Received: 5 January 2022 | Revised: 11 June 2022 | Accepted: 7 July 2022

DOI: 10.1111/ejn.15775

RESEARCH REPORT

EJN European Journal of Neuroscience FENS WILEY

Involvement of $GABA_A$ receptors containing α_6 subtypes in antisecretory factor activity on rat cerebellar granule cells studied by two-photon uncaging

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Funding information
Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale, Grant/Award Number: 20177XJCHX_003; Ministero dell'Università e della Ricerca, Grant/Award Number: RBAP11ETKA-005

Edited by: Marco Capogna

Abstract

The antisecretory factor (AF) is an endogenous protein that counteracts intestinal hypersecretion and various inflammation conditions in vivo. It has been detected in many mammalian tissues and plasma, but its mechanisms of action are largely unknown. To study the pharmacological action of the AF on different $GABA_A$ receptor populations in cerebellar granule cells, we took advantage of the two-photon uncaging method as this technique allows to stimulate the cell locally in well-identified plasma membrane parts. We compared the electrophysiological response evoked by releasing a caged GABA compound on the soma, the axon initial segment and neurites before and after administering AF-16, a 16 amino acids long peptide obtained from the amino-terminal end of the AF protein. After the treatment with AF-16, we observed peak current increases of varying magnitude depending on the neuronal region. Thus, studying the effects of furosemide and AF-16 on the electrophysiological behaviour of cerebellar granules, we suggest that $GABA_A$ receptors, containing the α_6 subunit, may be specifically involved in the increase of the peak current by AF, and different receptor subtype distribution may be responsible for differences in this increase on the cell.

KEYWORDS
2PE uncaging, antisecretory factor, cerebellar granule cells, $GABA_A$ receptor, patch-clamp technique, RuBi-GABA

1 | INTRODUCTION

The antisecretory factor (AF) is an endogenous 41-kDa protein expressed in different mammalian tissues and plasma (Johansson et al., 1995). It was identified, cloned and sequenced in the 1980s by Lange and Lönnroth and presents an inhibitory function against hypersecretion

Abbreviations: 2PE, two-photon excitation; AF, antisecretory factor; AF-16, 16 amino acids long peptide obtained from the amino-terminal end of the antisecretory factor protein; AIS, axon initial segment; CGC, cerebellar granule cells; E%, percentage enhancement effect; GABA, γ-aminobutyric acid; ROI, region of interest; RuBi-GABA, (bis(2,2'-Bipyridine-N,N')triphenylphosphine)-4-aminobutyric acid ruthenium hexafluorophosphate complex.

induced by enterotoxins, especially cholera toxin, or due to chronic inflammatory diseases (Lange & Lönnroth, 2001).

The antisecretory site, in the region between the amino acids 35 and 42, has the sequence (I)VCHSKTR. Four cysteine groups form disulphide bridges in the N-terminal region to guarantee the antisecretory activity (Jennische et al., 2008; Johansson et al., 1997).

Several peptides and fragments were synthesized from the recombinant protein; the AF peptide, formed by 16 amino acids (AF-16), is derived from amino acids 36–51. AF-16 is chemically the most stable and was conclusively selected for laboratory experiments.

Using the AF concept, clinical treatment of various human diseases has significantly improved patients' clinical outcomes (Lange & Lönnroth, 2001). Such diseases include Ménière's disease (Hanner et al., 2004, 2010), diarrhoeal diseases of various origins (Björck et al., 2000; Laurenius et al., 2003; Zaman et al., 2018), Mb Crohn (Eriksson, Shafazand, Jennische, Lönnroth, & Lange, 2003), ulcerative colitis (Eriksson, Shafazand, Jennische, & Lange, 2003) and suppression of elevated intracranial pressure after head trauma (Gatzinsky et al., 2020). The pathophysiology behind these clinical conditions varies widely, but they display various forms of transport derangement of the water and ion transport across the cellular membrane. These diseases represent a multitude of pathological mechanisms, and consequently, there is a general demand for a better understanding of a more detailed 'mechanism of action' of the AF protein. Such investigations must also include the mode of action of the smaller, various biologically active AF-peptides, which all emanate from the amino-terminal end of the original, full-length AF protein. These studies should primarily be accomplished by evaluating the influence on the cellular reactivity to AF administration in various in vitro test systems.

Previous works considered AF effect in GABAergic transmission (Bazzurro et al., 2018; Kim et al., 2005; Strandberg et al., 2014). GABA represents the principal inhibitory neurotransmitter of the mammalian central nervous system (CNS) and also acts on $GABA_A$ receptors in the enteric nervous system (ENS), albeit the enteric GABAergic neurons are principally interneurons releasing GABA as an excitatory neurotransmitter (Galligan, 2002; Krantis, 2000; Seifi et al., 2014).

It appears of interest to study in model systems with well-characterized $GABA_A$ receptors the effects of the AF in order to build a framework of knowledge allowing to conceive and test hypotheses about the roles of GABA in the AF anti-hypersecretory activity via $GABA_A$ receptors of enteric ganglia. Based on a long experience with $GABA_A$ receptors of cerebellar granule cells (CGCs) in primary cultures (Cupello et al., 2013; Robello et al., 1993), we studied the effects of AF on those $GABA_A$ receptors. In addition, in this context, the CGC model is interesting from another point of view. In fact, AF induction in patients, who suffer from Ménière's disease, has been shown to counteract the symptoms of that disease, especially vertigo (Hanner et al., 2004, 2010). Granule cells have an essential role in controlling the cerebellar output via Purkinje cells' innervation and, in particular, the inhibitory actions of Purkinje cells on lateral vestibular nuclei of the brain stem (Hydén et al., 2000; Ito, 1984; Ito et al., 1966). Thus, it appears interesting to investigate further the AF action on the $GABA_A$ receptor-mediated inhibitory brake of cerebellar granule neurons.

With the use of the patch-clamp technique, we demonstrated that AF-16 affects neuronal signalling in the rat CGCs in vitro (Bazzurro et al., 2018). Specifically, the results showed an increase in the peak current induced by GABA after AF-16 pretreatment. The effect depended on peptide concentration and incubation time: the higher the concentration and longer incubation time, the more the effect on $GABA_A$ receptors until saturation.

Here, we use a new approach to better understand the AF effect on $GABA_A$ receptors and precisely follow the receptor activation after AF-16 treatment, in a limited cell area, thanks to caged GABA photorelease; this technique allows selecting a neuronal region of interest to investigate the variations in the receptor modulation of that specific region.

This approach offers the possibility of studying $GABA_A$ receptors of specific sub-neuronal districts such as axons and potential axo-axonic synapses in situ in the actual tissue (Nathanson et al., 2019; Schmid et al., 1996).

Caged compounds are chemically modified neurotransmitters whose biological activity is inhibited until light activates them when and where needed. Once uncaged, they become active effectors and interact with their target inducing a response (Shi et al., 2014).

We chose RuBi-GABA, a commercial caged neurotransmitter described by Rial Verde et al. (2008), for studying the specific $GABA_A$ receptors modulation by AF-16. After characterizing the uncaging process both for 1- (1PE) and 2-photon excitation (2PE) by using electrophysiological measurements in CGCs (Cozzolino et al., 2020), we decided to investigate the role of AF in the $GABA_A$ receptor modulation by taking advantage of 2PE uncaging RuBi-GABA (Diaspro et al., 2005). This strategy allows photoreleasing femtoliter volumes of GABA in specific and confined cell regions to stimulate $GABA_A$ receptors only in that portion of the cell membrane.

We selected three regions on cerebellar granules (cell body, axon initial segment and neurite) and studied how

AF-16 affects receptors' response to GABA in those specific areas.

The rationale of the experiments was to understand which $GABA_A$ subtypes were involved in the receptor response after AF-16 incubation, considering that α_6 subunits mainly characterize CGCs $GABA_A$ receptors (Cupello et al., 2013; Gatta et al., 2009).

Because of this assumption, we focused on the α_6 subunit, analysing how furosemide, a pharmacological molecule that selectively blocks $GABA_A$ receptors containing this subunit (Korpi et al., 1995; Korpi & Lüddens, 1997), affects the electrophysiological response if administered with AF-16.

This strategy allowed us to infer AF-16 action on a selected $GABA_A$ receptor population.

2 | MATERIALS AND METHODS

2.1 | AF-16 peptide production

The AF-16 peptide formed by AF amino acid sequence 36–51 (VCHSKTRSNPENNVGL) was synthesized with solid-phase synthesis by Ross-Petersen AS, Copenhagen, Denmark, as described by Rapallino et al. (2003).

2.2 | Animals

Sprague–Dawley rats were housed in the Department of Pharmacy's animal unit, Section of Pharmacology and Toxicology of Genoa University. Animals were treated according to the E.U. Parliament and Council Directive of 22 September 2010 (2010/63/E.U.) and approved by the Italian Ministry of Health (COD. 75F11.N.6DX) D.M. 116/1992. We made all efforts to minimize animal suffering and reduce the number of animals used.

2.3 | CGC primary cultures

CGCs were prepared from 6- to 8-day-old Sprague–Dawley rats, following procedures described previously (Robello et al., 1993).

The cells were plated at a density of 1×10^6 per dish on 24-mm poly-L-lysine-coated glass coverslip and maintained in 90% Basal Medium Eagle, 10% Fetal Calf Serum (Sigma-Aldrich, St. Louis, MO, USA), 25-mM KCl, 2.0-mM glutamine, 100 μg/ml gentamicin at 37°C in a humidified 95% air 5% CO_2 atmosphere.

A 10 μM cytosine arabinoside (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium to prevent glial cell growth at 18–24 h from plating. The medium was renewed at 48 h, and 10 μM cytosine arabinoside was added again. We studied the cells, in vitro, from the fifth to the 10th day.

2.4 | Electrophysiology

The membrane currents were measured with the whole-cell patch-clamp technique, setting the holding potential at −80 mV and using an Axopatch 200 B (Axon Instruments, Burlingame, CA, USA) as previously described in Robello et al. (1993).

Pipettes were pulled from borosilicate glass capillaries type TW 150-3 (OD 1.5 mm ID 1.12 mm World Precision Instruments Inc., Sarasota, FL, USA) and using a P-30 puller (Sutter Instruments Co., Novato, CA, USA). We recorded the currents with a Labmaster D/A, A/D converter driven by pClamp 10 software (Axon Instruments, Burlingame, CA, USA). We analysed the experimental data with pClamp and SigmaPlot 12 (SYSTAT Software, San Jose, CA, USA).

We maintained the cells in the recording bath with the standard external solution (pH 7.4 with NaOH) containing (in mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose; we filled the glass pipettes with the internal solution that contained (in mM) 142 KCl, 10 HEPES, 2 EGTA, 2 $MgCl_2$, 3 ATP; we added tris base to adjust the pH to 7.3.

In some experiments, 5.0-μM biocytin conjugate with CF®640R (Biotium Inc., Fremont, CA, USA) was included in the internal solution to highlight the cell morphology.

In all experiments, the caged neurotransmitter RuBi-GABA (Tocris Cookson Ltd, Bristol, UK) and AF-16 were diluted in the external solution to reach the needed final concentration.

The solutions were applied to the cell bath by continuous perfusion (~3 ml/min gravity flow).

2.5 | Confocal imaging and two-photon uncaging

The optical set-up used for performing the experiments consists of an inverted three-channel laser-scanning confocal microscope (Leica TCS SP5) equipped with 458-, 476-, 488-, 514-, 543- and 633-nm excitation lines and a plan-apochromatic oil immersion objective ×63/1.4. To check the cell's position, we acquired images in transmitted light, using a 633-nm laser source, before and after the uncaging process using the 'LAS AF' software package (Leica Microsystems, Germany).

We performed two-photon excitation with a Ti: Sapphire laser (Chameleon II, Coherent Inc., Santa Clara, CA, USA), operating 100 fs pulses at 80 MHz of repetition frequency. We measured the average beam power at the objective focal point with a PM100A power metre (Thorlabs Inc., Newton, NJ, USA) (Diaspro & Robello, 2000).

We set up the bleach points' coordinates, laser power and exposure time using the FRAP Wizard Leica module, as previously described (Cozzolino et al., 2020).

3 | RESULTS

To evaluate the AF's effect in different and specific neuronal regions, we chose to study the AF-16 action on the soma, the axon initial segment (AIS) and neurites (Figure 1). We administered AF-16 at a concentration of 1.0 μM for 3 min to work with the lowest concentration granting the maximum enhancement effect on the peak current component as demonstrated in a previous work by Bazzurro et al. (Bazzurro et al., 2018).

Figure 2 shows the typical traces of chloride current evoked by RuBi-GABA photolyzed with 2PE at about 2 μm from the soma. During experiments, we kept on measuring the current while applying the following procedure. We perfused 10-μM RuBi-GABA in the petri-dish with CGCs, uncaged the compound (Figure 2a) and washed it away with the external solution. Then, we perfused 1.0-μM AF-16 for 3 min (Figure 2b), administered a 10-μM RuBi-GABA and 1.0-μM AF-16 solution and activated the 2PE beam again (Figure 2c). We repeated the same procedure in different neuron regions. We reported typical currents in Figure 3.

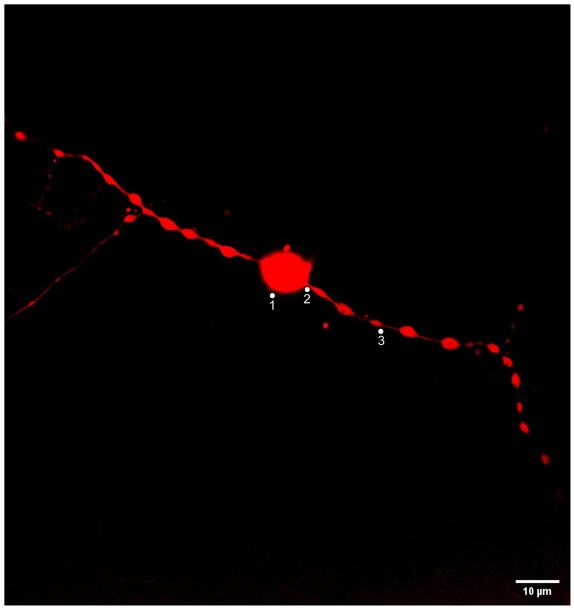

FIGURE 1 The image illustrates different uncaging points on soma (1), axon initial segment (AIS) (2) and neurite (3) of a cerebellar granule cell that has been processed to reveal biocytin conjugate with CF®640R during patch-clamp recording

The comparison of the currents evoked by RuBi-GABA before and after the treatment with AF-16, on the same cell, shows a significant increase in the peak current; thus, AF may facilitate the activation of $GABA_A$ receptors. We have quantified the AF action, in terms of percentage effect (E%), by applying the formula below to peak current data:

$$E\% = \left(\frac{I_{RuBi-GABA+AF} - I_{RuBi-GABA}}{I_{RuBi-GABA}} \right) \cdot 100,$$

where $I_{RuBi\text{-}GABA+AF}$ is the chloride peak current induced by uncaging RuBi-GABA in the presence of AF-16 and $I_{RuBi\text{-}GABA}$ is the one evoked by just photolyzing RuBi-GABA. The E% values, reported in Table 1, derive from averaging the E% calculated on n cells; data are reported as mean ± SEM and compared with controls by using Student's *t* test. In Table 2, we compared AF effect on the three neuronal regions under study (soma, AIS and neurite) by analysis of variance (ANOVA) test and on couples of regions (soma vs. AIS, soma vs. neurite, AIS vs. neurite) by post hoc Student's *t* test.

To understand which receptor subtypes may be involved in the AF mechanism of action, we performed experiments using furosemide, a selective blocker of α_6 subunit-containing $GABA_A$ receptors; we reported typical traces of current for the cell body in Figure 4.

We followed this protocol: We measured the current evoked by uncaging 10-μM RuBi-GABA (Figure 4a, trace [A]); after a 3-min incubation with 1.0-μM AF-16 (Figure 4a, trace [B]), we administered and photolyzed RuBi-GABA with 1.0-μM AF-16 (Figure 4a, trace [C]); then, after a 1-min wash-out with the external solution (Figure 4b, trace [B]), we perfused and uncaged a solution of RuBi-GABA with 1.0-mM furosemide (Figure 4b, trace [C]). We observed a significant decrease of the current peak, probably due to furosemide blocking of α_6 receptor subtypes.

Finally, after a second 3-min treatment with AF (Figure 4c, trace [B]), we dispensed RuBi-GABA with 1.0-μM AF-16 and 1.0-mM furosemide, uncaged it by activating a 2PE beam and registered a current peak (Figure 4c, trace [C]) comparable with the one acquired with only 1.0-mM furosemide in addition to uncaged RuBi-GABA (Figure 4b, trace [C]). Data indicate that in the presence of 1.0-mM furosemide, no effect by AF-16 is present.

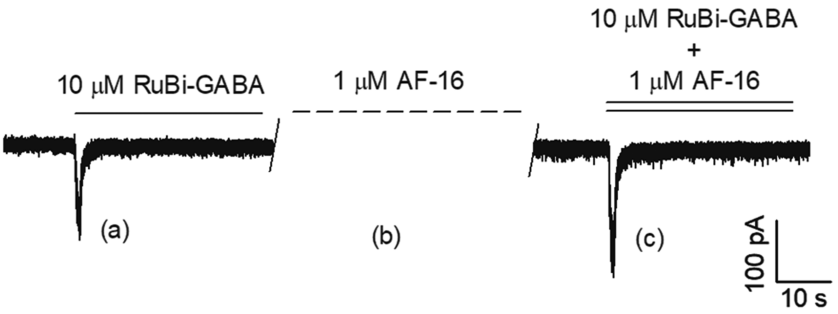

FIGURE 2 Typical chloride current measurements evoked by the uncaging of 10-μM RuBi-GABA (750 nm, 100 ms, 30 mW, −80 mV) at about 2 μm from the soma, demonstrating the current (pA) versus time (s). Firstly, 10-μM RuBi-GABA was uncaged (a), then, the neurons were incubated for 3 min with 1-μM AF-16 (b) and, finally, the current was recorded after the photorelease of 10-μM RuBi-GABA in combination with 1-μM AF-16 (c)

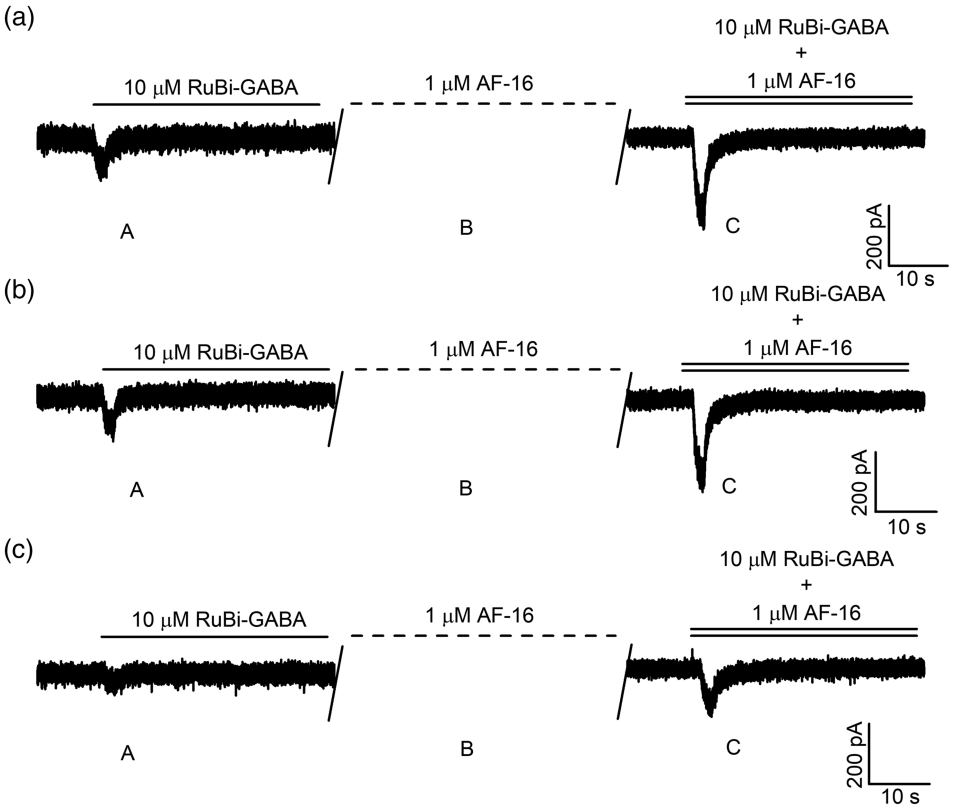

FIGURE 3 Example of chloride current traces evoked by the uncaging of 10-μM RuBi-GABA (750 nm, 100 ms, 30 mW, −80 mV) on soma (a), axon initial segment (AIS) (b) and neurite (c) on the same cell: (A) current trace after the photorelease of 10-μM RuBi-GABA, (B) incubation for 3 min with 1-μM AF-16 and (C) current measured after the uncaging of 10-μM RuBi-GABA in combination with 1-μM AF-16

TABLE 1 Percentage effect (E%) of AF-16 on photolyzed RuBi-GABA induced current after the incubation for 3 min with AF-16 on soma, AIS and neurite

	E% ± SEM	n	*p* values
Soma	36 ± 3	27	$p < 0.001$
AIS	26 ± 4	16	$p < 0.001$
Neurite	42 ± 4	17	$p < 0.001$

Note: The data are given as mean ± SEM; *n* is the number of experiments, *p* values calculated with Student's *t* test.
Abbreviations: AF, antisecretory factor; AIS, axon initial segment.

TABLE 2 Effect of 1-μM AF-16 on the soma-AIS-neurite (first row: ANOVA test), soma versus AIS, soma versus neurite and on AIS versus neurite (post hoc comparisons)

	p values
Soma-AIS-neurite	$p < 0.05$
Soma-AIS	$p < 0.05$
Soma-neurite	n.s.
AIS-neurite	$p < 0.01$

Note: n.s. is not significant.
Abbreviations: AF, antisecretory factor; AIS, axon initial segment; ANOVA, analysis of variance.

In Table 3, we reported the data as mean ± SEM, statistical significance versus control was calculated by Student's *t* test.

4 | DISCUSSION

In the past decades, various molecules, named caged neurotransmitters, have been developed, and now they are

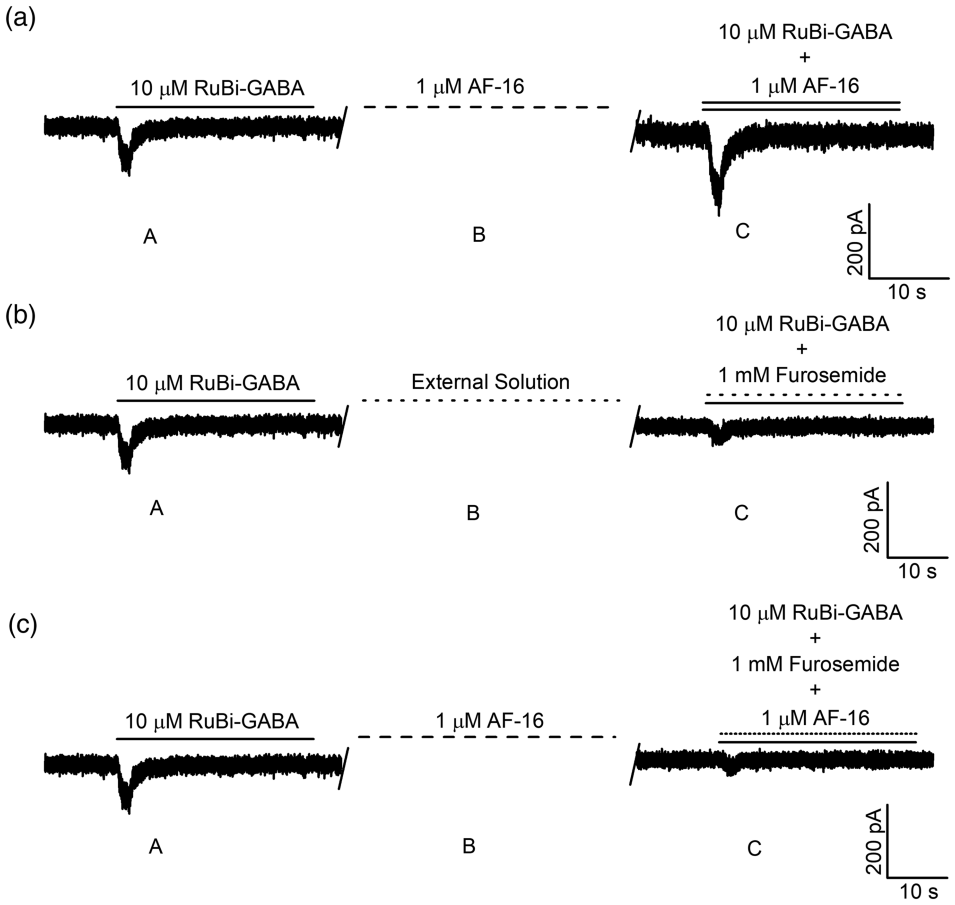

FIGURE 4 Example of chloride current traces evoked by uncaging 10-μM RuBi-GABA (750 nm, 100 ms, 30 mW, −80 mV) on the soma of the same cell. (a) and (c): (A) Current trace recorded after the photorelease of 10-μM RuBi-GABA, (B) incubation for 3 min with 1-μM AF-16 and (C) current measured after the uncaging of 10-μM RuBi-GABA in combination with 1-μM AF-16 and, only for trace c, 1 mM furosemide. (b): (A) Current trace recorded after the photorelease of 10-μM RuBi-GABA, (B) wash-out with external solution for 1 min and (C) current measured after the uncaging of 10-μM RuBi-GABA in combination with 1 mM furosemide

TABLE 3 Percentage effect (E%) of 1-μM AF-16 (first row), 1-mM furosemide (second row) and 1-mM furosemide + 1-μM AF-16 (third row) on 10-μM uncaged RuBi-GABA induced current after the incubation with AF-16 for 3 min

	E% ± SEM	*n*	*p* values
10-μM RuBi-GABA + 1-μM AF-16	45 ± 5	8	$p < 0.05$
10-μM RuBi-GABA + 1-mM furosemide	−65 ± 2	9	$p < 0.001$
10-μM RuBi-GABA + 1-mM furosemide + 1-μM AF-16	−64 ± 2	9	$p < 0.001$; n.s.*

Note: For each data series, the number of experiments (*n*) and the statistical significance (*p* values) of the results are reported. The data are given as mean ± SEM; statistical comparisons were analysed with Student's *t* test versus the control (no AF-16, no furosemide): *p* values. The last column reports the comparison between the 10-μM RuBi-GABA + 1-mM furosemide + 1-μM AF-16 and the 10-μM RuBi-GABA + 1-mM furosemide case (n.s.*: not significant). Abbreviation: AF, antisecretory factor.

widely used to study neurobiological processes such as synaptic transmission and plasticity.

Caged neurotransmitters like RuBi-GABA are valuable tools for neurophysiology studies because light activation allows controlling the timing, location and amplitude of neurotransmitter release.

This study takes advantage of the multiphoton uncaging method to determine how pharmacological molecules, particularly the AF-16 antisecretory peptide, modulate $GABA_A$ receptors in different regions of CGCs. The advantage of this method is in photoreleasing small volumes (in the order of fl) (Ellis-Davies, 2007) of neurotransmitter (i.e., GABA) in well-defined positions and thus stimulating small portions of the cell at a time. We applied 2PE uncaging to RuBi-GABA, a commercial caged-neurotransmitter, to study the variations of the chloride current associated with the activation of $GABA_A$ receptors in the proximity of the neuron soma, AIS and neurites (Figure 1).

We were interested in studying the AIS considering recent reports about the physiological importance of $GABA_A$ inhibitory synapses in the neuronal axonal initial segment (AIS) (Koike et al., 2013; Kriebel et al., 2011; Nathanson et al., 2019).

Schmid et al., in a previous work (Schmid et al., 1996), suggested the presence of axo-axonic synapses in rat cerebellar granule nerve terminals. Such synapses act on $GABA_A$ receptors with the pharmacological characteristics typical of granule cells: For example, they are not sensitive to classical benzodiazepines. We thus thought it would be interesting to test whether the AF peptides could affect axonal $GABA_A$ receptors.

Even if the AF mechanism of action is still partly unknown, AF-16 seems to facilitate the GABAergic transmission in the cerebellar granule neurons (Bazzurro et al., 2018). With this in mind, we investigated how AF-16 works in different cell regions and analysed the GABA activated chloride currents, and related the variations to differences in $GABA_A$ receptors distribution.

At first, to study the effect of AF-16 on electrophysiological behaviour, we compared data acquired by uncaging RuBi-GABA with 2PE with previous experimental data obtained by perfusing GABA in the petri dish with cerebellar granules (Bazzurro et al., 2018).

In both cases, AF-16 increases the peak current evoked by the neurotransmitter. The peaks determined with this new approach are lower than the peaks achieved with GABA only. The lower peaks might reflect the release of relatively few uncaged neurotransmitter molecules, which activates a lower number of receptors on the cell membrane.

The data suggest that AF-16 may act differently on the considered areas, implying a different effect on the receptor subtypes of $GABA_A$: In fact, the results showed significant differences in the effects of AF-16 in the AIS compared with the soma and neurites (Tables 1 and 2); in particular, the action seems more effective in the cell body and neurites, suggesting that AF-16 interacts with a specific subtype of $GABA_A$ receptors.

CGCs in primary culture are mainly composed of α_1, α_6, $\beta_{2/3}$, γ_2 and δ subunits. Previous studies (Cupello et al., 2013; Gatta et al., 2009) showed that the $GABA_A$ subtypes that contribute to the peak current are $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2 + \alpha_1\alpha_6\beta_{2/3}\gamma_2$ and $\alpha_6\beta_{2/3}$ incomplete receptors. So studying the peak current variations, induced by AF-16, means investigating its effects on these subtypes.

Because the AF-16 percentage effect is predominant in the neurites and in the cell body, the AF probably affects the receptor subtypes composed of the α_6 subunit, which is mainly expressed in the plasma membrane of the soma and neurites (Merlo et al., 2004).

In order to test this hypothesis, we performed measurements with the pharmacological molecule furosemide, a selective blocker of $GABA_A$ receptor subtypes composed of α_6 subunit (Korpi et al., 1995; Korpi & Lüddens, 1997).

We used a furosemide concentration of 1.0 mM for blocking only $\alpha_6\beta_2\gamma_2$ containing $GABA_A$ receptors, being the IC_{50} 10 μM, as shown by Korpi et al. (Korpi et al., 1995). In fact, to block $\alpha_1\beta_2\gamma_2$ subtypes, higher concentrations are necessary, being the $IC_{50} > 3$ mM (Korpi et al., 1995). When furosemide 1.0 mM was perfused with GABA, the peak current decreased.

AF-16 increases the peak current evoked by the activation of $GABA_A$ receptors after the photorelease of RuBi-GABA (Figure 4a), but furosemide almost entirely blocks the AF-16 effect (Figure 4b,c and Table 3).

The fact that AF selectively stimulates the activity of $GABA_A$ receptors of composition $\alpha_6\beta_{2/3}\gamma_2$, and not that of $GABA_A$ receptors of different subunit composition, is interesting. These receptors are specific for cerebellar granule neurons (Farrant & Nusser, 2005). They have specific pharmacology; they are not sensitive to 1,4-benzodiazepines. Particular pharmacology could be of limited interest if we think about the endogenous mechanisms of the nervous tissue in situ. However, it indicates a particular structure and biochemistry: In fact, the $\alpha_6\beta_{2/3}\gamma_2$ receptors have different characteristics from those $\alpha_1\beta_{2/3}\gamma_2$. They have a higher affinity for the endogenous ligand GABA (Sigel & Baur, 2000), different deactivation and different desensitization kinetics (Tia et al., 1996). This prevalence of the $\alpha_6\beta_{2/3}\gamma_2$ receptor in cerebellar granule neurons could be explained by the need for these receptors, which act by mediating phasic inhibition. Such receptors could act on top of the tonic inhibition mediated by $\alpha_1(\alpha_6)\beta_{2/3}\delta$, which are sensitive to very low extracellular concentrations of GABA ($\leq$0.5 μM). This feature may be essential for the proper functioning of the glomeruli structure in the cerebellum.

We have recent evidence of the presence of the $GABA_A$ receptor α_6 subunit in enteric ganglia neurons (own unpublished results). A specific action of local GABA on such neurons may be part of the antisecretory activity of AF. This aspect must be a subject for our future investigations.

5 | CONCLUSIONS

We applied the uncaging technique to study variations in the receptor responses in a region of interest (ROI) after treatment with AF.

The AF effect, previously tested in CGCs, facilitates the activity of $GABA_A$ receptors.

The uncaging method validates the data obtained with the classical electrophysiological techniques and verifies the importance of AF in several neuronal areas. Moreover, it highlighted a different action on different

ROIs, revealing a specificity on the $GABA_A$ receptor subtypes containing the α_6 subunit.

This topic could be extended to a deeper study on various receptor subtypes using a combination of other pharmacological molecules, for example, the benzodiazepine antagonist flumazenil. Such a combination might explain how the AF-16 effect may change in different parts of the neuron.

Moreover, advanced microscopy combined with selectively labeling $GABA_A$ receptor subunits could shed light on how AF modifies the distribution of receptors subtypes in different neuronal areas. Comparing the data acquired with electrophysiological measurements with high-resolution microscopy could significantly improve the comprehension of the AF-16 mechanism of action.

ACKNOWLEDGEMENTS

The study was supported by MUR (Ministero dell'Università e della Ricerca), DIFILAB, Grant/Award Number: RBAP11ETKA-005 and Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale (PRIN) Grant/Award Number: 20177XJCHX_003. Open Access Funding provided by Universita degli Studi di Genova within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.15775.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

ORCID

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REFERENCES

Bazzurro, V., Gatta, E., Cupello, A., Lange, S., & Robello, M. (2018). Antisecretory factor modulates $GABA_A$ receptor activity in neurons. *Journal of Molecular Neuroscience*, *64*(2), 312–320. https://doi.org/10.1007/s12031-017-1024-8

Björck, S., Bosaeus, I., Ek, E., Jennische, E., Lönnroth, I., Johansson, E., & Lange, S. (2000). Food induced stimulation of the antisecretory factor can improve symptoms in human inflammatory bowel disease: A study of a concept. *Gut*, *46*(6), 824–829. https://doi.org/10.1136/gut.46.6.824

Cozzolino, M., Bazzurro, V., Gatta, E., Bianchini, P., Angeli, E., Robello, M., & Diaspro, A. (2020). Precise 3D modulation of electro-optical parameters during neurotransmitter uncaging experiments with neurons in vitro. *Scientific Reports*, *10*(1), 13380. https://doi.org/10.1038/s41598-020-70217-5

Cupello, A., Di Braccio, M., Gatta, E., Grossi, G., Nikas, P., Pellistri, F., & Robello, M. (2013). $GABA_A$ receptors of cerebellar granule cells in culture: Interaction with benzodiazepines. *Neurochemical Research*, *38*, 2453–2462. https://doi.org/10.1007/s11064-013-1171-4

Diaspro, A., Chirico, G., & Collini, M. (2005). Two-photon fluorescence excitation and related techniques in biological microscopy. *Quarterly Reviews of Biophysics*, *38*(2), 97–166. https://doi.org/10.1017/S0033583505004129

Diaspro, A., & Robello, M. (2000). Two-photon excitation of fluorescence for three-dimensional optical imaging of biological structures. *Journal of Photochemistry and Photobiology. B*, *55*(1), 1–8. https://doi.org/10.1016/s1011-1344(00)00028-2

Ellis-Davies, G. (2007). Caged compounds: Photorelease technology for control of cellular chemistry and physiology. *Nature Methods*, *4*, 619–628. https://doi.org/10.1038/nmeth1072

Eriksson, A., Shafazand, M., Jennische, E., & Lange, S. (2003). Effect of antisecretory factor in ulcerative colitis on histological and laborative outcome: A short period clinical trial. *Scandinavian Journal of Gastroenterology*, *38*(10), 1045–1049. https://doi.org/10.1080/00365520310005064

Eriksson, A., Shafazand, M., Jennische, E., Lönnroth, I., & Lange, S. (2003). Antisecretory factor-induced regression of Crohn's disease in a weak responder to conventional pharmacological treatment. *Inflammatory Bowel Diseases*, *9*(6), 398–400. https://doi.org/10.1097/00054725-200311000-00013

Farrant, M., & Nusser, Z. (2005). Variations on an inhibitory theme: Phasic and tonic activation of $GABA_A$ receptors. *Nature Reviews. Neuroscience*, *6*(3), 215–229. https://doi.org/10.1038/nrn1625

Galligan, J. J. (2002). Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterology and Motility*, *14*(6), 611–623. https://doi.org/10.1046/j.1365-2982.2002.00363.x

Gatta, E., Cupello, A., Pellistri, F., & Robello, M. (2009). $GABA_A$ receptors of cerebellar granule cells in culture: Explanation of overall insensitivity to ethanol. *Neuroscience*, *162*(4), 1187–1191. https://doi.org/10.1016/j.neuroscience.2009.05.031

Gatzinsky, K., Johansson, E., Jennische, E., Oshalim, M., & Lange, S. (2020). Elevated intracranial pressure after head trauma can be suppressed by antisecretory factor-a pilot study. *Acta Neurochirurgica*, *162*(7), 1629–1637. https://doi.org/10.1007/s00701-020-04407-5

Hanner, P., Jennische, E., Lange, S., Lönnroth, I., & Wahlström, B. (2004). Increased antisecretory factor reduces vertigo in patients with Ménière's disease: A pilot study. *Hearing Research*, *190*(1–2), 31–36. https://doi.org/10.1016/S0378-5955(03)00368-X

Hanner, P., Rask-Andersen, H., Lange, S., & Jennische, E. (2010). Antisecretory factor-inducing therapy improves the clinical outcome in patients with Ménière's disease. *Acta Oto-Laryngologica*, *130*(2), 223–227. https://doi.org/10.3109/00016480903022842

Hydén, H., Rapallino, M. V., & Cupello, A. (2000). Unraveling of important neurobiological mechanisms by the use of pure, fully differentiated neurons obtained from adult animals. *Progress in Neurobiology*, *60*(5), 471–499. https://doi.org/10.1016/s0301-0082(99)00035-0

Ito, M. (1984). *The Cerebellum and Neural Control*. Raven Press.

Ito, M., Obata, K., & Ochi, R. (1966). The origin of cerebellar-induced inhibition of Deiters neurones. II. Temporal correlation between the trans-synaptic activation of Purkinje cells and the inhibition of Deiters neurons. *Experimental Brain Research*, *2*(4), 350–364. https://doi.org/10.1007/BF00234780

Jennische, E., Bergström, T., Johansson, M., Nyström, K., Tarkowski, A., Hansson, H. A., & Lange, S. (2008). The peptide AF-16 abolishes sickness and death at experimental encephalitis by reducing increase of intracranial pressure. *Brain Research*, *1227*, 189–197. https://doi.org/10.1016/j.brainres.2008.05.083

Johansson, E., Lange, S., & Lönnroth, I. (1997). Identification of an active site in the antisecretory factor protein. *Biochimica et Biophysica Acta*, *1362*(2–3), 177–182. https://doi.org/10.1016/s0925-4439(97)00066-5

Johansson, E., Lönnroth, I., Lange, S., Jonson, I., Jennische, E., & Lönnroth, C. (1995). Molecular cloning and expression of a pituitary gland protein modulating intestinal fluid secretion. *The Journal of Biological Chemistry*, *270*(35), 20615–20620. https://doi.org/10.1074/jbc.270.35.20615

Kim, M., Wasling, P., Xiao, M.-Y., Jennische, E., Lange, S., & Hanse, E. (2005). Antisecretory factor modulates GABAergic transmission in the rat hippocampus. *Regulatory Peptides*, *129*(1–3), 109–118. https://doi.org/10.1016/j.regpep.2005.01.018

Koike, M., Tanida, I., Nanao, T., Tada, N., Iwata, J., Ueno, T., Kominami, E., & Uchiyama, Y. (2013). Enrichment of GABARAP relative to LC3 in the axonal initial segments of neurons. *PLoS ONE*, *8*(5), e63568. https://doi.org/10.1371/journal.pone.0063568

Korpi, E. R., Kuner, T., Seeburg, P. H., & Lüddens, H. (1995). Selective antagonist for cerebellar granule cell-specific GABA type A receptor. *Molecular Pharmacology*, *47*(2), 283–289.

Korpi, E. R., & Lüddens, H. (1997). Furosemide interactions with brain $GABA_A$ receptors. *British Journal of Pharmacology*, *120*(5), 741–748. https://doi.org/10.1038/sj.bjp.0700922

Krantis, A. (2000). GABA in the mammalian enteric nervous system. *News in Physiological Sciences*, *15*, 284–290. https://doi.org/10.1152/physiologyonline.2000.15.6.284

Kriebel, M., Metzger, J., Trinks, S., Chugh, D., Harvey, R. J., Harvey, K., & Volkmer, H. (2011). The cell adhesion molecule neurofascin stabilizes axo-axonic GABAergic terminals at the axon initial segment. *The Journal of Biological Chemistry*, *286*(27), 24385–24393. https://doi.org/10.1074/jbc.M110.212191

Lange, S., & Lönnroth, I. (2001). The Antisecretory factor: Synthesis, anatomical and cellular distribution, and biological action in experimental and clinical studies. *Int Rev of Cytol*, *210*, 39–75. https://doi.org/10.1016/s0074-7696(01)10003-3

Laurenius, A., Wängberg, B., Lange, S., Jennische, E., Lundgren, B. K., & Bosaeus, I. (2003). Antisecretory factor counteracts secretory diarrhoea of endocrine origin. *Clinical Nutrition*, *22*(6), 549–552. https://doi.org/10.1016/s0261-5614(03)00057-8

Merlo, F., Balduzzi, R., Cupello, A., & Robello, M. (2004). Immunocytochemical study by two photon fluorescence microscopy of the distribution of $GABA_A$ receptor subunits in rat cerebellar granule cells in culture. *Amino Acids*, *26*(1), 77–84. https://doi.org/10.1007/s00726-003-0006-z

Nathanson, A. J., Davies, P. A., & Moss, S. J. (2019). Inhibitory synapse formation at the axon initial segment. *Frontiers in Molecular Neuroscience*, *12*, 266. https://doi.org/10.3389/fnmol.2019.00266

Rapallino, M. V., Cupello, A., Lange, S., & Lönnroth, I. (2003). Antisecretory factor peptide derivatives specifically inhibit [^{3}H]-γ-amino-butyric acid/$^{36}Cl^-$ out→in permeation across the isolated rabbit Deiters' neuronal membrane. *Acta Physiologica Scandinavica*, *179*(4), 367–371. https://doi.org/10.1111/j.1365-201X.2003.01173.x

Rial Verde, E. M., Zayat, L., Etchenique, R., & Yuste, R. (2008). Photorelease of GABA with visible light using an inorganic caging group. *Front Neural Circuits*, *2*, 2. https://doi.org/10.3389/neuro.04.002.2008

Robello, M., Amico, C., & Cupello, A. (1993). Regulation of $GABA_A$ receptor in cerebellar granule cells in culture: Differential involvement of kinase-activities. *Neuroscience*, *53*, 131–138. https://doi.org/10.1016/0306-4522(93)90291-m

Schmid, G., Bonanno, G., & Raiteri, M. (1996). Functional evidence for two native $GABA_A$ receptor subtypes in adult rat hippocampus and cerebellum. *Neuroscience*, *73*(3), 697–704. https://doi.org/10.1016/0306-4522(96)00085-1

Seifi, M., Brown, J. F., Mills, J., Bhandari, P., Belelli, D., Lambert, J. J., Rudolph, U., & Swinny, J. D. (2014). Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon. *The Journal of Neuroscience*, *34*(31), 10361–10378. https://doi.org/10.1523/JNEUROSCI.0441-14.2014

Shi, D. D., Trigo, F. F., Semmelhack, M. F., & Wang, S. S.-H. (2014). Synthesis and biological evaluation of bis-CNB-GABA, a photoactivatable neurotransmitter with low receptor interference and chemical two-photon uncaging properties. *Journal of the American Chemical Society*, *136*(5), 1976–1981. https://doi.org/10.1021/ja411082f

Sigel, E., & Baur, R. (2000). Electrophysiological evidence for the coexistence of α_1 and α_6 subunits in a single functional $GABA_A$ receptor. *Journal of Neurochemistry*, *74*(6), 2590–2596. https://doi.org/10.1046/j.1471-4159.2000.0742590.x

Strandberg, J., Lindquist, C., Lange, S., Asztely, F., & Hanse, E. (2014). The endogenous peptide antisecretory factor promotes tonic GABAergic signaling in CA1 stratum radiatum interneurons. *Frontiers in Cellular Neuroscience*, *8*, 1–9. https://doi.org/10.3389/fncel.2014.00013

Tia, S., Wang, J. F., Kotchabhakdi, N., & Vicini, S. (1996). Distinct deactivation and desensitization kinetics of recombinant $GABA_A$ receptors. *Neuropharmacology*, *35*(9–10), 1375–1382. https://doi.org/10.1016/s0028-3908(96)00018-4

Zaman, S., Aamir, K., Hanson, L. Å., & Lange, S. (2018). High doses of Antisecretory factor stop diarrhea fast without recurrence for six weeks post treatment. *International Journal of Infectious Diseases*, *71*, 48–52. https://doi.org/10.1016/j.ijid.2018.03.015

How to cite this article: Bazzurro, V., Gatta, E., Angeli, E., Cupello, A., Lange, S., Jennische, E., Robello, M., & Diaspro, A. (2022). Involvement of $GABA_A$ receptors containing α_6 subtypes in antisecretory factor activity on rat cerebellar granule cells studied by two-photon uncaging. *European Journal of Neuroscience*, *56*(5), 4505–4513. https://doi.org/10.1111/ejn.15775