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SHORT REPORT

Co-expression of VGLUT1 and VGAT sustains glutamate and GABA co-release and is regulated by activity in cortical neurons

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ABSTRACT

In adult neocortex, VGLUT1 (also known as SLC17A7), the main glutamate vesicular transporter, and VGAT (also known as SLC32A1), the γ-aminobutyric acid (GABA) vesicular transporter, are co-expressed in a subset of axon terminals forming both symmetric and asymmetric synapses, where they are sorted into the same vesicles. However, the functional consequence of this colocalization 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 treatment with glutamate receptor antagonists decreased the amount of 'mixed' synapses, whereas reducing spontaneous inhibition by treatment with bicuculline increased them. These synapses might contribute to homeostatic regulation of excitation–inhibition balance.

KEY WORDS: VGAT, VGLUT1, Co-release, GABA, Glutamate

INTRODUCTION

Glutamate (Glu) and γ-aminobutyric acid (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–VGLUT3 (also known as SLC17A7, SLC17A6 and SLC17A8, respectively) (Fremeau et al., 2004; Gras et al., 2002; Takamori, 2006) and VGAT (also known as SLC32A1), respectively (McIntire et al., 1997; Sagné et al., 1997; Takamori et al., 2000).

We have recently shown that in the 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 have subsequently been reported in the 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 might 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 (Beltrán and Gutiérrez, 2012), evoking both Glu and GABA post-synaptic responses (Münster-Wandowski et al., 2013; Walker et al., 2001). In the adult hippocampus, mossy fibers can transiently release GABA only after periods of enhanced excitability (Gutiérrez, 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 six 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 (evoked excitatory postsynaptic currents, eEPSCs), which are blocked by selective Glu receptor antagonists (100 µM APV + 20 µM CNQX) (Fig. 1A), and pure GABA-mediated inhibitory responses (evoked inhibitory postsynaptic currents, eIPSCs), which are sensitive to $GABA_A$ receptor blockers (20 µM bicuculline) (Fig. 1B), 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 Glu 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,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).

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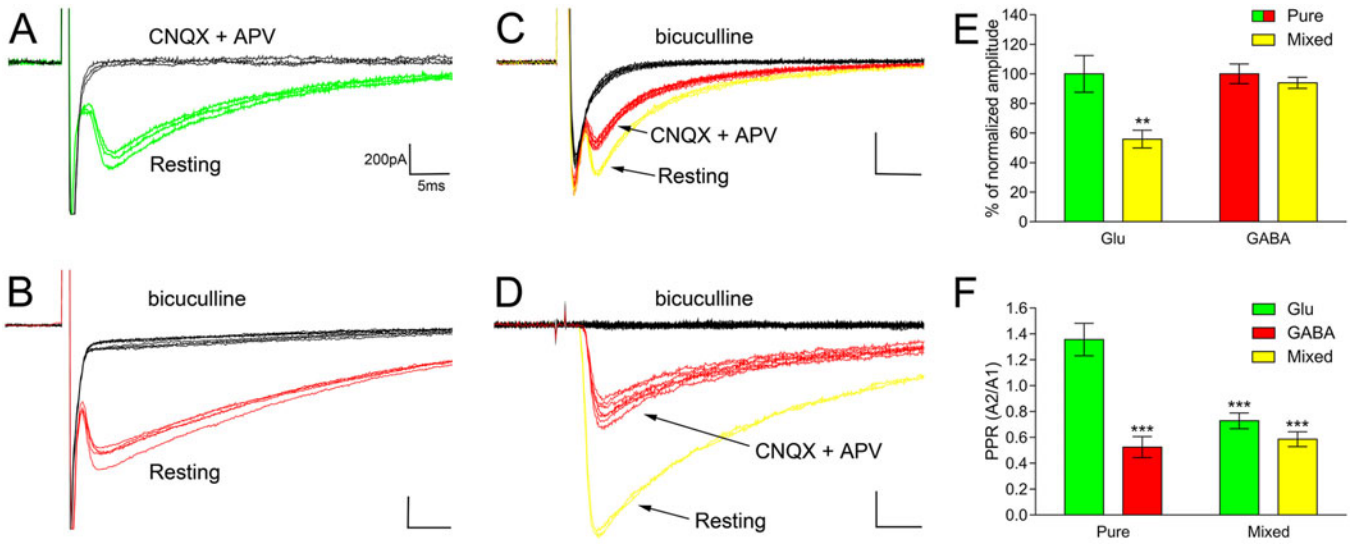

Fig. 1. Glutamate and GABA co-release from synaptic and autaptic terminals. (A–D) Representative traces of evoked responses in pure glutamatergic synapses (A), pure GABAergic synapses (B), mixed autapses (C) and mixed synapses (D) recorded in cortical neurons at 15–20 DIV. Treatments are as indicated. (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. Results are mean±s.e.m.. $^{**}P<0.01$; $^{***}P<0.001$. $n=8$ out of 18 for

Quantification of the paired-pulse ratio (PPR) revealed that whereas glutamatergic and GABAergic synapses underwent paired-pulse facilitation (PPF) (PPR=1.35692±0.12514; mean±s.e.m.; 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 colocalization of VGLUT1 and VGAT in the same vesicles (Fattorini et al., 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 and VGAT co-expression was prominent in inhibitory neurons. To this end, we studied VGLUT1 and VGAT double-positive 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-positive (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, $P=0.072$) (Fig. 2).

In hippocampus, mossy fibers can transiently release GABA following enhanced excitability (Gutiérrez, 2000). We therefore evaluated whether VGLUT1 and VGAT co-expression in cortical axon terminals is sensitive to variations in the excitation–inhibition balance. For this purpose, we used two different protocols: in the first, we reduced spontaneous excitation for 1 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 1 week by administering bicuculline (100 µM) (Swann et al., 2007). Results showed that after 1 week of reduced excitation the percentage of mixed terminals decreased to 38.35±2.99% of controls for VGLUT1-positive (VGLUT1+) terminals ($n=17,569$) and to 33.15±6.46% for VGAT+ terminals ($n=12,205$) ($P<0.05$ and $P<0.01$, respectively; Fig. 3A,C). By contrast, after 1 week of disinhibition, the percentage of VGLUT1+ puncta ($n=10,813$) co-expressing VGAT increased up to 216.96±3.73% ($P<0.05$) compared to controls, whereas that of VGAT+ puncta ($n=5907$) co-expressing VGLUT1 increased up to 153.98±33.78% compared to controls (Fig. 3B,C). These results indicate that the extent of Glu and GABA co-expression is regulated in an activity-dependent mode in order to potentiate the degree of inhibition upon excessive neuronal activity.

Glu and GABA co-release mediated by VGLUT1 and VGAT colocalization has been demonstrated in hippocampal mossy fibers during early development (Safiulina et al., 2006) and in pathological conditions (Gutiérrez, 2000). In addition, Glu and GABA co-release mediated by VGLUT2 and 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 have demonstrated that in the 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 and VGAT co-expression had functional consequences was still undefined. The present study shows that mixed glutamatergic and GABAergic responses can be recorded from cultured cortical neurons, functionally indicating the occurrence of Glu and GABA co-release from neurons co-expressing VGLUT1 and VGAT; these responses exhibited PPD. This latter feature and the observation that in fast-spiking-enriched cultures the percentage of mixed terminals is increased compared to controls seem to suggest that VGLUT1 and VGAT co-expression occurs more often in terminals displaying GABAergic features. The present results thus show for the first time that Glu

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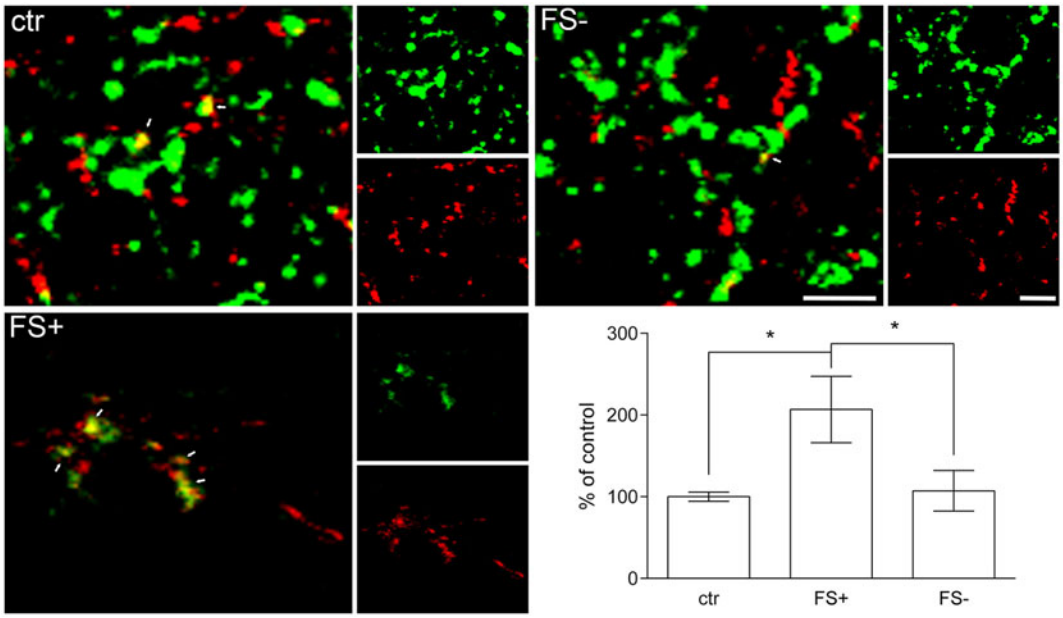

Fig. 2. VGLUT1 and VGAT colocalization is highest in fast spiking interneurons. VGLUT1 (green) and VGAT (red) in control (ctr), fast-spiking-enriched (FS+) and -depleted (FS−) cortical cultures. Large panels show merged images from the three types of cultures, whereas small panels show the same image as single channels. Scale bars: 5 μm. The graph illustrates the percentage of VGAT+ puncta expressing VGLUT1 in FS+ and FS− cultures compared to controls. Values (mean±s.e.m.) are normalized to controls. *$P<0.05$. $n=13,343$ VGAT+ puncta for controls; $n=5502$ for FS+; and $n=5548$ for FS−.

and GABA co-release mediated by VGLUT1 and VGAT colocalization occurs also in adult cortical neurons.

We also showed that the amount of synapses co-expressing VGLUT1 and VGAT is regulated in an activity-dependent manner. By preventing systemic overexcitability by downregulating synaptic activity, this population of mixed synapses might play a role in regulating excitation–inhibition balance in cortical microcircuits. Interestingly, Glu and 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

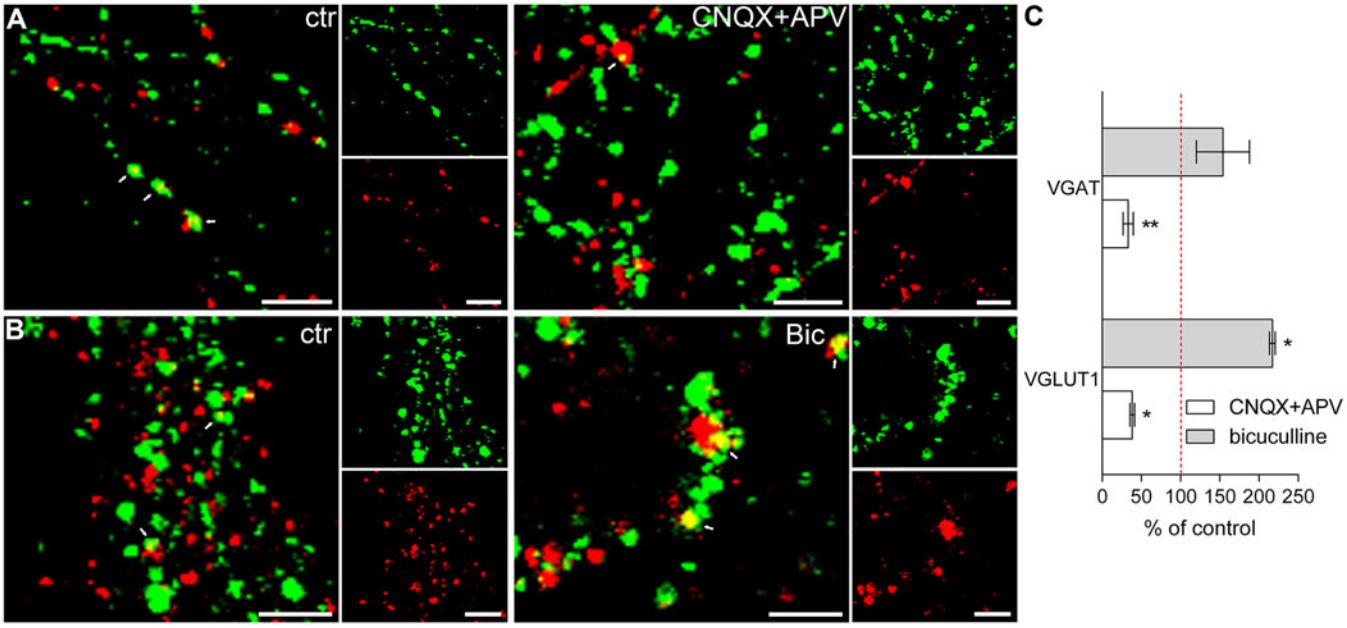

Fig. 3. Altering excitation-inhibition balance changes the degree of VGLUT1-VGAT colocalization. (A) VGLUT1 (green) and VGAT (red) in cortical cultures with reduced excitation. The image on the left (ctr) is a frame from control cultures, the image on the right (CNQX+APV) is from cultures treated for 1 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 1 week with bicuculline 100 μM. Small panels show the same fields as single channels. Scale bars: 5 μm. (C) Quantification of VGLUT1 and VGAT colocalization. Values (mean±s.e.m.) refer to the percentages of respective controls. *$P<0.05$; **$P<0.01$. $n=17,569$ VGLUT1+ puncta for CNQX+APV and 8785 for controls; $n=12,205$ VGAT+ puncta for CNQX+APV and 7123

activity-dependent regulation of Glu and GABA co-release, induced by excitation–inhibition unbalance, might contribute to regulating mood and cognition in both normal and pathological conditions (e.g. depression, epilepsy and 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 suffering.

Cortical cell cultures

Primary neuronal cultures from cerebral cortex were obtained from E18 Sprague-Dawley rats as described by previously (Banker and Cowan, 1977; Bartlett and Banker, 1984). Pregnant animals were killed 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 medium (Invitrogen, Gibco, Carlsbad, CA) 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 and kainate antagonist; Tocris Bioscience, 20 µM) were added to the medium after 14 DIV for 1 week. Medium containing freshly prepared inhibitors was replaced 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 1 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 4% paraformaldehyde (PFA) for 15 min and processed for immunocytochemistry. At the level of visual appearance, control and treated cultures were indistinguishable.

Fast-spiking interneuron cultures

To isolate Kv3.1b-expressing cells, ~4×10^7 super-paramagnetic polystyrene beads covalently bound to affinity-purified sheep anti-rabbit IgG (Dynal Biotech, Oslo, Norway) were washed in PBS containing 0.1% BSA and subsequently incubated with 20 µg of rabbit anti-Kv3.1b antibody (Härtig et al., 1999) in 1 ml PBS containing 0.1% BSA overnight at 4℃ 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 with fetal calf serum (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 with FCS. The beads were enzymatically detached from the cells (0.1% trypsin, 7 min at 37℃), 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% 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 100 mV depolarization pulse (1 ms) in presynaptic cell. We tested synaptic connectivity by applying at least 15 sweeps, each of them separated by 5 s. The inhibitory or excitatory nature of the presynaptic neurons was routinely determined by application of selective receptor blockers (100 µM APV plus 20 µM CNQX, or 20 µM 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 $MgCl_2$, 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 s.

Antibodies

Primary antibodies were: anti-VGAT (rabbit; Synaptic System, Goettingen, Germany; 131003, 1:1000; Takamori et al., 2000) and anti-VGLUT1 (guinea pig; Chemicon Millipore, Billerica, MA; AB5905, 1:1000; Melone et al., 2005) antibodies. Secondary antibodies were FITC-conjugated anti-guinea pig IgG (Vector Laboratories, Burlingame, CA, 1:100) and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, 1:200) antibodies.

Colocalization studies

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

Statistical analysis

Unless otherwise stated, average data are expressed as mean±s.e.m. *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 a paired or unpaired Student's *t*-test or, in case of more than two experimental groups, by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison tests.

Competing interests

The authors declare no competing or financial interests.

Author contributions

G.F., M.M. and F.C. designed the experiments; G.F., F.A. and E.M. performed experiments and analyzed data; G.F., F.A., E.M., M.M. and F.C. discussed the data; F.C. and M.M. wrote the paper.

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