Cannabinoid type 2 receptors mediate a cell type-specific self-inhibition in cortical neurons

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Abbreviations: CB1R and CB2R, cannabinoid receptors type 1 and 2; SSI, slow self-inhibition; GIRK channel, G protein-coupled inward-rectifying K+ channel; GPCR, G protein-coupled receptors; DSI, depolarization-induced suppression of inhibition; DSE, depolarization-induced suppression of excitation; CNS, central nervous system; APs, action potentials; 2-AG, 2-arachidonoylglycerol; LTS, low–threshold spiking interneurons; NBC, sodium/bicarbonate co-transporter; PC, pyramidal cell; RSNPC, regular spiking non-pyramidal cell; FS, fast spiking interneuron; ACSF, artificial cerebrospinal fluid; $V_{m}$, membrane potential; NE, noladin ether;
Abstract

Endogenous cannabinoids are diffusible lipid ligands of the main cannabinoid receptors type 1 and 2 (CB₁R and CB₂R). In the central nervous system endocannabinoids are produced in an activity-dependent manner and have been identified as retrograde modulators of synaptic transmission. Additionally, some neurons display a cell-autonomous slow self-inhibition (SSI) mediated by endocannabinoids. In these neurons, repetitive action potential firing triggers the production of endocannabinoids, which induce a long-lasting hyperpolarization of the membrane potential, rendering the cells less excitable. Different endocannabinoid receptors and effector mechanisms have been described underlying SSI in different cell types and brain areas. Here, we investigate SSI in neurons of layer 2/3 in the somatosensory cortex. High-frequency bursts of action potentials induced SSI in pyramidal cells (PC) and regular spiking non-pyramidal cells (RSNPC), but not in fast-spiking interneurons (FS). In RSNPCs the hyperpolarization was accompanied by a change in input resistance due to the activation of G protein-coupled inward-rectifying K⁺ (GIRK) channels. A CB₂-specific agonist induced the long-lasting hyperpolarization, whereas preincubation with a CB₂-specific inverse agonist suppressed SSI. Additionally, using cannabinoid receptor knockout mice, we found that SSI was still intact in CB₁-deficient but abolished in CB₂-deficient mice. Taken together, we describe an additional SSI mechanism in which the activity-induced release of endocannabinoids activates GIRK channels via CB₂Rs. These findings expand our knowledge about cell type-specific differential neuronal cannabinoid receptor signaling and suggest CB₂-selective compounds as potential therapeutic approaches.
Abstract

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1 Introduction

The endocannabinoid system is one of the main neuromodulatory systems in the vertebrate central nervous system (CNS). Endocannabinoids are membrane-derived lipid molecules that mainly, albeit not exclusively, exert their effects by acting via G protein-coupled receptors (GPCR) (Kano, 2009). While CB₁Rs are one of the most widely expressed GPCRs in the CNS, CB₂Rs were traditionally referred to as peripheral endocannabinoid receptors, since their expression was primarily detected in cells of the immune system (Munro et al., 1993).

The best-studies effects of endocannabinoids in the CNS are two forms of short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and excitation (DSE) (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). In both DSI and DSE, endocannabinoids are produced by the postsynaptic cell and retrogradely activate presynaptic CB₁Rs. Further, numerous forms of CB₁R-dependent synaptic long-term plasticity have been described, with endocannabinoids being involved in both long-term potentiation (Gómez-Gonzalo et al., 2015; Maglio et al., 2017; Wang et al., 2016) and long-term depression (Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Safo and Regehr, 2005; Sjöström et al., 2003). However, in recent years several publications have provided functional evidence for the presence of CB₂Rs in cells of the CNS, where CB₂Rs exert inhibitory effects (García-Gutiérrez et al., 2013, 2012; Gong et al., 2006; Kim and Li, 2015; Onaivi, 2007; Stempel et al., 2016).

In addition to their synaptic effects, a plethora of non-synaptic and cell-autonomous forms of endocannabinoid modulation exists (Bacci et al., 2004; den Boon et al., 2012; Stempel et al., 2016). For example, layer 2/3 pyramidal neurons and layer 5 low-threshold spiking (LTS) interneurons in the somatosensory cortex express a CB₁R-dependent form of self-inhibitory plasticity, namely SSI (Bacci et al., 2004; Marinelli et
In both cell types, trains of action potentials (APs) were described to induce the production of the endocannabinoid 2-arachidonoylglycerol (2-AG) and subsequent CB₁R activation within the same cell. This leads to a G protein-dependent opening of GIRK channels, which hyperpolarizes the cell. In contrast, in hippocampal CA3 pyramidal cells, activation of CB₂Rs has been shown to cause a long-lasting hyperpolarization of the cells that alters local network rhythms (Stempel et al., 2016). This form of self-inhibition is phenotypically similar to cortical SSI but depends on the downstream modulation of the sodium/bicarbonate co-transporter (NBC).

At present, it is not known if SSI in different types of cortical neurons exclusively depends on CB₁Rs or whether CB₂Rs may also contribute to its induction. Furthermore, it is not clear exactly which cell types are capable of inducing SSI and by which mechanism it is implemented.

In this study, we investigate cell-autonomous SSI in neocortical neurons of the mouse somatosensory cortex layer 2/3. We show that trains of APs evoke a long-lasting hyperpolarization in pyramidal cells (PCs) and regular spiking non-pyramidal cells (RSNPCs), but not in fast spiking interneurons (FS). In RSNPCs, this self-inhibition is exclusively mediated by activation of CB₂R, demonstrated both by pharmacological tools and knockout (KO) mice, and leads to a hyperpolarization via activation of GIRK channels. Our findings add to the understanding of the highly complex function of the endocannabinoid neuromodulatory system, and provide additional evidence for functional expression of CB₂R in the CNS.
2 Materials and Methods

2.1 Ethical Statement and Animal Handling

Animal husbandry and experimental procedures were performed in accordance with the guidelines of local authorities (Berlin, Germany), the German Animal Welfare Act, and the European Council Directive 86/609/EEC. CB₁R- and CB₂R-deficient mice (Buckley et al., 2000; Zimmer et al., 1999) were maintained on a C57BL/6n genetic background, and homozygous KO mice and their wild-type (WT) littermates were obtained from heterozygous breeding. Animals were housed on a 12:12h reversed day-night cycle with food and water ad libitum.

2.2 Preparation of brain slices

Coronal slices were prepared from the somatosensory cortex of C57BL6/n mice, CB₁R or CB₂R KO mice and their WT-littermates aged postnatal day 21-35. Animals were anesthetized with isoflurane and decapitated. Brains were removed and transferred to ice-cold sucrose-based artificial cerebrospinal fluid (sACSF) containing in mM: 87 NaCl, 26 NaHCO₃, 50 sucrose, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 3 MgCl₂, 0.5 CaCl₂. Tissue blocks were mounted on a vibratome (Leica VT 1200S, Leica Microsystems), cut at 300 μm thickness, and stored in an interface chamber. The interface chamber was perfused with ACSF containing in mM: 119 NaCl, 26 NaHCO₃, 10 glucose, 2.5 KCl, 1 NaH₂PO₄, 2.5 CaCl₂ and 1.3 MgCl₂. Slices were incubated for at least 60 min before recordings started. All ACSF solutions were equilibrated with carbogen (95% O₂ and 5% CO₂).

2.3 Slice electrophysiology

Whole-cell current-clamp recordings were performed in layer 2/3 of somatosensory cortex with a KMeSO₃-based intracellular solution (containing in mM: 130 KMeSO₃, 10
KCl, 10 HEPES, 4 NaCl, 4 Mg-ATP, 0.5 Na-GTP, 5 Na-Phosphocreatine, 0.1 % Biocytin), using a Multiclamp 700B amplifier (Molecular Devices). Data were low-pass filtered at 3 kHz and sampled at 10 kHz. Neurons were identified visually with infrared differential interference contract (IR-DIC) optics on an Olympus BX-51 WI microscope. Interneurons were differentiated from PCs based on two criteria: a lack of apical dendrite projecting towards the pial surface, and horizontally orientated and spherical shaped somata compared to the pyramidal shaped somata of PCs. Experiments were only performed if cells had a resting membrane potential more hyperpolarized than -55 mV (without correction for liquid junction potential) and a series resistance below 25 MΩ. Bridge balance and pipette capacitance compensation was performed throughout the recording. Cells were characterized by recording their membrane response and firing pattern by applying hyperpolarizing and depolarizing current steps (-200 to + 600 pA, increment: 40 pA, 1 s). Fast-spiking interneurons showed high frequency AP firing (>200 Hz) with no frequency adaptation. Both RSNPCs and PCs showed moderate spiking frequency (20 – 60 Hz) and increasing inter-spike intervals during the depolarization step. The AP slope ratio was calculated by dividing maximal positive slope with the maximal negative slope of the AP. Before inducing SSI, we manually adjusted the membrane potential to -60 mV by continuous somatic current injection and recorded a stable baseline for 2 min. Cells that did not reach a stable baseline were excluded. SSI was induced either by eliciting AP trains with 2 ms long somatic current injection (10 AP trains, 20 s inter-train interval; 50 APs/train at 100 Hz) or by bath application of the CB₂R agonist HU-308 (1 µM, Tocris). Other pharmacological agents (10 µM SCH23390, Tocris; 10µM S0589, Sigma-Aldrich; 1 µM SR 144528, Tocris) were applied to the bath and brain slices were preincubated for at least 10 min before recordings were performed. The input resistance was monitored by a 400 ms test-pulse of -40 pA every 20 s.
2.4 Data analysis and statistics

Data were recorded and analyzed with Igor Pro (Wavemetrics) and Neuromatic software. Changes in membrane potential ($\Delta V_m$) were calculated by subtracting the average membrane potential of 2 min, 60 s after the last AP train, from the baseline membrane potential. Changes in input resistance after SSI were calculated by normalizing the average input resistance after SSI induction to the average baseline input resistance. Cells were classified as responsive when $\Delta V_m$ was higher than three times the standard deviation of the baseline. Sample size is given as the number of recorded cells ($n$). D’Agostino-Pearson normality test was performed to test individual datasets for normal distribution of the data points. Normally distributed datasets were compared using a two-tailed Student’s t-test. Stated $p$-values refer to comparison of hyperpolarization amplitude ($\Delta V_m$) between different datasets by using Student’s t-test, unless otherwise stated. If datasets were not normally distributed a Mann-Whitney test was performed to compare the groups. Data are presented as mean ± standard error of the mean (SEM). Box plots are shown as median with 25th and 75th percentile.

2.5 Morphological reconstruction

Biocytin-containing intracellular solution (0.1 % Biocytin) was used for post-hoc identification of the recorded neuron. After the recording, the brain slices were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C before subsequent visualization with streptavidin conjugated with Alexa-488 (RRID:AB_2315383). Stained slices were imaged with a laser confocal microscope (Leica SP5 on a Leica DMI 6000) using a 20x or 63x objective and a z-step size of 1 µm. Morphological reconstruction was performed using Neutube (Feng et al., 2015) and Fiji software (Schindelin et al., 2012).
3 Results

3.1 Cell type-specific hyperpolarization

We performed whole-cell patch-clamp recordings from different cell types of layer 2/3 in the somatosensory cortex. Based on their appearance in the IR-DIC image, we identified PCs by their eponymous soma shape and a prominent apical dendrite projecting towards the pial surface (Fig. 1A). Interneurons were differentiated by their horizontally orientated or spherical shaped somata and the absence of a prominent apical dendrite (Fig. 1B, 1C). Interneurons were further differentiated according to their firing properties (Table 1) into RSNPCs (Fig. 1B) and FS interneurons (Fig. 1C). Physiological properties and firing patterns of PCs and RSNPCs were very similar (Table 1). In contrast, FS had a lower input resistance and showed a characteristic firing pattern (shorter AP half-width and high frequency firing), which allowed distinguishing these neurons from PCs and RSNPCs (Table 1).

Trains of APs elicited long-lasting SSI in PCs (Fig. 1D, G, J: $\Delta V_m$: -4.1 ± 1.5 mV) and in RSNPCs (Fig. 1E, H, K: $\Delta V_m$: -5.6 ± 1.1 mV), but not in FS interneurons (Fig. 1F, I, L: $\Delta V_m$: -0.7 ± 0.5 mV).

According to our criteria (see Methods), 73 % (8/11) of PCs and 71% (15/21) of RSNPCs exhibited a significant hyperpolarization after AP trains, whereas none of the FS were responsive. Here, both responsive and non-responsive cells were included in averaged values and statistics (Fig. 1). Taken into account only responsive cells, PCs hyperpolarize by -6.0 ± 1.6 mV and RSNPCs by -7.6 ± 1.0 mV (data not shown).

Thus, trains of APs induced a long-lasting hyperpolarization exclusively in regular spiking cells (PCs and RSNPCs) in layer 2/3 of the somatosensory cortex, but not in FS interneurons. We also assessed the stability of SSI and found that the SSI induced
hyperpolarization of the membrane potential in RSNPCs was stable and lasted for the entire recording period of up to 40 min after induction (Suppl. Fig. 1A). Additional AP trains with an interval of two minutes that were applied after SSI had stabilized did not lead to any further significant additional hyperpolarization of the cells (Suppl. Fig. 1B and C).

3.2 Mechanism underlying long-lasting hyperpolarization in regular spiking non-pyramidal neurons

We have previously shown that trains of APs induce a cell-autonomous CB$_2$R-dependent SSI in hippocampal PCs by activation of a sodium/bicarbonate co-transporter (NBC) (Stempel et al. 2016). In contrast, both layer 2/3 PCs and layer 5 interneurons of the somatosensory cortex utilize an alternative mechanism in which activation of CB$_1$Rs induces a GIRK channel-driven hyperpolarization (Bacci et al., 2004; Marinelli et al., 2009).

The cellular mechanisms of SSI have not been characterized before in layer 2/3 RSNPCs, despite the fact that of all cells in layer 2/3, these show the most pronounced SSI (Fig. 1). Thus, we focused on RSNPCs to further investigate the SSI mechanism: in RSNPCs, the magnitude of hyperpolarization correlated with the decrease in input resistance (Fig. 2A), indicating an increase in ion channel conductance. Preincubation with an inhibitor of NBC (10 µM S0589) did not alter SSI in RSNPCs ($\Delta V_m$: -4.4 ± 3.4 mV; n = 13; p = 0.4 compared to control condition; data not shown). In contrast, preincubation with a GIRK channel blocker (10 µM SCH23390) prevented the long-lasting hyperpolarization (Fig. 2B-D: control $\Delta V_m$: -5.6 ± 1.1 mV; 15/21 responsive cells; SCH23390 $\Delta V_m$: -0.2 ± 1.1 mV; 1/11 responsive cells). Additionally, application of SCH23390 after SSI induction strongly
depolarized the cells and increased input resistance, reversing the AP-induced effects (Fig. 2E-F). In contrast, only a weak baseline depolarization occurred when SCH23390 was applied to non-stimulated RSNPCs (Fig. 2G).

Thus, the AP-induced hyperpolarization in RSNPCs is mediated via activation of GIRK channels and not by the NBC.

**3.3 CB₂ receptors mediate SSI**

SSI was previously characterized as an endocannabinoid-dependent mechanism, in which either CB₁Rs (Bacci et al., 2004; Marinelli et al., 2009) or CB₂Rs (Stempel et al., 2016) induce a long-lasting hyperpolarization after periods of AP firing, via different mechanisms. The specific CB₂R agonist HU-308 (1 µM) mimicked the AP-induced hyperpolarization, whereas application of the endocannabinoid Noladin ether (NE, 300 nM), which displays selectivity for CB₁Rs over CB₂Rs (Hanus et al., 2001), did not cause a hyperpolarization (Fig. 3A and B: HU-308 \( \Delta V_{m} \): -6.0 ± 1.6 mV, 8/11 responding cells; NE: \( \Delta V_{m} \): -0.4 ± 0.8 mV, 3/10 responding cells). Additionally, preincubation with the CB₂R inverse agonist SR 144528 (1 µM) prevented the AP-induced long-lasting hyperpolarization (Fig. 3C and D: control \( \Delta V_{m} \): -5.6 ± 1.1 mV, 15/21 responsive cells; SR 144528: \( \Delta V_{m} \): -0.8 ± 0.6 mV, 3/15 responsive cells) indicating the involvement of CB₂R in SSI.

In order to verify this finding, we used transgenic KO mice lacking CB₁R or CB₂R (CB₁R KO and CB₂R KO) and their corresponding littermates (Buckley et al., 2000; Zimmer et al., 1999) to further disentangle the involvement of the major cannabinoid receptors in AP-induced SSI. In both CB₁R KO mice and WT-littermates, trains of APs elicited a long-lasting hyperpolarization of similar magnitude in RSNPCs (Fig. 4A – C: CB₁R KO: \( \Delta V_{m} \): -3.7 ± 0.9 mV, 12/17 responsive cells; CB₁R WT: \( \Delta V_{m} \): -5.2 ± 1.5 mV, 7/10 responsive cells). In
contrast, CB2R-deficient mice showed a marked reduction of SSI, both in the SSI amplitude (Fig. 4D and E: CB2R KO: ΔV_m: -0.4 ± 0.6 mV; CB2R WT: ΔV_m: -3.6 ± 0.8 mV) as well as in the number of responding cells (Fig. 4F: CB2R KO: 2/12; CB2R WT: 9/12).

Corresponding phenotypes were also observed in recordings of PCs in transgenic CB-R knockout animals: in CB2R-deficient mice and their WT littermates trains of APs induced SSI of similar magnitude (CB1R WT: ΔV_m: -3.9 ± 0.9 mV; CB1R KO: ΔV_m: -4.2 ± 1.5 mV; Suppl. Fig. 2). In contrast, the genetic deletion of CB2Rs abolishes SSI also in PCs (CB2R WT: ΔV_m: -6.1 ± 1.7 mV; CB2R WT: ΔV_m: -0.5 ± 0.6 mV; Suppl. Fig. 2).

Finally, we tested the specificity of the CB2R agonist HU-308 for inducing a long-lasting hyperpolarization in RSNPCs. In CB1R-deficient mice as well as in their corresponding littermates, HU-308 application mimicked AP-induced SSI while it failed to hyperpolarize RSNPCs in CB2R-deficient mice (Fig. 4G – I: CB1R KO: ΔV_m: -4.9 ± 1.9 mV 7/10 responsive cells; CB1R WT: ΔV_m: -5.5 ± 1.6 mV, 7/10 responsive cells; CB2R KO: ΔV_m: 0.5 ± 1.5 mV, 0/8 responsive cells; CB2R WT: ΔV_m: -4.1 ± 1.8 mV, 7/11 responsive cells). These experiments rule out potential off-target effects of HU-308 in the induction of SSI, and underlie establish its specificity for CB2R at a concentration of 1 µM.

Taken together, both pharmacological intervention and genetic ablation of CB2Rs confirm the involvement of CB2Rs in SSI of layer 2/3 regular spiking cells, providing strong evidence that cell-autonomous activation of CB2Rs and downstream GIRK channel opening is mediating the AP-induced self-inhibition in these cell types.
Discussion

Here, we show that trains of APs induce SSI in RSNPCs and PCs in layer 2/3 of the somatosensory cortex but not in FS interneurons. This cell type-specific expression of SSI was also described for layer 5 of the somatosensory cortex, where only LTS interneurons, but not FS neurons exhibited SSI (Bacci et al., 2004). In RSNPCs of layer 2/3 we investigated the underlying mechanism in detail using pharmacological tools as well as CB₁R- and CB₂R-deficient mice. We find that SSI is selectively mediated by CB₂Rs in both RSNPCs as well as PCs. This is somewhat unexpected as CB₁Rs have been previously implicated in SSI of PCs in layer 2/3 of somatosensory cortex (Marinelli et al., 2009).

Several recent studies have described the role of CB₂Rs in cellular auto-inhibition: In hippocampal pyramidal neurons CB₂Rs mediate SSI after trains of APs (Stempel et al., 2016). Additionally, intracellular CB₂Rs were also shown to reduce firing frequency in PCs of the prefrontal cortex (den Boon et al., 2012). Furthermore, application of CB₂R agonists hyperpolarizes dissociated dopaminergic neurons of the ventral tegmental area and inhibits spiking (Zhang et al., 2014). Together these findings illustrate that CB₂R activation can lead to modifications in excitability in several different cell types and in different brain regions.

What are the signaling events downstream of the endocannabinoid receptors that lead to SSI? So far, several mechanisms have been identified: In hippocampal PCs SSI is mediated by CB₂R-induced NBC transporter activation (Stempel et al., 2016). In contrast, CB₁R-mediated GIRK channel activation was described as the mechanism responsible for SSI in layer 2/3 PCs and layer 5 LTS interneurons (Bacci et al., 2004; Marinelli et al., 2009). In the present study, we demonstrate a different pathway for SSI in RSNPCs of layer 2/3 and show that APs lead to the activation of CB₂Rs, resulting in the opening of GIRK channels and hyperpolarization of the membrane potential.
In addition to the cell type-specific preference for endocannabinoid receptor subtypes in SSI activation, divergent intracellular transduction pathways are also employed to hyperpolarize the membrane potential. Given the variability of receptor and receptor subtype expression across different classes of neurons, it is not surprising that multiple mechanisms and downstream signaling cascades are involved in phenomena such as SSI (Arey, 2014). Further, several studies have shown that CB₂Rs activation can lead to selective utilization of different transduction pathways (Atwood et al., 2012; Dhopeshwarkar and Mackie, 2016). Thus, cell type specific variations of the intracellular signaling machinery may determine which transduction pathway is implemented after agonist binding.

Due to the low CB₂R expression levels in neuronal cells under physiological conditions, it has been a challenging task to study CNS effects of CB₂R. Unspecific CB₁R-pharmacology (Stempel et al., 2016) and CB₂R antibodies of insufficient specificity (Cécyre et al., 2014; Marchalant et al., 2014) have previously impeded a convincing discrimination between CB₁R- and CB₂R-mediated effects in the CNS. However, in recent years, evidence accumulated suggesting that both CB₁- and CB₂Rs serve divergent physiological effects. Stempel et al. (Stempel et al., 2016) and Chen et al. (Chen et al., 2017) proposed that CB₁Rs seem to be mainly involved in modulation of synaptic functions while CB₂R activation results in postsynaptic inhibition. Additionally, microglial CB₂R expression was shown to be involved and upregulated in a variety of pathological conditions including neuroinflammation (Carlisle et al., 2002; Zoppi et al., 2014), stroke (Yu et al., 2015; Zarruk et al., 2012), Parkinson’s disease (Concannon et al., 2016, 2015), Alzheimer’s (Benito et al., 2003) and Huntington’s disease (Palazuelos et al., 2009). Also, neuronal CB₂R expression is increased in neuropathic pain (Svíženská et al., 2013) and drug addiction (Zhang et al., 2016). Manipulation of CB₂R expression in CA1 PC or microglia was shown to induce distinct behavioral phenotypes in mice: while microglial CB₂Rs
were involved in contextual fear memory, overexpression or disruption of CB₂Rs in PC lowered anxiety levels or enhanced spatial working memory, respectively (Li and Kim, 2017). Moreover, constitutive deletion of CB₂Rs induces a schizophrenic phenotype in mice (Ortega-Alvaro et al., 2011), increases aggressive behavior (Rodríguez-Arias et al., 2015) and modulates drug-seeking behavior for ethanol (Ortega-Álvaro et al., 2015) and nicotine (Navarrete et al., 2013). Furthermore, neuronal CB₂Rs modulate oscillatory activity – more specific theta-gamma-coupling – in the hippocampal formation (Stempel et al., 2016).

According to these data, Pacher and Machoulam (Pacher and Mechoulam, 2011) suggested that CB₂R signaling might represent a protective system that prevents tissue and cell damage. In line with this, the CB₂R mediated auto-inhibition described here may represent a cell-autonomous feedback loop preventing neurons from damage due to excessive excitability. In this study, the SSI-induced hyperpolarization is indiscriminately observed in both types of regular spiking neurons. In contrast, fast spiking interneurons do not show this phenomenon, which argues in favor of a protective role against intolerable amounts of excitation. In addition, on a more speculative note, it is likely that RSNPCS belong to a group of interneurons (5-HT₃A receptor containing), which preferentially synapse onto other interneurons (Tremblay et al., 2016). Thus, silencing RSPNCs would lead to disinhibition of interneurons, effectively adding to the excitation protection of PCs. The long-term stability of SSI after induction is a further indication that it might occur in specific events where neurons must be prevented from excessive activity levels for a longer period of time.

In terms of the physiological relevance of the phenomenon under study, it has been shown before that SSI can also be induced with more naturally spaced activity patterns than the induction patterns used in this study: physiological spike trains from in vivo
recordings were applied in slices and induced SSI in CA3 pyramidal neurons of similar magnitude like the more artificial AP trains (Stempel et al., 2016). Similarly, Marinelli et al. could reliably induce SSI in cortical PC with spike trains of lower (10 – 50 Hz) frequencies (Marinelli et al., 2009). In this context it is noteworthy that for somatosensory cortex layer 2/3 regular spiking pyramidal neurons, firing frequencies of up to 60 Hz have been reported (Kinnischtzke et al., 2012). Therefore, SSI can already be induced by activity patterns of neurons that can occur in vivo. However, the specific role of SSI under physiological conditions has to be addressed experimentally in more detail.

Together with the lack of psychoactive effects upon CB₂R activation and other CB₁R activation-related side effects (Pertwee, 2012), these findings highlight that CB₂Rs represent an excellent target for drug-discovery research for multiple pathological conditions.

5. Conclusion

Our study describes a new mechanism by which SSI is implemented in neocortical neurons. We show that CB₂R activation leads to a GIRK channel mediated cell-autonomous hyperpolarization and provide further evidence for functional CB₂Rs in the CNS and supporting their role in regulation of neuronal excitability. Future studies combining different techniques will aid in disentangling the different roles of CB₁Rs and CB₂Rs, resulting in a better understanding of their functions and helping the discovery of specific therapeutic targets for different pathological conditions.
Acknowledgments

This work was supported by the NeuroCure Cluster of Excellence (grand number EXC 257), the Bernstein Center for Computational Neuroscience (grand number 01GQ1001A), the Deutsche Zentrum für neurodegenerative Erkrankungen, the Einstein Foundation and the Deutsche Forschungsgemeinschaft (grand numbers SFB958, SPP1926). The study was conceived and designed by D.S. and A.S., who also wrote the paper. A.S. performed all experiments with the help of D.P., R.P.S., and A.S. analyzed all experiments. A.V.S., J.B and B.R.R. contributed to validation and formal analysis as well as reviewing and editing of this study.

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Captions

Fig. 1. Trains of APs induce cell type-specific hyperpolarization in regular firing neurons in layer II/III of somatosensory cortex.

A – C) Characteristic cell morphology and firing pattern of a PC (A), an RSNPC (B) and a FS interneuron (C), visualized by post-hoc biocytin staining and reconstruction. Scale bar: 50 µm; arrow heads depict the direction of the pial surface. Insets show neuron-type-specific firing patterns evoked by depolarizing current injection (scale bars: 20 mV, 0.2 s).

D – I) Single cell examples (D – F) and time course of the average membrane potential (G – I) before, during and after ten trains of APs (black lines) in the different cell types.

J – L) Individual magnitudes of the AP-train induced hyperpolarization. Trains of APs induce SSI in PCs (D, G, J; n = 11) and in RSNPCs (E, H, K; n = 21), but not in FS interneurons (F, I, L; n = 6).
Fig. 2. AP-induced hyperpolarization is accompanied by a reduction in input resistance that is mediated by activation of GIRK-channels in RSNPCs.

A) The amplitude of the hyperpolarization ($\Delta V_m$) correlates with the reduction in input resistance (normalized to the pre-AP average; $r^2 = 0.81$, $p < 0.0001$). Inset: example traces of -40 pA testpulses before and after AP trains; scale bars: 0.2 s, 5 mV; filled circle in the plot depicts example recording.

B) Inhibition of AP-induced hyperpolarization by preincubation with the GIRK channel blocker SCH23390 (10 µM; B; black lines mark AP stimulation). C) SCH23390 reduced the average SSI magnitude (**p = 0.0005, Student’s t-test; control $n = 21$; SCH23390 $n = 11$) as well as percentage of hyperpolarizing cells (D).

E) Single cell example of the depolarization induced by application of SCH23390 (10 µM) after AP-induced SSI, compared to a control recording without SCH23390 application.

F) Summary of the SCH23390 effect on the membrane potential after SSI induction ($n = 5$).

G) Application of SCH2330 on non-stimulated cells (on baseline) causes only a minor depolarization (*p = 0.0159 Mann-Whitney test; on baseline: $n = 4$; after SSI: $n = 5$) compared to the effect after SSI.
**Fig. 3. Pharmacological experiments indicate involvement of CB$_2$R in RSNPC SSI.**

A) Time course of the average membrane potential in response to application of the specific CB$_2$R agonist HU-308 (1 µM) and the endocannabinoid NE (300 nM) that displays a selectivity for CB$_1$Rs over CB$_2$Rs. Agonist application is indicated by the black line.

B) Individual magnitudes of agonist-induced hyperpolarization. NE does not cause a hyperpolarization (** $p = 0.005$, Mann-Whitney rest; HU-308: $n = 10$; NE: $n = 11$).

C) Time course of the average membrane potential before, during (black lines) and after AP trains in presence or absence of the CB$_2$R inverse agonist SR 144528 (1 µM).

D) Individual magnitudes of AP-induced hyperpolarization in the presence of SR 144528. Preincubation with SR 144528 prevents AP-induced hyperpolarization (** $p = 0.0019$, Mann-Whitney rest; SR 144528: $n = 15$; control: $n = 21$).

**Fig. 4. AP-induced hyperpolarization in RSNPCs is absent in CB$_2$R-deficient mice but present in CB$_1$R-deficient mice.**

A – C) AP-induced SSI in RSNPCs of CB$_2$R-deficient mice is indistinguishable from SSI in WT-littermates ($p = 0.4$ Student’s $t$-test; CB$_2$R KO $n = 17$; CB$_2$R WT $n = 10$). A) Time course of the average membrane potential in WT (open circles) and CB$_2$R KO mice (black circles). B) Overview on individual magnitudes of AP-induced hyperpolarization. C) Percentage of cells in which AP trains evoked hyperpolarization. AP trains are indicated by black bars.

D – F) Trains of APs failed to induced SSI in CB$_2$R-deficient mice compared to WT-littermates (** $p = 0.0029$ Student’s $t$-test; CB$_2$R KO $n = 12$; CB$_2$R WT $n = 12$). D) Time
course of the average membrane potential in WT (open circles) and CB₂R KO mice (black circles). E) Individual magnitudes of the AP-induced hyperpolarization. F) Percentage of cells in which AP trains evoked a hyperpolarization. AP trains are indicated by black bars.

G – I) HU-308 (1 µM) hyperpolarized RSNPCs in CB₁R deficient mice and WT-littermates of transgenic animals (p = 0.8 Student’s t-test; CB₁R KO n = 10; CB₁R WT n = 10), but not in CB₂R-deficient mice (* p = 0.046; CB₂R KO n = 8; CB₂R WT n = 11). G) Exemplary time course of the membrane potential of RSNPCs in response to HU-308 application (black line). Note the hyperpolarization in the CB₁R KO (black circles), and the lack of hyperpolarization in the CB₂R KO (black squares). H) Individual magnitudes of agonist-induced hyperpolarization in different genotypes. I) Percentage of cells in which agonist application evoked hyperpolarization.
Table 1: cell properties of cortical neurons in somatosensory cortex layer 2/3

<table>
<thead>
<tr>
<th></th>
<th>PCs (11)</th>
<th>RSNPCs (21)</th>
<th>FSs (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential [mV]</td>
<td>-81.9 ± 2.0</td>
<td>-80.6 ± 1.2</td>
<td>-65.2 ± 1.9</td>
</tr>
<tr>
<td>Input resistance [MΩ]</td>
<td>153.1 ± 11.6</td>
<td>189.3 ± 13.87</td>
<td>80.6 ± 9.7</td>
</tr>
<tr>
<td>AP half-width [ms]</td>
<td>1.0 ± 0.08</td>
<td>1.6 ± 0.1</td>
<td>0.3 ± 0.19</td>
</tr>
<tr>
<td>AP threshold [mV]</td>
<td>-33.9 ± 1.3</td>
<td>-36.0 ± 1.0</td>
<td>-46.8 ± 2.6</td>
</tr>
<tr>
<td>AP slope ratio</td>
<td>4.5 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>AHP [mV]</td>
<td>-16.7 ± 0.7</td>
<td>-15.0 ± 0.5</td>
<td>-18.1 ± 0.6</td>
</tr>
<tr>
<td>Maximal firing frequency [Hz]</td>
<td>36.8 ± 2.3</td>
<td>41.17 ± 2.3</td>
<td>360 ± 39.9</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM, PC: pyramidal cell; RSNPC: regular spiking non-pyramidal cell; FS: fast spiking interneuron; AHP: Afterhyperpolarization; numbers of recorded cells are displayed in parentheses.
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