VGLUT1/VGAT CO-EXPRESSION SUSTAINS GLUTAMATE-GABA CO-RELEASE AND IS REGULATED BY ACTIVITY

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Running title: Glutamate-GABA co-release in neocortex

JCS Advance Online Article. Posted on 6 March 2015

ABSTRACT

In adult neocortex, VGLUT1, the main glutamate vesicular transporter, and VGAT, the GABA vesicular transporter, are co-expressed in a subset of axon terminals forming both symmetric and asymmetric synapses, where they are sorted to the same vesicles. However, the functional consequence of this co-localization in cortical neurons has not been clarified. Here, we tested the hypothesis that cortical axon terminals co-expressing VGLUT1 and VGAT can evoke simultaneously monosynaptic glutamate and GABA responses and investigated whether the amount of terminals co-expressing VGLUT1 and VGAT is affected by perturbations of excitation-inhibition balance. In rat primary cortical neurons, we found that a proportion of synaptic and autaptic responses were indeed sensitive to consecutive application of selective glutamate and $GABA_A$ receptor blockers. These "mixed" synapses exhibited paired-pulse depression. Notably, reducing the activity of the neuronal network by glutamate receptor antagonists decreased the amount of "mixed" synapses, whereas reducing spontaneous inhibition by bicuculline increased them. These synapses may contribute to homeostatic regulation of excitation/inhibition balance.

Keywords: VGAT, VGLUT1, Co-release, GABA, Glutamate

INTRODUCTION

Glutamate (Glu) and GABA mediate most of the excitatory and inhibitory synaptic transmission in central nervous system (Cherubini and Conti, 2001; Conti and Weinberg, 1999); they are taken up and accumulated in synaptic vesicles by specific vesicular transporters named VGLUT1-3 (Fremeau et al., 2004; Gras et al., 2002; Takamori, 2006) and VGAT, respectively (McIntire et al., 1997; Sagne et al., 1997; Takamori et al., 2000).

We have recently shown that in adult neocortex VGLUT1 and VGAT are co-expressed in a subset of axon terminals forming both symmetric and asymmetric synapses, where VGLUT1 and VGAT are sorted to the same vesicles. Moreover, vesicles expressing the heterotransporters participate in the exo-endocytotic cycle (Fattorini et al., 2009). Similar observations were subsequently reported in hippocampus and cerebellum (Zander et al., 2010). Based on our understanding of the role of vesicular neurotransmitter transporters, the most likely interpretation of these results is that Glu and GABA may be co-released from this sub-population of nerve terminals (Fattorini et al., 2009). This possibility is supported by studies in the developing hippocampus, where mossy fibers co-express VGLUT1 and VGAT (Safiulina et al., 2006) and co-release Glu and GABA (Beltran and Gutierrez, 2012), evoking both Glu and GABA post-synaptic responses (Munster-Wandowski et al., 2013; Walker et al., 2001). In the adult hippocampus, mossy fibers can transiently release GABA only after periods of enhanced excitability (Gutierrez, 2000).

Here we tested the hypothesis that cortical axon terminals can evoke simultaneously monosynaptic Glu and GABA responses and that the amount of terminals co-expressing VGLUT1 and VGAT is affected by perturbations of excitation-inhibition balance.

RESULTS AND DISCUSSION

We first investigated whether cortical axon terminals can sustain co-release of Glu and GABA. To this end, we performed paired-recording of monosynaptically connected neurons in low-density cultures of rat primary cortical neurons (n=18; from 12 experimental series of 6 dishes each). Stimulation of an action potential in a presynaptic cell resulted in receptor-mediated excitatory or inhibitory postsynaptic currents in the postsynaptic cell. Besides pure Glu-mediated excitatory responses (eEPSCs), which are blocked by selective Glu receptor antagonists (100µM APV + 20µM CNQX) (Fig. 1A), and pure GABA-mediated inhibitory responses (eIPSCs), which are sensitive to $GABA_A$ receptor blockers (20µM bicuculline) (Fig. 1 B), we found that a proportion of synaptic (n=8 out of 18) and autaptic (n=4 out of 9) responses were not completely inhibited by either glutamate or GABA receptor blockers, being instead sensitive only to consecutive application of Glu and $GABA_A$ receptor antagonists. These data indicate that Glu and GABA are co-released

following presynaptically evoked action potentials (Fig. 1C and D). The amplitude of evoked responses in mixed synapses was significantly lower than that of "pure" glutamatergic or GABAergic responses, as shown in Fig 1E by normalizing the "mixed" evoked potentials to "pure" glutamatergic (left) or "pure" GABAergic (right) responses. "Mixed" synapses displayed different functional properties, as revealed by the analysis of short term plasticity, where two synaptic responses are evoked by closely spaced presynaptic stimuli (Antonucci et al., 2013). Quantitation of paired-pulse ratio (PPR) revealed that whereas glutamatergic and GABAergic synapses undergo paired-pulse facilitation (PPF) (PPR=1,35692 ± 0,12514; (Antonucci et al., 2013)) and paired-pulse depression (PPD) (PPR=0,52457 ± 0,08087; (Debanne et al., 1996)), respectively, all mixed synapses exhibited PPD (PPR=0,58597 ± 0,05726) (Fig. 1F). In addition, the glutamatergic components of mixed synapses (i.e., the current residual to bicuculline administration) exhibited PPD in all cases (PPR=0,72841 ± 0,06077) (Fig. 1F), in line with the co-localization of VGLUT1 and VGAT in the same vesicles (Fattorini et., 2009).

These results suggest that synapses co-releasing GABA and Glu, display a short term plasticity functional profile more similar to that of GABAergic neurons, which are characterized by a higher probability of release. Therefore, we investigated whether VGLUT1-VGAT co-expression was prominent in inhibitory neurons. To this end, we studied VGLUT1-VGAT+ terminals in phenotypically-differentiated cultures enriched in fast-spiking GABAergic interneurons (FS+) (Berghuis et al., 2004) compared to cultures depleted in fast-spiking GABAergic interneurons (FS-) and control cultures. The percentage of VGAT+ terminals co-expressing VGLUT1 was 206.8 ± 40.7% in FS+ cultures (5502 VGAT+ puncta); and 107.2 ± 24.9% in FS- cultures (5548 VGAT+ puncta) compared to controls (ANOVA, p=0.072) (Figure 2).

In hippocampus, mossy fibers can transiently release GABA following enhanced excitability (Gutierrez, 2000). We therefore evaluated whether VGLUT1/VGAT co-expression in cortical axon terminals is sensitive to variations of excitation-inhibition balance. For this purpose, we used two different protocols: in the first, we reduced spontaneous excitation for one week (starting at 14 DIV), using APV (100 µM) and CNQX (20 µM) (Bacci et al., 2001); in the second, again in 14 DIV cultures, we reduced spontaneous inhibition for one week administering bicuculline (100 µM) (Swann et al., 2007). Results showed that after one week of reduced excitation the percentage of "mixed" terminals decreased to 38.35 ± 2.99% of controls for VGLUT1 positive (+) terminals (n=17569) and to 33.15 ± 6.46% for VGAT+ terminals (n=12205) ($p<0.05$ and $p<0.01$ respectively; Fig. 3A and C). Conversely, after one week of disinhibition, the percentage of VGLUT1+ puncta (n=10813) co-expressing VGAT increased up to 216.96 ± 3.73% ($p<0.05$) compared to controls,

while that of VGAT+ puncta (n=5907) co-expressing VGLUT1 increased up to 153.98 ± 33.78% compared to controls (Fig. 3B and C). These results indicate that the extent of Glu/GABA co-expression is regulated in an activity-dependent mode in order to potentiate the degree of inhibition upon excessive neuronal activity.

Glu/GABA co-release mediated by VGLUT1/VGAT co-localization has been demonstrated in hippocampal mossy fibers during early development (Safiulina et al., 2006) and in pathological conditions (Gutierrez, 2000). In addition, Glu/GABA co-release mediated by VGLUT2/VGAT co-expression has been recently reported in terminals originating from basal ganglia (Shabel et al., 2014) and ventral tegmental area (Root et al., 2014) which form synapses on lateral habenular neurons. Although in a previous study we demonstrated that in neocortex VGLUT1 and VGAT are co-expressed in a subset of axon terminals forming both symmetric and asymmetric synapses, that VGLUT1 and VGAT are sorted to the same vesicles and that, at synapses expressing the vesicular heterotransporters, these vesicles participate in the exo-endocytotic cycle (Fattorini et al., 2009), whether VGLUT1/VGAT co-expression has functional consequences was still undefined. The present study shows that mixed glutamatergic-GABAergic responses can be recorded from cultured cortical neurons, functionally indicating the occurrence of Glu/GABA co-release from neurons co-expressing VGLUT1 and VGAT; these responses exhibited PPD. This latter feature and the observation that in fast-spinking enriched cultures the percentage of mixed terminals is increased compared to controls seem to suggest that VGLUT1/VGAT co-expression occurs more often in terminals displaying GABAergic features. The present results thus show for the first time that Glu/GABA co-release mediated by VGLUT1/VGAT co-localization occurs also in adult cortical neurons.

We also showed that the amount of synapses co-expressing VGLUT1/VGAT is regulated in an activity-dependent manner. By preventing systemic overexcitability *via* a downregulation of synaptic activity, this population of mixed synapses may play a role in regulating excitation-inhibition balance in cortical microcircuits. Interestingly, Glu/GABA co-release on habenular neurons has been associated with the pathophysiology of mood disorders (Root et al., 2014; Shabel et al., 2014). It is thus tempting to speculate that activity-dependent regulation of Glu/GABA co-release, induced by excitation-inhibition unbalance, may contribute to regulating mood and cognition in both normal and pathological conditions (e.g., depression, epilepsy, schizophrenia).

MATERIALS and METHODS

Animals

All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 27/2010,, and the Italian Legislation (L.D. no 26/2014). All efforts were made to minimize the number of animals used and their sufferings.

Cortical cell cultures

Primary neuronal cultures from cerebral cortex were obtained from E18 Sprague-Dawley rats as described by Banker and Cowan (Banker and Cowan, 1977) and Bartlett and Banker (Bartlett and Banker, 1984). Pregnant animals were sacrified by cervical dislocation under CO_2 anesthesia, and the fetuses removed and put into ice-cold Hanks balanced salt solution. After dissection of cortices, cells were dissociated and plated on poly-L-lysine-treated coverslips in Neurobasal (Invitrogen, Gibco, Carlsbad, CA, USA) with 2% B27 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 200 mM glutamine, 10 nM glutamate. After 3 days in vitro (DIV), half of the culture medium was replaced with fresh medium without glutamate.

Pharmacological treatments. APV (competitive NMDA antagonist; Tocris Bioscience, Bristol, UK, 100 µM) and CNQX (competitive AMPA/kainate antagonist; Tocris Bioscience, 20 µM) were added to the medium after 14 DIV for one week. Medium containing freshly prepared inhibitors was substituted every 2 days (Bacci et al., 2001). Chronic disinhibition was achieved by adding bicuculline methiodide (competitive $GABA_A$ antagonist; Tocris Bioscience, 100 µM) to the culture medium for one week (Swann et al., 2007).

Sister cultures from the same animals were randomly assigned to control and experimental groups in all experiments. After treatments neurons were fixed in PFA4% for 15 min and processed for immunocytochemistry. At the visual appearance control and treated cultures was indistinguishable.

Fast-Spiking interneurons cultures

To isolate Kv3.1b-expressing cells, approximately 4 x 10^7 super-paramagnetic polystyrene beads covalently bound to affinity-purified sheep anti-rabbit IgG (Dynal Biotech, Oslo, Norway) were washed in PBS containing 0.1% bovine serum albumin (BSA) and subsequently incubated with 20 µg of rabbit anti-Kv3.1b antibody (Hartig et al., 1999) in 1 mL PBS containing 0.1% BSA overnight at 4 °C under continuous agitation. Anti-Kv3.1b-conjugated beads were then collected with a magnetic particle concentrator (Dynal Biotech), washed three times in PBS containing 0.1% BSA, and mixed with the suspension of cells in DMEM/FCS. This mixture was incubated for 90 min at room temperature under continuous agitation. Cells bound to the anti-Kv3.1b antibody-conjugated beads were isolated by placing the suspension in a magnetic particle concentrator for 2–3 min unbound cells were also collected. Isolated cells were then washed in DMEM / FCS. The beads were enzymatically detached from the cells (0.1% trypsin, 7 min at 37 °C), and loose beads

were magnetically separated (2–3 min). Isolated cells (FS+), unbound cells (FS-), and control cells were plated on poly-d-lysine-coated coverslips at a density of 50.000–100.000 cells/well in 24-well plates and maintained in glia-conditioned medium containing B27 supplement (2%; Invitrogen) (Berghuis et al., 2004).

At 14 DIV, neurons were fixed in 4% paraformaldheyde (PFA) for 15 min, and processed for immunocytochemistry.

Electrophysiology

Evoked currents were recorded in isolated pairs of rat primary cortical neurons in low-density cultures (15-20 DIV). During recordings, neurons were held at -70 mV and synaptic transmission (eEPSC or eIPSC) was evoked by a 100mV depolarization pulse (1 ms) in presynaptic cell. We tested synaptic connectivity by applying at least 15 sweeps, each of them separated by 5 sec. The inhibitory or excitatory nature of the presynaptic neurons was routinely determined by application of selective receptor blockers (100µM APV + 20µM CNQX, or 20µM bicuculline) to unambiguously identify the presynaptic neuronal phenotype. Recording pipettes were filled with the following intracellular solution (in mM): 130 K-gluconate, 10 KCl,1 EGTA, 10 HEPES, 2 MgCl2, 4 MgATP, and 0.3 Tris-GTP. Paired pulse ratio (PPR=P2/P1) was recorded by applying pairs of action potentials separated by an Inter- Stimulus Interval (ISI) of 50 ms presented every 5 sec.

Antibodies

Primary antibodies. Anti-VGAT (rabbit; Synaptic System, Goettingen, Germany 131003, 1:1000 (Takamori et al., 2000)); anti-VGLUT1 (guinea pig; Chemicon Millipore, Billerica, MA, USA, AB5905, 1:1000 (Melone et al., 2005)).

Secondary antibodies. FITC (anti-guinea pig; Vector Laboratories, Burlingame, CA, USA, 1:100); Cy3 (anti-rabbit; Jackson ImmunoResearch, West Grove, PA, USA, 1:200).

Co-localization studies

Fixed cells were detergent-permeabilized and labeled with a mixture of anti-VGLUT1 and anti-VGAT. Appropriate secondary antibodies were used (see above). Samples were rinsed and observed in a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) (pixel size 0.15µm). Analysis was performed as described (Fattorini et al., 2009).

Statistical analysis

Unless otherwise stated, average data are expressed as mean ± SEM. *n* refers to the number of elements analyzed. Statistical analysis was performed using SigmaStat 3.5 (Jandel Scientific). After testing whether data were normally distributed or not, the appropriate statistical test has been used. Data were analyzed by paired/unpaired Student's t-test or, in case of more than 2 experimental groups, by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison tests.

ACKNOWLEDGEMENTS

Supported by grants from Italian Ministry of University and Research (PRIN 2010-2011) to FC and MM, from Università Politecnica delle Marche (FC and GF); from Italian Ministry of Research and Education program "FIRB giovani" 2010 (RBFR10ZBYZ to FA), from CNR Research Project on Aging (Regione Lombardia Project MbMM-conv18099/RCC) to MM, and from INRCA IRCCS intramural funds (to FC).

AUTHOR CONTRIBUTIONS

GF, MM and FC designed the experiments, GF, FA and EM performed experiments and analyzed data, GF, FA, EM, MM and FC discussed the data, FC and MM wrote the paper.

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FIGURE LEGENDS

Fig. 1. Glutamate and GABA co-release from synaptic and autaptic terminals. (A-D) Representative traces of evoked responses in "pure" glutamatergic synapses (A), "pure" GABAergic synapses (B), "mixed" autapses (C) and "mixed" synapses (D) recorded in DIV 15-20 cortical neurons. (E) Analysis of the amplitude of evoked responses measured in mixed synapses normalized to "pure" glutamatergic (left) or "pure" GABAergic (right) responses. (F) Quantification of short term plasticity experiments (PPR= A2/A1) at glutamatergic (*green*), GABAergic (*red*), and mixed (yellow) synapses. In the "mixed" columns, green indicates the residual glutamatergic component of mixed currents. **p<0.01; ***p<0.001.

Figure 2 – VGLUT1-VGAT co-localization is highest in fast spiking interneurons

VGLUT1 (green) and VGAT (red) in control (ctr), fast-spiking enriched (FS+) and depleted (FS-) cortical cultures. Large panels show merged images from the three types of cultures, whereas small panels show the same image in single channels. Scale bars 5µm.

The graph illustrate the percentage of VGAT+ puncta expressing VGLUT1 in FS+ and FS- cultures compared to controls. Values (mean ± SEM) are normalized to controls. *p<0.05.

Fig. 3. Altering excitation-inhibition balance changes the degree of VGLUT1-VGAT co-localization. (A) VGLUT1 (green) and VGAT (red) in cortical cultures with reduced excitation. The image on the left (ctr) is a frame from control cultures, the image on the right (CNQX+APV) from cultures treated for one week with a mixture of APV 100 µM and CNQX 20 µM. Scale bars 5 µm. (B) VGLUT1 (green) and VGAT (red) in cortical cultures with reduced inhibition. The image on the left (ctr) is a frame from control cultures, the image on the right (Bic) is from cultures treated for one week with bicuculline 100 µM. Small panels show the same fields in single channels. Scale bars 5 µm. (C) Quantification of VGLUT1 and VGAT co-localization. Values (mean ± SEM) refer to the percentages of respective controls. *p<0.05; **p<0.01.