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Complete List of Authors:	Rebosio, Claudia; University of Genoa, Department of Pharmacy, Section of Pharmacology and Toxicology Balbi, Matilde; University of Genoa, Department of Pharmacy, Section of Pharmacology and Toxicology Passalacqua, Mario; University of Genoa, Department of Experimental Medicine, Section of Biochemistry Ricciarelli, Roberta; University of Genoa, Department of Experimental Medicine, Section of General Pathology Fedele, Ernesto; University of Genoa, Department of Pharmacy, Section of Pharmacology and Toxicology
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Presynaptic GLP-1 receptors enhance the depolarization-evoked release of Glutamate and GABA in the mouse cortex and hippocampus.

Claudia Rebosio[a]*, Matilde Balbi[a]*, Mario Passalacqua[b], Roberta Ricciarelli[c], Ernesto Fedele[a,d†]

[a]Department of Pharmacy, Section of Pharmacology and Toxicology, University of Genova, Genova, Italy;

[b]Department of Experimental Medicine, Section of Biochemistry and Italian Institute of Biostructures and Biosystems, University of Genova, Genova, Italy;

[c]Department of Experimental Medicine, Section of General Pathology, University of Genova, Genova, Italy;

[d]Centre of Excellence for Biomedical Research, University of Genova, Genova, Italy.

*equally contributed

[†]Corresponding author: Ernesto Fedele, Department of Pharmacy, Section of Pharmacology and Toxicology, University of Genova, Viale Cembrano 4, 16148 Genova, Italy. Tel. +39 0103532659. Fax +39 010

E-mail address: fedele@difar.unige.it

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Abbreviations: Aβ, amyloid β; cAMP, 3':5' cyclic adenosine monophosphate; DDP-4, dipeptidyl peptidase IV; EPAC 2, exchange protein activated by cAMP type 2; GLP-1, glucagon-like peptide-1; GLP-1Rs, GLP-1 receptors; 6-OHDA, 6-hydroxydopamine; LTP, long-term potentiation; PKA, protein kinase A; PPF, paired pulse facilitation.

Abstract

Glucagon-like peptide-1 receptors (GLP-1Rs) have been shown to mediate cognitive-enhancing and neuroprotective effects in the central nervous system. However, little is known about their physiological roles on central neurotransmission, especially at the presynaptic level. Using purified synaptosomal preparations and immunofluorescence techniques, here we show for the first time that GLP-1Rs are localized on mouse cortical and hippocampal synaptic boutons, in particular on glutamatergic and GABAergic nerve terminals. Their activation by the selective agonist exendin-4 was able to increase the release of either [^{3}H]D-aspartate or [^{3}H]GABA in a concentration-dependent fashion (1-100 nM). These effects were abolished by 10 nM of the selective GLP1-R antagonist exendin-3 (9-39) and were prevented by the selective adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (10 μM), indicating the involvement of classic GLP-1Rs coupled to G_s protein stimulating cAMP synthesis.

Our data demonstrate the existence and activity of presynaptic receptors for GLP-1 that could represent additional mechanisms by which this neurohormone exerts its effects in the CNS.

Introduction

Glucagon-like peptide-1 (GLP-1) is a hormone belonging to the incretin family that has been extensively investigated for its peripheral effects on the regulation of glucose homeostasis [1].

In response to nutrient ingestion, ileal L cells process proglucagon to produce and secrete GLP-1, which then exerts its insulinotropic effects by stimulating GLP-1 receptors (GLP-1R) located onto pancreatic β-cells. GLP-1R is a G protein coupled receptor (GPCR), which signals by activating adenylyl cyclase and the subsequent production of 3':5' cyclic adenosine monophosphate (cAMP). In turn, cAMP recruits either protein kinase A (PKA) or exchange protein activated by cAMP type 2 (EPAC 2) that promotes intracellular Ca^{2+} elevation. In addition, the cAMP/PKA pathway also reduces Kv channel currents, thus delaying β-cell repolarization. The overall effect induced by GLP-1 through these mechanisms is to amplify the glucose-induced release of insulin. Moreover, the incretin hormone also stimulates proliferation of β-cells and increases insulin gene transcription and insulin synthesis. Besides, the GLP-1/GLP-1R system is also known to exert pleiotropic effects in many other tissues and organs (e.g. liver, kidney, heart, muscle, bone).

GLP-1 is then rapidly degraded by dipeptidyl peptidase IV (DDP-4) that acts at its N-terminus to produce inactive GLP-1(9-36) amide and GLP-1(9-37).

In recent years, the GLP-1/GLP-1R system in the central nervous system has attracted the attention of neuroscientists, especially for its pro-mnesic and neuroprotective effects that could be therapeutically exploited in different neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) [2,3].

Indeed, it has been consistently reported that activation of GLP-1Rs with DDP-4 resistant GLP-1 agonists, such as (Val^8)GLP-1 and liraglutide, facilitates hippocampal long-term potentiation (LTP) [4,5], the generally accepted electrophysiological substrate of memory formation and consolidation, whereas their genetic deletion impairs this synaptic plasticity process and severely affects memory functions [6]. More interestingly from a translational point of view, a large body of evidence has accumulated showing that GLP-1R stimulation is able to rescue the impairment of LTP and

memory induced by amyloid β (Aβ), the small peptide involved in the AD pathocascade, and to decrease Aβ plaques, tau hyperphosphorylation and synaptic loss in rodent models of the human disease [7-15].

GLP1-R agonists have been found to rescue also the neurochemical and functional deficits in mice modelling human PD. As a matter of fact, it has been reported that the GLP1-1R agonist exendin-4 reversed the loss of dopaminergic neurons, the reduction of striatal dopamine concentrations and the circling behaviour caused by the 6-hydroxy-dopamine (6-OHDA)-induced denervation of the nigrostriatal pathway, an effect that has been attributed to the GLP-1R ability to stimulate adult neurogenesis, to inhibit apoptosis and to contrast neuroinflammation [16,17]. Similar results on histological, neurochemical and behavioural analysis were obtained with GLP-1R agonists in a different rodent model of PD [18].

On the basis of these results, the GLP-1R agonists exenatide and liraglutide have been trialled in small cohorts of AD and PD patients with promising results [19-21].

Despite these clear-cut evidences, little is known on the physiological effects of GLP-1R on central neurotransmission. In the CNS, neuropeptides and neurohormones mainly modulate, rather than mediate, neurotransmission and they often do this by regulating neurotransmitter release through activation of presynaptic receptors [22,23].

In the present study, we provide histochemical and functional evidence showing that mouse cortical and hippocampal GABAergic and glutamatergic nerve terminals are endowed with presynaptic GLP-1Rs whose activation is able to increase the release of the respective aminoacidergic neurotransmitters.

Methods

Animals

C57BL/6J adult male mice (2-3 months of age, 25-30 g weigh) were used. Animals were bred in the animal care facility at the Department of Pharmacy (University of Genova, Genova, Italy) and

housed at constant temperature (22 ± 1C) and relative humidity (50%), under a regular light–dark schedule (light 7 AM–7 PM). Food and water were freely available. All experiments were carried out in accordance with the European Union Directive 2010/63/EU for animal experiments and the ARRIVE guidelines, and were approved by the Ethical Committee of the University of Genoa (protocol n° 09/02/2016 OPBA). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

Preparation of purified synaptosomes

Purified synaptosomes were prepared on Percoll® gradients (Sigma-Aldrich, St Louis, MO, USA) essentially according to Nakamura et al., 1993 [24], with minor modifications. Briefly, cerebral cortex or hippocampi were homogenized in sucrose (0.32 M, 1:10 weight/volume) buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue homogenizer (clearance 0.25 mm, 12 updown strokes in about 1 min). The homogenate was first spun at 1,000 g for 5 min (4 °C) to remove nuclei and debris. The supernatant was then stratified on a discontinuous Percoll® gradient (6%, 10%, and 20% v/v in Tris-buffered sucrose) and spun at 33,500 g for 5 min (4 °C). The purified synaptosomal fraction (layer between 10% and 20% Percoll) was collected, washed and re-suspended in physiological medium (NaCl 140 mM; KCl 3 mM; $MgSO_4$ 1.2 mM; NaH_2PO_4 1.2 mM; HEPES 10 mM; glucose 10 mM; pH 7.4).

Immunoblot analysis

Purified synaptosomes were lysed in ice-cold RIPA buffer containing 1% protease inhibitor cocktail (total volume of lysis buffer: 600 µl for cortical and 300 µl for hippocampal synaptosomes). After lysis, samples were centrifuged at 10,000 g for 10 min at 4°C and the resulting supernatants were subjected to immunoblot analysis for the evaluation of GLP-1R expression (20 µg protein/lane). Immunoblots were done according to standard methods, using a rabbit polyclonal antibody to GLP-1R (1µg/ml) from Abcam (Cambridge, UK;) and a mouse monoclonal antibody to β-actin

(1:10,000) from Sigma (MO, USA). Anti-mouse and anti-rabbit secondary antibodies were coupled to horseradish peroxidase (Amersham, NJ, USA). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's instructions (Amersham). Chemiluminescence was monitored by exposure to film and the signals were analyzed under non saturating conditions with an image densitometer (Bio-Rad laboratories, CA, USA).

Release experiments

Release experiments have been carried out as previously reported [25]. Cortical or hippocampal purified synaptosomes have been incubated at 37 °C for 15 min with 0.03 μM [^{3}H]D-Aspartate (a non metabolizable marker of glutamatergic nerve terminals) or with 0.02 μM [^{3}H]GABA (Perkin-Elmer, Milan, Italy) in the presence of 50 μM amino-oxyacetic acid to prevent GABA metabolism. Next, identical aliquots of synaptosomal suspension (were layered on microporous filters placed at the bottom of parallel superfusion chambers (Superfusion System, Ugo Basile, Comerio, Varese, Italy) maintained at 37 °C and superfusion was started at a flow rate of 0.5 mL/min. After 36 min of superfusion to equilibrate the system, samples were collected as follows: two 3-min samples (t = 36–39 min and t = 45–48 min; basal release) before and after one 6-min sample [t = 39–45 min; K^+-evoked release]. A 90-sec period of depolarization was applied at t = 39 min of superfusion. Depolarization was performed by using 15 mM ([^{3}H]D-ASP) or 12 mM ([^{3}H]GABA) KCl, substituting for an equimolar concentration of NaCl. Synaptosomes were exposed to the GLP-1R agonist exendin-4 (Tocris Bioscience, Milan, Italy) concomitantly with the KCl depolarizing stimulus, whereas the selective GLP-1R antagonist exendin-3 (9-39) (Tocris Bioscience, Milan, Italy) or the selective adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (Sigma Aldrich, Milan, Italy) when used, were present 8 min before and during the depolarization. Drug concentrations were chosen on the basis of their reported affinities for the GLP-1R and of functional studies reported in the literature. Appropriate controls were always run in parallel. At the end of the experiment, collected fractions and superfused synaptosomes were counted for radioactivity by

liquid scintillation counting. The efflux of radioactivity in each fraction was expressed as a percentage of the total radioactivity present in synaptosomes at the onset of the fraction collection (fractional rate). Depolarization-evoked neurotransmitter overflow was calculated by subtracting the transmitter content of the two 3-min fractions (spontaneous release) from that in the 6-min fraction collected during and after the KCl depolarization pulse.

Since the depolarization-evoked overflows measured under control conditions in the different experiments were not different, they were pooled.

Data in the figures have been expressed as percentages of controls (KCl-evoked overflow) and are reported as mean ± SEM of the number of experiments reported in the figure legends. Differences have been analyzed by one-way ANOVA followed by Dunnett's or Tukey-Kramer multiple comparison test, where appropriate, and considered significant at the level of $p<0.05$, at least.

Confocal microscopy localization of GLP-1Rs

Cortical and hippocampal purified synaptosomes (50 µg of protein) [26] were fixed with 2% paraformaldehyde for 15 min, washed with Phosphate Buffered Saline (PBS; 3x5min), permeabilized with 0.05% Triton X-100 for 5 minutes and incubated overnight at 4 C° with primary antibodies diluted in PBS containing 3% bovine serum albumin. The following antibodies were used: rabbit anti-GLP-1R (1:500; Abcam, Cambridge; UK), guinea pig anti-VGLUT1 (1:1000; Merk Millipore; Milan; Italy) and guinea pig anti-VGAT (1:500; Synaptic System, Gottigen; Germany).

After washing with PBS, synaptosomes were incubated for 60 min at room temperature with Alexa Fluor 488 and 633 secondary antibodies conjugates (1:1000; Life Technologies Corporation, Carlsbad, CA, USA) in PBS containing 0.5% bovine serum albumin.

Images were acquired using a Leica TCS SP confocal microscope (Leica Wetzlar, Germany) equipped with 488 and 633 excitation lines. Sequential acquisition was performed to avoid crosstalk between color channels. Spatial co-localization was determined using Manders' coefficients

calculated with Jacop plugin in ImageJ 1,46 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), after background subtraction. Overlay is depicted in white to make the co-localization stand out. Data were collected analyzing 10 to 12 non-overlapping fields from three independent experiments and are expressed as mean ± SEM.

Results

GLP-1Rs are expressed in purified cortical and hippocampal synaptosomes.

Before starting the functional study on the effects of GLP-1Rs on neurotransmitter release, we performed a western blot analysis to evaluate whether these receptors were present in our synaptosomal preparations. As shown in Fig. 1, the rabbit polyclonal anti-GLP-1R antibody used in our analysis clearly detected a band of approximately 53 kDa (the predicted molecular weight for this receptor) in both purified cortical and hippocampal synaptosomes.

Activation of GLP-1Rs enhances the depolarization-evoked release of [^{3}H]D-aspartate from purified cortical and hippocampal synaptosomes

Under our superfusion conditions, the spontaneous release of [^{3}H]D-aspartate from purified cortical synaptosomes amounted to 0.533 ± 0.034% of total radioactivity (n=20 independent experiments). When synaptosomes where depolarized with a 90-sec pulse of 15 mM KCl, the release was significantly increased and the overflow amounted to 2.151 ± 0.142% of total radioactivity (n=20 independent experiments).

As shown in Fig. 2A, exposure to the selective GLP-1R agonist exendin-4 (1-30 nM) during the KCl-induced depolarization was able to further increase the release of [^{3}H]D-aspartate in a concentration dependent fashion (max. increase 37% vs controls).

The effect of exendin-4 (10 nM) on the depolarization-evoked overflow was completely prevented when synaptosomes were co-perfused with an equimolar concentration (10 nM) of the selective GLP-1R antagonist exendin-3 or with the selective adenylyl cyclase inhibitor 2',5'-

dideoxyadenosine (10 μM) (Fig. 2B). Both the GLP-1R antagonist and the adenylyl cyclase inhibitor were devoid of any effect on their own (Fig. 2B)

In the case of superfused purified hippocampal synaptosomes, the spontaneous [^{3}H]D-aspartate release was 0.853 ± 0.051% (n=11 independent experiments) and the 15 mM KCl-evoked overflow was 2.833 ± 0,255 (n=11 independent experiments). Also in this case, exendin-4 (1-30 nM) was able to enhance the KCl-induced release (max. increase 43% vs controls) in a concentration dependent and exendin-3 sensitive manner (Fig. 3).

Activation of GLP-1Rs enhances the depolarization-evoked release of [^{3}H]GABA from purified cortical and hippocampal synaptosomes

The spontaneous release of [^{3}H]GABA from superfused purified cortical synaptosomes amounted to 2.516 ± 0.312% and the 12 mM KCl-induced overflow was 4.553 ± 0.360% (n=11 independent experiments). Activation of GLP-1Rs with exendin-4 (10-100 nM) resulted in the potentiation of the [^{3}H]GABA release elicited by KCl depolarization, with a maximal effect of approximately 30% reached at the highest concentration tested (Fig. 4A). The enhancing effect of 30 nM exendin-4 was completely prevented when synaptosomes were co-exposed to the GLP-1R antagonist exendin-3 (10 nM) or to the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (10 μM), which were ineffective on their own (Fig. 4B).

Similar results were obtained in the hippocampus. In this brain region, the [^{3}H]GABA spontaneous release from superfused purified synaptosomes was 4.067 ± 0.792% and the 12 mM KCl-evoked overflow was 5.195 ± 0.366 (n=5 independent experiments). Exendin-4 was able to enhance the release of [^{3}H]GABA by approximately 33% at the concentration of 30 nM and this effect was abolished by exendin-3 (10 nM; Fig. 5).

GLP-1Rs are localized onto cortical and hippocampal glutamatergic and GABAergic nerve terminals

To further confirm that presynaptic GLP-1Rs are present on synaptic boutons of glutamate and GABA neurons, we carried out an immunofluorescence analysis on purified preparations of cortical and hippocampal synaptosomes.

To this purpose, we have labelled synaptosomes with an anti-GLP-1R antibody and with anti-VGLUT1 or anti-VGAT antibodies to identify glutamatergic and GABAergic synaptosomes, respectively.

The co-localization analysis showed that 40 ± 2% of glutamatergic nerve terminals expresses GLP-1Rs in the cerebral cortex, whereas in the hippocampus the value was 20 ± 3% (Fig. 6).

In the case of GABAergic nerve terminals, the same type of co-localization analysis resulted in values of 17 ± 4% and 34 ± 1% for the cerebral cortex and hippocampus, respectively (Fig. 7).

Discussion

Our immunological and functional experiments demonstrate for the first time the existence of release-regulating presynaptic receptors for the neurohormone GLP-1 on mouse glutamatergic and GABAergic nerve terminals. Their activation results in the enhancement of the depolarization-induced release of the two neurotransmitters.

From a methodological point of view, the functional data showing that activation of GLP-1Rs increases the release of glutamate and GABA from depolarized synaptosomes in superfusion are already indicative of the localization of these receptors on the respective nerve terminals.

In fact, when synaptosomes are plated as a quasi-monolayer on microporous filters and up-down superfused with physiological solutions at a defined flow rate, the released transmitters are immediately removed by the superfusion fluid before they can accumulate and activate presynaptic auto- and hetero-receptors present on the plasma membrane, thus excluding the possibility of indirect effects. Therefore, the scenario is that of nerve terminals having their membrane targets

(receptors, transporters, etc.) free of endogenously released agonists. Each of these targets can, however, be selectively activated by adding the appropriate ligand to the superfusion medium at the desired concentration. Therefore, any effect of selective drugs on the release of a given neurotransmitter can be exclusively attributed to the action on the target present on the nerve terminal releasing that transmitter, thus permitting its unequivocal localization. Indeed, the immunofluorescence analyses carried out on the purified synaptosomal preparations have confirmed that GLP-1Rs are localized on cortical and hippocampal glutamatergic and GABAergic nerve endings. Furthermore, the functional effects that we have observed were specifically mediated by classical GLP-1Rs, being prevented by the selective antagonist exndin-3 (9-39) and by blocking their known transduction mechanism using the selective adenylyl cyclase inhibitor 2',5'-dideoxyadenosine.

Although it is now clear that stimulation of GLP-1Rs can be beneficial to AD and PD in terms of nootropic effects and neuroprotection [2,3] only few studies have investigated the cellular and molecular mechanisms by which the GLP-1/GLP-1R system influences central neurotransmission, especially at the presynaptic level.

Using the push-pull cannula or the microdialysis techniques, it has been shown that in vivo infusion of GLP-1(7-36) amide was able to increase the extracellular levels of glutamate, glutamine and/or aspartate in the basal ganglia [27] or in the ventromedial hypothalamus of conscious rats [28]. In addition, intraperitoneal (IP) administration of the GLP-1R agonist exendin-4 for 7 days was able to enhance the L-DOPA induced release of dopamine (DA) in the striatum of 6-hydroxydopamine lesioned rats [29]. On the other hand, intracerebroventricular injection of GLP-1 or of the GLP-1R agonist exendin-4 resulted in a significant decrease of extracellular levels of serotonin (5-HT), aspartate, glutamate, glutamine, glycine, GABA, tryptophan and tyrosine in the rat hypothalamus [30]. Finally, systemic administration of exendin-4 attenuated the ethanol-, amphetamine- and cocaine-induced elevation of extracellular DA levels in the rat nucleus [31,32]. Although these in vivo studies highlighted the role of GLP-1Rs in modulating neurotransmitter release, they could not

discriminate between agonist direct and indirect effects, whether presynaptic GLP-1Rs were involved and, in some cases (e.g. glutamate, aspartate and GABA release), they could not even identify the cellular source of neurotransmitter release (i.e. neurons or astrocytes). To the best of our knowledge, there is only one functional study that has reported the presence of presynaptic GLP-1Rs able to enhance 5-HT release from hypothalamic synaptosomes [33].

Our data on GLP-1R-induced glutamate and GABA release are in accordance with those reported in different electrophysiological studies. In fact, it has been shown that activation of GLP-1Rs increased the firing activity of hippocampal CA1 pyramidal neurons, possibly by enhancing glutamate release from presynaptic sites [34]. Moreover, GLP-1R agonists enhanced paired pulse facilitation (PPF) at Schaffer collateral/CA1 pyramidal neuron synapses in the hippocampus, an effect suggesting a presynaptic site of action [8]. Interestingly, PPF was impaired in GLP-1R KO mice, further supporting the role of presynaptic GLP-1Rs in modulating neurotransmitter release [6]. Also, GLP-1 and exendin-4 increased the frequency, but not the amplitude, of miniature excitatory post-synaptic currents (mEPSCs) of hypocretin neurons in mouse hypothalamic slices, suggesting presynaptic modulation of glutamate release from afferent fibres [35].

Similarly, $GABA_A$ receptor-mediated spontaneous inhibitory post-synaptic current (sIPSCs) in hippocampal CA3 pyramidal neurons are increased by GLP-1 and exendin-4 in an exendin 3 (9-39)- and TTX-sensitive manner [36,37]. Again, these results indicate that GLP-1R activation can evoke GABA release from presynaptic elements.

With regard to pro-cognitive effects, it has been consistently shown that administration of selective agonists or overexpression of GLP-1Rs improve learning and memory under physiological or pathological conditions, such as Alzheimer's disease [9,11,12,38-40], whereas memory deficits manifest when these receptors are knocked out [6,38]. As for the mechanism of action for those memory-enhancing effects, several lines of evidence have demonstrated that activation of GLP-1Rs, in different experimental settings, can enhance or rescue hippocampal long-term potentiation (LTP), a synaptic plasticity phenomenon generally accepted to represent the electrophysiological substrate

of learning and memory [4,5,8,9,11,13,39]. On the contrary, LTP is impaired in GLP-1R KO mice [6].

Since the presynaptic component of LTP expression requires glutamate release, our results, showing that activation of presynaptic GLP-1Rs enhances the release of the excitatory neurotransmitter, could represent one of the mechanisms by which GLP-1 exerts its physiological effects on learning and memory formation.

On the other hand, the GLP-1R-mediated enhancement of GABA release is expected to result in $GABA_A$-mediated hyperpolarization of post-synaptic neurons, which is known to prevent LTP induction. However, it has been shown that during high frequency stimulation of synaptic activity to induce LTP, GABAergic synaptic inhibition decreases due to the GABA-induced activation of $GABA_B$ auto-receptors, which dampen GABA release itself [41,42]. Thus, we can speculate that, in the intact synapse, the GLP-1R-induced increase of GABA release might activate a similar mechanism to facilitate LTP induction and memory formation.

As introduced above, neuroprotection is another important research field in which GLP-1R agonists are being investigated with very promising results. In this view, and bearing in mind the excitotoxic properties of glutamate, it would seem difficult to reconcile the increase of glutamate release caused by the activation of GLP-1Rs with their neuroprotective effects.

The research of the last 15 years, however, has highlighted the critical role of the subunit composition of glutamatergic NMDA receptors in mediating excitotoxic and neuroprotective effects. In fact, it is now well established that the stimulation of GluN2B-containing NMDA receptors, which are mostly localized extrasynaptically, triggers excitotoxic-mediated cell death, whereas GluN2A-containing NMDA receptors, which predominate at the synaptic level, mediate neuroprotection through the activation of specific molecular pathways [43-51]. Since in the present study we show that GLP-Rs are located on the glutamatergic nerve terminals, their stimulation is expected to increase glutamate release in the synaptic cleft, thus activating synaptic GluN2A NMDA receptors that favour neuroprotection.

As for GABA, the neuroprotective properties of increased GABAergic neurotransmission have long been known [52,53] and, therefore, the increase of GABA release by presynaptic GLP-1Rs could well be a mechanism that explains part of the neuroprotection achieved with the stimulation of these receptors.

In conclusion, our data, demonstrating that cortical and hippocampal glutamatergic and GABAergic nerve terminals are endowed with presynaptic release-regulating GLP-1Rs, provide additional information on the biochemical mechanisms by which GLP-1 can exert its physiological roles in the central nervous system.

Acknowledgements and conflict of interest disclosure

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Authors declare no conflicts of interest.

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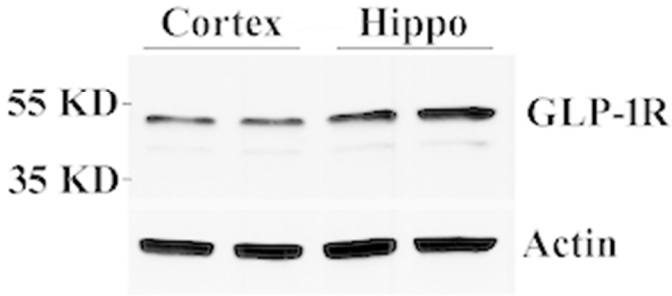

Figure 1. Immunoblot analysis showing the presence of GLP-1 receptors in purified synaptosomal preparation of mouse cerebral cortex and hippocampus. Representative immunoblot analysis (cropped images) performed on protein extracts (20 μg/lane) from purified synaptosomes. β-actin signal represents the internal loading control.

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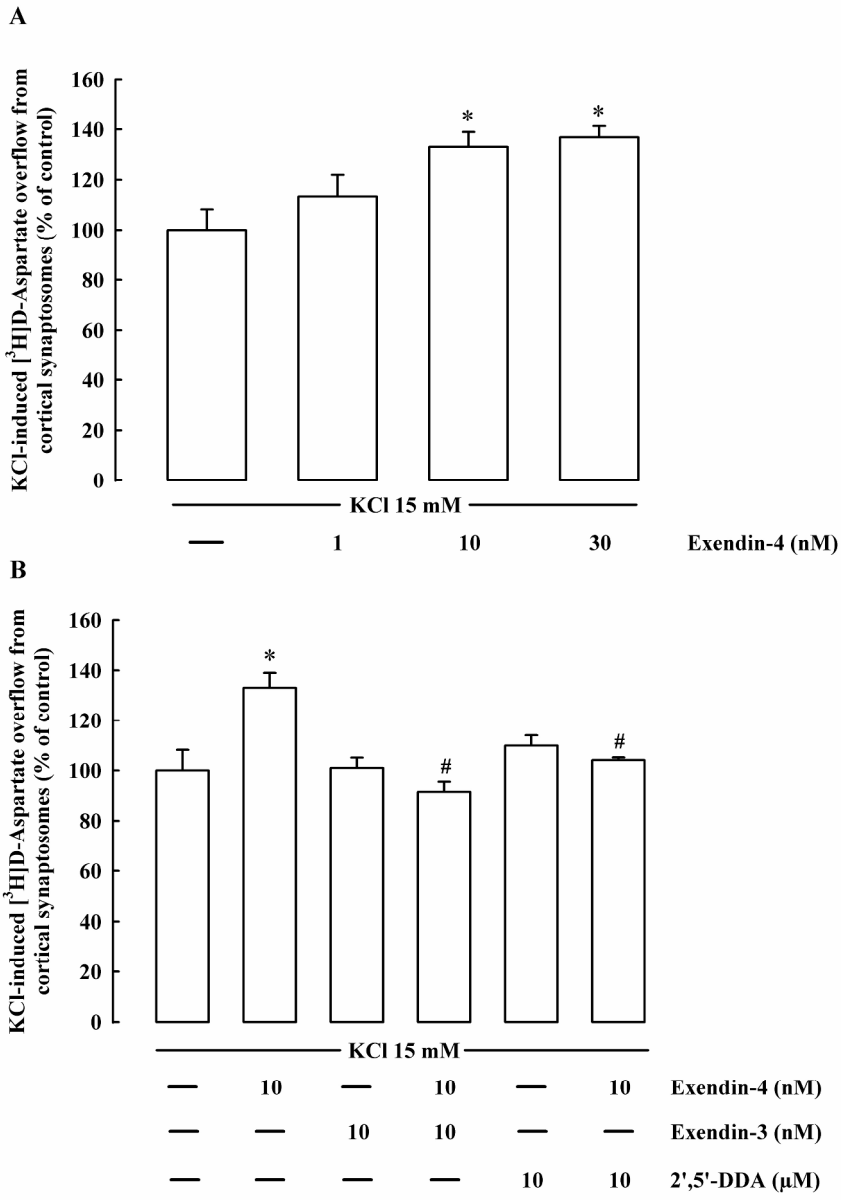

Figure 2. Activation of GLP-1 receptors enhances the KCl-induced release of [3H]D-aspartate from purified mouse cerebral cortex synaptosomes. Panel A. Concentration-response curve of the GLP-1R agonist exendin-4. Panel B. The effect of exendin-4 (10 nM) was completely prevented by the selective GLP-1R antagonist exendin-3 (9-39) or by the adenylyl cyclase inhibitor 2',5' dideoxyadenosine (2',5'-DDA). Data regarding the release evoked by KCl under control conditions were very similar and, therefore, have been pooled. Each bar represents mean ± SEM of: KCl (n=20); exendin-4 1 nM (n=3), 10 nM (n=8), 30 nM (n=7); exendin-3 (9-39) 10 nM (n=3); exendin-3 (9-39) 10 nM + exendin-4 10 nM (n=4); 2',5' DDA 10 μM (4); 2',5' DDA 10 μM + exendin-4 10 nM (n=4). *p < 0.05 vs KCl alone; #p < 0.05 vs exendin-4.

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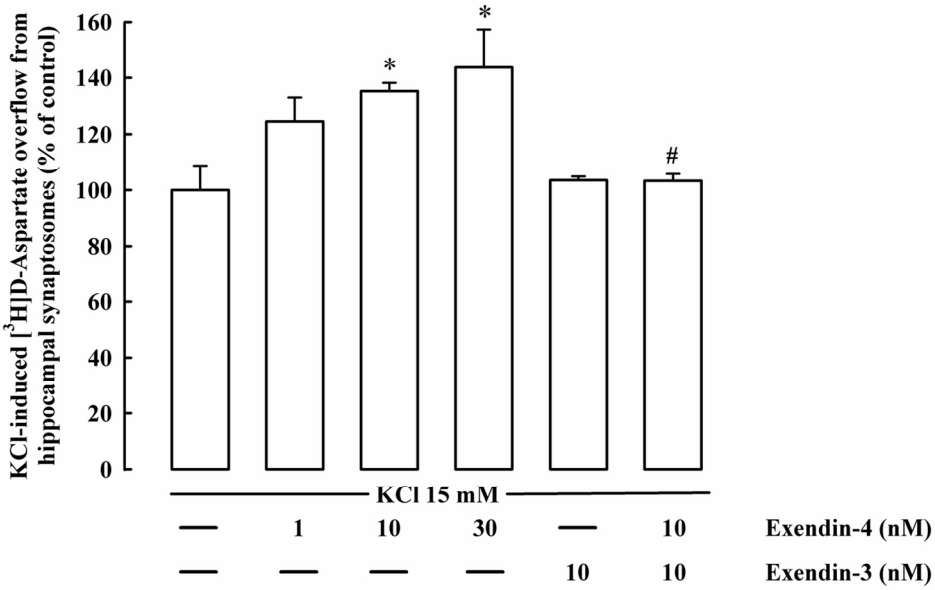

Figure 3. Activation of GLP-1 receptors enhances the KCl-induced release of [3H]D-aspartate from purified mouse hippocampal synaptosomes. Concentration-response curve of exendin-4 and antagonism by exendin-3 (9-39). Each bar represents mean ± SEM of: KCl (n=11); exendin-4 1 nM (n=3), 10 nM (n=7), 30 nM (n=3); exendin-3 (9-39) 10 nM (n=3); exendin-3 (9-39) 10 nM + exendin-4 10 nM (n=4). *p < 0.05 vs KCl alone; #p < 0.05 vs exendin-4.

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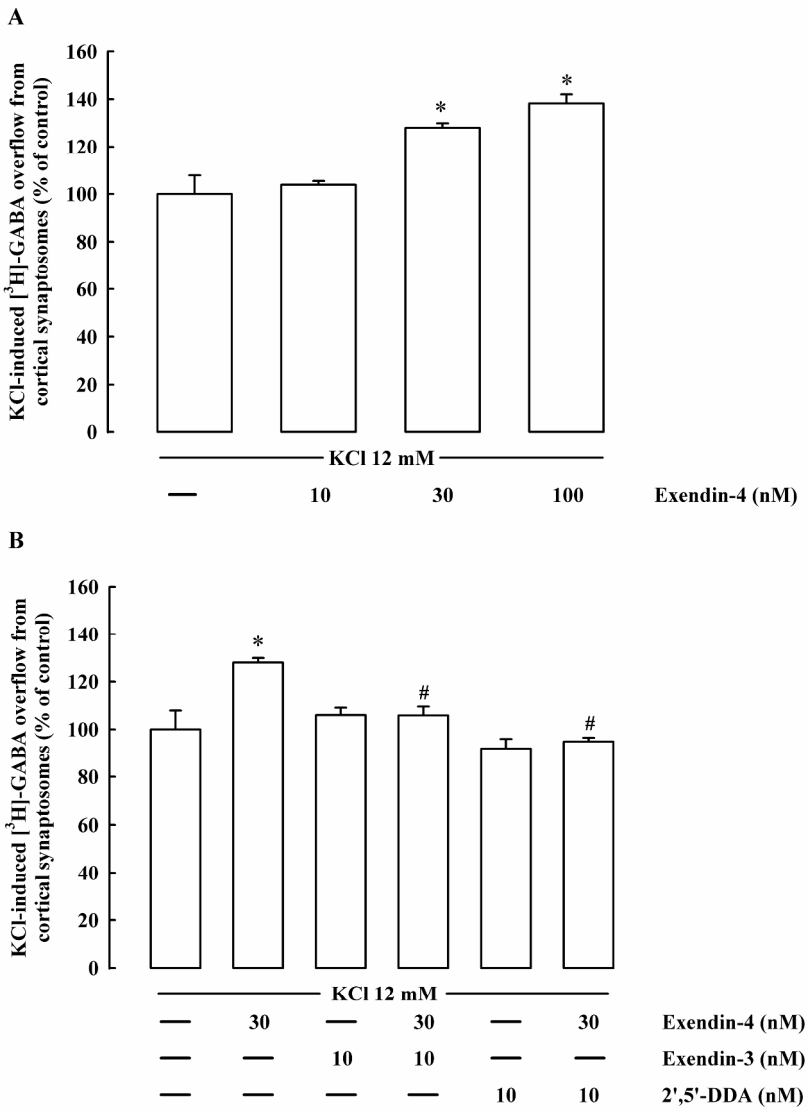

Figure 4. Activation of GLP-1 receptors enhances the KCl-induced release of [3H]GABA from purified mouse cerebral cortex synaptosomes. Panel A. Concentration-response curve of the GLP-1R agonist exendin-4. Panel B. The effect of exendin-4 (10 nM) was completely prevented by the selective GLP-1R antagonist exendin-3 (9-39) or by the adenylyl cyclase inhibitor 2',5' dideoxyadenosine (2',5'-DDA). Data regarding the release evoked by KCl under control conditions were very similar and, therefore, have been pooled. Each bar represents mean ± SEM of: KCl (n=11); exendin-4 10 nM (n=4), 30 nM (n=10), 100 nM (n=5); exendin-3 (9-39) 100 nM (n=6); exendin-3 (9-39)10 nM + exendin-4 10 nM (n=10); 2',5' DDA 10 μM (n=4); 2',5' DDA 10 nM + exendin-4 10 nM (n=3). *p < 0.05 vs KCl alone; #p < 0.05 vs exendin-4.

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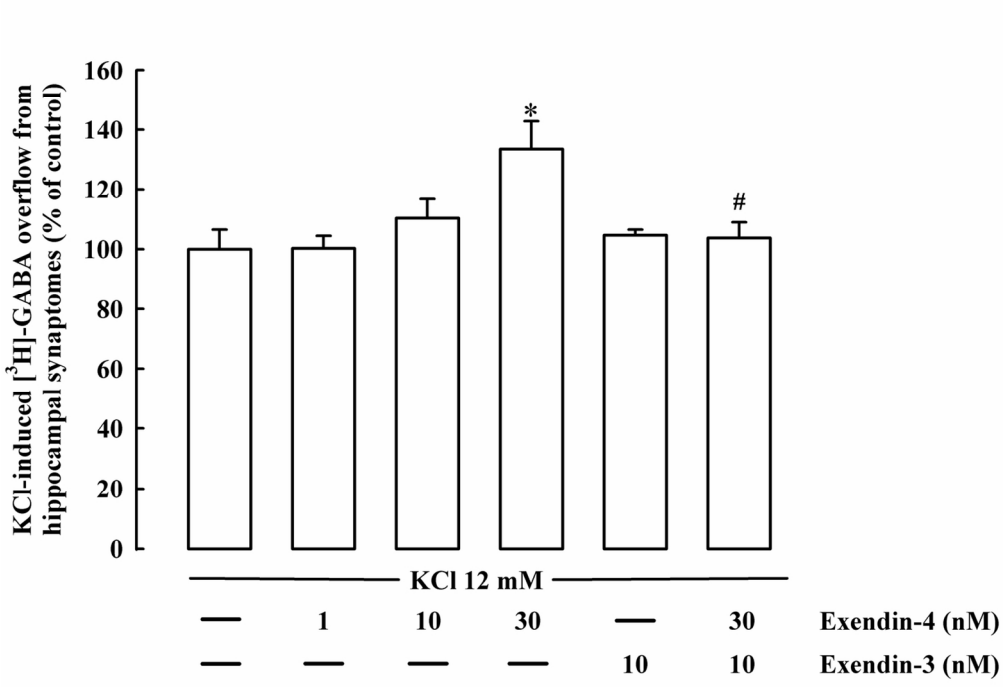

Figure 5. Activation of GLP-1 receptors enhances the KCl-induced release of [3H]GABA from purified mouse hippocampal synaptosomes. Concentration-response curve of exendin-4 and antagonism by exendin-3 (9-39). Each bar represents mean ± SEM of: KCl (n=5); exendin-4 1 nM (n=4), 10 nM (n=4), 30 nM (n=5); exendin-3 (9-39) 10 nM (n=3); exendin-3 (9-39) 10 nM + exendin-4 10 nM (n=4). *p < 0.05 vs KCl alone; #p < 0.05 vs exendin-4.

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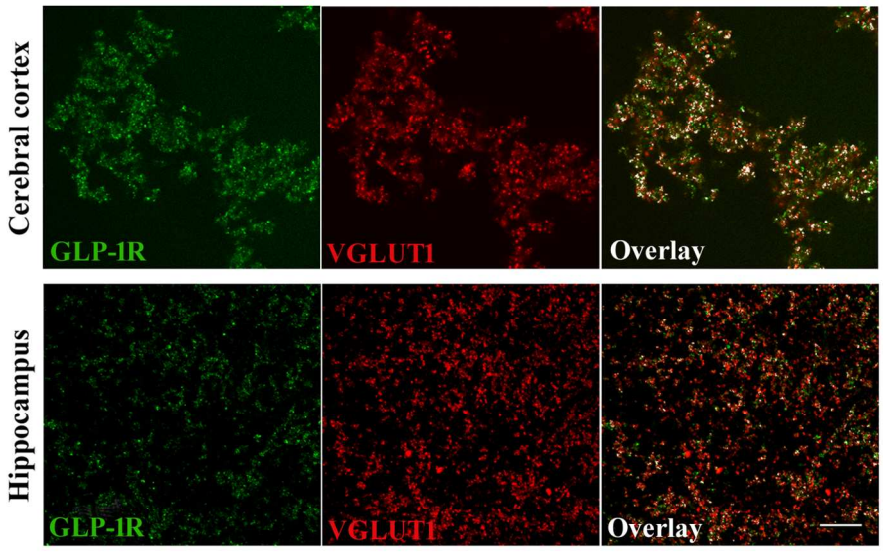

Figure 6. Immunofluorescence localization of GLP-1 receptors on purified cortical and hippocampal glutamatergic synaptosomes. Synaptosomes were fixed with paraformaldehyde and then incubated with anti-GLP-1R (green) and anti-VGLUT1 (red) antibodies overnight followed by incubation with Alexa Fluor 488 and 633 secondary antibodies conjugates. Overlay is depicted in white to make the co-localization stand out. Upper panels, cerebral cortex; lower panels, hippocampus. Images are representative of three independent experiments. Scale bar: 20 μm.

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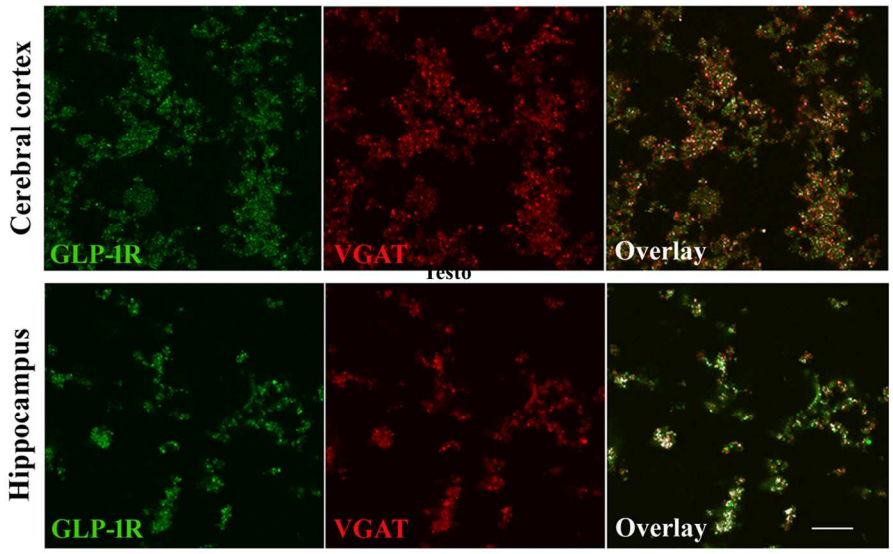

Figure 7. Immunocytochemical localization of GLP-1 receptors on purified cortical and hippocampal GABAergic synaptosomes. Synaptosomes were fixed with paraformaldehyde and then incubated with anti-GLP-1R (green) and anti-VGAT (red) antibodies overnight followed by incubation with Alexa Fluor 488 and 633 secondary antibodies conjugates. Overlay is depicted in white to make the co-localization stand out. Upper panels, cerebral cortex; lower panels, hippocampus. Images are representative of three independent experiments. Scale bar: 20 μm.

140x93mm (300 x 300 DPI)