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Corresponding Author: Dr. Luca Raiteri, Ph.D.

Corresponding Author's Institution: University of Genoa

First Author: Cristina Romei

Order of Authors: Cristina Romei; Chiara Sabolla; Luca Raiteri, Ph.D.

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*Title page

High-affinity GABA uptake by neuronal GAT1 transporters provokes release of [^{3}H]GABA by homoexchange and through GAT1-independent Ca^{2+}-mediated mechanisms

Cristina Romei[a], Chiara Sabolla[a], Luca Raiteri[a,b,c,*]

[a] *Department of Pharmacy, Pharmacology* and *Toxicology Section, University of Genoa, Genoa, Italy*

[b] *Center of Excellence for Biomedical Research. University of Genoa, Genoa, Italy*

[c] *National Institute of Neuroscience, Genoa, Italy*

* Corresponding author. Department of Pharmacy, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4 – 16148 Genoa, Italy. Tel.: +39 010 3532093; FAX: +39 010 3993360.

E-mail address: lraiter@pharmatox.unige.it (Luca Raiteri)

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CGP37157, 7,chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one; GAT1, GABA transporter type 1; $InsP_3R$, inositol(1,4,5)trisphosphate receptor; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea; NCX, Na^+/Ca^{2+} exchanger; NPPB, 5-nitro-2-(phenylpropylamino)benzoic acid; SKF89976A, 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxilic acid; VSCCs, voltage-sensitive Ca^{2+} channels.

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Cristina Romei[a], Chiara Sabolla[a], Luca Raiteri[a,b,c,*]

[a] *Department of Pharmacy, Pharmacology* and *Toxicology Section, University of Genoa, Genoa, Italy*

[b] *Center of Excellence for Biomedical Research. University of Genoa, Genoa, Italy*

[c] *National Institute of Neuroscience, Genoa, Italy*

* Corresponding author. Department of Pharmacy, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4 – 16148 Genoa, Italy. Tel.: +39 010 3532093; FAX: +39 010 3993360.

E-mail address: lraiter@pharmatox.unige.it (Luca Raiteri)

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ABSTRACT

High-affinity uptake of GABA into nerve terminals may have functions other than recapture of the neurotransmitter. Synaptosomes purified from mouse cerebellum were prelabelled with [^{3}H]GABA and then superfused with GABA and drugs selective for some presynaptic targets. Influx of GABA through GAT1 transporters stimulated efflux of [^{3}H]GABA in a concentration-dependent manner (EC_{50} = 3 μM). The efflux of the transmitter occurred in part by GAT1 reversal through the so called homoexchange. The ion fluxes (particularly Na^+ influx) accompanying GABA uptake triggered intraterminal Ca^{2+} signals through both plasmalemmal Na^+/Ca^{2+} exchangers, sensitive to KB-R7943 or to ifenprodil and mitochondrial Na^+/Ca^{2+} exchangers, sensitive to CGP37157. These Ca^{2+} signals likely facilitated GABA release from nerve terminals via niflumic acid- and NPPB-sensitive anion channels. The results show that GABA, at concentrations corresponding to the high-affinity uptake, can evoke GABA release which occurs in part by the expected GAT1-mediated homoexchange, while the transporter-independent component of the GABA uptake-evoked GABA release takes place by hitherto unsuspected mechanisms which include Na^+/Ca^{2+} exchangers and anion channels. The significance of the novel function of the GABA high-affinity uptake here identified deserves further multidisciplinary investigation.

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1. Introduction

Transporters for neurotransmitters have been attributed for long time only one function: reuptake of the transmitters just released by exocytosis into the synapse, in order to terminate the activation of postsynaptic receptors and prevent their excessive stimulation. The mechanisms of action of important drugs, like antidepressants, are based on their ability to block the reuptake of neurotransmitters by selective plasma membrane transporters. Subsequently, it was observed that neurotransmitter transporters, as other transporters, are bidirectional and can, under some conditions, work in the inside-out direction by a mechanism known as carrier-mediated release or release by transporter reversal (see, for reviews, Attwell et al., 1993; Belhage et al., 1993; Levi and Raiteri, 1993; Richerson and Wu, 2003). Well known psychostimulant drugs, like the amphetamines, produce their major effects by causing release of biogenic amines by transporter reversal (see, for reviews, Robertson et al., 2009; Sitte and Freissmuth, 2010). There is increasing evidence that neurotransmitter transporters can exhibit, in addition, functions other than reuptake and release by reverse transport.

In general, neurotransmitter transporters can modulate excitability of neurons indirectly, by controlling through uptake the extracellular concentrations of neurotransmitters at pre- and postsynaptic receptors. Besides this indirect mode of regulation, some transporters can affect neuronal excitability directly, through receptor-independent, transporter-mediated, conductances.

In an electrophysiological study with cultured ventral mesencephalic cells containing dopamine (DA) neurons, Ingram et al. (2002) showed that substrates of the DA transporter (DAT), such as DA and amphetamine, enhanced the firing activity of DA neurons independently of receptors. The currents were Na^+-dependent and could be blocked by the

DAT inhibitor cocaine, suggesting that they are a property of the DAT protein. Ingram et al. (2002) concluded that DAT-associated conductances elicited by DAT substrates directly modulate neuronal excitability and may mediate release of DA by mechanisms that include DAT reversal.

Many reports describe examples of indirect receptor-mediated processes occurring when GABA transporters GAT1 are inhibited and extracellular GABA concentrations are consequently increased. A direct effect of GAT1 currents on the action potential rate of neurons was observed in the mouse midbrain periaqueductal grey by Bagley et al. (2005) during opioid withdrawal. In an in vitro system mimicking opioid withdrawal, a cation current mediated by GAT1 could be recorded. More recently, Bagley et al. (2011) found that, during opioid withdrawal, GAT1 currents directly stimulated GABA release via depolarization of presynaptic GABAergic nerve terminals.

To discriminate between receptor-dependent indirect and receptor-independent direct mechanisms in native tissue preparations requires relatively complex experimental strategies. In particular, direct mechanisms need to be studied by preventing indirect effects with receptor antagonists and other synaptic neurotransmission blockers. Moreover, it is necessary to identify neurons belonging to a well defined family. The above examples of direct modulation of neuronal excitability are in a way reminiscent of the mechanisms of feedback control of transmitter release brought about by presynaptic autoreceptors (Starke et al., 1989; Langer, 1993; Raiteri, 2001), which can conveniently be studied by the technique of superfused synaptosomes (see, for a review, Raiteri and Raiteri, 2000). We here assumed that superfused synaptosomes might represent an appropriate technical approach to characterize the mechanisms by which direct activation of transporters can mediate transmitter release. Our assumption may in part be justified considering that,

because of the dimension of nerve terminals, transporter-associated conductances can not be studied by electrophysiological techniques.

To our knowledge, events possibly occurring in GABAergic nerve terminals following high-affinity uptake of GABA through GAT1 have not been investigated in detail. In the present work, purified mouse cerebellar nerve terminals were selectively prelabelled with [^{3}H]GABA and exposed to external GABA in conditions in which the effects of GABA on [^{3}H]GABA release are due to *direct* action of GABA on GABAergic nerve terminals. The results show that the GABA-evoked release was more complex than the expected GAT1-mediated homoexchange between external and internal GABA; moreover, some data are in a way surprising considering that processes generally associated to pathological conditions can be activated by physiological concentrations of GABA.

2. **Materials and Methods**

2.1.Animals

Adult male Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light/dark schedule (light 7.00 am to 7.00 pm). Food and water were freely available. Experimental procedures and animal care complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Italian Ministry of Health in accordance with Decreto Ministeriale 116/1992 (protocol number 29823-9 of 9/12/2010). All experimental procedures have been performed according to 'ARRIVE' guidelines for reporting research. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable results.

2.2. Isolation and purification of nerve endings

Animals were killed by cervical dislocation and the cerebellum was quickly removed. The tissue was homogenized in 10 vol. of 0.32 M sucrose buffered at pH 7.4 with Tris–HCl, using a glass-Teflon tissue grinder (clearance 0.25 mm, 24 up-down strokes in about 2 min). The homogenate was centrifuged (5 min, 1000 g at 4°C) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10% and 20% in Tris-buffered sucrose) and centrifuged at 33,500 g for 5 min. The band between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in a physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 10;

HEPES, 10; pH adjusted to 7.4 with NaOH. All the above procedures were performed at 0–4°C.

2.3. Experiments of release

Synaptosomes were incubated at 37°C for 15 min with [^{3}H]GABA (0.015 μM). At the end of incubation, aliquots of the synaptosomal suspension (about 25 μg protein) were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C and superfused with standard medium at a rate of 0.5 ml/min (Raiteri and Raiteri, 2000). After 36 min of superfusion with standard medium, to equilibrate the system, four 3 min fractions were collected. Synaptosomes were exposed to GABA at the end of the first fraction collected (t = 39 min). SKF89976A, KB-R7943, ifenprodil, dantrolene, 2-APB, niflumic acid and NPPB were introduced 9 min before GABA; CGP37157 was added 19 min before GABA. When appropriate, Ca^{2+} was omitted from the superfusion medium at t = 20 min of superfusion. The Ca^{2+}-free medium contained 8.8 mM $MgCl_2$, substituting for an isoosmotic amount of NaCl. Fractions collected and superfused filters were counted for radioactivity.

2.4. Calculations

Neurotransmitter released in each fraction collected was expressed as a percentage of the radioactivity content of synaptosomes at the start of the respective collection period (fractional rate x 100). Drug effects were evaluated by calculating the ratio between the efflux in the third fraction collected (in which the maximum effect of GABA was generally reached)

and that of the first fraction. This ratio was compared to the corresponding ratio obtained under control conditions. Appropriate controls were always run in parallel.

2.5. Statistics

All data are given as means ± SEM Statistical comparison of data was performed by one-way ANOVA followed by Dunnett's test. Differences were regarded as statistically significant for $P < 0.05$.

2.6. Materials

[^{3}H]GABA (specific activity: 3.31 x 10^{12} Bq/mol) was purchased from Perkin Elmer (Boston, MA, USA). Percoll®, GABA, SKF89976A and niflumic acid were from Sigma Chemical Co. (St. Louis, MO, USA). KB-R7943, dantrolene, 2-APB, CGP37157, NPPB and ifenprodil were from Tocris Bioscience (Bristol, UK). Some of these drugs, in particular niflumic acid, NPPB and CGP37157, when added to the superfusion medium at relatively high concentrations, exhibited sometimes unspecific releasing activities on their own and could not be utilized to establish their maximal inhibitory potential.

3. Results

3.1. GABA provoked release of [^{3}H]GABA in a concentration-dependent manner

Purified mouse cerebellar synaptosomes, prelabelled with [^{3}H]GABA, were exposed in superfusion to varying concentrations (0.03-30 μM) of unlabeled GABA. Externally added GABA provoked release of [^{3}H]GABA in a concentration-dependent manner (Fig. 1). The basal release was potentiated by about 150% by 1 μM GABA and by more than 400% when GABA was added at 3 μM, the concentration corresponding to the EC_{50} of the transmitter.

3.2. The effect of GABA was prevented by the GAT1 transporter inhibitor SKF89976A

The releasing effect of 10 μM GABA (about 800 % over basal) was prevented by SKF89976A (3-30 μM), a selective inhibitor of the GABA transporter of GAT1 type (Fig. 2). The GABA-evoked release was insensitive to bicuculline, a $GABA_A$ receptor antagonist (not shown), indicating that the evoked release of [^{3}H]GABA was caused by GABA uptake through GAT1.

3.3. The effect of GABA was prevented by Ca^{2+} omission or by KB-R7943, but not by dantrolene or 2-APB

Figure 3 shows that the GABA uptake-evoked [^{3}H]GABA release was significantly inhibited when Ca^{2+} ions were omitted from the superfusion solution. The releasing effect of 10 μM GABA was significantly prevented by 5 μM KB-R7943, an inhibitor of the plasmalemmal Na^+/Ca^{2+} exchangers (NCXs) when they work in reverse to import Ca^{2+} ions.

Because KB-R7943 was reported to be only relatively selective for reverse plasmalemmal NCXs (see, for references, Romei et al., 2011), we tested ifenprodil which has recently been found to inhibit reverse Na^+/Ca^{2+} exchangers in neurons (Brittain et al., 2012). When added at 5 μM, the drug significantly inhibited the GABA-evoked [^{3}H]GABA release by 20% (629 ± 43 vs 786 ± 59; n = 5). Figure 3 also shows that neither dantrolene (10 μM), a ryanodine receptor inhibitor, nor 2-APB (30 μM), an inhibitor of inositoltrisphosphate receptors ($InsP_3Rs$), modified the GABA-evoked [^{3}H]GABA release.

3.4. The stimulatory effect of GABA was prevented by the anion channel inhibitors niflumic acid and NPPB

The release of [^{3}H]GABA caused by 10 μM GABA was significantly prevented by niflumic acid (20 and 30 μM) or by NPPB (10 μM), two widely employed inhibitors of anion channels (Fig. 4). We also tested DCPIB, a selective blocker of volume-regulated anion channels. However, when added under basal conditions (before the addition of the GABA stimulus) at 10-20 uM, concentrations reported to inhibit anion channels (see, for instance, Bowens et al., 2013), DCPIB produced massive release of tritium and could not be employed in our system. Lower concentrations of the compound were ineffective (data not shown).

3.5. The effect of GABA was inhibited by CGP37157, niflumic acid or NPPB, in the absence of external Ca^{2+} ions

Because families of anion channels were shown to be dependent on intracellular Ca^{2+}, experiments were performed in the absence of external Ca^{2+} in order to ascertain the involvement of intraterminal Ca^{2+} signals other than ryanodine or $InsP_3R$ activation. As

illustrated in Fig. 5, the effect of GABA was significantly prevented by CGP37157, an inhibitor of Na^+/Ca^{2+}exchangers present on intraterminal mitochondria. The GABA effect, in Ca^{2+}-free solutions, was inhibited by niflumic acid or NPPB. Interestingly, the inhibitions caused by CGP37157 (10 μM) and NPPB (30 μM), added in combination, did not show additivity (Fig. 5), consistent with the involvement of mitochondrial Ca^{2+} in the opening of anionic channels.

Finally, a set of experiments were carried out to establish if the evoked release of [^{3}H]GABA included exocytotic components. The effect of GABA was not affected in synaptosomes that had been preincubated with tetanus toxin (50 nM; 90 min) or with okadaic acid (100 nM; 90 min), two exocytosis inhibitors (10 μM GABA: 774 ± 69% ; 10 μM GABA with tetanus toxin: 794 ± 85%; 10 μM GABA with okadaic acid: 799 ±93%; n = 3).

4. Discusssion

The major findings of the present investigation are that high-affinity activation by GABA of GAT1 transporters provokes release of [^{3}H]GABA from nerve terminals by different mechanisms. The release of [^{3}H]GABA preaccumulated into purified cerebellar mouse nerve endings was stimulated by external GABA in a concentration-dependent manner. The EC_{50} of the GABA effect obtained in the present study amounted to about 3 μM, well in keeping with the K_m values calculated from studies of high-affinity GABA uptake performed in several laboratories. The characteristics of the superfusion technique employed (parallel monolayers of synaptosomes placed on microporous filters and up-down superfused in conditions minimizing indirect effects; see Raiteri and Raiteri, 2000, for technical details) allow to assume that external GABA acted directly on the terminals releasing [^{3}H]GABA.

The releasing effect of 10 μM GABA could be totally prevented when the selective neuronal GAT1 inhibitor SKF89976A was added to the superfusion solution, indicating that GABA uptake by GAT1 represented the only external trigger for GABA release. As to the mode of exit of the transmitter, the information from the literature suggested the process of GAT1-mediated GABA homoexchange as the one most probably involved (Levi and Raiteri, 1974; 1978).

Neurotransmitter release by transporter reversal has been the object of several investigations (for references, see Introduction). Because this process is critical for the mechanism of action of drugs like the amphetamines, the vast majority of the studies have been focused on the transporters for biogenic amines, particularly the transporters for DA, DAT, and for serotonin (SERT). Some excellent review articles describe the story of amphetamine-induced monoamine efflux (Robertson et al, 2009; Sitte and Freissmuth, 2010). To explain the amphetamine-induced DA release via DAT, the facilitated -exchange diffusion

model was proposed by Fisher and Cho (1979), who hypothesized that amphetamine is transported intracellularly as a DAT substrate and this is followed by a counter-transport of DA extracellularly. During several years, the relatively simple model of facilitated-exchange diffusion, also known as 'revolving door' model, has evolved into a complex mechanism whereby the amphetamine-induced amine release by reverse transport involves multiple processes which make transporter-mediated influx mechanistically different from transporter-mediated efflux (Robertson et al., 2009; Sitte and Freissmuth, 2010).

As to non-aminergic transporters, such as glutamatergic and GABAergic transporters, their ability to perform carrier-mediated release has long been known (Attwell et al., 1993; Belhage et al., 1993; Levi and Raiteri, 1993; Richerson and Wu, 2003). However, the intimate mechanisms of the substrate-induced release by transporter reversal have rarely been investigated. Interestingly, two reports on glutamate transporters (EAATs; Funicello et al., 2004; Colleoni et al., 2008) showed for the first time that EAAT-mediated substrate uptake and substrate-induced EAAT-mediated reverse transport are independent events, which, according to the authors, would be inconsistent with the classic model of facilitated-exchange diffusion. In the case of GABA, there seems not to exist clear evidence that the GABA-evoked GABA release (GABA homoexchange) consists of an influx component mechanistically different from the efflux component. A very recent report on the amphetamine actions at SERT demonstrates that phosphatidylinositol-4,5-bisphosphate (PIP_2) binding to SERT is required for the amphetamine-evoked SERT-mediated serotonin release, but is not a requirement for serotonin uptake (Buchmayer et al., 2013). These results strengthen the view that inward and outward transport of biogenic amines are distinct mechanistic phenomena (Robertson et al., 2009; Sitte and Freissmuth, 2010). In contrast, PIP_2 was found unable to regulate substrate efflux through the GABA transporter GAT1, indicating that the results obtained with the amine transporters do not apply to GAT1,

although the latter belongs to the neurotransmitter: sodium symporter family (Buchmayer et al., 2013).

Neurotransmitter release by transporter reversal has been described as an external Ca^{2+} -independent process (Adam-Vizi, 1992; Bernath, 1992; Attwell et al., 1993; Belhage et al., 1993; Levi and Raiteri, 1993; Richerson and Wu, 2003; Wu et al., 2006). On the other hand, in the present work, a fraction of the GABA-evoked release of GABA was found to be significantly inhibited when Ca^{2+} ions were removed from the superfusion solution. This unexpected finding prompted us to analyze in some detail the Ca^{2+}-dependent fraction of GABA release.

The possibility that GAT1 activation provoked vesicular release by classical voltage-sensitive Ca^{2+} channel (VSCC)-dependent exocytosis (S□dhof, 1995) is unlikely, because the evoked release was insensitive to the exocytosis inhibitors tetanus toxin (Schiavo et al., 2000) or okadaic acid (Baldwin et al., 2003). Additional mechanisms of neurotransmitter release need to be considered. External Ca^{2+} can evoke VSCC-independent transmitter release, if cytosolic Na^{+} concentrations rise enough to permit reversal of plasmalemmal Na^{+}/Ca^{2+} exchangers (NCXs; Blaustein and Lederer, 1999; Annunziato et al., 2004). Evidence was provided for vesicular exocytotic release independent of external Ca^{2+} but triggered by Ca^{2+} ions mobilized from intraterminal stores (for a review, see Berridge, 1998). Internal Ca^{2+}can be mobilized from different pools, depending on the stimulus applied to nerve endings and it can mediate transmitter release by different modes (Luccini et al., 2008; Romei et al., 2011). Cytosolic Na^{+} increases can lead to transmitter release by mobilizing internal Ca^{2+} through mitochondrial Na^{+}/Ca^{2+}exchangers (Rizzuto and Pozzan, 2006; Palty et al., 2012). Furthermore, some experimental conditions been shown able to activate anion channels through which some transmitters can be released (Raiteri et al., 2005; Lee et al., 2010; Peng et

al., 2010; Saransaari and Oja, 2010; Romei et al., 2012) by a mechanism that is not exocytotic and does not involve reversal of transporters.

Considering all this information on the different mechanisms of transmitter release, it seemed important to investigate the possible effects on GABA release of ion fluxes, particularly of the Na^+ ions entering the cytosol with GABA uptake. In fact, this localized Na^+ influx could trigger intraterminal events leading to release of GABA not only by the Na^+-dependent GAT1 reversal (homoexchange in our case), but also by modes different from reverse transport.

The GABA uptake-evoked GABA release was in part sensitive to KB-R7943 and to ifenprodil, two inhibitors of the plasmalemmal Na^+/Ca^{2+} exchangers when they work in the reverse (Iwamoto et al., 1996; Brittain et al., 2012) and import Ca^{2+} in exchange with cytosolic Na^+ ions. It seems plausible to assume that this process occurs as a consequence of the increase in cytosolic Na^+ accompanying the uptake of GABA through GAT1 (Blaustein and Lederer, 1999). Because the inhibition of the evoked GABA release caused by removal of external Ca^{2+} was quantitatively very similar to that produced by KB-R7943, it can be proposed that Ca^{2+} import by the reversal of plasmalemmal NCXs largely explains the external Ca^{2+}-dependency of the GABA uptake-evoked GABA release. Of note, activation of plasmalemmal NCX reversal has generally been associated to pathological conditions. The present finding that NCX reverse was triggered by low micromolar GABA implies that the mechanism can have physiological implications, as recently proposed in a study on the cerebellar parallel fiber to Purkinje neuron synapse (Roome et al., 2013).

The GABA release evoked by GABA in the absence of external Ca^{2+} was significantly prevented by CGP37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger (Hernàndez-SanMiguel et al., 2006). Also in this case, the influx of Na^+ ions accompanying the uptake of GABA through GAT1 is likely to provoke localized increases in cytosolic Ca^{2+}. This could

however occur by a mechanism different from that involving plasmalemmal NCXs, i.e. by activation of mitochondrial Na^+/Ca^{2+}exchangers.

Neither the Ca^{2+}ions entering the cytosol by the reverse plasmalemmal NCXs nor the Ca^{2+} ions mobilized from the intraterminal mitochondria performed Ca^{2+}-induced Ca^{2+} release (CICR) because the GABA uptake-evoked GABA release was not affected by the inositoltrisphosphate inhibitor 2-APB or by the ryanodine receptor inhibitor dantrolene, indicating no involvement of Ca^{2+} mobilized from the endoplasmic reticulum.

Finally, the GABA-evoked GABA release was significantly inhibited by niflumic acid or by NPPB, two inhibitors of anion channels. Cell membranes possess families of various Cl^- /anion channels (see, for instance, Frings et al., 2000). Efflux through anion channels was reported to occur with anionic compounds, particularly with glutamate (Tuz et al., 2004; Milanese et al., 2010; Pasantes-Morales and Vazquez-Juarez, 2012), but it was described also in the case of GABA, a zwitterionic compound. It was reported that the portion of GABA present in anionic form at physiological pH could permit the permeation of the transmitter through intracellular Ca^{2+}-dependent anion channels (Lee et al., 2010; Peng et al., 2010; Milanese et al., 2014). Activation of anion channels often occurs in pathological conditions, including ischemia (see, for instance, Saransaari and Oja, 2008, 2010; Oja and Saransaari, 2013). Again, our observation that low micromolar concentrations of GABA caused GABA release through anion channels suggests that the process could play a role also in physiologically relevant conditions.

The process of the GABA-evoked GABA release we propose could be schematized as shown in Fig. 6. Neuronal GAT1 transporters situated on GABAergic nerve terminals take up GABA and release GABA in part by GAT1 reversal. The influx of Na^+ accompanying GABA uptake causes increases in cytosolic Ca^{2+} by two ways: (i) reversal of plasmalemmal NCX and (ii) mobilization of Ca^{2+} from intraterminal mitochondria through activation of mitochondrial

Na^+/Ca^{2+} exchangers. These Ca^{2+} signals permit the opening of anion channels through which GABA can be released (possibly) at extrasynaptic locations where the transmitter would exert presently unknown modulatory effects.

To conclude, robust release from one GABAergic nerve terminal can be elicited by uptake of low micromolar GABA into the same terminal, implying that the function of high-affinity GABA uptake may not only be to remove the transmitter from the extracellular space. In addition to perform uptake, many transporters, including GAT1, are electrogenic and the transporter-associated currents may directly affect neuronal excitability and modulate transmitter release (Ingram et al., 2002; Bagley et al., 2005; 2011; Veruki et al., 2006). In the present work, while the influx of GABA occurs exclusively by GAT1, the intraterminal transmitter can exit in part by GAT1 reversal. Reversal of GABA transporters is widely believed to occur only under pathological conditions. However, the work carried out in Richerson's laboratory has shown that the reverse potential of GAT1 under physiologically relevant conditions is near the resting potential of neurons and that GAT1 reversal can occur relatively easily (Wu et al., 2001; 2007). The authors conclude that GAT1-mediated GABA release can contribute to phasic inhibition and that GAT1 can finely regulate tonic inhibition (Wu et al., 2007; Ransom et al., 2013). Because the carrier-mediated component of the evoked release here observed is triggered by low micromolar GABA, it might have physiological significance. The major novelty of the present work is that a significant portion of the GABA-evoked GABA efflux involves hitherto unsuspected mechanisms such as plasmalemmal Na^+/Ca^{2+} exchangers, mitochondrial Na^+/Ca^{2+} exchangers and anion channels. Clearly, the functional significance of the GABA uptake-evoked GABA release that is not GAT1-mediated needs to be further investigated, particularly considering that the release is stimulated by physiological concentrations of GABA.

Conflict of interest

The authors declare that they have no conflict of interests.

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Legends to the figures

Fig. 1 Release of [^{3}H]GABA evoked by GABA from purified mouse cerebellar synaptosomes. Synaptosomes were exposed in superfusion to varying concentrations of GABA. The effect of GABA was evaluated by performing the ratio between the efflux in the third fraction collected and that of the first fraction. This ratio was compared to the corresponding ratio obtained under control conditions. Results are expressed as percent potentiation with respect to the basal efflux. Data are means ± SEM of 3–7 experiments in triplicate (three superfusion chambers for each experimental condition).

Fig. 2 Effect of the GABA transporter blocker SKF89976A on the release of [^{3}H]GABA evoked by 10 μM GABA. The drug was introduced 9 min before GABA. Results are expressed as percent potentiation with respect to the basal efflux. Means ± SEM of 3 experiments performed in triplicate are reported. $^*P < 0.01$ vs. the control value representing the effect of GABA in standard medium and in the absence of drugs (one-way ANOVA followed by Dunnett's test).

Fig. 3 Effects of Ca^{2+} omission, KB-R7943, dantrolene and 2-APB on the release of [^{3}H]GABA induced by 10 μM GABA. When appropriate, Ca^{2+} was omitted from the superfusion medium 19 min before introduction of GABA. KB-R7943, dantrolene and 2-APB

were introduced 9 min before GABA. Results are expressed as percent potentiation with respect to the basal release. Data are means ± SEM of 3–6 experiments performed in triplicate $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the control value representing the effect of GABA in standard medium and in the absence of drugs (one-way ANOVA followed by Dunnett's test).

Fig. 4 Effects of niflumic acid and NPPB on the release of [^{3}H]GABA induced by 10 μM GABA. Niflumic acid and NPPB were introduced 9 min before GABA. Results are expressed as percent potentiation with respect to the basal release. Data are means ± SEM of 3–6 experiments performed in triplicate. $^{*}P < 0.05$ vs. the effect of GABA in the absence of drugs (one-way ANOVA followed by Dunnett's test).

Fig. 5 Effects of CGP37157, niflumic acid and NPPB on the release of [^{3}H]GABA induced by 10 μM GABA in nominal absence of external Ca^{2+}. Ca^{2+} was omitted from the superfusion medium 19 min before addition of GABA. CGP37157 was added 19 min before GABA. Niflumic acid and NPPB were added 9 min before GABA. Results are expressed as percent potentiation with respect to the basal release. Data are means ± SEM of 3–5 experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the effect of GABA in the absence of drugs (one-way ANOVA followed by Dunnett's test).

Fig. 6 Schematic representation of the GABA-evoked [^{3}H]GABA release. GABA is taken up into GABAergic nerve terminals by GAT1 transporters. The influx of GABA provokes efflux of GABA in part by GAT1 reversal. A significant portion of the transmitter is released by

permeation through anion channels (AC) activated by Ca^{2+} ions originating from both plasmalemmal Na^{+}/Ca^{2+} exchangers (pNCX) and mitochondrial Na^{+}/Ca^{2+} exchangers (mNCX).

Figure 1
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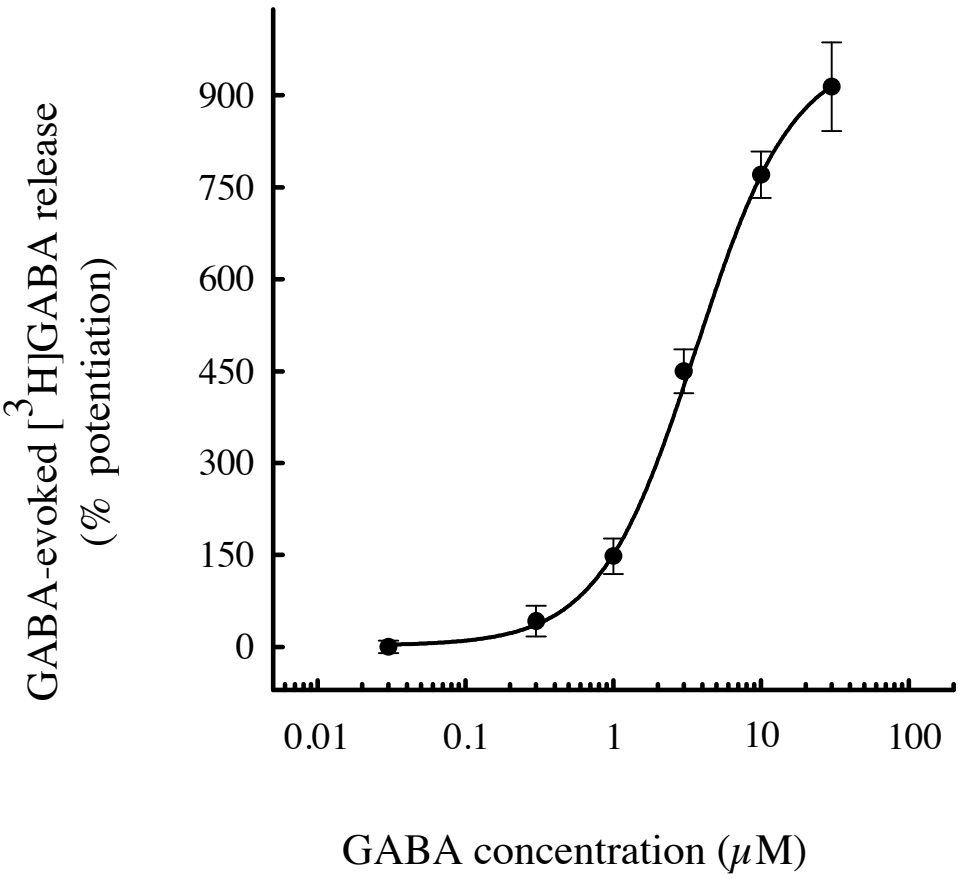

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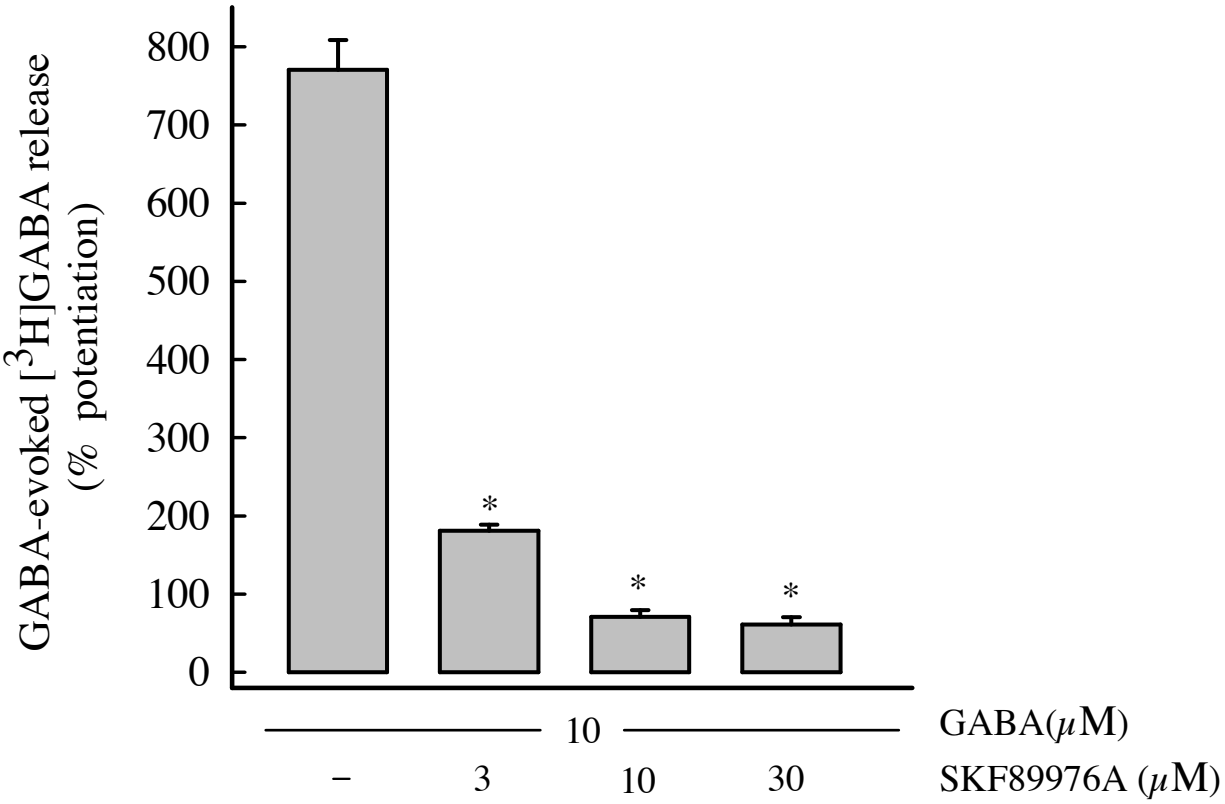

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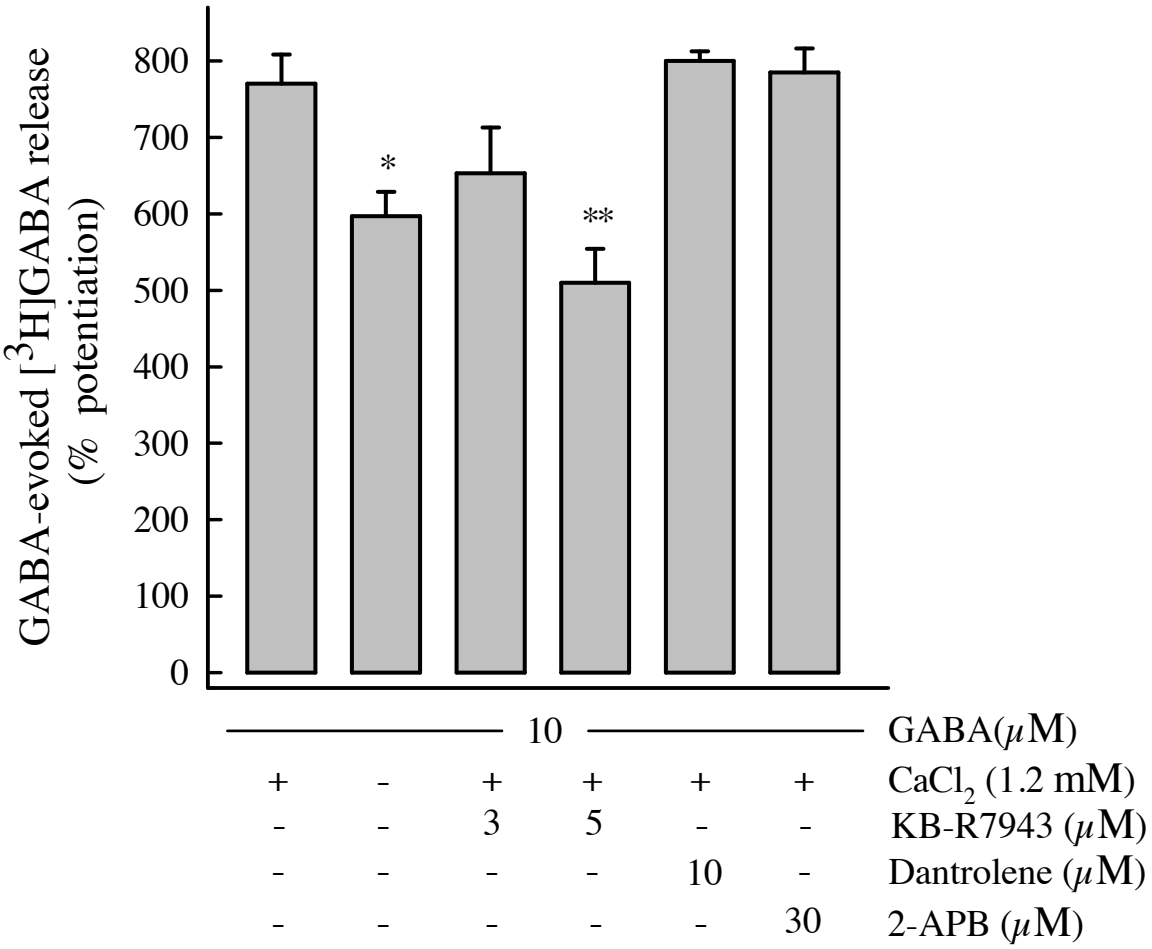

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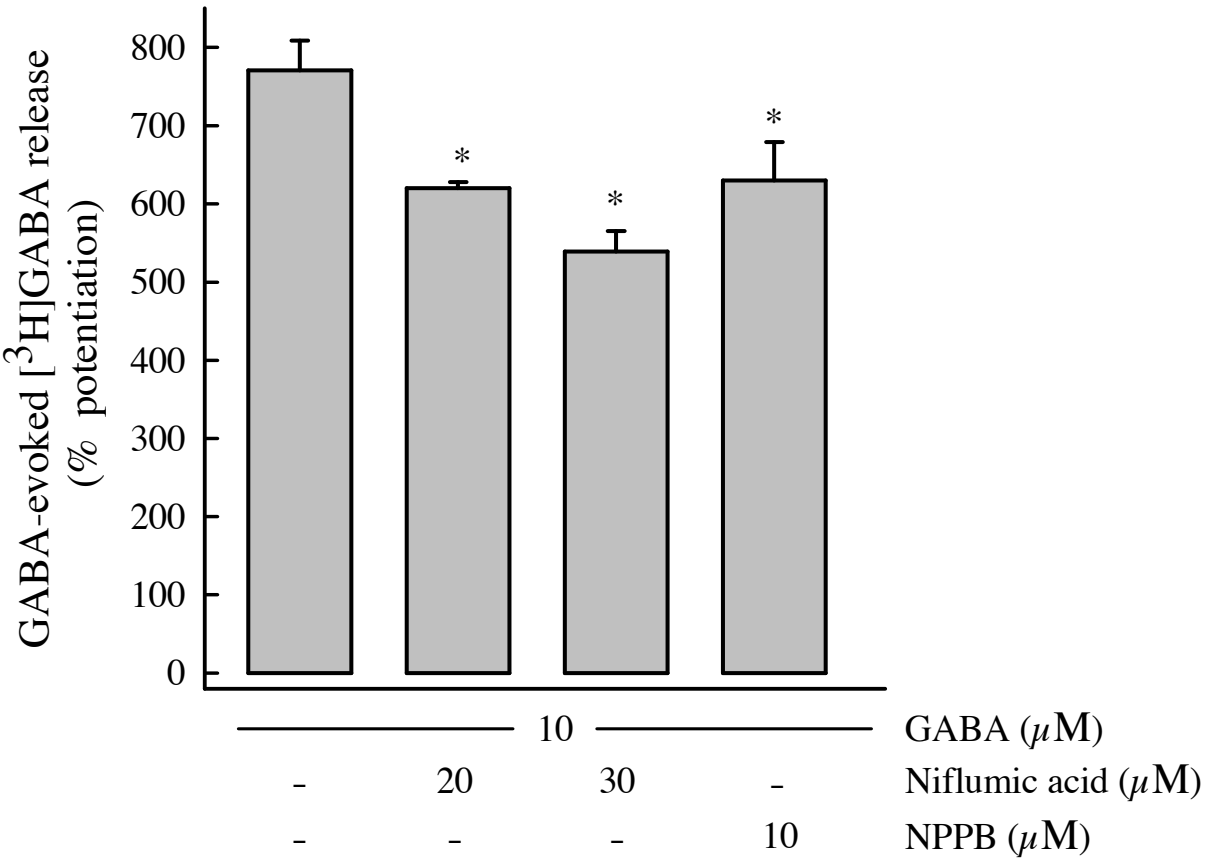

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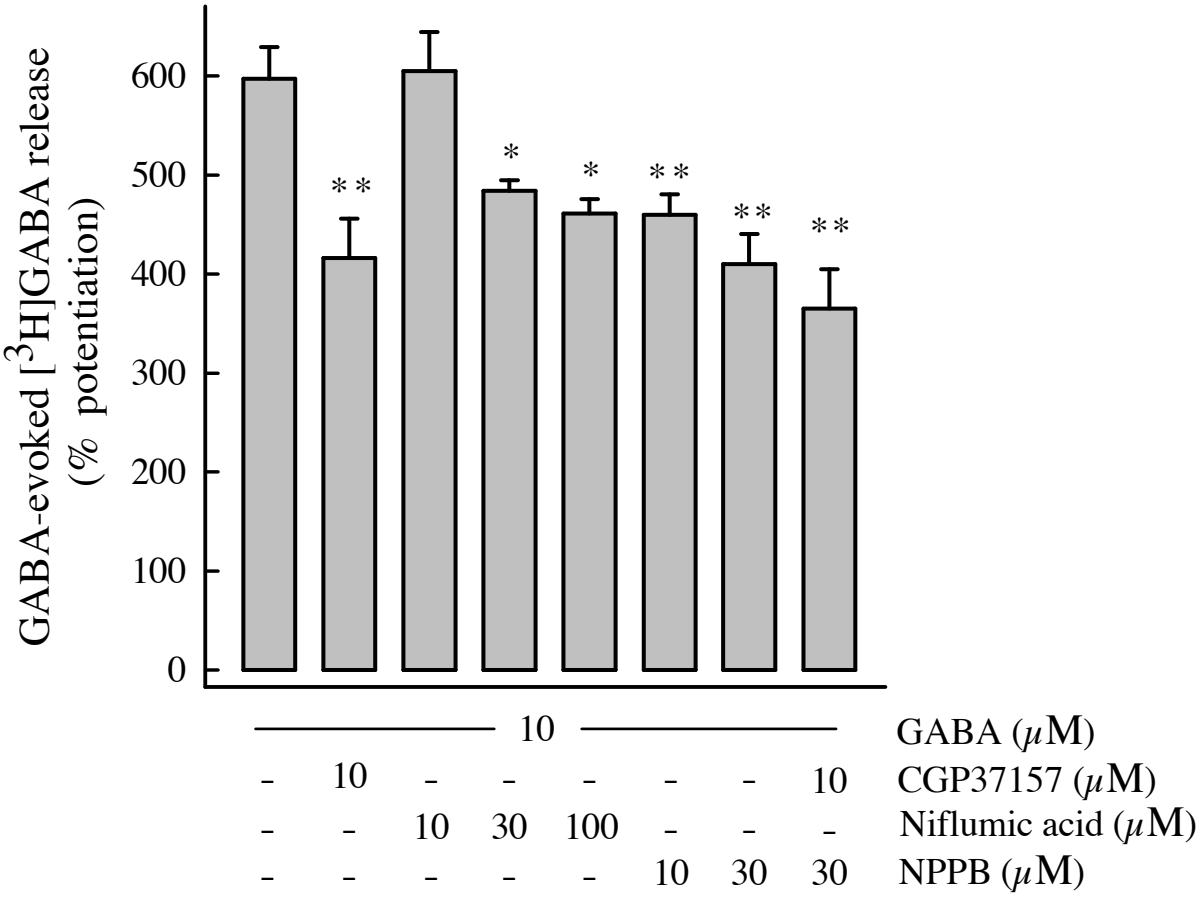

Figure 6

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