Unravelling a novel role for Cannabidivarin in the modulation of subventricular zone postnatal neurogenesis

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Conflict of Interest
The authors declare no competing interests.

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Abbreviations
5'-IRTX, 5'-iodoresiniferotoxin; BrdU, 5-bromo-2'-deoxyuridine; CBDV, Cannabidivarin; CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2; DIV, day in vitro; ICC, immunocytochemistry; MBP, myelin basic protein; NG2, neuron/glia antigen 2; NSA, neurosphere assay; NSPC, neural stem/progenitor cell; OPC, oligodendrocyte progenitor cell; PI, propidium iodine; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; SVZ, subventricular zone; TRPV1, transient receptor potential cation channel subfamily V member 1; NeuN, neuronal nuclei.
Abstract

Postnatal neurogenesis has been shown to rely on the endocannabinoid system. Here we aimed at unravelling the role of Cannabidivarin (CBDV), a non-psychoactive cannabinoid, with high affinity for the non-classical cannabinoid receptor TRPV1, on subventricular zone (SVZ) postnatal neurogenesis. Using the neurosphere assay, SVZ-derived neural stem/progenitor cells (NSPCs) were incubated with CBDV and/or 5’-iodoresiniferotoxin (TRPV1 antagonist), and their role on cell viability, proliferation, and differentiation were dissected. CBDV was able to promote, through a TRPV1-dependent mechanism, cell survival, cell proliferation and neuronal differentiation. Furthermore, pulse-chase experiments revealed that CBDV-induced neuronal differentiation was a result of cell cycle exit of NSPCs. Regarding oligodendrocyte differentiation, CBDV inhibited oligodendrocyte differentiation and maturation. Since our data suggested that the CBDV-induced modulation of NSPCs acted via TRPV1, a sodium-calcium channel, and that intracellular calcium levels are known regulators of NSPCs fate and neuronal maturation, single cell calcium imaging was performed to evaluate the functional response of SVZ-derived cells. We observed that CBDV-responsive cells displayed a two-phase calcium influx profile, being the initial phase dependent on TRPV1 activation. Taken together, this work unveiled a novel and untapped neurogenic potential of CBDV via TRPV1 modulation. These findings pave the way to future neural stem cell biological studies and repair strategies by repurposing this non-psychoactive cannabinoid as a valuable therapeutic target.

Keywords

Adult neurogenesis; Cannabidivarin; TRPV1; Neural stem cells; Oligodendrogenesis; Subventricular Zone
1. Introduction

Adult neural stem/progenitor cells (NSPCs) can be found in the subventricular zone (SVZ) lining the lateral walls of the lateral ventricles (Bond et al. 2015). Importantly, adult NSPCs have self-renewing properties and are tri-potent, having the capacity to generate neurons, astrocytes and oligodendrocytes (Soares et al. 2020). In the SVZ, besides originating neuroblasts (Luskin and Boone 1994), a minority of NSPCs enter the oligodendroglial lineage (Suzuki and Goldman 2003; Menn et al. 2006) by originating oligodendrocyte progenitor cells (OPCs) that migrate into the surrounding cortex and white matter, differentiating into myelinating oligodendrocytes (Aguirre and Gallo 2004; Butti et al. 2019).

Adult NSPCs are mostly quiescent in vivo (Cavallucci et al. 2016). The balance between quiescence and activity regulates, not only the rate of cytogenesis, but also the long-term maintenance of the NSPC pool (Urbán et al. 2019; Cheung and Rando 2013). Therefore, finding and exploiting modulators of NSPCs that can boost postnatal neurogenesis and oligodendrogenesis is imperative.

Several studies have shown that neurogenesis is heavily modulated by the endocannabinoid system, (Rodrigues et al. 2017; Galve-Roperh et al. 2013; Zimmermann et al. 2018; Xapelli et al. 2013; Molina-Holgado et al. 2007; Ferreira et al. 2018; Bravo-Ferrer et al. 2017). A growing body of scientific and clinical data has been attesting the potential of medical-cannabis to ameliorate symptoms of several pathologies (Pacher et al. 2006; Pertwee 2005; Di Marzo and De Petrocellis 2006; Schlag et al. 2021; Hall and Degenhardt 2009; Castaneto et al. 2014; Ebbert et al. 2018). Notwithstanding, the chronic and abusive consumption of Cannabis sp. has also been associated with detrimental health effects, such as cognitive and memory impairments (Cohen et al. 2020; Figueiredo et al. 2020). Thus, one of the major challenges of cannabis research is to find ways to prevent the negative side-effects associated with cannabis-based medicines (Hall and Degenhardt 2009; Grant et al. 2018; Connor et al.
One possible alternative to overcome this issue can be related with the modulation of the non-classical cannabinoid receptors, such as the transient receptor potential cation channel subfamily V member 1 (TRPV1). This sodium-calcium ion channel receptor, belongs to the endovanilloid system and is associated with thermoregulation and nociception (Tominaga and Tominaga 2005; Caterina et al. 1997). Importantly, several studies demonstrated that TRPV1 is a potential target for the regulation of cell proliferation and apoptosis (Stock et al. 2014; Kong et al. 2010; Czaja et al. 2008). Previous works have highlighted that, in both mouse and rat models, TRPV1-activation was shown to promote cell death, inhibited cell proliferation and impaired neuronal maturation (Czaja et al. 2008; Kong et al. 2010). Accordingly, TRPV1 knockout (KO) mice present higher levels of NSPC proliferation when compared to wild type (Stock et al. 2014). In contrast, other works reported a TRPV1- dependent increase of neuronal differentiation and promotion of axonal and neurite growth in dorsal root ganglia cultures (Frey et al. 2018; Goswami et al. 2007). TRPV1 can also be activated by cannabidivarin (CBDV), a phytocannabinoid that has negligible affinity for cannabinoid type 1 and 2 receptors (CB1R and CB2R) (Rosenthaler et al. 2014; Hill et al. 2013). CBDV has also been described as an agonist of TRPV2 and TRPA1 (De Petrocellis et al. 2011), as an inverse agonist of GPR6 (Laun et al. 2019) as well as an allosteric antagonist of GPR55 (Anavi-Goffer et al. 2012). However, there is a broad consensus in the literature suggesting that its biological activity is mostly via a TRPV1- dependent mechanism of action (Iannotti et al. 2014; Straiker et al. 2021; Huizenga et al. 2019; Thornton et al. 2020; Muller et al. 2019).

Given the role of TRPV1 in regulating cell death, cell proliferation and neuronal differentiation and, that CBDV is a non-psychoactive cannabinoid, it is relevant to thoroughly understand how this emerging cannabinoid can modulate neurogenesis and how it could be used as a viable drug for brain repair strategies. Therefore, with this work
we aimed at unravelling how CBDV modulate NSPCs fate and whether those effects are mediated by TRPV1.

2. Material and methods

2.1. Ethical statement

All experiments followed the European Community (86/609/EEC; 2010/63/EU; 2012/707/EU) and Portuguese (DL 113/2013) legislation for the protection of animals used for scientific purposes. The protocol was approved by the “iMM’s institutional Animal Welfare Body – ORBEA-iMM and the National competent authority – DGAV (Direção Geral de Alimentação e Veterinária).”

2.2. Animals

C57BL/6J females were kept in standard housing, grouped in pairs, while males were single housed. All animals were kept on a 12h light/dark cycle, with food and water provided ad libitum. No breeding attempts were made before sexual maturity was reached, at 8 weeks of age. Breeding trios were used until one-year old of age or 10 successful mating sessions, in order to maximise breeding success.

All efforts were made to minimize animal suffering and stress, and to use the minimum number of animals, according to standard and ethical procedures. All animals were given access to hiding places, in the form of disposable igloos or cardboard tubes, as well as proper nesting material and wooden pellets for chewing.

Experiments were performed ex-vivo with biological material obtained from postnatal day 1 to 3 (P1-3) C57BL/6J mice and subsequently maintained in in vitro conditions. A minimum of 3 pups was required to perform one SVZ cell culture and a minimum of 3 independent cultures was required to perform statistical analysis. All pups per litter were used per SVZ cell culture.
2.3. SVZ Cell Cultures

SVZ Neurospheres (3D clusters of clones of NSPCs) were prepared from early postnatal (P1-3) C57BL/6J mice as previously described (Soares et al. 2020). In brief, P1-3 C57BL/6J mice were decapitated with a single incision with sharp scissors at the base of the brainstem. SVZ fragments were dissected out from 450μm-thick coronal brain slices. All collected tissue was pooled and digested with 0.05% Trypsin-EDTA (#25300054, Gibco™) in Hanks' Balanced Salt Solution (HBSS, #14175095, Gibco™) and mechanically dissociated with a P1000 pipette. Single-cell suspension was resuspended in serum-free medium (SFM), composed of Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 + GlutaMAX™ supplement (DMEM+GlutaMAX™, #31331028, Gibco™) supplemented with 100U/mL penicillin and 100μg/mL streptomycin (#15070063, Gibco™), 1% B-27™ (#17504044, Gibco™) and growth factors (10ng/mL epidermal growth factor (EGF, #PHG0311, Gibco™) and 5ng/mL bovine fibroblast growth factor-2 (bFGF-2, #13256-029, Gibco™)) (proliferative conditions). SVZ cells were seeded in 60mm diameter Petri dishes (#430166, Corning) and maintained for six days in a 95% air/5% CO₂ humidified atmosphere at 37°C. Resulting neurospheres were seeded for 24h onto 12mm glass coverslips (#631-1577P, VWR) coated with 100μg/mL poly-D-lysine (PDL, #P7886, Sigma-Aldrich) in SFM devoid of growth factors (differentiative conditions). After 24h (day 0), the medium was renewed with or without (control) a range of pharmacological treatments for 48h or 7 days.

2.4. Pharmacological treatments and experimental setting

Since CBDV has very weak affinity for both CB1R and CB2R (Rosenthaler et al. 2014) and it has been proposed to activate TRPV1 (Iannotti et al. 2014), we evaluated its effects on SVZ-derived NSPC survival, proliferation and differentiation and studied TRPV1-dependency using a selective TRPV1 antagonist (5’-Iodosiresiniferatoxin, 5’-IRTX).
Table 1 – Pharmacological treatments

<table>
<thead>
<tr>
<th>Drug</th>
<th>Purity</th>
<th>Biological Activity</th>
<th>Concentration used</th>
<th>Ki for CB1R/CB2R</th>
<th>Ki for TRPV1</th>
<th>Company</th>
<th>#</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannabidivarin 2-((1S,6S)-3-methyl-6-(prop-1-en-2-yl)cyclohex-2-enyl)-5-propylbenzene-1,3-diol</td>
<td>≥98%</td>
<td>TRPV1 agonist and GPR55 antagonist</td>
<td>100nM 300nM 1µM</td>
<td>CB1R: 14711nM CB2R: 574.2nM</td>
<td>Not Known</td>
<td>THC Pharm GmbH The Health Concept</td>
<td>-</td>
<td>(Anavi-Goffer et al. 2012; Iannotti et al. 2014; Rosenthaler et al. 2014)</td>
</tr>
<tr>
<td>5’-Iodoresiniferatoxin 6,7-Deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzene acetate)</td>
<td>&gt;99%</td>
<td>TRPV1 antagonist</td>
<td>300nM</td>
<td>Not applicable</td>
<td>5.8nM</td>
<td>Alomone Labs</td>
<td>I-800</td>
<td>(Wahl et al. 2001)</td>
</tr>
</tbody>
</table>

Plated neurospheres were exposed to three increasing concentrations of CBDV (drug concentration-response studies) (Table 1). These were established based on previous studies with cannabinoids (Rodrigues et al. 2017; Xapelli et al. 2013; Stanslowsky et al. 2017; Compagnucci et al. 2013) since the Ki for CBDV for TRPV1 is not known. Additionally, to study if the effect seen by CBDV was TRPV1-dependent, the TRPV1 antagonist 5’-IRTX was used. This drug is a potent vanilloid receptor antagonist, 40-fold more potent than the prototypical TRPV1 antagonist capsazepine (Wahl et al. 2001). Whenever cells needed to be co-treated with the antagonist (at 300nM), they were incubated 30 minutes prior to the treatment with CBDV. All drugs were dissolved in Dimethyl sulfoxide (DMSO, #D2650, Merck Life Sciences) at a stock solution of [50mM] CBDV and [10mM] 5’-IRTX. Fresh dilutions were prepared on the day of the pharmacological tests with SFM-DMEM+GlutaMAX™ devoid of growth factors.

2.5. Total RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from DIV7 SVZ-derived cells, subjected to the pharmacological treatments mentioned above, using TRIzol™ Reagent (#15596026, Invitrogen™) (Vilain et al. 2012). Cells were scraped into a tube containing 1mL TRIzol™ and manually dissociated with a P1000 pipette After mixing with 200µL of chloroform, and vortexing for...
15 seconds, a centrifugation at 12,000 × g for 10 minutes at 4°C was performed to collect the aqueous phase, to which equal volume of isopropyl alcohol was added. After a centrifugation at 17,500 × g, for 10 minutes at 4°C, RNA pellet was washed in sequential cycles of decreasing volumes of 75% ethanol (400µL–100µL). RNA pellet was air dried, for 15 minutes at room temperature (RT) and resuspended in 10µL of nuclease-free water followed by an incubation for 10 minutes at 55°C. RNA purity and concentration were obtained using Nanodrop 2000 Spectrophotometer (NanoDrop Technologies LLC). DNA contaminations were eliminated with DNase I recombinant (#04716728001; Roche Applied Science) following the manufacturer’s instructions. All samples were stored at −80°C until use.

cDNA was prepared from 1000ng total RNA using NZY Reverse Transcriptase (#MB12402; NZYTech) according to manufacturer’s instructions. Real-time RT-PCR was performed using a SensiFastTM SYBR® Hi-ROX kit (#BIO-92020; Bioline USA Inc.) in an Applied Biosystems QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific Inc.). Primer sequences are listed in Table 2. Relative gene expression was calculated based on the standard curve and normalized to the level of hypoxanthine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and expressed as fold change from controls. Primers were designed using the Reference Genome GRCm39 (Ensembl Assembly Genome C57BL_6NJ_v1; Accession: GCA_001632555.1) taken from the Ensembl Release 100 (April 2020).
Table 2 – Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trpv1</td>
<td>FW ACT CTT ACC ACA CAG CAG CC</td>
</tr>
<tr>
<td></td>
<td>RV GCC CAA TTT GCA ACC AGC TA</td>
</tr>
<tr>
<td>Mki67</td>
<td>FW CCT TTG CTG TCC CCG AAG A</td>
</tr>
<tr>
<td></td>
<td>RV GGC TTC TCA TCT GTT GCT TCC T</td>
</tr>
<tr>
<td>Tubb3</td>
<td>FW GTG AAG TCA GCA TGA GGG AGA T</td>
</tr>
<tr>
<td></td>
<td>RV AGG TTC CAA GTC CAC CAG AAT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>FW CCC CTT CAT TGA CCT CAA CTA C</td>
</tr>
<tr>
<td></td>
<td>RV CCT CAC CCC ATT TGA TGT TAG T</td>
</tr>
</tbody>
</table>

2.6. Evaluation of Cell viability, Cell proliferation and Cell differentiation under differentiative conditions and immunocytochemistry (ICC) assays

2.6.1. Cell viability

To investigate the effect of the different pharmacological treatments on cell viability, SVZ cells were exposed, at DIV2, to 3μg/mL of propidium iodide (PI, # P4170, Sigma-Aldrich) for 30min before fixation. PI is a fluorescent intercalating agent that binds to DNA in cells that have a compromised cell membrane, thus is useful to differentiate healthy cells from necrotic or late apoptotic cells (Lecoeur 2002). Cells were processed for ICC, as mentioned in 2.5.5.

2.6.2. Cell proliferation

Cell proliferation was assessed at DIV2 by co-incubating cells with 10μM of 5-bromo-2'-deoxyuridine (BrdU) (#B5002, Sigma-Aldrich) in the last 4h of the pharmacological treatments. BrdU is a synthetic thymidine analogue able to substitute thymidine in the DNA double chain during the S Phase of the cell cycle (Kee et al. 2002). Cells were prepared for ICC, as mentioned in 2.5.5. BrdU was unmasked by permeabilizing cells in PBS with 1% Triton™ X-100 at RT for 30min. DNA was denatured in 1M HCl for 20min at 37ºC. See antibodies on Table 3.
2.6.3. Cell differentiation

To assess cell differentiation, cells were fixed at two different timepoints, DIV2 and DIV7, and handled for ICC, as mentioned in 2.5.5. Neuronal and oligodendroglial lineages were evaluated using the respective antibodies on Table 3.

2.6.4. Pulse-chase experiments

SVZ-derived cells were given a BrdU pulse for the first 24h of drug treatments followed by a chase of 6 days (without BrdU) in the absence (control) or presence of our pharmacological treatments. 7-day treated SVZ-derived cells, were fixed and prepared for ICC according to the protocol described in 2.5.5. For antibodies see Table 3.

2.6.5. ICC assay

Cells were fixed in 4% PFA for 30 minutes and washed with PBS at RT. An incubation with PBS with 0.5% Triton X-100 (#X100, Sigma-Aldrich) and 3% bovine serum albumin (BSA, #MB04602, NZYTech) for 1.5h was performed to block nonspecific binding sites, followed by an overnight incubation with the primary antibodies (Table 3). The next day, after several washes with PBS, the corresponding secondary antibodies (Table 3) were incubated for 1h at RT, followed by nuclei counterstaining with Hoechst 33342 (12μg/mL in PBS, #H1399, Invitrogen™) and mounting in Mowiol fluorescent medium (#324590, Sigma-Aldrich).
### Table 3 – Primary and Secondary antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Dilution</th>
<th>Company</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>Mouse</td>
<td>1:500</td>
<td>Dako</td>
<td>M0744</td>
</tr>
<tr>
<td>NeuN</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Cell Signalling Technology</td>
<td>12943</td>
</tr>
<tr>
<td>NeuN</td>
<td>Mouse</td>
<td>1:500</td>
<td>Merck Milipore</td>
<td>MAB377</td>
</tr>
<tr>
<td>NG2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Merck Milipore</td>
<td>B5320</td>
</tr>
<tr>
<td>MBP</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Cell Signalling Technology</td>
<td>78896S</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Dilution</th>
<th>Company</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488 anti-rabbit</td>
<td>Donkey</td>
<td>1:500</td>
<td>Life Technologies</td>
<td>A21206</td>
</tr>
<tr>
<td>Alexa Fluor® 488 anti-mouse</td>
<td>Donkey</td>
<td>1:500</td>
<td>Life Technologies</td>
<td>A21202</td>
</tr>
<tr>
<td>Alexa Fluor® 568 anti-mouse</td>
<td>Donkey</td>
<td>1:500</td>
<td>Life Technologies</td>
<td>A10037</td>
</tr>
<tr>
<td>Alexa Fluor® 568 anti-rabbit</td>
<td>Donkey</td>
<td>1:500</td>
<td>Life Technologies</td>
<td>A10042</td>
</tr>
</tbody>
</table>

#### 2.7. Evaluation of cell proliferation under proliferative conditions

SVZ neurospheres were prepared according to the protocol mentioned in 2.2 by growing SVZ cells in proliferative conditions (SFM supplemented with EGF and bFGF-2) in the presence or absence (control condition) of CBDV (1µM). SVZ-derived cells were allowed to form neurospheres and the analysis of proliferation occurred after 5 days in culture. Neurosphere size (diameter, $D_N$, and area, $A_N$) was used as an indirect indicator of cell proliferation (Mori et al. 2006).

#### 2.8. Microscopy

Immunofluorescence images were captured using an AxioCamMR3 monochrome digital camera (Carl Zeiss Inc.) mounted on a Zeiss Axiovert 200M inverted widefield fluorescence microscope (Carl Zeiss Inc.), with a 40x EC Plan-NeoFluar (NA 0.75) objective. Images were obtained using the software AxioVision 4 (Carl Zeiss Inc.), stored and analysed in an uncompressed 8-bit Tiff (.tiff) format. One pixel corresponds to 0.25μm and the captured image size was 1388 x 1040 pixels.

Phase contrast images of neurospheres were captured using an AxioCam 208c colour digital camera (Carl Zeiss Inc.) mounted on a Zeiss Primovert inverted widefield microscope (Carl Zeiss Inc.), with a 4x Plan Achromat (NA 0.1) Ph0 objective. Images
were obtained using the software Zen 3.2 (Blue edition) (Carl Zeiss Inc.), stored and
analysed in an uncompressed Carl Zeiss Image (.czi) format. One pixel corresponds to
0.925μm and the captured image size was 3840 x 2160 pixels.

2.9. Calcium imaging

SVZ-derived cells were cultured as mentioned in 2.2 and cells were seeded in μ-ibidi 8
well plates (#80826, ibidi). The Ca^{2+} imaging assay was performed in SVZ cells 7 days
after seeding using a Zeiss Axiovert 135TV inverted microscope with epifluorescent
optics and equipped with a high-speed multiple excitation fluorimetric system (Lambda
DG4, with a 175W Xenon arc lamp), according to (Rodrigues et al. 2017; Marques et al.
2019).

Data was recorded by a cooled CCD camera (Photometrics CoolSNAP). Before
measurements, cell medium was replaced by warm standard buffer containing 119mM
NaCl, 2mM Ca^{2+}, 2mM MgCl_{2}, 5mM KCl, 25mM HEPES, pH 7.4 (adjusted with NaOH).
Cells were loaded with the Ca^{2+} sensitive dye Fura-2 AM (5μM, #47989, Life
Technologies) for 45min before performing the intracellular calcium measurements. After
45min, Fura-2 AM was removed and replace by warm standard buffer (as described
above) and experiments were performed at room temperature.

In order to evaluate the calcium response to the drugs, at 100s, an incubation with 1μM
CBDV was performed for 700s. If the treatments required co-incubation with the
antagonist 5'-IRTX, after the incubation with Fura-2 AM and 30min before performing the
intracellular calcium measurements, cells were incubated with the antagonist at 300nM.
At 800s, 2μM Ionomycin was added to record maximum response of cells (data not
shown), as a positive control for cell response. Recordings ended after 900s.

Responses were recorded by a ratiometric method, in which image pairs were obtained
every 5s by exciting the preparations at 340 and 380nm. Fura-2 AM has an absorbance
at 340nm if bound to calcium, and at 380nm if not, while the emission wavelength is
maintained at 510nm. The magnitude of the changes in the emission fluorescence of Fura-2 AM were taken as a measure of the changes in intracellular calcium concentration, as response to the drug stimulation.

2.10. Statistical analysis

Every independent experiment (\(n\)) corresponds to one independent SVZ neurosphere culture from one litter of C57BL/6J mice at P1-3. A minimum of 3 independent cultures was required to perform statistical analysis. Statistical analyses were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Inc.), unless stated otherwise. Significance is reported as: ns: \(p>0.05\); \(*p<0.05\); \(**p<0.01\); \(***p<0.001\); \(****p<0.0001\) when compared with control. § For comparisons of the co-incubation of drugs against the respective drug.

2.10.1. For qRT-PCR experiments

SVZ-derived cells were grown in triplicates and each independent culture is considered \(n=1\). Values were normalized to the control expression of GAPDH expression for each experiment. Data presented as Mean ± standard error of the mean (SEM) and the control was set to 1. Statistical significance was obtained using a Two-tailed Unpaired t-test.

2.10.2. For the studies of cell viability, cell proliferation, neuronal/oligodendroglial differentiation, and pulse-chase experiments

All experiment measurements were performed at the border of SVZ neurospheres, where migrating cells form a pseudo-monolayer. Each condition was measured in three different coverslips. Percentages of immunoreactive cells were calculated from cell counts of five independent microscopic fields in each triplicated coverslip (representing \(n=1\)) with a 40x objective (=100-200 cells per field). All experiments were analysed in a blind fashion and the obtained data was normalised to each corresponding control.
Normalisation of data was obtained by transformation using the $Y = Y \times K$ function in GraphPad Prism, with $K = \text{control group mean}/100$. Each individual experiment ($n$) was normalised to the respective control for that experiment. Normal distribution of data was tested using the Shapiro-Wilk normality test, for small sized samples ($n<50$) (Mishra et al. 2019). Data is presented as Mean ± SEM from the indicated number of independent cultures. For drug concentration-response curve experiments, statistical significance was obtained using a One-way ANOVA followed by Dunnett’s multiple comparisons post-hoc test. For the experiments with combination of drugs, statistical significance was obtained using a Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. For the pulse-chase experiments, statistical significance was obtained using a Two-tailed Unpaired t-test. Outliers were identified and removed from the analysis by Mean ± (2 × standard deviation).

2.10.3. For the neurosphere growth assay

Neurosphere diameter ($D_N$, in µm) was measured using the Region of Interest (ROI) Manager tool from (Fiji Is Just) Image J version 2.3.0/1.53q for Windows OS (64-bit). The $D_N$ and the projected area ($A_N$, in µm$^2$) of each neurosphere were used as an indirect measure of cell proliferation. The size of the neurospheres was defined as an equivalent circle diameter, $D_N = 2(A_N/\pi)^{1/2}$.

Neurospheres of $D_N < 30\mu m$ were excluded from the analysis because they were mainly single or paired cells. Data is presented as a violin plot with the median, 25% and 75% quartiles or as a bar chart representing the percentage of neurospheres binned according to size. The number of neurospheres considered for analysis in each condition ($n$) is from two independent cell cultures. Normal distribution of data was tested using the Kolmogorov–Smirnov test ($n>50$) (Mishra et al. 2019). Statistical significance was obtained using a Two-tailed Unpaired t-test. For the analysis of the comparison of proportions, statistical significance was obtained using the chi-square test using the MedCalc Software Ltd (MedCalc Software Ltd 2023), according to (Campbell 2007;
Outliers were identified and removed from the analysis by Mean ± (2 × standard deviation).

2.10.4. For calcium imaging experiments

Images were collected and analysed using MetaFluor Fluorescence Ratio Imaging Software (Molecular Devices). Regions of interest were acquired by delineating the profile of the cells and averaging the fluorescence intensity inside the delineated area. Statistical significance was obtained after peak determination by the analysis of the area under the curve. Peak amplitudes were calculated by subtracting the baseline level to the maximum peak intensity. Experiments were performed at least in triplicate, except stated otherwise. The number of responsive cells is designated by n. A cell was considered responsive when its maximum recorded response was greater than the average of the responses for all cells for each condition. Data is expressed as Mean ± SEM or as a bar chart representing the proportion of responsive vs non-responsive cells. Statistical significance was obtained using a Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. For the analysis of the comparison of proportions, statistical significance was obtained using the chi-square test using the MedCalc Software Ltd (MedCalc Software Ltd 2023), according to (Campbell 2007; Richardson 2011).

3. Results

3.1. TRPV1 expression is increased by CBDV.

To evaluate the expression of TRPV1 in our culture model, SVZ neurospheres were incubated for 7 days in vitro (DIV7) under differentiative conditions, in the presence or absence of CBDV, and further processed for qRT-PCR. We observed that not only TRPV1 is expressed in SVZ-derived cells, but also its expression is increased in the presence of CBDV, when compared to the control condition (t(6)=5.487, p=0.0015; Ctrl: 1.0-fold; CBDV 1µM: 2.457±0.363-fold; n=3-5; ***p<0.001) (Fig. S1).
3.2. CBDV promotes cell viability via a TRPV1-dependent mechanism of action.

The effect of CBDV in cell viability was studied using a drug concentration-response curve in SVZ-derived cells. For that, SVZ neurospheres were incubated for 2 days in vitro (DIV2) under differentiative conditions with increasing concentrations of CBDV (100nM - 1µM) and, 30 minutes before fixing, cells were incubated with Propidium Iodide (PI) to label late apoptosis or necrosis (Lecoeur 2002) (Fig. 1A, 1D).

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**Fig. 1.** Cannabidivarin promotes cell viability under differentiative conditions.

(A) Schematic representation of the protocol used to evaluate cell viability. (B) Bar graphs depict the percentage of PI+ cells treated with CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 15.72% PI+ cells). n=4; **p<0.01. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (C) Bar graphs depict the percentage of PI+ cells co-treated with 5’-iRTX and CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100%. n=7; ns: p>0.05; **p<0.01; ****p<0.0001; §§§§p<0.0001. Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. (D) Representative fluorescent images of cells positive for PI (in red) and Hoechst 33342 staining (blue nuclei) at DIV2. Scale bar=50µm.

At DIV2, CBDV-treated cells, at the highest concentration, showed a reduction in the percentage of PI+ cells when compared to the control condition, therefore promoting cell survival (F(3,12)=9, p=0.0016; Ctrl: 100±0.0112%; CBDV 1µM: 76.63±2.379%; n=4; **p<0.01) (Fig. 1B, 1D). To evaluate whether the effects of CBDV upon cell viability were TRPV1-dependent, cells were incubated with the TRPV1 antagonist 5’-iodoresinofetoxin (5’-IRTX) at 300nM for 30 minutes, before being co-incubated with the
highest concentration of CBDV (1µM). For CBDV-induced cell survival, the decrease in cell death was blocked in the presence of the antagonist, revealing that CBDV-induced cell survival is TRPV1-dependent (CBDV×5'-IRTX: F(3,50)=23.19, p<0.0001; CBDV 1µM: 73.76±6.068%; 5'-IRTX 300nM + CBDV 1µM: 119.4±7.555%; n=7; §§§§p<0.0001) (Fig. 1C, 1D). 5'-IRTX alone, in turn, had no effect on cell viability when compared to the control condition (Fig. 1C, 1D).

Taken together, this data demonstrates that CBDV is able to regulate the viability of SVZ-derived cells via a TRPV1-dependent mechanism of action.

3.3. CBDV promotes cell proliferation in a TRPV1-dependent mechanism of action.

To study the effect of CBDV in cell proliferation, SVZ neurospheres were incubated with increasing concentrations of CBDV (100nM - 1µM) for DIV2 under differentiative conditions and, 4h before fixing, cells were incubated with 5-bromo-2'-deoxyuridine (BrdU) to label proliferating cells (Fig. 2A, 2E).

Fig. 2. Cell proliferation is increased with Cannabidivarin-treatment under differentiative conditions.

(A) Schematic representation of the protocol used to evaluate cell proliferation. (B) Bar graphs depict the percentage of BrdU+ cells treated with CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 6.39% BrdU+ cells). n=4-7; ****p<0.0001. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (C) Bar graphs depict the percentage of BrdU+ cells co-treated with 5'-IRTX and CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100%. n=3-7; ns: p>0.05; ****p<0.0001; §§§§p<0.0001. Two-way ANOVA followed by Bonferroni
multiple comparisons post-hoc test. (D) Bar graph depicts the levels of Ki67 mRNA expression. Values were normalized to the control expression of GAPDH expression for each experiment. Data presented as Mean ± SEM and the control was set to 1. n=6; **p<0.01. Unpaired t test. (E) Representative fluorescent images of cells positive for BrdU (in green) and Hoechst 33342 staining (blue nuclei) at DIV2. Scale bar=50μm.

CBDV-treated cells, at the highest concentration, showed an increase in the percentage of BrdU+ cells when compared to the control condition (F(3,17)=8.937, p=0.0009; Ctrl: 100±0.00182%; CBDV 1μM: 172.6±11.360%; n=4-7; ****p<0.0001) (Fig. 2B, 2E). Importantly, when the antagonist 5’-IRTX was co-incubated with CBDV, the increase in CBDV-induced cell proliferation was blocked (CBDV×5’-IRTX: F(3,41)=56.25, p<0.0001; CBDV 1μM: 172.6±11.360%; 5’-IRTX 300nM + CBDV 1μM: 64.36±0.6442%; n=3-7; §§§§p<0.0001) (Fig. 2C, 2E). Incubation with the antagonist 5’-IRTX alone had no effect on NSPC proliferation (Fig. 2C, 2E).

To test whether the CBDV-induced proliferative properties in NSPCs were long-lasting and to further assess cell proliferation by a different technique at a different time-point, the expression of Ki67 mRNA, a nuclear protein associated with cellular proliferation, was evaluated in SVZ-derived cells at DIV7. Corroborating the BrdU assays at DIV2, a significant increase in Ki67 expression levels was observed in CBDV-treated cells (t(10)=3.552, p=0.0053; Ctrl: 1.0-fold; CBDV 1μM: 1.862±0.242-fold; n=6; **p<0.01) (Fig. 2D).

Furthermore, cell proliferation was also evaluated under proliferative conditions through the assessment of neurosphere size (Fig. 3A, 3E). In agreement with the results obtained under differentiative conditions, CBDV significantly increased the size of neurospheres, both in terms of diameter (DN, Fig. 3B) and area (AN, Fig. 3C), when compared to the control condition (DN: t(3829)=7.658, p<0.0001; Ctrl: 94.36 (70.81 to 124.0)μm, n=2110; CBDV 1μM: 100.5 (75.49 to 140.1)μm, n=1721; ****p<0.0001; AN: t(3900)=8.629, p<0.0001; Ctrl: 7143 (3987 to 12526)μm², n=2143; CBDV 1μM: 8177 (4535 to 16195)μm², n=1759; ****p<0.0001). A more in-depth analysis of the DN revealed that CBDV-treated neurospheres generate more neurospheres with a DN > 300μm than
control condition ($\chi^2(1, N=3969) = 20.761, p<0.0001; \text{Ctrl}: 1.37\%, n=2177; \text{CBDV 1µM}: 3.58\%, n=1792$) (Fig. 3D, 3E; Table S1).

**Fig. 3. Neurosphere size is increased with Cannabidivarin-treatment under proliferative conditions.**

(A) Schematic representation of the protocol used to evaluate neurosphere size. (B) Violin plot representing the variation of neurosphere diameter ($D_N$). Data presented as median, 25% and 75% quartiles. The total number of neurospheres analysed (n) is for Ctrl: 2110, CBDV: 1721, from two independent cultures, ****p<0.0001. Unpaired t test. (C) Violin plot representing the variation of neurosphere area ($A_N$). Data presented as median, 25% and 75% quartiles. The total number of neurospheres analysed (n) is for Ctrl: 2143, CBDV: 1759, for two independent cultures, ****p<0.0001. Unpaired t test. (D) Bar graph representing the percentages of neurospheres distributed according to the size-binning categories, ns: p>0.05; **p<0.01; ****p<0.0001. Chi-square test. (E) Representative phase contrast images of neurospheres after 5 days in culture. Scale bar=100μm.

Thus, these results demonstrate that CBDV promotes cell proliferation in both proliferative and differentiative conditions in a TRPV1-dependent mechanism of action.
3.4. CBDV promotes neuronal differentiation in a TRPV1-dependent mechanism of action.

Neuronal differentiation was evaluated in SVZ neurospheres treated with increasing concentrations of CDBV (100nM - 1µM), for DIV2 or DIV7, under differentiative conditions. For that, immunocytochemistry (ICC) assays against NeuN, a marker for mature neurons, were performed (Fig. 4A, 4F).

Fig. 4. Neuronal differentiation is increased by Cannabidivarín treatment.

(A) Schematic representation of the protocol used to evaluate neuronal differentiation. (B) Bar graphs depict the percentage of NeuN+ cells treated with CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 3.468% NeuN+ cells). n=4; *p<0.05. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (C) Bar graphs depict the percentage of NeuN+ cells treated with CBDV for DIV7. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 11.143% NeuN+ cells). n=4; *p<0.05; ****p<0.0001. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (D) Bar graphs depict the percentage of NeuN+ cells co-treated with 5'-I-RTX and CBDV for DIV7. Data presented as Mean ± SEM and the control was set to 100%. n=4; ns: p>0.05; ****p<0.0001; §§§§p<0.0001. Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. (E) Bar graph depicts the levels of βIII-tubulin mRNA expression. Values were normalized to the control expression of GAPDH for each experiment. Data presented as Mean ± SEM and the control was set to 1. n=4; ***p<0.001. Unpaired t test. (F) Representative fluorescent images of cells positive for NeuN (in green) and Hoechst 33342 staining (blue nuclei) at DIV7. Scale bar=50µm.
Our results showed that the highest concentration of CBDV increased the percentage of NeuN+ cells at DIV2, when compared to the control condition ($F_{(3,12)}=5.422$, $p=0.0137$; Ctrl: 100±0.00583%; CBDV 1µM: 223.7±44.690%; $n=4$; *$p<0.05$) (Fig. 4B).

Importantly, at DIV7, the effect of the highest concentration of CBDV in neuronal differentiation was still detected ($F_{(3,12)}=24.67$, $p<0.0001$; Ctrl: 100±0.00139%; CBDV 1µM: 264.8±28.930%; $n=4$; ****$p<0.0001$) (Fig. 4C, 4F). Furthermore, at this time point, blocking TRPV1 with 5’-IRTX alone had no significant differences in the percentage of neurons when compared to the control condition. Notwithstanding, CBDV-induced neuronal differentiation was blocked with TRPV1 antagonist (CBDV×5’-IRTX: $F_{(3,26)}=62.16$, $p<0.0001$; CBDV 1µM: 228.1±30.480%; 5’-IRTX 300nM + CBDV 1µM: 122.1±4.984%; $n=4$; §§§$p<0.001$) (Fig. 4D, 4F).

To further understand the effects of CBDV and Capsaicin on the degree of neuronal maturation, the mRNA expression levels of βIII-tubulin, a marker for immature neurons, was evaluated in SVZ neurospheres at DIV7 under differentiative conditions. In fact, in CBDV-treated cells, the mRNA expression of βIII-tubulin was found significantly increased ($t_{(6)}=8.177$, $p=0.0002$; Ctrl: 1.0-fold; CBDV 1µM: 2.263±0.154-fold; $n=4$; ***$p<0.001$) (Fig. 4E).

These results highlight the importance of TRPV1 in neuronal differentiation process, namely on its impact in regulating the early stages of NSPC pool and maturation of newborn neurons.

3.5. CBDV-induced neuronal differentiation is a result of cell cycle exit of NSPCs.

To further clarify whether the CBDV-induced increase in neuronal differentiation relies on their effects in controlling proliferating and/or cell cycle exit of NSPCs, neurospheres, under differentiative conditions, were subjected to CBDV treatment and exposed to a
pulse of BrdU for the first 24h. The drugs, however, remained in culture until DIV7, the
time at which cells were co-labelled against BrdU and NeuN (Fig. 5A, 5F).

Fig. 5. CBDV promotes NSPC exit of the cell cycle and differentiation into neurons.

(A) Schematic representation of the protocol used to evaluate differentiation of neurons from proliferative
NSPCs. (B) Bar graphs depict the percentage of BrdU−NeuN+ cells out of total cells. (C) Bar graphs depict
the percentage of BrdU+NeuN+ cells out of NeuN+. (D) Bar graphs depict the percentage of BrdU+NeuN+
cells out of BrdU−. (E) Bar graphs depict the percentage of BrdU−NeuN+ out of total cells. (B-E) Values were
normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was
set to 100% (corresponding to (B) 2.490% BrdU−NeuN− cells out of total cells; (C) 15.731% BrdU−NeuN−
cells out of NeuN−; (D) 38.416% BrdU+NeuN+ cells out of BrdU−; (E) 13.746% BrdU−NeuN+ out of total cells);
n=5; ns: p>0.05; ***p<0.001. Unpaired t test. (F) Representative fluorescent images of cells positive for NeuN
(in green), BrdU (in red) and Hoechst 33342 staining (blue nuclei) at DIV 7. Arrows represent double-positive
cells for BrdU/NeuN. Scale bar=50μm.

Surprisingly no significant changes in the percentage of BrdU−NeuN+ cells (out of total
cells) were observed in CBDV condition (t(8)=0.1553, p=0.8804) (Fig. 5B, 5F). When
looking at the percentage of neurons that differentiated from proliferating cells
(BrdU+NeuN+ cells (out of NeuN+)) (Fig. 5C, 5F), again no differences were observed
suggesting that the differentiated BrdU^+NeuN^+ neurons represent only a small fraction of the total population of neurons. Concomitantly, when looking at the percentage of proliferating cells that differentiated into neurons (BrdU^+NeuN^+ cells (out of BrdU^+)) (Fig. 5D, 5F) no differences were observed when comparing to the control condition ($t(8)=1.760$, $p=0.1164$), suggesting that CBDV-induced proliferative cells are not responsible for the increased percentage of neurons observed after this treatment. However, an increase in the percentage of BrdU^−NeuN^+ cells (out of total cells) by CBDV treatment was observed ($t(8)=5.865$, $p=0.0004$; Ctrl: 100±0.01139%; CBDV 1µM: 148.2±8.213%; n=5; ***$p<0.001$), revealing that CBDV induced the arrest and exit of cell cycle in NSPCs and stimulates their differentiation into neurons (Fig. 5E, 5F).

In conclusion, our BrdU/NeuN experiments clearly show that CBDV-increased NeuN^+ cells result from NSPCs that exit the cell cycle.

3.6. CBDV inhibits oligodendrocyte differentiation and maturation.

Since cannabinoids have been shown to regulate oligodendrocyte differentiation in the SVZ (Arévalo-Martín et al. 2007), we aimed at understanding if CBDV would share this effect and if TRPV1 was involved. To do so, neurospheres, under differentiative conditions, were treated with CBDV for DIV2 and DIV7, and an ICC against Neuron/glia antigen 2 (NG2), a marker for oligodendrocyte progenitor cells (OPC), was performed (Fig. 6A, 6F). Notably, CBDV did not induced significant changes in the percentage of NG2^+ cells at DIV2 when compared to the control condition ($F_{(3,12)}=0.1647$, $p=0.9181$) (Fig. 6B). However, at DIV7, the highest concentration of CBDV ($F_{(3,12)}=12.12$, $p=0.0006$; Ctrl: 100±0.0107%; CBDV 1µM: 72.61±3.506%; n=4; **$p<0.01$) significantly reduced the percentage of OPCs (Fig. 6C, 6F). Therefore, given the reduction in the percentage of OPCs, we hypothesized that CBDV could be promoting of oligodendrocyte maturation. Thus, an ICC was performed in the same conditions at DIV7, but this time against Myelin Basic Protein (MBP), a marker for mature myelinating oligodendrocytes (Fig. 6A, 6G). Surprisingly, the highest concentration of CBDV diminished the percentage of MBP^+ cells
**(F\(3,12\))=10.80, p=0.0010; Ctrl: 100±0.00372%; CBDV 1µM: 44.65±3.506%; n=4; p<0.01 (Fig. 6D, 6G).

In addition, and strikingly, blocking TRPV1 with the antagonist 5'-IRTX was able to increase the percentage of MBP+ cells, when compared to the control condition (F\(3,12\))=13.25, p=0.0004; Ctrl: 100±0.00134%; 5'-IRTX 300nM: 137.3±7.918%; n=4; p<0.01 (Fig. 6E, 6G), suggesting that TRPV1 inhibition per se promotes MBP differentiation and maturation. As such, when co-incubating 5'-IRTX with CBDV, an increase in the percentage of myelinating oligodendrocytes was detected when comparing with CBDV alone (CBDV×5'-IRTX: F\(3,28\)=21.79, p<0.0001; CBDV 1µM: 51.81±8.299%; 5'-IRTX 300nM + CBDV 1µM: 89.34±15.55%; n=4; p<0.01) (Fig. 6E, 6G).

These results reveal a potential for TRPV1 in the modulation of oligodendrocyte differentiation and maturation.
Fig. 6. Oligodendroglial differentiation and maturation is inhibited by Cannabidivarin treatment. (A) Schematic representation of the protocol used to evaluate oligodendrocyte differentiation. (B) Bar graphs depict the percentage of NG2+ cells treated with CBDV for DIV2. Values were normalized to the control mean for each experiment. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 13.24% NG2+ cells). n=4; ns: p>0.05. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (C) Bar graphs depict the percentage of NG2+ cells treated with CBDV for DIV7. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 6.943% NG2+ cells). n=4; **p<0.01. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (D) Bar graphs depict the percentage of MBP+ cells treated with CBDV for DIV7. Data presented as Mean ± SEM and the control was set to 100% (corresponding to 1.395% of MBP+ cells). n=4; **p<0.01. One-way ANOVA using Dunnett’s multiple comparison post-hoc test. (E) Bar graphs depict the percentage of MBP+ cells co-treated with CBDV and 5’-IRTX for DIV7. Data presented as Mean ± SEM and the control was set to 100%. n=4; p>0.05; **p<0.01; ***p<0.001; §§§p<0.001. Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. (F) Representative fluorescent images of cells positive for NG2 (in green) and Hoechst 33342 staining (blue nuclei) at DIV7. Scale bars=50μm. 3.7. CBDV-responsive cells display a two-phase calcium influx profile. Since CBDV acts via TRPV1, a sodium-calcium channel, and that intracellular calcium levels are known regulators of NSPCs fate and neuronal maturation, single cell calcium imaging was performed to evaluate the functional response of SVZ-derived cells at DIV7 exposed, or not (Movie A1) to CBDV. Cells were also exposed to a prior incubation/non-incubation with the TRPV1 antagonist, 5’-ITRX, to further clarify the role of TRPV1 activation in response to these drugs (Fig. 7A). Surprisingly, incubation with 1µM CBDV at 100 seconds elicited two types of calcium influx responses in SVZ-derived cells. One short initial response (∼85 seconds after the CBDV was added to cells), that corresponds to TRPV1 activation, which is blocked by the antagonist 5’-ITRX, and a second long-lasting response (∼550 seconds after CBDV was added to cells). Specifically, in the first response, CBDV elicited a moderate increase in the calcium influx, with a peak, followed by a subtle decline (F(3,976)=200.5, p<0.0001; Ctrl peak ratio: 1.014±0.00173, n=365; CBDV 1µM peak ratio: 1.266±0.0260, n=120 responsive cells; ****p<0.0001). In contrast, in cells co-exposed to CBDV and 5’-ITRX, the initial calcium influx was blocked (CBDV×5’-ITRX: F(3,612)=145.4, p<0.0001; CBDV 1µM peak ratio: 1.266±0.0260, n=120; 5’-ITRX 300nM + CBDV 1µM peak ratio: 1.049±0.00598, n=166 responsive cells; §§§§p<0.0001). Unexpectedly, an increase in
calcium influx, starting at around 500 seconds, was also observed, peaking at ≈ 650s, after CBDV was added, lasting until the end of the experiment. Of note, when compared to the control condition, the co-exposure with the antagonist, resulted in a slight increase of calcium influx at the peak (CBDV × 5'-IRTX: F(3,612) = 145.4, p < 0.0001; Ctrl peak ratio: 1.014 ± 0.00173, n = 365; 5'-IRTX 300nM + CBDV 1μM peak ratio: 1.049 ± 0.00598, n = 166 responsive cells; **p < 0.01) (Fig. 7B, 7D, 7E, 7F, Movie A2, Movie A4). No significant differences in the calcium profile were detected when comparing the calcium influx of 5'-IRTX exposed cells with the control condition at the peak (Movie A3). Similarly, in the last 100 seconds of responses, no differences in the calcium influx were observed when comparing control with 5'-IRTX alone (Movie A3). Moreover, the calcium influx induced by CBDV was higher than the influx induced by the co-exposure with the antagonist in the last 100 seconds of responses (CBDV × 5'-IRTX: F(3,377) = 115.7, p < 0.0001; CBDV 1μM ratio: 1.224 ± 0.0160, n = 120; 5'-IRTX 300nM + CBDV 1μM: 1.136 ± 0.00908, n = 166 responsive cells; §§§p < 0.0001). Furthermore, the influx induced by both CBDV or by co-exposure with the antagonist did not reach levels close to the control baseline in the last 100 seconds (F(3,741) = 178.9, p < 0.0001; CBDV × 5'-IRTX: F(3,377) = 115.7, p < 0.0001; Ctrl ratio: 0.9964 ± 0.00368, n = 365; CBDV 1μM ratio: 1.224 ± 0.0160, n = 120; ****p < 0.0001; 5'-IRTX 300nM + CBDV 1μM ratio: 1.136 ± 0.00908, n = 166 responsive cells; ****p < 0.0001) (Fig. 7C, 7D, 7E, 7F, Movie A2, Movie A4).

Interestingly, out of all the analysed cells, no differences were found between the percentage of responsive and non-responsive cells. In more detail, when comparing cells that responded to CBDV to those that were co-incubated with the antagonist, the proportion of responsive cells was equivalent (Χ² (1, N = 973) = 0.839, p = 0.3596; %Responsive CBDV 1μM: 26.85%; %Responsive 5'-IRTX 300nM + CBDV 1μM: 31.56%). Concomitantly, the proportion on non-responsive cells also presented no differences (Χ² (1, N = 973) = 2.580, p = 0.1082; %Non-Responsive CBDV 1μM: 73.15%; %Non-Responsive 5'-IRTX 300nM + CBDV 1μM: 68.44%) (Fig. 7G).
In conclusion, CBDV was able to induce two types of response. One, in the first 100s after CBDV exposure, that was blocked by 5'-IRTX, and another that was not blocked by the antagonist in the last 100s of the experimental protocol.

**Fig. 7.** CBDV-induced calcium influx profile is dependent of TRPV1 activation in SVZ-derived cells.

(A) Schematic representation of the protocol used to evaluate calcium influx. (B) Ratio of calcium influx at peak maxima from T=100-200 seconds, after CBDV exposure. (C) Ratio of calcium influx at peak maxima in the last 100s of experiment, after CBDV exposure. (B)(C) The responding cells were analysed in each well (120-365 cells) and normalized to the first point of the baseline for each data set. n=3; ns: p>0.05; **p<0.01; ****p<0.0001. Two-way ANOVA followed by Bonferroni multiple comparisons post-hoc test at peak maxima. (D) Representative scatter plot that depicts a single-cell variation of calcium influx throughout the time (in seconds) in response to 1µM CBDV. (E) Representative fluorescent intensity ratio of calcium influx (colour code, blue for a low calcium level, green for intermediate, yellow for medium high, and red for high). (F) Representative matrix of single-cell calcium response to a pulse of 1µM CBDV. Each row represents time-points, and the columns represent the mean values of calcium influx of most responsive cells in each condition. The heatmap graph represents a colour-code variation (purple/blue for a low calcium level, green for intermediate, yellow for medium high, and red for high) of the mean values of calcium influx of the most responsive cells from a total of 973 cells analysed in these conditions. (G) Stacked bar graphs representing the percentage of responsive versus non-responsive cells influx upon a pulse of 1µM CBDV. A cell was considered responsive when its maximum recorded response was greater than the average of the responses for all cells for each condition. ns: p>0.05; Chi-square test.
4. Discussion

In this study we describe the proneurogenic effects of CBDV, *in vitro*, as a direct effect of TRPV1 activation. CBDV positively regulates cell survival, cell proliferation and neuronal differentiation in a TRPV1-dependent mechanism of action. Furthermore, inhibition of oligodendrogenesis by CBDV was also observed, which was blocked with TRPV1 antagonist.

Although the affinity of CBDV for TRPV1 is yet uncertain, since the $K_i_{\text{TRPV1}}$ for CBDV has not been yet determined, the concentrations of CBDV used in this study were based on previous works made with other endo-, phyto- and synthetic cannabinoids (Xapelli *et al.* 2013; Stanslowsky *et al.* 2017; Compagnucci *et al.* 2013; Rodrigues *et al.* 2017). In an elegant study by Petrocellis and colleagues (De Petrocellis *et al.* 2011), they determined the $EC_{50}$ for CBDV for several receptors, namely TRPV1, TRPV2 and TRPA1. Their study has served as the base of several others that further clarified the pharmacology of this drug. Despite that CBDV was reported to have a $EC_{50}$ for TRPA1 (0.42±0.01µM) lower than for TRPV1 (3.6±0.7µM), it is possible that these results might be influenced by the methodology applied to determine the $EC_{50}$ (MarÉchal 2011). In their study, HEK-293 cells were transfected using recombinant rat TRPA1, rat TRPV2 and human TRPV1. While, not only these cells do not physiologically express these receptors (Costanzo *et al.* 2020; Starkus *et al.* 2019), the constructs used were from different species, thus the calcium changes, used to determine the $EC_{50}$, might not totally translate into physiological responses. Furthermore, another study, also using HEK-293 cells, transfected with recombinant human TRPV1, calculated the $EC_{50}$ of CBDV for TRPV1 at 56µM (Starkus *et al.* 2019).

While TRPV1 expression in the central nervous system (CNS) has been controversial (reviewed in Kauer and Gibson 2009), In our model, SVZ-derived cells do not only express high levels of TRPV1, as previously described (Stock *et al.* 2014; Zhai *et al.*
2020; Ramírez-Barrantes et al. 2016), but its mRNA expression is significantly increased in the presence of CBDV.

A not well-understood function of TRPV1 is its role in regulating cell death and proliferation. Particularly, the majority of these studies have been carried out using cancer cell models. These highlighted the potential of several TRPV1 modulators, such as Capsaicin, the prototypic TRPV1 agonist, as inducers of cell death (Stock et al. 2012; Hou et al. 2019; Díaz-Laviada and Rodríguez-Henche 2014), and inhibitors of cell proliferation (Li et al. 2021; Lin et al. 2013; Weber et al. 2016). Thus, ideal candidates as anti-cancer drugs. In our study, we have found that CBDV, in a TRPV1-dependent manner, was able to promote cell viability and proliferation in SVZ-derived NSPCs. Despite that our data contrasts with what was previously shown using cancer cell lines, it aligns with other works that have evaluated the effects of cannabinoids upon cell death and proliferation (Pacher and Mackie 2012; Yang et al. 2020; Stone et al. 2021; Molina-Holgado et al. 2002; Xapelli et al. 2013; Rodrigues et al. 2017; Bravo-Ferrer et al. 2017; Bockmann et al. 2022). Interestingly, anandamide, an endogenous cannabinoid and TRPV1 agonist, has been shown to display similar results to the ones obtained in our study (Stock et al. 2012; Hofmann et al. 2014). In fact, regarding cell death, incubation with anandamide alone did not to induce cell death in the GL261 glioma cell line (Stock et al. 2012). In another study, it promoted endothelial cell proliferation in a TRPV1-dependent mechanism of action, since blocking TRPV1 with the antagonist SB366791 inhibited that effect (Hofmann et al. 2014). These works support the idea that TRPV1, via cannabinoids, might play a dual role in the mediation of cell death and cell proliferation.

The transmission of electrical signals between neurons in brain networks and circuits is essential to normal brain function. In an oversimplistic model, the neurosphere assay, cultured NSPCs may differentiate into neurons as a consequence of external neural activity that requires a calcium flux (Deisseroth et al. 2004). Indeed, our work shows that
after a 7-day exposure to CBDV, NSPCs exit the cell cycle and differentiate mostly into neurons. While the mechanisms regarding cell cycle arrest induced by TRPV1 have not yet been fully understood, our results further support the idea that CBDV halts NSPC cell cycle. Thus, the modulation of TRPV1 might offer an innovative strategy to study all these important aspects of well-functioning neural precursors.

Interestingly, our data has shown that CBDV inhibited oligodendroglial maturation and differentiation. Previous works suggested that oligodendrocyte differentiation and myelinating abilities could be modulated by cannabinoids, which are responsible for promoting OPC survival and maturation (Molina-Holgado et al. 2002; Rodgers et al. 2013). TRPV1 is expressed in OPCs and myelinating oligodendrocytes (Moreno-Luna et al. 2021) suggesting that the development of these cells could be modulated by agonists and antagonists of this channel. Therefore, our results agree with published data whilst not reflecting a positive effect on oligodendrogenesis mediated by cannabinoids. The co-incubation with the antagonist 5'-IRTX, was able to reverse the loss of mature oligodendrocytes induced by CBDV. It should, however, be noted that the percentage of myelinated oligodendrocytes in the presence of both drugs is in between that observed in the presence of each drug alone, therefore additive effects cannot be discarded. Importantly, the incubation of 5'-IRTX alone was able to significantly increase the percentage of myelinating oligodendrocytes. One study linked the effect of 5'-IRTX with the inflammatory response induced my TRPV1 on microglial cells both in vitro and in samples of cerebrospinal fluid of patients with multiple sclerosis (MS). Upon incubation with 5'-IRTX, the proinflammatory cytokines tumour necrosis factor and interleukin-6, which were elevated after TRPV1 activation by Capsaicin, were significantly reduced (Bassi et al. 2019). Our data extends these findings by suggesting that 5'-IRTX not only can have anti-inflammatory properties, but also a pro-oligodendrogenic potential. This response of 5'-IRTX can serve up as a proof of concept for future studies aiming at evaluating the neuroimmune modulatory responses of this drug as well as its pro-
oligodendrogenic effects in MS. Further studies, clearly outside the scope of this work, are necessary to better understand the role of CBDV as a modulators of oligodendrocyte differentiation and maturation.

Spontaneous calcium oscillations play important roles in CNS development, neural induction, axon guidance, growth cone morphology, migration, and proliferation (Goswami and Hucho 2007; Komuro and Rakic 1996; Gu et al. 1994; Weissman et al. 2004). Regarding CBDV, SVZ-derived cells displayed two types of response, one short initial response that corresponds to TRPV1 activation and which is blocked by prior incubation with 5’-IRTX and a long-lasting response that was not fully blocked by the antagonist, possibly suggesting the involvement of a metabotropic receptor. Therefore, the effects in postnatal SVZ neurogenesis might require the activation of both TRPV1 and a putative metabotropic receptor. This polymodal response corresponds to the initial peak of calcium influx observed, prompted by TRPV1, that might act as a trigger for the adjacent cell responses, which are maintained, long term, via a metabotropic mechanism. Thus, when the antagonist 5’-IRTX is present, the trigger is ablated, and the CBDV-elicited calcium response has a delayed onset, as observed.

Furthermore, one study compared the calcium responses elicited by Cannabidiol, to which CBDV is a propyl analogue (Thomas and ElSohly 2016), with the prototypic TRPV1 agonist Capsaicin (Bisogno et al. 2001). The calcium influx response evoked by Capsaicin was 100-fold higher than the one elicited by Cannabidiol (Bisogno et al. 2001). A recent work highlighted that in cultures derived from rat-dorsal root ganglion neurons, 1µM of Cannabidiol was able to induce a very low calcium response (Anand et al. 2020).

In our study, CBDV-elicited a calcium response higher than the Cannabidiol reported by Anand and colleagues, and much lower than the one evoked by Capsaicin reported by Bisogno and colleagues. This is relevant since the observed differences regarding the effects of CBDV on cell viability and proliferation might be related to the different magnitudes of calcium influx elicited by different TRPV1 agonists. Indeed, other works
have linked the concentration of calcium entering the cell, upon TRPV1 activation, to several molecular pathways that regulate cell homeostasis (Touska et al. 2011; reviewed in Zhai et al. 2020). The pro-apoptotic effects of TRPV1, as seen in cancer studies, may involve the mitochondria (reviewed in Juárez-Contreras et al. 2020). The influx of calcium into the mitochondria results in the depolarization of the mitochondrial membrane and subsequent activation of pro-apoptotic pathways, with higher levels of calcium influx and higher rates of cell death (Kim et al. 2006). On the other hand, TRPV1-dependent cell proliferation has also been linked to ATP release and to the activation of the purine receptor P2Y2, with a limited role for calcium influx (Denda et al. 2010). Neuronal differentiation has been implicated with calcium influx, with increased levels being linked to a faster differentiation and more mature neurons (Spoerri et al. 1990; Holliday et al. 1991). Thus, the concentration of calcium influx elicited by CBDV is critical for the maintenance and regulation of cellular homeostasis, with higher concentrations of calcium leading to cell death and inhibition of cell proliferation, as seen for Capsaicin (reviewed in Zhai et al. 2020), and lower concentrations promoting cell survival, proliferation and a slower rate of differentiation, as suggested for CBDV (Ramírez-Barrantes et al. 2016).

5. Conclusions

Based on the multifactorial responses evoked by CBDV, the data herein described provides new insights on the action of this cannabinoid upon TRPV1, with impact in the modulation of postnatal neurogenesis. Despite that the pharmacodynamics of this cannabinoid are still not entirely clear, there is a strong interest by both researchers and clinicians in repurposing CBDV as a viable medicine, with several clinical trials already undergoing studies for several disorders, ranging from autism spectrum disorders (ClinicalTrials.gov Identifier: NCT03537950 2018; ClinicalTrials.gov Identifier: NCT03202303 2017; ClinicalTrials.gov Identifier: NCT03849456 2019) to androgenetic alopecia (ClinicalTrials.gov Identifier: NCT04842383 2021). The fact that CBDV is a non-
psychoactive cannabinoid makes this cannabinoid a promising candidate for further studies. Given the significant impact of neurological disorders worldwide, the outcome of such studies has the potential to serve as a cornerstone for advancing brain repair strategies by utilizing NSPCs and cannabinoids as therapeutic options.

CRediT authorship contribution statement

Conceptualization: DML, AMS, SX; methodology: DML, SSS, SV and SX; investigation: DML, RS, SSS, JM, RSR and JBM; formal analysis: DML and SSS; project administration: AMS, SS and SX; writing – original draft: DML and SX.; writing – review& editing: DML, RS, SSS, JM, RSR, JBM, SV, AMS, SS and SX; funding acquisition: SS, MJD and SX; resources: SV, SS and SX; visualization: DML; supervision: SX.

Conflict of Interest

The authors declare no competing interests.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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