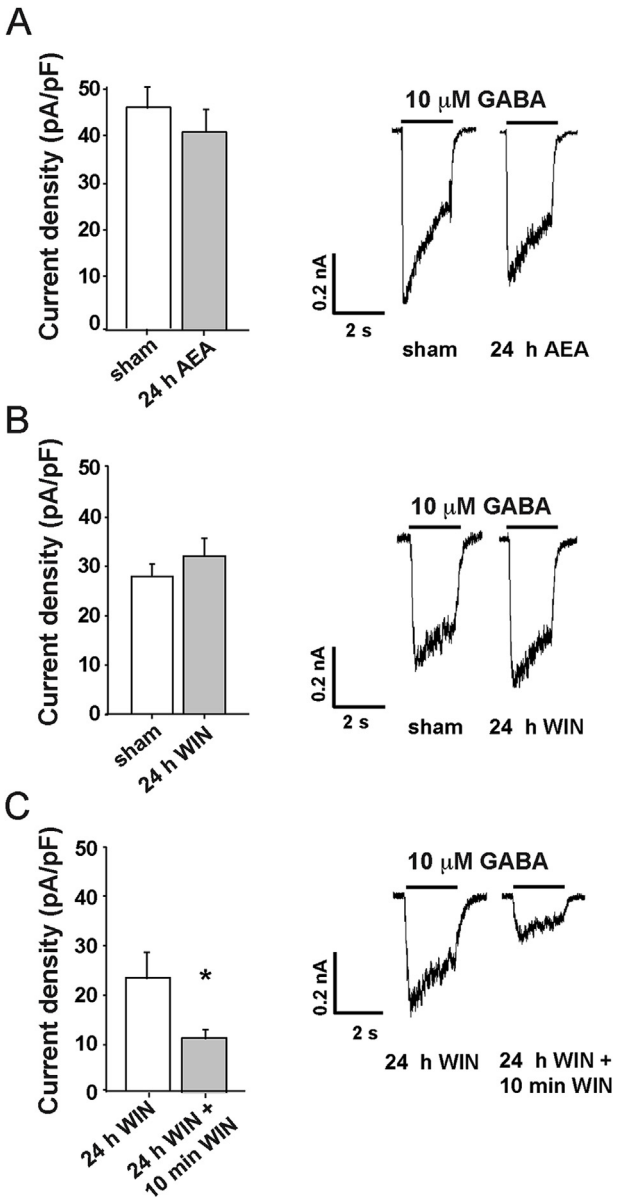

Fig. 2. Effect of sustained application of WIN 55,212-2 or AEA on $GABA_A$-evoked currents. **A**: histograms show average current density values of GABA-mediated responses induced by 2 s pulse application of GABA (10 μM) to sham neurons or to neurons after 24 h pre-treatment with AEA (0.5 μM); n = 26, 18 cells, respectively. On the right, examples of GABA currents in sham or after 24 h AEA. **B**: histograms show average current density values of GABA-mediated response induced by 2 s pulse application of GABA (10 μM) to TG neurons in sham or after 24 h pre-treatment with WIN 55,212-2 (5 μM); n = 40, 37 cells, respectively. On the right, representative traces of GABA responses in sham and after 24 h WIN 55,212-2. **C**: histograms show average current density values of GABA-mediated responses induced by 2 s pulse application of GABA (10 μM) to TG neurons from cultures pre-treated with WIN 55,212-2 (5 μM) for 24 h, or after a 24 h incubation with WIN 55,212-2 (5 μM) followed by washout and 10 min re-application of the same drug; n = 3, same cells; *p = 0.0264 vs 24 h WIN 55,212-2, paired Student's t-test. On the right, representative traces of GABA responses from 24 h WIN 55,212-2 treated neurons or 24 h WIN 55,212-2 + 10 min WIN 55,212-2 treated cells. Data are expressed as mean ± SEM.

major band of 53 kDa in brain samples, which corresponds to the non-glycosylated form of the receptor (Song and Howlett, 1995, Fig. 3 B). The 53 kDa band was expressed by TG lysates (Fig. 3 B, right lane), thus validating the presence of CB1 receptors in our system. The expression specificity was further supported by the absence of such a band in HEK293 cell lysates used as negative control (Fig. 3 B, left). To assess whether primary cultures of trigeminal ganglia retained the characteristics of the *in vivo* tissue, we also explored CB1 expression *in situ* by performing immunohistochemistry on slices from adult mouse TG. Fig. 3 C shows that CB1 extensively co-localized with β-tubulin III, while non-neuronal cells were CB1 immunonegative. Fig. 3 D indicates that CB1 expression was detected in small, medium and large sized neurons with predominance in medium size neurons that represent the largest neuronal population.

Immunocytochemistry showed that 24 h incubation of cultured TG with 0.5 μM AEA did not change the percentage of β-tubulin III labeled neurons with CB1 immunoreactivity (85 ± 2%), as compared to the untreated (sham) controls (83 ± 3%; see examples in Fig. 3E). Furthermore, prolonged exposure of cultured TG to 0.1 μM AM251 was ineffective in changing the percentage of CB1 positive neurons (88 ± 2% vs 83 ± 3%, AM251 treated and sham cultures, respectively) (Fig. 3 E). For these experiments, cell counts were performed over a total of n = 455 (sham), 352 (AEA) and 320 (AM251) neurons, from 6 (sham) or 7 (AEA, AM251) coverslips obtained from 3 mice per condition. We further performed western blotting of immunoprecipitated TG culture lysates to quantify whether the prolonged treatment with either AEA or AM251 would change the total amount of CB1 protein. As quantified in Fig. 3 F, there was no significant difference in receptor expression by sham or AEA (or AM251) treated cultures. Further tests to examine the compartmentalization of CB1 receptors with the biotinylation method demonstrated that the expression of membrane bound CB1 receptors was likewise unaffected by sustained application of AEA or AM251 (Fig. 3 G). We concluded that the expression of CB1 in culture was stable and not largely up or down-regulated by agonist/antagonist treatment. Thus, lack of cannabinoid-mediated inhibition of $GABA_A$ currents after long exposure to CB1 agonists was not apparently due to loss of CB1 expression.

3.3. Conserved function of CB1 in culture

Activated CB1 inhibit AC to decrease cAMP levels (Howlett, 1985; Howlett et al., 2002; Howlett and Fleming, 1984). This effect is a standard readout for CB1 functionality assessed under basal or FSK stimulated activity of AC. Application (30 min) of AEA (0.5 μM) or WIN 55,212-2 (5 μM) significantly decreased basal cAMP levels with the respect to the sham as shown in Fig. 4. Interestingly, 24 h applied AEA or WIN 55,212-2 similarly decreased cAMP concentrations (Fig. 4). The co-application of the selective CB1 antagonist AM251 (0.1 μM) with AEA for 24 h prevented depression of cAMP levels, confirming that the effects on AC were mediated by activating CB1 (Fig. 4). We also incubated TG cultures with AM251 for 24 h and observed a significant increase in cAMP concentration compared to sham (Fig. 4).

Fig. 5 compares the ability of cannabinoid agonists to modulate 30 min FSK-stimulated cAMP synthesis. Fig. 5 A shows that 1 μM FSK significantly enhanced cAMP levels and that this effect was suppressed when FSK was co-applied with AEA (0.5 μM) or WIN 55,212-2 (5 μM). Fig. 5 B indicates that 24 h incubation with AEA or WIN 55,212-2 was still effective in preventing the cAMP rise caused by subsequent (30 min) application of FSK, thus keeping this second messenger at concentration similar to sham. Furthermore, 24 h co-application of AM251 with AEA enabled full expression of the cAMP level stimulation by 30 min FSK (Fig. 5 B).

Fig. 5 C shows that, in analogy with the data with the cAMP assay, pretreatment with AEA for 24 h prevented the facilitation of the GABA-evoked currents by a short application of FSK. Overall, these findings indicate that acutely activated CB1 inhibited basal and FSK-enhanced production of cAMP, and that this effect was well preserved even when CB1 was treated with an agonist for 24 h. Lack of inhibition by sustained AEA application on GABA currents was not apparently due to loss of CB1 activity or cAMP insensitivity.

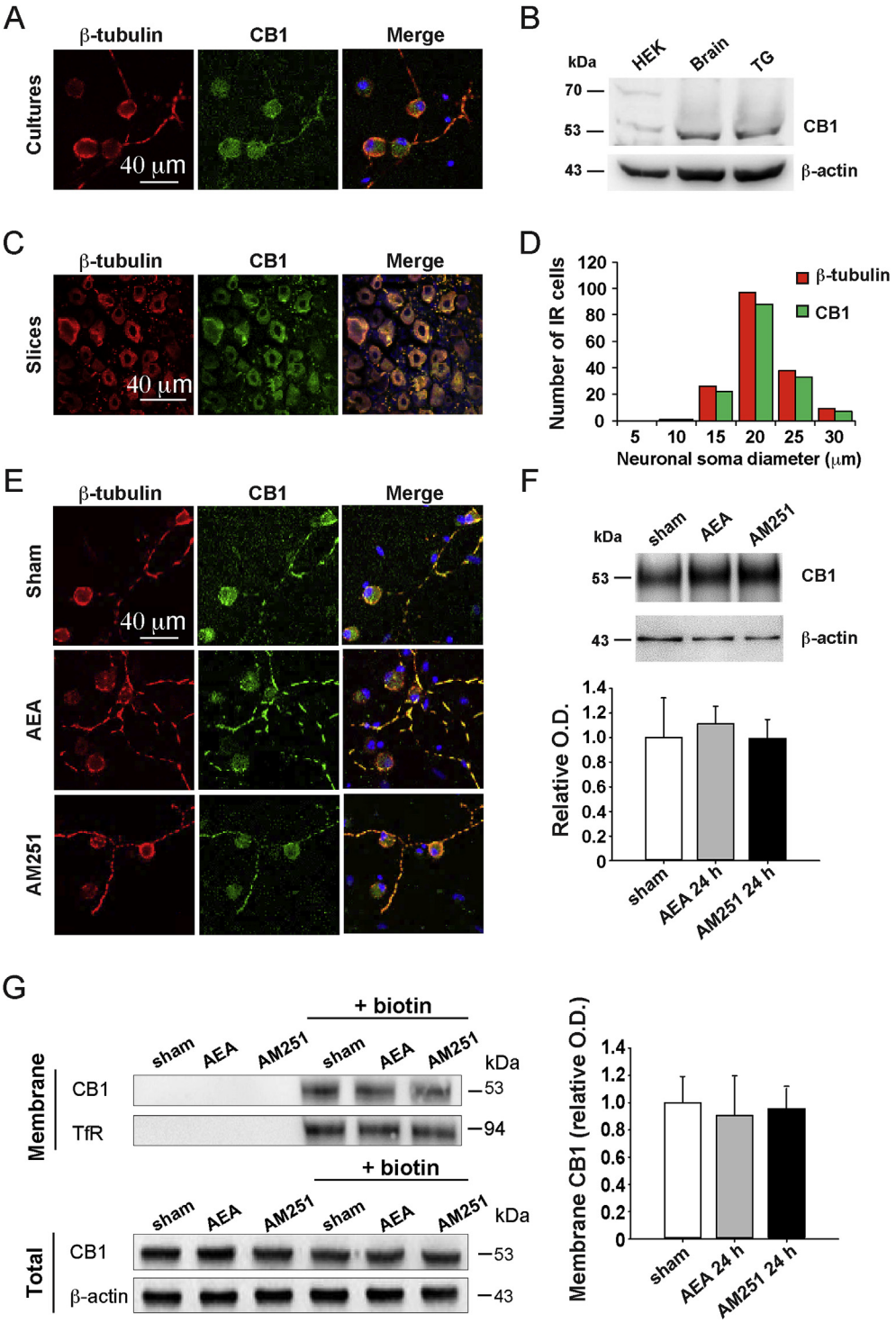

Fig. 3. CB1 receptor expression in mouse TG. A: representative images of CB1 receptors (green) co-localizing with β-tubulin III (red) in TG cultured neurons. Nuclei are counterstained with DAPI (blue); cell counts were performed over a total of n = 455 neurons from 6 coverslips obtained from 3 mice. **B**: representative western immunoblots show a 53 kDa band, corresponding to the non-glycosylated form of the CB1 protein (Song and Howlett, 1995), in brain (positive control) and TG lysates, with no expression in HEK cells (negative control). Lower lane shows actin loading controls; n = 2 mice. **C**: representative images of TG slices cut from P90 mice with extensive co-localization of CB1 receptors (green) with β-tubulin III (red) labeled neurons. Nuclei are counterstained with DAPI (blue). **D**: histograms show the distribution of the soma diameters of CB1 positive neurons in mouse TG slices; n = 171 neurons. **E**: representative images of sham (top row), 24 h AEA (0.5 μM, middle row), or 24 h AM251 (0.1 μM, bottom row) treated TG cultures. Nuclei are counterstained with DAPI (blue); cell counts were performed over a total of n = 455 (sham), 352 (AEA) and 320 (AM251) neurons, from 6 (sham) or 7 (AEA, AM251) coverslips obtained from 3 mice per condition: no significant difference was observed. **F**: representative immunoblots (top) and related quantification (bottom) of the amount of CB1 protein in sham, 24 h AEA (0.5 μM) or 24 h AM251 (0.1 μM) treated TG cultures, normalized to sham; samples were collected from 15 mice in 5 independent experiments. **G**: biotinylation experiments (see example on the left) show distribution of CB1 receptors at membrane level and in the whole lysate after 24 h (see protocol for data in **F**). Marker for membrane localization is the transferrin receptor (TfR). Note similar expression of membrane or whole cell CB1 receptor after sustained application of AEA or AM251. The bar graph (right) quantifies these data, n = 5 independent experiments. Data are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Modulation of AC activity

While acutely-applied FSK significantly enhances GABA responses (Eroli et al., 2018, Fig. 5 C), Fig. 6 A shows that 24 h pre-treatment with FSK left $GABA_A$-mediated currents similar to sham. This observation might have suggested uncoupling of the cAMP system from $GABA_A$ receptors. To clarify this issue, we next tested whether RLP, that increases cAMP levels via selective inhibition of the cAMP catalytic enzyme phosphodiesterases (Ye et al., 2001), could modulate GABA-evoked currents. Fig. 6 B shows that RLP (100 μM, 24 h pre-incubation) significantly enhanced GABA-evoked currents by an average of 29%.

Fig. 6 C shows that 24 h pre-treatment with FSK (20 μM) was not accompanied by a rise in cAMP levels and was not due to cytotoxicity, since no cell loss was observed as evaluated with the number of nuclei stained with DAPI (Fig. 6 D). Likewise, no neuronal loss was detected when neurons were immunostained for β-tubulin III (Fig. 6 E). Furthermore, acute application (30 min) of AEA after 24 h pre-treatment with FSK retained its ability to depress GABA-mediated currents (Fig. 6 F), suggesting that AC had remained sensitive to CB1 modulation. Finally, prolonged application of 100 μM RLP increased cAMP concentration (Fig. 6 G) and was not cytotoxic (Fig. 6D and E).

Hence, while TG cultures could persistently express high levels of

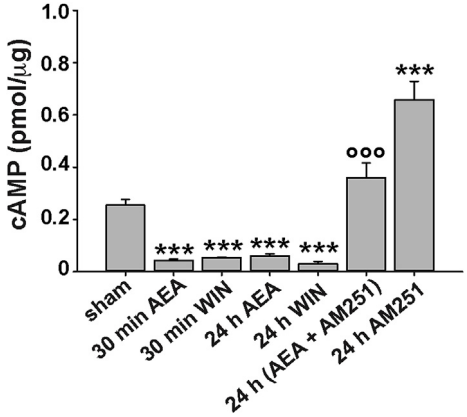

Fig. 4. CB1–mediated decrease of basal cAMP concentration. Histograms show average cAMP values after applying AEA (0.5 μM) or WIN 55,212-2 (5 μM) for 30 min or 24 h. Twenty-four h co-application of AEA with 0.1 μM AM251 prevents cAMP fall. CB1 shows constitutive activity, as indicated by the significant rise of cAMP in 24 h AM251 treated TG cultures; n = 12 mice from 4 independent experiments; ***p ≤ 0.001 vs sham, °p ≤ 0.001 vs 24 h AEA, Mann-Whitney Rank Sum test. Data are expressed as mean ± SEM.

cAMP in association with enhanced GABA currents, the disappearance of FSK effectiveness on cAMP and GABA responses seemed likely associated with loss of FSK intrinsic activity in accordance with previous reports on the time limited action of this compound (Puhar et al., 2008).

Because cAMP-activated PKA is not only an important effector of $GABA_A$ subunit phosphorylation but it also exerts negative feedback on cAMP synthesis (Chen et al., 1997; Defer et al., 2000; Iwami et al., 1995), we tested the effect of the PKA inhibitor 14–22 (PKI; 10 μM; 30 min). Thus, while 30 min application of PKI elicited a modest increase in cAMP levels (Fig. 7A), Fig. 7 B shows that 30 min co-application of PKI and FSK strongly increased cAMP levels in TG cultures as compared to the FSK control. Interestingly, despite the superadditive effect of PKI and FSK, no potentiation of GABA currents was observed (Fig. 7 C), probably because PKI prevented the PKA-mediated phospshorylation of $GABA_A$ receptors subserving the current enhancement.

4. Discussion

The principal result of the present study is the demonstration that sustained application of CB1 agonists could not inhibit $GABA_A$ receptors of putative nociceptive neurons in contrast with the previously demonstrated GABA inhibition following short term application of the same agents (Eroli et al., 2018). This phenomenon was apparently distinct from mere desensitization of CB1 receptors because their inhibition of cAMP synthesis persisted and raised the question of any longterm impact of this second messenger on $GABA_A$ receptor activity. Our data add new complexity to the issue of CB1 modulation of $GABA_A$ receptor mediated responses because enhancement (Li et al., 2009) or inhibition (Eroli et al., 2018; Kang-Park et al., 2007) has been previously reported. It is, however, noteworthy that follow-up of GABA responses after prolonged administration of cannabinoids has not been extensively investigated before. This is somewhat surprising as, for instance, frequent cannabis smokers who are given free access to cannabis under controlled laboratory conditions, can smoke up to 16 cigarettes/day (Lee et al., 2015), thus creating a condition for prolonged interaction of CB1 receptors and neurotransmitters, including GABA.

4.1. CB1 maintain expression and functionality after prolonged exposure to AEA or WIN 55,212-2

As reported by several studies (Angelotti et al., 1993; Connelly et al., 2013; Kapur and Macdonald, 1996; Vithlani et al., 2011) and confirmed by our previous experiments (Eroli et al., 2018), activation of AC and cAMP synthesis enhances responses evoked by GABA. While the current sustained application of cannabinoid agonists may not mimic the pattern of repeated human self-administration of cannabinoids, it

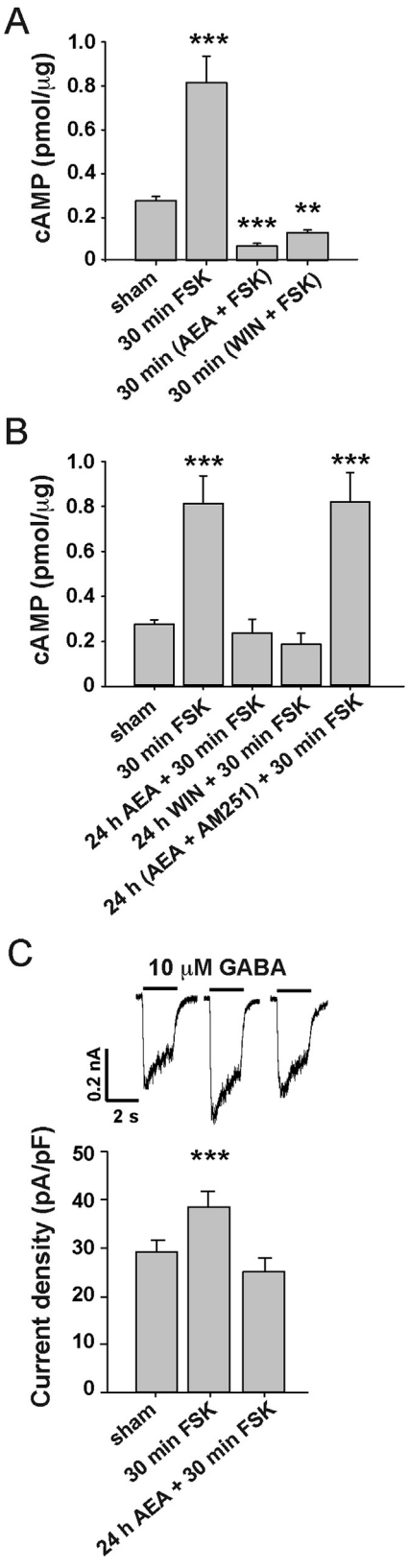

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Fig. 5. CB1–mediated inhibition of FSK–induced cAMP production and enhancement of GABA–evoked responses. A: histograms show average cAMP levels quantified with ELISA assay. Thirty min applied FSK (1 μM) increases cAMP, an effect prevented by the acute co-application of AEA (0.5 μM) or WIN 55,212-2 (5 μM); **p ≤ 0.01, ***p ≤ 0.001 vs sham, Mann-Whitney Rank Sum test. **B**: histograms show average cAMP levels quantified with ELISA assay. Twenty-four h pre-application of either AEA or WIN 55,212-2 inhibits FSK-induced cAMP increase, an effect prevented when AEA was applied together with AM251 (0.1 μM); n = 15 mice from 5 independent experiments; ***p ≤ 0.001 vs sham, Mann-Whitney Rank Sum test. **C**: histograms show average current density values of GABA-mediated responses induced by 2 s pulse application of GABA (10 μM) to TG neurons treated with FSK (20 μM, 30 min) or AEA (0.5 μM, 24 h) followed by 30 min FSK. Note that pre-treatment with AEA prevents FSK-induced increase of GABA-evoked responses; n = 8, 8, 9 cells; ***p = 0.0001 vs sham, Student's t-test. Inset shows representative traces of GABA-mediated currents in sham, 30 min FSK and 24 h AEA + 30 min FSK treated TG cultures, respectively. Data are expressed as mean ± SEM.

has been reported that, after experimental lesion to the nervous system, the level of endocannabinoids is persistently increased (up to 24 h; Garcia-Ovejero et al., 2009), a phenomenon suggested to occur also in other pathological conditions like neuroinflammation and neurodegeneration (Bisogno and Di Marzo, 2007). Furthermore, cannabinoid metabolites can linger for many hours or even days in humans with potential for sustained CB1 activity (Schlienz et al., 2018).

Thus, the present electrophysiological data show that after 24 h incubation of TG cultures with AEA or the more stable cannabinoid agonist WIN 55,212-2 GABA-evoked responses were similar to control, even if such cannabinoid inhibition was restored after prolonged washout. The present data, therefore, differ from a previous report on cultured hippocampal neurons that showed downregulation of CB1 and $GABA_A$ receptor expression after 24 h cannabinoid application (Deshpande et al., 2011). While there is no evidence for a direct protein-protein interaction between CB1 and $GABA_A$ receptors (unlike the $GABA_B$ and CB1 receptor interaction; Cinar et al., 2008), our data suggested that loss of effect on GABA currents might point to possible uncoupling between CB1 receptors and AC, or between AC and $GABA_A$ receptors. The simplest explanation would have been that CB1 receptors became inactivated due to desensitization, internalization or transcriptional downregulation as reported by previous studies (Martin et al., 2004; Sim-Selley, 2003). Nevertheless, immunocytochemistry and western blotting showed that, despite the 24 h application of AEA, CB1 immunopositive TG neurons, which represented about the 80% of the neuronal population, did not change the amount of the expressed protein globally or at membrane level. Previous *in vitro* (Coutts et al., 2001; Hsieh et al., 1999) and *in vivo* (Rubino et al., 2000a) studies have shown that AEA is less efficacious in promoting CB1 internalization and degradation, when compared to other synthetic cannabinoids like WIN 55,212-2. Nonetheless, in our experiments, prolonged application of AEA or WIN 55,212-2 yielded similar loss of inhibition of GABA responses, yet remained efficacious to inhibit AC even in the presence of FSK (via an AM251-sensitive mechanism) as much as when these drugs were applied for 30 min. Thus, on mouse TG neurons in culture, inactivation of CB1 receptors appeared to be minimal and inconsistent to explain the current findings.

The question of specificity of the CB1 receptor antibody has been a vexed issue (Grimsey et al., 2008; Morozov et al., 2013) and requires caution when interpreting data. In accordance with Saper's suggestions to establish antibody specificity and related Barry's criteria (Saper, 2009; Burry, 2011), our present experiments have shown that the CB1 antibody was correctly immunoblotted for a protein of the expected mw (Fig. 3 B), and that in immunohistochemical tests the antibody staining was fully prevented by coapplication of its blocking peptide (Suppl Fig 1 A). Furthermore, the latter experiments indicate that there was no autofluorescence in the cells (see Supplemental Fig 1 A) and previous studies have demonstrated that the secondary antibodies alone do not produce a fluorescent signal (Vilotti et al., 2016). Importantly, our recent data have shown that acute application of the CB1 agonist AEA induces a rapid depression of GABA-mediated current, a phenomenon prevented by the CB1 antagonist AM251 (Eroli et al., 2018): this effect, therefore, supports the expression of CB1 receptors by TG neurons.

Interestingly, 24 h applied AM251 significantly increased cAMP levels: because CB1 may show constitutive activity due to their G-protein coupling in the absence of any agonist (Losonczy et al., 2004; Pertwee, 2005), this phenomenon suggests that AM251 acted as an inverse agonist on basal CB1 activity. As indicated by Howlett et al. (2011), caution should be exerted to assume constitutive activity in the presumed absence of cannabinoid agonists that might actually be endogenously present even in minute amounts. Studies of recombinant $GABA_A$ receptors expressed by oocytes have shown that AM251 can act directly on $GABA_A$ receptors as positive allosteric modulator (Baur et al., 2012): this phenomenon is, however, absent at the 0.1 μM concentration (Baur et al., 2012).

4.2. AC activity and $GABA_A$ receptors

It is well established that cAMP-dependent modulation of $GABA_A$ receptors is an important mechanism to regulate the operation of this neurotransmitter (McDonald et al., 1998; Moss and Smart, 1996), implying a dual modulation by cAMP on GABA-mediated currents depending on the intracellular level of this second messenger. In particular, cAMP-stimulated PKA activity is believed to phosphorylate the serine 408 and 409 of the β3 subunit amply expressed in ganglia (Maddox et al., 2004), including TG (Hayasaki et al., 2006; Kramer and Bellinger, 2013), and to enhance GABA receptor function (McDonald et al., 1998). The role of β3 subunits appears to facilitate the targeting of α subunits to the cell membrane and their relocation (Connolly et al., 1996; Ives et al., 2002), thus conferring response stability (Kanematsu et al., 2006). The longterm effects of these mechanisms had not, however, been extensively investigated, at least at sensory ganglion level. Thus, it was not anticipated that, despite significant fall in intracellular cAMP concentration after long application of cannabinoids, GABA receptor mediated responses were similar to control. Because prolonged application of RLP enhanced cAMP and GABA response, it appeared that the link (at least in the positive modulatory direction) between these two effects was not necessarily labile over time. We propose that continuous depression of PKA activity by CB1Rs would be compensated by upregulation of PKC activity to bring back GABA receptor function to basal level. An alternative hypothesis is that, although PKA remains one key effector of $GABA_A$ receptor phosphorylation and its facilitation (Angelotti et al., 1993; Kapur and McDonald, 1996; McDonald et al., 1998), this enzyme also exerts a negative feedback over further cAMP synthesis (Chen et al., 1997; Defer et al., 2000; Iwami et al., 1995), thus leading to self-control over excessive production of this second messenger. Hence, the PKA inhibitor PKI 14–22 strongly synergized with FSK to increase cAMP, yet it prevented a change in GABA currents probably because it inhibited the phosphorylation process responsible for it.

In the case of 24 h FSK, lack of facilitation of GABA-evoked currents was associated with no significant change in the cAMP level, despite robust increment in both parameters when FSK was applied for just 30 min. The loss of FSK effect accords with previous studies showing that FSK-mediated increase in cAMP concentration peaks after 30–45 min, and largely fades with a variable time-course depending on the cells examined (Florio et al., 1999; Puhar et al., 2008) perhaps because of negative feedback mechanisms based on phosphodiesterase hyperactivity (Rochais et al., 2004), and/or limited stability of FSK in solution (Yamamura et al., 1991; Wang et al., 2016). Lack of FSK action did not depend on any cytotoxic effect. Although we observed that the vast majority of small-medium size TG neurons express $GABA_A$ and CB1 receptors, future studies are required to find out if the modulatory role of cannabinoids is preferentially exerted on a distinct subgroup of

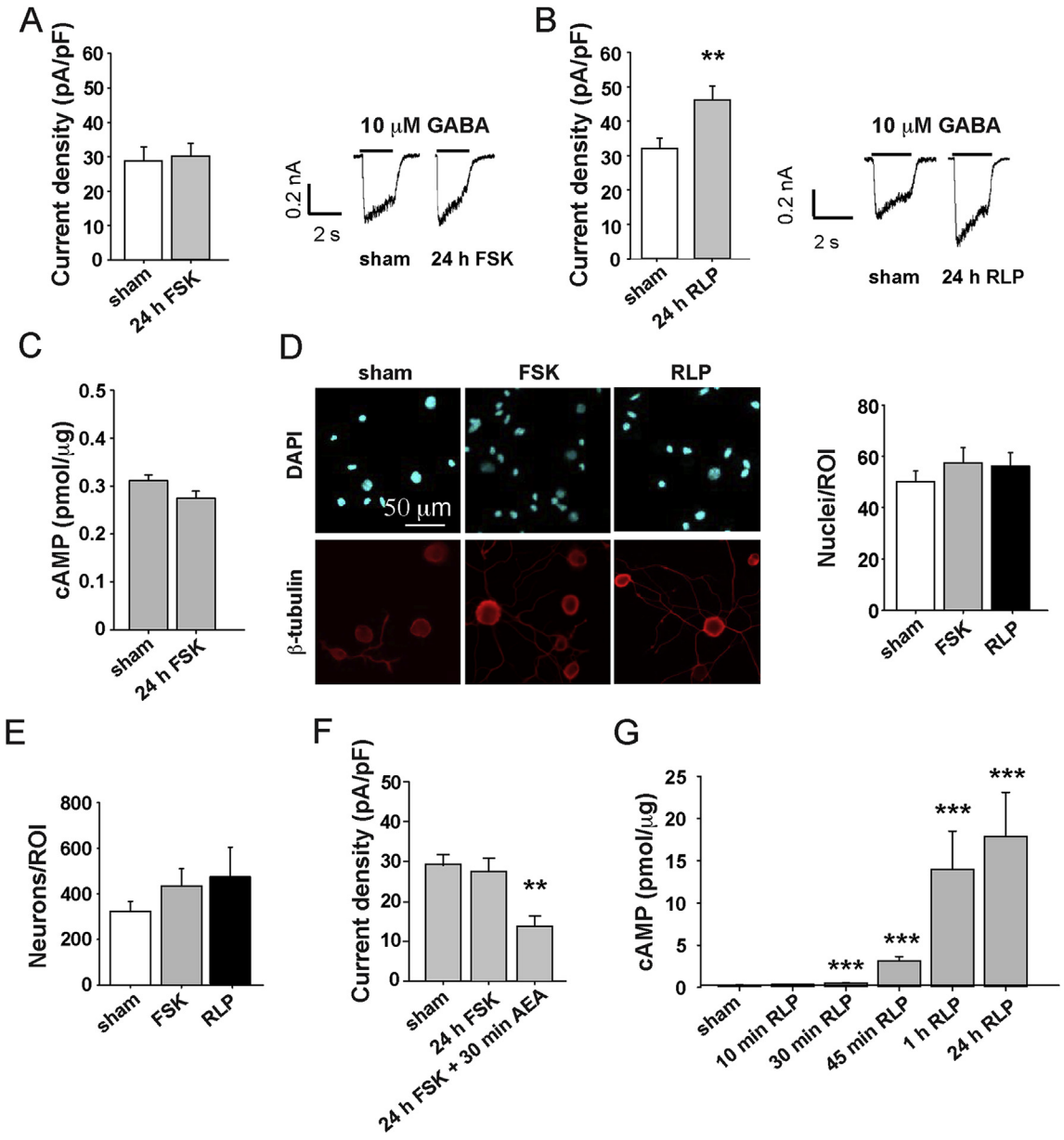

Fig. 6. Effects of sustained FSK or RLP application to TG neurons. A: histograms show average current density values of GABA-mediated responses induced by 2 s pulse application of GABA (10 μM) to sham or FSK (20 μM, 24 h) treated TG neurons; n = 32, 34 cells, respectively (sample records are shown on the right). **B**: histograms show average current density values of GABA-mediated responses induced by GABA (10 μM) on neurons treated with RLP (100 μM, 24 h; sample records are on the right); n = 49, 52 cells, respectively; **p = 0.007 vs sham, Mann-Whitney Rank Sum test. **C**: histograms show average cAMP levels in sham or FSK (20 μM, 24 h) treated TG cultures; n = 6 mice from 2 independent experiments. **D**: representative images of TG cultures treated with 24 h FSK (20 μM) or RLP (100 μM). Red pseudocolor indicates β-tubulin III, while blue shows DAPI staining. Average number of DAPI-stained nuclei in a region of interest (ROI, 600 μm × 450 μm) of sham, FSK (20 μM, 24 h) or RLP (100 μM, 24 h) treated TG cultures is shown in bar graph; counts were performed on n = 904, 1035 and 842 total nuclei. Average number of β-tubulin III positive neurons (**E**) in a ROI (7 mm × 6.750 mm); counts were performed on n = 1943, 2610 and 2370 neurons. All data are from 6, 6, and 5 coverslips from 3 mice per condition (sham, FSK, RLP) respectively. **F**: histograms show average current density values of responses induced GABA (10 μM) on sham, FSK (20 μM, 24 h) or FSK (24 h) + AEA (0.5 μM, 30 min) treated neurons; n = 8, 5, 5 cells, respectively; **p ≤ 0.01 vs 24 h FSK, Student's t-test. **G**: histograms show average cAMP levels in sham or RLP (100 μM) treated TG cultures. The sustained increase of cAMP concentration induced by RLP persists even at 24 h; n = 9 mice from 3 experiments; ***p ≤ 0.001 vs sham, Mann-Whitney Rank Sum test. Data are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$GABA_A$ receptor expressing a certain subunit composition.

4.3. A scheme to account for the regulation of $GABA_A$ receptors

In keeping with previous studies (Angelotti et al., 1993; Kapur and McDonald, 1996; McDonald et al., 1998), we propose that, in trigeminal sensory ganglia, over a short time span, the activity of $GABA_A$ receptors was controlled, in a positive or negative fashion, by the intracellular cAMP concentration. Nonetheless, the link between cAMP and GABA responses was lost after longterm cannabinoid application. It seems unlikely that genomic changes in $GABA_A$ receptor structure had occurred because the cannabinoid effect was reversible after 30 min wash. One possibility is that increased turnover of GABA receptors during sustained depression of cAMP might have led to membrane expression of distinct receptor assemblies less sensitive to cAMP and PKA mediated inhibition: while testing this hypothesis requires future,

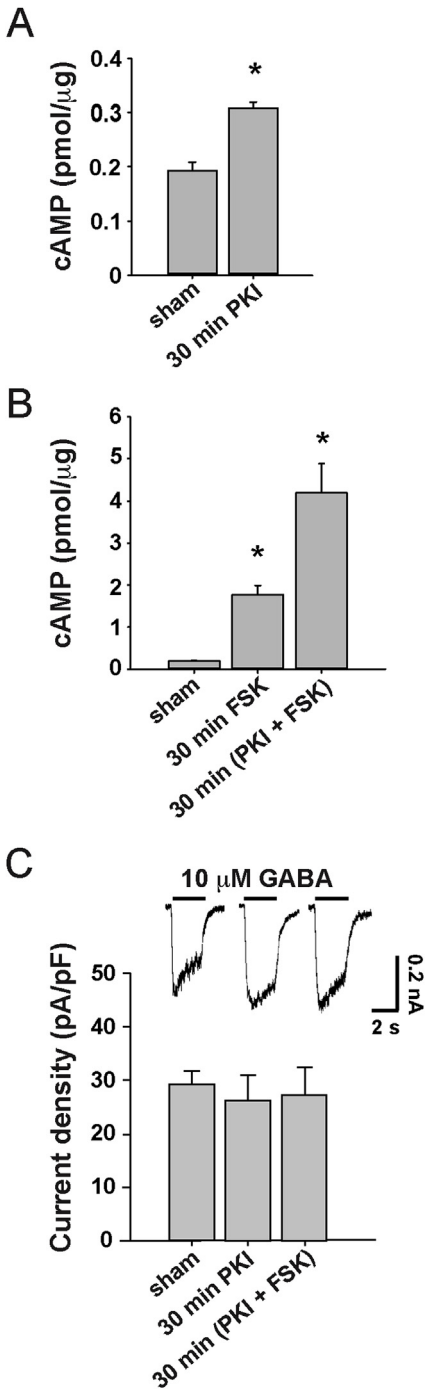

Fig. 7. Effects of PKI on cAMP levels and GABA-evoked currents. A: histograms show average cAMP levels in sham or PKI (10 μM, 30 min) treated TG cultures; n = 3 mice; *p ≤ 0.05 vs sham, Wilcoxon-Signed Rank test. **B**: histograms show average cAMP levels in sham, FSK (1 μM, 30 min) or PKI (10 μM, 30 min) + FSK (1 μM, 30 min) treated TG cultures. Note the superadditive effect of PKI and FSK in enhancing cAMP production; n = 3 mice; *p ≤ 0.05 vs sham, Wilcoxon-Signed rank test. **C**: histograms show average current density values of GABA-mediated responses of sham, PKI (10 μM, 30 min) or (PKI + FSK (20 μM)) 30 min treated neurons; n = 8, 8, 8 cells, respectively. At the top, representative traces of GABA-induced currents in sham, 30 min PKI or 30 min (PKI + FSK). Data are expressed as mean ± SEM.

dedicated studies, it is worth mentioning that the GABA responses generated by neurons after prolonged application of AEA or WIN 55,212-2 were similar to control, implying that there was no apparent change in the receptor functionality. Another possibility is that PKA possessed intrinsic activity independent from cAMP (Ji et al., 2012; Kohr et al., 2010) to support $GABA_A$ receptor phosphorylation. Further work will be necessary to clarify these issues.

5. Conclusions

Cannabis has been used for centuries by humans for its analgesic properties. However, chronic consumption of cannabinoids leads to the development of tolerance to their effects, and may produce dependence in humans and animals (Lichtman and Martin, 2005; Sim-Selley, 2003). For example, mice develop tolerance to cannabinoid antinociceptive effects after 1 d (Bass and Martin, 2000). Likewise, rapid antinociceptive tolerance has also been documented in rats (Rubino et al., 2000b). This phenomenon implies cellular and molecular adaptation at the level of cannabinoid receptors and their downstream effectors (Lichtman and Martin, 2005; Martin et al., 2004; Sim-Selley, 2003) even if the underlying processes are incompletely understood particularly as far as the central nervous system is concerned. Because part of the analgesic action of cannabinoids is mediated by peripheral nociceptors (Agarwal et al., 2007), our data suggest that, at least after a 24 h exposure to cannabinoids, in mouse TG the CB1 receptor expression and function remained unscathed. This observation, while excluding adaptive mechanisms in the AC/cAMP/PKA system over this timeframe, points to compensatory changes in $GABA_A$ receptor function that became reversibly insensitive to depression by cannabinoids or cAMP.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.01.007.

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