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Involvement of the GABAergic system in excitotoxic damage caused by AMPA in oligodendrocytes

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USED ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA: analysis of variance
Bid: BH3 interacting-domain death agonist
BSA: bovine serum albumin
CaMK: calmodulin kinases
cAMP: cyclic adenosine monophosphate
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CREB: cAMP- responsive element-binding protein
CTZ: ciclotiazide
DAPI: 4′,6-diamidino-2-phenylindole
DIV: days *in vitro*
FBS: setal bovine serum
GABA: γ--aminobutyric acid
$GABA_AR$: $GABA_A$ receptor
$GABA_BR$: $GABA_B$ receptor
GalC: galactocerebroside C
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GRP-17: gadd-related protein 17
GPCR: g-protein coupled receptor
IMDM: Iscove's modified Dulbecco's medium
JNK: c-Jun N-terminal kinase
MAG: myelin associated glycoprotein
MBP: myelin basic protein
MOG: myelin oligodendrocyte glycoprotein
NGS: normal goat serum
NMDAR: N-methyl-D-aspartate receptor
NT3: neurotrophin-3
mGluR: metabotropic glutamate receptor
OL: oligodendrocyte
OPC: oligodendrocyte progenitor cell
Pacs-2: phosphofurin acidic cluster sorting protein
PBS: phosphate-buffered saline
PDL: poly-D-lysine
PDGFRα: platelet-derived growth factor receptor α
PKA: protein kinase A
PKC: protein kinase C
PLP: proteolipid protein
SEM: standard error of the mean
t-Bid: truncated Bid
TBST: tris buffer saline with tween 20
TOMM-20: translocase of outer mitochondrial membrane 22
WB: western blot

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1. INTRODUCTION

Two types of cells can be found in the central nervous system (CNS): neurons, which are responsible for action potential transmission, and glial cells, which protect neurons and offer them metabolic and physical support, among other functions (Shimizu et al., 2017; Pepper et al., 2018). Among glial cells, we find macroglia, comprising astrocytes, oligodendrocytes (OLs) and ependymal cells, and microglia, which are phagocytic cells derived from macrophages (Knott & Molnar, 2005).

Among them, OLs are the cells responsible for axon myelination in the CNS, providing the high electrical resistance and low capacitance that is essential for adequate saltatory impulse propagation (Simons & Nave, 2015; Pepper et al., 2018). Their equivalents in the peripheral nervous system are Schwann cells (Knott & Molnar, 2005).

Myelination consists in a complex sequence of events that can be separated into several steps: proliferation and migration of oligodendrocyte progenitor cells (OPCs), recognition of target axons and axon-glia signaling, differentiation of OPCs into myelinating OLs, membrane outgrowth and axonal wrapping, trafficking of membrane components, myelin compaction, and node formation (Simons & Nave, 2015). As we see, to complete the task of creating myelin sheaths around axons, OLs have to differentiate from OPCs, following a highly regulated process (Barateiro et al., 2015).

This differentiation process can be divided into four cellular stages: OPCs, pre-OLs, immature OLs and mature or myelinating OLs (**Figure 1**) (Barateiro et al., 2015). During the process, OPCs will change their morphological and functional characteristics, and they will express specific markers that enable us to identify each developmental stage. To start, OPCs have a high proliferative and migratory capacity and express specific markers like platelet-derived growth factor receptor α (PDGFRα) and proteoglycan NG2. In this progenitor state, OPCs present a bipolar morphology with few short processes. As they differentiate into pre-OL, they will start to express other markers such as the gadd-related protein-17 (GRP-17). Later on, they will lose the expression of NG2 markers and start expressing markers characteristic from immature OLs, for instance, the galactocerebroside C (GalC). These immature OLs already present long ramified branches that will end up wrapping around the axons. Finally, mature OLs synthesize the major myelin structural proteins, for example myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG) and myelin OL glycoprotein (MOG), and proceed to form myelin sheaths around axons (Zhang, 2001; Barateiro et al., 2015).

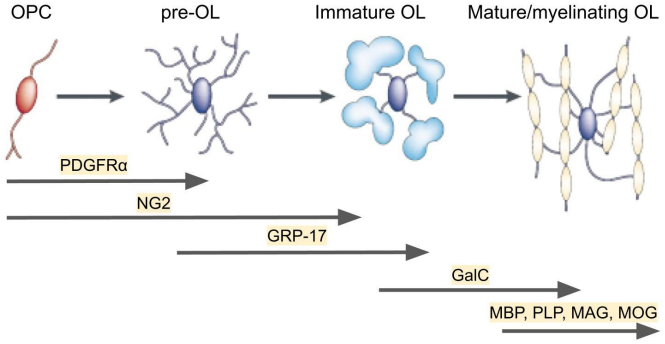

Figure 1. Differentiation stages of the oligodendroglial lineage. Schematic depiction of OPC differentiation stages showing their morphological features and the most representative markers expressed in each stage. Adapted from Zhang, 2001.

Therefore, myelination is closely related to OPC differentiation, being this process strictly controlled by inter-signalling between OPC/OLs and neurons, through the action of neurotransmitters and growth factors. However, the molecular mechanisms that regulate this communication have not been completely defined yet. As for now, we know that both OPC and OL, express several neurotransmitter-related receptors, including AMPA and GABA receptors (Patneau et al., 1994; Barateiro et al., 2015).

AMPA signalling is thought to be mostly excitatory (Kantamneni, 2015), and it has been demonstrated that activation of AMPA/kainate inhibits PDGF-induced proliferation and promotes OPC differentiation (Patneau et al., 1994), and therefore the progress of myelination. On the other hand, OPCs/OLs also express the GABAergic receptors $GABA_AR$ and $GABA_BR$, which transmit mostly inhibitory signals (Pastor et al., 1995; Barateiro et al., 2015). $GABA_ARs$ are ionotropic receptors that allow the passing of Cl^- and HCO_3^- anions, whereas $GABA_BRs$ are metabotropic receptors coupled to G proteins (GPCRs) (Kantamneni, 2015).

As could be expected, these excitatory and inhibitory signalling pathways are balanced in normal situations, but can become unbalanced in pathological contexts. Moreover, it has been proven that glutamatergic and GABAergic signalling crosstalk in cells, to preserve the equilibrium, and in some cases controlling the activity of these receptors has therapeutic potential (Kantamneni, 2015). Also, GABARs have a strong impact on OPC differentiation and myelin related protein expression, as well as on the survival of myelinating OLs (Hamilton et al., 2016; Serrano-Regal et al., 2020a; Serrano-Regal et al., 2020b).

Given that failure of efficient remyelination in diseases like multiple sclerosis is related to the incapacity of OPCs to efficiently differentiate, understanding the mechanisms that control this differentiation process is essential to identify therapeutic strategies that promote myelin repair (Weider et al., 2018; Serrano-Regal et al., 2020b). In addition, cell dead provoked by AMPA excitotoxicity is also of great relevance in demyelinating illnesses (Canedo-Antelo et al., 2018), and considering the capacity of GABA to regulate OL functions and survival it would be interesting to study the relationship between these two receptor families.

Excitotoxicity is the cell death caused by excessive glutamatergic signalling that happens when receptor agonists, such as AMPA, are in a pathologically high concentration, and can affect many cell lineages, including OLs. This overactivation of AMPA/kainate receptors leads to an aberrant increase in intracellular Ca^{2+}, whose accumulation within the mitochondria increases reactive oxygen species (ROS) production, provoking the activation of initiator caspases, as caspase-8, which later leads to the activation of executioner caspases like 9 and 3, culminating in the execution of the intrinsic apoptotic cascade. In addition, this excitotoxicity-mediated cell death can also follow non-apoptotic/necrotic pathways (Sánchez-Gómez et al., 2003; Sánchez-Gómez et al., 2011; Barateiro et al., 2015). The

apoptotic process can be observed via subcellular localization of specific proteins which take part in it, for example Pacs-2, normally found in the cytosol, which translocates to the mitochondria in response to apoptotic inducers. Pacs-2 transports the protein Bid to the mitochondria, where it will be transformed into truncated Bid (t-Bid), to increase the permeability of the mitochondrial membrane, allowing the release of cytochrome c and the activation of the executioner caspase-3, thereby causing cell death (Simmen et al., 2005). There are several signaling pathways that affect or modulate the excitotoxic response, although the exact mechanisms that mediate AMPA and GABAR-associated response in OLs remains unclear.

It has been reported that $GABA_BR$ activation leads to the PI3K/Akt/Src-kinases signalling pathway in neurons, where it plays an important role in anti-apoptotic/pro-survival cellular processes (Tu et al., 2010) and could exert the same effect in glial cells. This pathway, activated following $GABA_BR$ stimulation, provokes an increase of intracellular cAMP, which prompts the expression of the transcription factor cyclic cAMP-response element binding protein (CREB) (Shiga et al., 2005). This fact is related with PKA activation, one of the kinases responsible for the phosphorylation and subsequent activation of CREB (Shiga et al., 2005), together with other kinases such as Akt, protein kinase C (PKC) and calmodulin kinases (CaMK) (Zhou et al., 2000; Wen et al., 2010). Once phosphorylated, CREB can activate the transcription of anti-apoptotic proteins. This, taken together with the phosphorylation and activation of Akt and its effect on other targets, has a strong anti-apoptotic effect in cells.

Taking all this information into account, the possible impact of GABAR on the reduction or prevention of AMPA-mediated excitotoxicity could help to develop new therapies to treat numerous neurological disorders characterized by this imbalance between excitatory and inhibitory signalling, such as demyelinating diseases (Ishii et al., 2019).

2. HYPOTHESIS AND OBJECTIVES

Considering the aforementioned importance of the balance between GABA and AMPA signaling, and therefore its therapeutic potential, we hypothesized that activation of GABARs can exert a protective effect on oligodendroglial cells subjected to excitotoxic damage. For that, the general objective is to elucidate the impact of GABAergic system modulation on AMPA-mediated excitotoxicity in OPCs *in vitro*. With that aim, the following specific objectives were set:

- To evaluate the effect of pharmacological $GABA_AR$ and $GABA_BR$ activation on the viability of OPCs treated with AMPA.
- To analyze the impact of $GABA_BR$ activation in AMPA-mediated apoptotic events.
- To assess the relation of Akt/CREB signaling pathways in $GABA_BR$ and AMPA crosstalk.

3. MATERIALS AND METHODS

All experiments were conducted with the approval of the internal Animal Ethics Committee of the University of the Basque Country (UPV/EHU) and the European Communities Council Directive.

3.1. Rat brain OPC culture

Highly enriched OPCs were isolated from mixed glial cultures obtained from newborn (P0–P2) Sprague-Dawley rat brain cortices as previously described (Canedo-Antelo et al., 2018). First, brains were removed from the skulls and the cortices were isolated and enzymatically digested by incubation in Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}) containing 0.25% trypsin and 4% DNAse for 15 min at 37ºC. The enzymatic reaction was stopped by adding Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS, Hyclone), and the suspension was centrifuged at 200 *g* for 5 min. Then, cells were mechanically dissociated by passage through two different sized needles (21 G and 23 G) and centrifuged at 200 *g* for 5 min. Finally, cells were resuspended in IMDM with 10% FBS, plated in 75 cm^2 flasks (pre-treated overnight with 1 μg/mL poly-d-lysine, Sigma-Aldrich), and maintained at 37ºC in a humidified atmosphere with 5% CO2. The medium was changed after 24 h and then every 3-4 days. In these flasks we obtained a mixed culture of astrocytes, microglia and OPCs. After 2 weeks, OPCs were isolated from the mixed glial cultures. Initially, microglia was removed by shaking the flasks at 400 rpm for 1 h. Then, OPCs were detached from the confluent astrocyte monolayer in the culture by shaking the flasks overnight at 300-400 rpm. The following day, the resultant OPC suspension was filtered using a 10 μm nylon mesh, then placed on a petri dish (Thermo Fisher Scientific) and incubated for 30 min for the remaining microglia to stick to the dish. After that, the OPC suspension was passed again through a 10 μm filter and then centrifuged at 200 *g* for 10 min. Cells were resuspended in SATO+ medium (OL differentiation medium) composed by: Dulbecco's modified Eagle's medium (Gibco) supplemented with 5 μg/mL insulin, 100 μg/mL transferrin, 62.5 ng/mL progesterone, 40 ng/mL sodium selenite, 16 μg/mL putrescine, 1 mg/ml bovine serum albumin (BSA), 63 μg/mL N-acetyl-cysteine, 2 mM L-glutamine, 30 ng/mL triiodothyronine, 40 ng/mL thyroxine (all from Sigma-Aldrich), 100 U/mL penicillin/streptomycin (Lonza), 10 ng/mL ciliary neurotrophic factor (CNTF) and 1 ng/mL neurotrophin-3 (NT3) (both from Peprotech).

3.2. Cell viability assay

For the study of cell viability, OPCs were seeded in 24-well plates at a density of 10^4 cells/well. After 24 h *in vitro* (1 day *in vitro*, 1 DIV), cells were treated for 30 min with 50 μM baclofen or 20 μM muscimol. Cells were then treated with 100 μM cyclothiazide (CTZ) for 10 min before incubation with 10 μM AMPA for 10 or 30 min. The medium was subsequently removed and cells were incubated overnight in AMPA-free fresh SATO+ medium (alone, with baclofen or muscimol). OPC viability was assessed 24 h later (at 2 DIV) by loading cells with 1 μM calcein-AM (Molecular Probes; Invitrogen) for 30 min and the fluorescence emitted by the calcein probe was measured using a

Synergy-H4 Hybrid Reader fluorimeter (Bio-Tek Instruments Inc., Winooski, VT, USA) as indicated by the supplier (485 nm excitation wavelength and 530 nm emission wavelength). After fluorescence measurement, cells were immediately fixed by substituting their medium for PBS with 4% paraformaldehyde (PFA) for 20 min at room temperature. They were subsequently rinsed with PBS and stored in PBS with 0.02% azide at 4ºC until imaged. Representative images from fixed cells were acquired using a Zeiss Axioplan2 fluorescence microscope (Department of Neuroscience, Faculty of Medicine and Nursing, UPV/EHU).

3.3. Immunocytochemistry

To evaluate the subcellular localization of Pacs-2, OPCs were seeded in 14 mm-diameter coverslips located in 24-well plates at a density of 10^4 cells/well. After 3 DIV, cells were treated with CTZ for 10 min and with AMPA for 30 min in presence or absence of baclofen as described above, then fixed with PBS with 4% PFA as also described above. To prepare cells for immunofluorescence, they were first rinsed with PBS and subsequently blocked for 1 h using PBS with 4% normal goat serum (NGS) and 0.1% Triton X-100 (Sigma-Aldrich). After that, they were incubated sequentially overnight at 4ºC with specific primary antibodies: goat anti-Pacs2 (1:100; Santa Cruz Technologies); rabbit anti-TOMM20 (1:300; Santa Cruz Technologies) and mouse anti-MBP (1:500; Biolegends). Primary antibodies were detected incubating the cells for 1 h at room temperature with Alexa Fluor secondary antibodies: donkey anti-goat 488 (1:500; Invitrogen); goat anti-rabbit 594 (1:500; Invitrogen); goat anti-mouse 647 (1:500; Invitrogen). DAPI was used (4 µg/mL; Sigma-Aldrich) to identify cell nuclei. Finally, coverslips were washed three times with PBS and mounted on slides using fluorescent mounting medium (Prolong Gold Antifade Reagent; ThermoFisher Scientific). Images were acquired with a Leica TCS STED CW SP8 super-resolution confocal microscope (Achucarro Basque Center for Neuroscience).

3.4. Protein extraction

For the analysis of activated signaling pathways total protein from $8x10^5$ cells/well was obtained from 1 DIV OPCs after incubation in fresh SATO+ medium (alone or with baclofen) for 30 min, and subsequent exposure to 100 µM CTZ for 10 min and 10 µM AMPA for 10 or 30 min. For protein extraction, cells were washed with ice-cold PBS, followed by mechanical scraping with 60 µL of RIPA buffer (50 mMTris, pH 7.5, 150 mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS, 1% NP-40, 1 mM EDTA) supplemented with Halt™ protease and phosphatase inhibitor cocktail and EDTA (Thermo Fisher Scientific, USA) per pair of duplicate wells. In this way, proteins were extracted 10 or 30 min after initial addition of AMPA. The obtained lysates were stored at -20ºC until used.

To analyze the signalling pathways at longer times, total protein was obtained from 2 DIV OPCs following the same steps as above but with one change: all cells were incubated with AMPA for 30 min, then the medium was changed back again to fresh SATO+ (alone or with baclofen). Finally, protein extraction was performed 10 or 30 min after the last medium change as described. In this way,

proteins were obtained 40 or 60 min after initial addition of AMPA. The lysates were stored at -20ºC until used.

3.5. Western Blot

To analyze the activation of signaling molecules, their phosphorylation state was assessed by Western Blot. Lysates from protein extractions were boiled in sample buffer for 8 min, and separated by electrophoresis using Criterion TGX Precast Any kD gels (Bio-Rad, Hercules, CA, USA) and transferred to Trans-Blot Turbo Midi Nitrocellulose Transfer Packs (Bio-Rad). After electroblotting on nitrocellulose membranes, these were blocked with Tris-buffered saline with Tween20 (TBST) with 5% PhosphoBlocker™ (for phosphorylated proteins) or 5% BSA (for other proteins) as blocking reagent for 1 h at room temperature. Then, blots were incubated overnight at 4ºC in the same solution with specific primary antibodies: mouse anti-CREB, rabbit anti-phosphoCREB, rabbit anti-Akt, rabbit anti-phosphoAkt, rabbit anti-GAPDH (all antibodies used at 1:1,000; from Cell Signaling). The following day, after washing with TBST, the membranes were incubated in blocking solution with HRP-conjugated secondary antibodies goat anti-rabbit or sheep anti-mouse (1:2,000, Sigma) and proteins were visualized enhancing the chemiluminescence signal using the Supersignal West Dura or Femto detection kit, according to the manufacturer's instructions (Thermo Scientific). Images were acquired with a ChemiDoc XRS system (Bio-Rad) and protein bands were quantified with ImageLab (Bio-Rad) software.

3.6. Statistical analysis

All data are shown as the mean ± standard error of the mean (SEM). The statistical analysis was performed using the GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, California, USA). Statistical comparisons between multiple experimental groups were made using one-way analysis of variance (ANOVA) with Fisher's LSD test where $p < 0.05$ was considered statistically significant. All experiments were performed in duplicate, and the values provided here are the mean of at least three independent experiments.

4. RESULTS

4.1. Baclofen and muscimol reduce AMPA-provoked OPCs death

First of all, our goal was to determine whether $GABA_AR$ or $GABA_BR$ activation had a protective effect in OPCs exposed to AMPA provoked excitotoxicity. For that, cells were incubated in the absence or presence of $GABA_BR$ selective agonist baclofen, or $GABA_AR$ selective agonist muscimol for 30 min, and then exposed to 10 μM of AMPA (in the presence of 100 μM CTZ; excitotoxic stimulus) for 10 or 30 min. Viability was assessed 24 h later to check alterations in AMPA-provoked cell death (**Figure 2**). In each condition, viability of 100% was considered from cells not treated with AMPA and the term control refers to cells treated with AMPA alone.

As we can observe in **Figure 2A,** after 10 min of AMPA exposure cell death was not significantly modified by baclofen (14.6 ± 4.4%) or muscimol treatment (6.61 ± 9.71%), compared to control (AMPA alone; 8.87 ± 3.61%). However, as can be seen in **Figure 2B**, at 30 min the detected cell death was significantly lower in OPCs treated with baclofen (-0.66 ± 1.42%) or muscimol (-2.29 ± 3.26%) than on control cells (9.59 ± 2.31%). These changes were corroborated by the fluorescence images acquired from the OPCs used for the viability assays after fixation, shown in **Figure 2C**. Therefore, these results confirm the protective role of $GABA_AR$ and $GABA_BR$ agonists against AMPA-provoked excitotoxicity in OPCs *in vitro*. Nevertheless, due to the increasing interest in $GABA_BRs$ and their role on OL functionality (Hamilton et al., 2016; Serrano-Regal et al., 2020b), we focused on baclofen for the rest of the work.

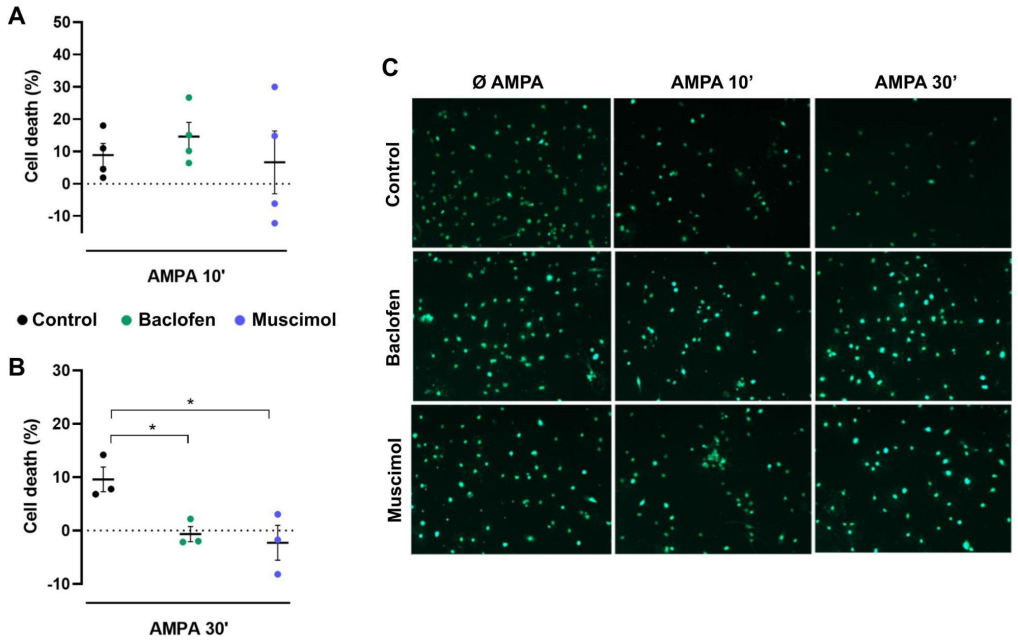

Figure 2. Baclofen and muscimol-induced protection from AMPA-provoked OPC death. (A, B) Percentage of cell death in OPCs treated in the absence or presence of baclofen (50 µM) or muscimol (20 µM) following treatment with AMPA (10 µM) for 10 **(A)** or 30 **(B)** min. 0% of cell death was considered in each case from no AMPA addition. Data are shown as the mean ± SEM of at least 3 independent experiments. *p < 0.05 versus control. **(C)** Representative images of OPCs from viability assays loaded with calcein (green) and stained with DAPI (blue).

4.2. Baclofen blocks AMPA-induced translocation of Pacs-2 to mitochondria

To further analyze the impact of baclofen treatment on AMPA-mediated cell death, an immunocytochemistry analysis was performed to verify the subcellular location of Pacs-2 in OPCs treated with AMPA for 30 min in the absence or presence of baclofen. With that aim, Pacs-2 was visualized in combination with the mitochondrial marker TOMM20 and the mature OL marker MBP (**Figure 3**). The fluorescence signals of Pacs-2 and TOMM20 were compared to see the colocalization score in both conditions. As can be seen qualitatively in **Figure 3A**, Pacs-2 and TOMM20 colocalization is higher in cells treated with AMPA in absence of baclofen. Moreover, as shown in

Figure 3B, the Pearson's R value is also higher in the case of OPCs treated with AMPA in absence of baclofen, and Manders' values are also consistent with this, especially Manders' M2 value, which expresses how much Pacs-2 signal is superposed with mitochondrial signal. In contrast, these values are significantly lower in OPCs treated with AMPA in presence of baclofen.

These results suggest that, in these conditions, AMPA-induced OPC death is mainly apoptotic, as evidenced by the change in localization of Pacs-2 from cytosol to mitochondria, and that baclofen acts by blocking this translocation, therefore hindering caspase activation and promoting cell-survival.

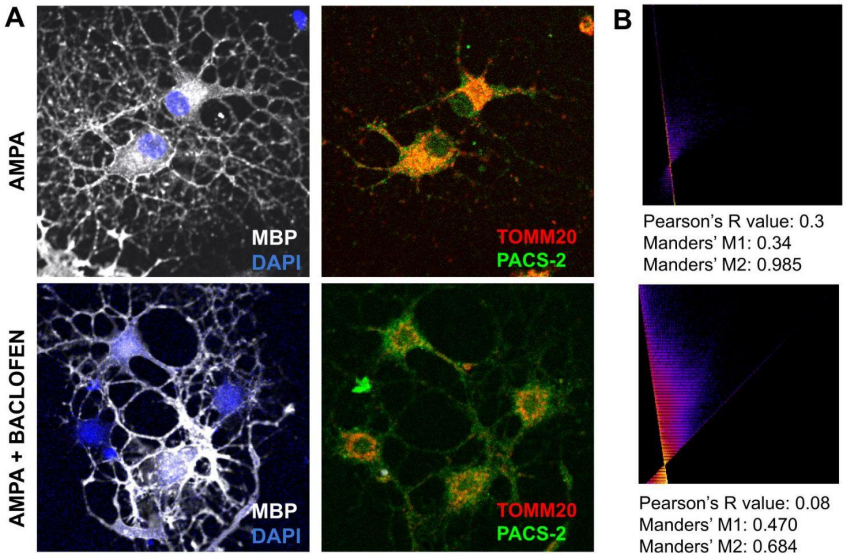

Figure 3. Baclofen blocks AMPA-induced translocation of Pacs-2 to mitochondria. (A) Cells were exposed to AMPA in absence or presence of baclofen and then immunostained to detect the subcellular locations of Pacs-2 (in green), mitochondrial marker TOMM20 (in red), OL marker MBP (in grey) and nuclei, marked with DAPI (in blue). **(B)** Diagram showing the colocalization of Pacs-2 and TOMM20 for both cases and their Pearson's R value and Manders' M1 and M2 values.

4.3. Baclofen increases AMPA-induced phosphorylation of CREB in OPCs

CREB is a transcription factor related to the activation of several anti-apoptotic genes, so with the aim of checking whether the baclofen-mediated protective effect occured through the activation of CREB, we analyzed changes in its phosphorylation levels of OPCs exposed to AMPA, in absence or presence of baclofen at different time points.

First, we analyzed the phosphorylation levels of CREB in cells exposed to AMPA for 10 min or 30 min with or without baclofen, **Figure 4**. In both cases, CREB phosphorylation is significantly increased 10 min after initial AMPA addition, as it increases to 1,281 ± 251.8% after addition of AMPA in absence of baclofen; and to 1,672 ± 707.9% after addition of AMPA in presence of baclofen. Also, as we can see in **Figure 4B**, this increase in phosphorylation levels at 10 min after AMPA addition is slightly higher in cells treated in presence of baclofen, compared to the cells without baclofen.

At 30 min after AMPA treatment, CREB phosphorylation seems to be slightly potentiated compared to cells not treated with AMPA, but no significant differences are detected in phosphorylation levels

between cells treated in absence or presence of baclofen (712.9 ± 197% from control for cells without baclofen and 653.6 ± 221.9% from control for cells with baclofen).

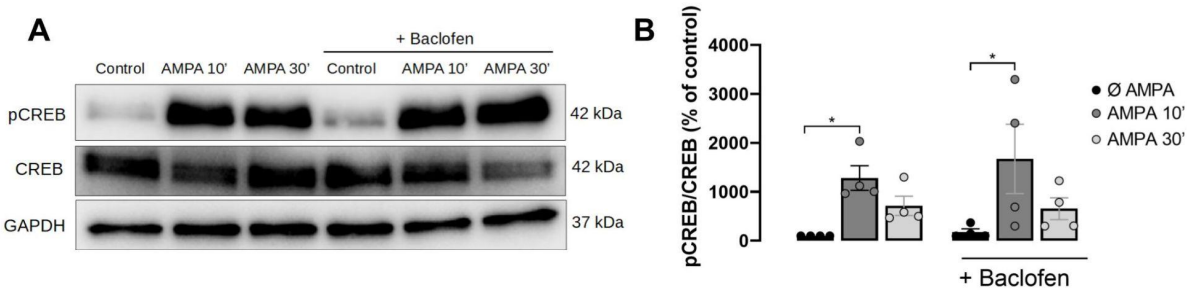

Figure 4. AMPA-induced tendency towards increase in CREB phosphorylation slightly potentiated by baclofen in OPCs at short time points. (A) Representative blots showing pCREB, CREB and GAPDH expression of OPCs treated in the absence or presence of baclofen (50 μM) and untreated or treated with AMPA for 10 or 30 min. **(B)** Histograms represent the values of optical density of the pCREB/CREB ratio normalized to control. GAPDH was analyzed as load control. Data are shown as the mean ± SEM of 3 independent experiments. *p < 0.05 versus control.

Then, we modified the experimental design with the goal of assessing CREB phosphorylation levels at longer time frames. For that, we exposed OPCs from all experimental conditions to AMPA for 30 min, and then maintained the cells in absence of AMPA for 10 or 30 min, that is, 40 or 60 min after the initial addition of AMPA (**Figure 5**). We can observe that AMPA also increased CREB phosphorylation in this case, along with a potentiator effect induced by baclofen 40 min after initial addition of AMPA. Taken together, these data seem to indicate that AMPA enhances CREB phosphorylation and activation. In addition, baclofen seems to potentiate this phosphorylation, provoking pCREB levels to be higher especially at 10 min after AMPA treatment.

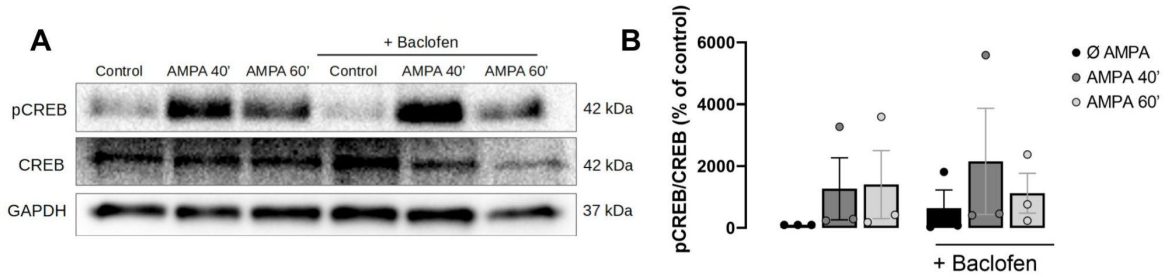

Figure 5. AMPA-induced increase in CREB phosphorylation slightly potentiated by baclofen in OPCs at long time points. (A) Representative blots showing pCREB, CREB and GAPDH expression of OPCs treated in the absence or presence of baclofen (50 μM) and untreated or treated with AMPA for 30 min and whose total proteins were extracted 40 or 60 min after initial addition of AMPA. **(B)** Histograms represent the values of optical density of the pCREB/CREB ratio normalized to control. GAPDH was analyzed as load control. Data are shown as the mean ± SEM of 3 independent experiments.

4.4. Baclofen reduces AMPA-induced phosphorylation of Akt at longer times in OPCs

After evaluating changes in CREB activation, we focused on the analysis of another protein described as relevant in AMPA and GABAR-mediated signaling, Akt. We first analyzed Akt phosphorylation in OPCs treated with or without baclofen and stimulated with AMPA for 10 or 30 min (**Figure 6**). We

can see that AMPA treatment seemed to slightly reduce Akt phosphorylation, although in a non-significant way, while baclofen treatment did not induce further effects.

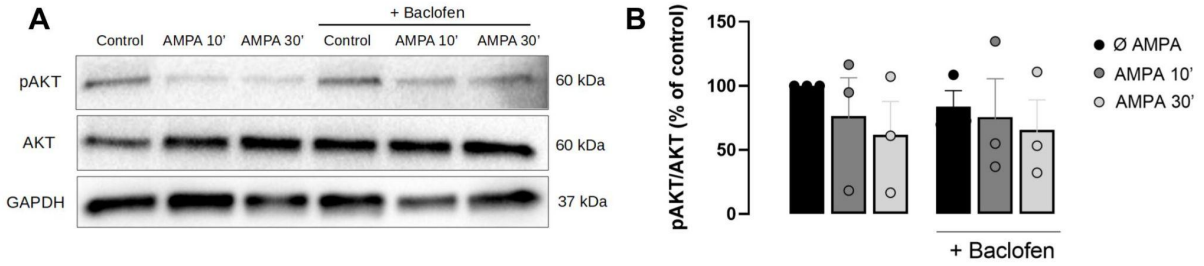

Figure 6. Baclofen does not alter the phosphorylation levels of Akt after short exposure to AMPA in OPCs. (A) Representative blots showing pAkt, Akt and GAPDH expression of OPCs treated in the absence or presence of baclofen (50 μM) and untreated or treated with AMPA for 10 or 30 min. **(B)** Histograms represent the values of optical density of the pAkt/Akt ratio normalized to control. GAPDH was analyzed as load control. Data are shown as the mean ± SEM of 3 independent experiments.

To continue with the study of Akt, we analyzed its activation at longer time frames, as previously described (**Figure 7**). The phosphorylation levels of Akt are significantly increased in cells without baclofen (165 ± 27.2% of control) at 60 min after initial addition of AMPA, and this increase is reduced in the case of the cells treated with baclofen (109 ± 14.5% of control). These data suggest that AMPA induced activation of Akt is blocked by baclofen treatment at 60 min after initial addition of AMPA.

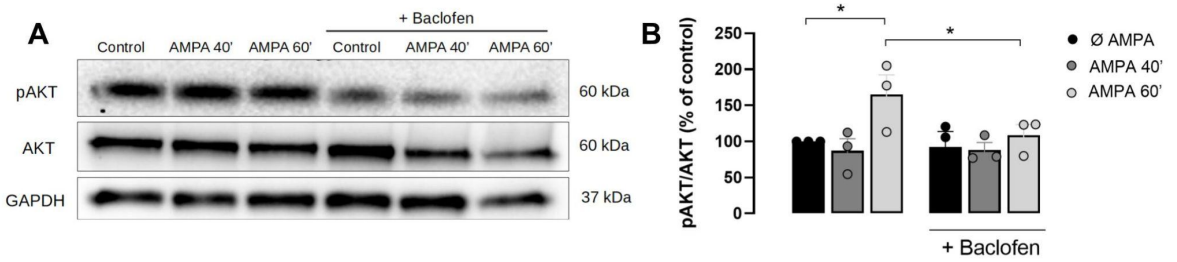

Figure 7. Baclofen reduces the phosphorylation levels of Akt after exposure to AMPA in OPCs at long time points. (A) Representative blots showing pAkt, Akt and GAPDH expression of OPCs treated in the absence or presence of baclofen (50 μM) and untreated or treated with AMPA for 30 min and whose total proteins were extracted 40 or 60 min after initial addition of AMPA. **(B)** Histograms represent the values of optical density of the pAkt/Akt ratio normalized to control. GAPDH was analyzed as load control. Data are shown as the mean ± SEM of 3 independent experiments. *p < 0.05 versus control.

5. DISCUSSION

In this study, we evaluated the effect of $GABA_BR$ and $GABA_AR$ pharmacological activation in OPCs exposed to AMPA excitotoxicity *in vitro*, and then we elucidated some of the proteins involved in the process. To begin, we confirmed that selective activation of $GABA_AR$ and $GABA_BR$ has a protective effect in OPCs exposed to 10 μM of AMPA *in vitro* (**Figure 2**), which induces a moderate activation of the receptors and causes excitotoxic cell death. Therefore, the activation of these receptors may lead to anti-apoptotic signalling pathways. Because of recent findings showing the crucial role of $GABA_BR$

signalling in OPCs (Serrano-Regal et al., 2020a), we centered on $GABA_BR$, although it would also be of great interest to elucidate the mechanisms of $GABA_AR$ activation-associated protective role.

To analyze how baclofen exerts its protective role, we analyzed the changes in Pacs-2 localization, and the activation state of CREB and Akt, as mentioned. The localization results of Pacs-2 in the cells indicated that AMPA excitotoxicity promoted the translocation of Pacs-2 to the mitochondria, therefore activating the apoptotic cascade (**Figure 3**). These results are in line with previous studies which showed that OL death initiated by AMPA stimulation was dependent on the severity of the excitotoxic insult, and that it followed intrinsic or extrinsic apoptotic pathways when the insult was moderate, like in this case, but that it could also follow necrotic pathways when the insult was more extreme (Sanchez-Gomez et al., 2003). In our case the excitotoxic insult was moderate, so we observe that cell-death follows mainly apoptotic pathways, which baclofen seems to hinder, by blocking the translocation of Pacs-2 to the mitochondria.

To continue, the first protein whose activation state we analyzed was CREB, because it has been described to regulate the transcription of several pro-survival/anti-apoptotic genes (Sato-Bigbee et al., 1994; Lonze & Ginty, 2002) and it is thought to reduce Ca^{2+} permeability in OLs, therefore reducing the activation of apoptosis (Deng et al., 2006). Our data show that AMPA increases CREB phosphorylation and therefore its activation (**Figures 4 and 5**), which is consistent with previous studies (Deng et al., 2006). We saw that this CREB activation reached its peak at around 10 min after initial addition of AMPA (**Figure 4**), and then it gradually descended. Moreover, our data suggests that this activation was enhanced and elongated when cells were treated with baclofen (**Figure 5**), which could mean that the observed protective effect of baclofen is mediated by this increased activation of CREB and molecules that it regulates. This hypothesis would be consistent with the findings of Deng et al., who found that overexpression of active CREB had a protective impact on OLs exposed to excitotoxicity (Deng et al., 2006).

To continue we analysed Akt, which has also been characterized as having pro-survival effects (Caravatta et al., 2008). In fact, it is known that Akt phosphorylates various proteins implicated in cell death and survival, for example CREB, caspase-9 and Bad (Zhou et al., 2000; Caravatta et al., 2008), modulating the fate of the cell. In the intrinsic apoptotic cascade, it has been proved that cell death can be inhibited by Akt, which is thought to act downstream of cytochrome c, blocking the caspase activation mediated by it, though the exact mechanism remains unclear (Zhou et al., 2000). Additionally, previous studies have found that AMPA reduces Akt activation, therefore inactivating its pro-survival role (Canedo-Antelo et al., 2016), and our results are in line with this fact. Nevertheless, we found that Akt activation was not altered by baclofen at short time frames (**Figure 6**). However, CREB can be phosphorylated by various other kinases, for instance PKC or CaMK, so the activation of Akt might not be crucial for CREB activation at this time point (Zhou et al., 2000; Caravatta et al., 2008; Wen et al., 2010).

In contrast, at 60 min after AMPA addition, baclofen did have an effect, as we observed that cells treated in presence of baclofen had lower Akt phosphorylation levels compared to cells treated in absence of baclofen, that is, we found that baclofen reduced AMPA-induced Akt phosphorylation at longer time frames (**Figure 7**). This might occur because, even if initial Akt activation and its pro-survival role decrease when OPCs are exposed to excitotoxicity, if the excitotoxic stimulus lasts longer, Akt can be reactivated, but this time with a pro-apoptotic effect (Benbrook & Masamha, 2011). This means that Akt interacts with several upstream regulators and downstream effectors creating a complex network of positive and negative feedbacks, and upon prolonged hyperactivation of Akt, its role can change from pro-survival to pro-apoptotic. These data taken together indicate that baclofen mediates its protective effect at longer time frames by hindering this pro-apoptotic Akt activation, but further research is needed.

Besides, as future directions, $GABA_BRs$ have been described to affect the expression, activity and signaling of glutamate receptors (Kantamneni, 2015), so elucidating which proteins take part in this crosstalk would also be of great interest. On the other hand, as it has already been mentioned, it would be of great value to complete the study with the analysis of $GABA_AR$, as we saw a protective effect when treating OPCs with muscimol.

All in all, these data suggest that the protective effect of baclofen against excitotoxicity in OPCs is mainly mediated by CREB at short time frames, but also mediated by Akt inhibition at longer time frames. In order to gain a better understanding of this pro-survival effect, it would be highly valuable to elucidate which molecules are being activated or inhibited upstream and downstream of these two proteins.

6. CONCLUSIONS

Overall, this project provides evidence of the potential protective effect of GABAR activation against AMPA-provoked excitotoxicity in OPCs. The conclusions obtained are the following:

- Baclofen acts on $GABA_BRs$ to promote OPC survival when exposed to AMPA-provoked excitotoxicity *in vitro*, and muscimol activation of $GABA_ARs$ also promotes this survival.
- This excitotoxic cell death follows mainly apoptotic pathways, as evidenced by the change in the subcellular location of Pacs-2, and baclofen acts by reducing this toxic displacement.
- CREB activation is a key mediator of this baclofen-induced protective role at short time frames, and, at longer time frames, Akt activation blockage also seems to be involved in this protective effect of baclofen.

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