Biochemical and Biophysical Research Communications 404 (2011) 228–232

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of complexin II in astrocytes: A possible regulator of glutamate release in these cells

Alan S. Hazell *, Dongmei Wang

Department of Medicine, University of Montreal, Montreal, Quebec, Canada H2X 3J4

ARTICLE INFO

Article history:
Received 23 October 2010
Available online 24 November 2010

Keywords:
Vesicular release
SNARE
Cell culture
Excitotoxicity
Neurotransmitter
Exocytosis

ABSTRACT

Complexins are a family of SNARE complex-binding proteins which regulate neurotransmitter release by playing a crucial role in triggering fast exocytosis at the synapse. Current evidence indicates astrocytes can release glutamate via a vesicular mechanism similar to that at nerve terminals and thereby modulate synaptic activity. In addition, components of the biochemical machinery associated with synaptic release have been identified in these cells. However, whether complexins are also present in astrocytes and may therefore participate in the vesicular release of glutamate is a key issue that is yet to be determined. In the present study we therefore examined if astrocytes express complexin I (Cpx I) and/or complexin II (Cpx II). Our results indicate these cells contain Cpx II but not Cpx I in primary culture. In addition, serum deprivation for 24 h led to a 2.6-fold increase in Cpx II, suggesting this protein is responsive to insults. These findings point to Cpx II being a likely key modulator of synaptic activity at the level of these glial cells. Given the considered involvement of complexins in neurologic and psychiatric illness, astrocytic Cpx II represents a potentially important therapeutic target for the future treatment of such maladies.

1. Introduction

A key function of astrocytes is the efficient removal of glutamate, the major excitatory neurotransmitter in brain, from the extracellular space following release from excitatory nerve terminals [1] via glutamate transporters, a process that is instrumental in maintaining normal interstitial levels of this amino acid [2]. Sustained elevation in extracellular glutamate levels causes over-stimulation of glutamate receptors that can result in neuronal cell death.

Complexin I (Cpx I) and complexin II (Cpx II) constitute part of a family of four small and highly charged proteins (Cpx I–IV), three of which are present in brain (Cpx I–III) [3–6]. Cpx I is expressed in axosomatic (inhibitory) synapses, while Cpx II is localized in axodendritic and axospinous synapses, of which the majority are excitatory [7,8]. A number of studies to date indicate that complexins are important regulators of neurotransmitter release. At the presynaptic terminal, they compete with the chaperone protein α-SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) for binding to SNAP receptors (SNAREs) [9]. These SNAREs consist of the synaptic vesicle protein synaptobrevin-2 as well as the synaptic membrane proteins SNAP-25 and syntaxin-1 [5,9,10]. Prior to vesicular release into the synaptic cleft, these membrane proteins form a stable core complex; interaction of complexins with the SNARE complex influence its stability [9] by promoting the direct assembly of all three SNARE proteins, and which involves an interaction of the transmembrane regions of both syntaxin and synaptobrevin [11]. Most recently, evidence suggest that release of complexins from the SNARE complex by its competition with another synaptic vesicle protein, synaptotagmin-1, triggers fast exocytosis and may explain the speed and efficiency of this process [12].

Despite the important role of astrocytes in glutamate clearance from the extracellular space, studies have established these cells also have the ability to release glutamate in a calcium-dependent manner similar to that found at the synaptic terminal [13,14]. Evidence also suggest this glutamate release occurs via a vesicular-mediated process [14,15], and in a similar fashion to that found at the synaptic terminal [16,17]. In addition, it is now evident that astrocytes contain SNARE proteins and vesicular glutamate transporters, chemical machinery consistent with a functional role in the vesicular release of glutamate and vesicle refilling [18,19].

Since astrocytes can release glutamate using the same type of machinery present at the synaptic terminal, it is important to examine the possible involvement of complexins in astrocytes, given its considered key role in neuronal vesicular release, an important issue that remains unexplored at the present time.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) and dibutyryl cyclic AMP (dbCAMP) were purchased from Sigma–Aldrich Ltd.

* Corresponding author. Address: NeuroRescue Laboratory, Hôpital Saint-Luc (CHUM), 1058 St-Denis, Montreal, Quebec, Canada H2X 3J4. Fax: +1 514 412 7737.
E-mail address: alan.stewart.hazell@umontreal.ca (A.S. Hazell).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.098

(Oakville, ON, Canada), and fetal calf serum and horse serum was obtained from Invitrogen Canada, Inc. (Burlington, ON, Canada). Protease inhibitor cocktail, 3,3′-diaminobenzidine (DAB), and mouse monoclonal antiserum against β-actin were purchased from Sigma–Aldrich Ltd. (Oakville, ON, Canada). Goat polyclonal antisera against glial fibrillary acidic protein (GFAP), α-internexin, synaptophysin, and myelin oligodendrocyte glycoprotein (MOG) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antisera against CD11b/c was purchased from Cederlane Laboratories Ltd. (Burlington, ON, Canada). Alexa Fluor 488 and Alexa Fluor 596 secondary antibodies and Prolong Gold AntiFade reagent were purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada). Polyvinylidene difluoride (PVDF) membranes and broad-range protein markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence (ECL) kits were purchased from New England Nuclear (Boston, MA, USA) and X-OMAT autoradiography film was purchased from Kodak (Ile des Soeurs, Quebec, Canada). All other materials and chemicals were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada).

2.2. Astrocyte cultures

Primary astrocyte cultures from newborn rats were prepared using a modification of the method of Booher and Sensenbrenner [20], according to Hazell and colleagues [21]. Briefly, cerebral cortices were removed and the tissue was dissociated, passed through sterile nylon sieves, and then suspended in DMEM containing 10% fetal calf serum. Approximately 0.25×10^6 cells/ml were seeded in 35 mm culture dishes, which were maintained in an incubator at 37 °C provided with a mixture of 5% CO_2 and 95% air. After 2 weeks, cells attained confluency, at which point the fetal calf serum was replaced by horse serum and they were then exposed constantly to dcAMP. Cultures were grown for a total of 3–5 weeks, during which the medium was changed twice a week. At least 95% of cells were determined to be astrocytes based on GFAP immunocytochemistry.

2.3. Immunoblotting

Astrocytes were harvested in buffer containing 50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate (pH 8.0), and protease inhibitor cocktail and centrifuged at 10,000 *g* for 10 min at 4 °C. Protein content of all samples was determined by the method of Lowry and colleagues [22] using bovine serum albumin as the standard. Aliquots (50 μg) were then subjected to SDS–polyacrylamide gel electrophoresis (8% polyacrylamide) and the proteins subsequently transferred to PVDF membranes by wet transfer at 20 V over 24 h. The transfer buffer consisted of 48 mM Tris (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol. Membranes were subsequently incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 5% nonfat dried milk, and 0.1% Tween-20) followed by incubation with antisera against Cpx I (1:1000), Cpx II (1:1000), GFAP (1,1000), α-internexin (1:100), synaptophysin (1:500), MOG (1:200), CD11b/c (OX-42, 1:500), or β-actin (1:10,000). Reblocking was followed by incubation with horseradish peroxidase-coupled anti-rabbit, anti-mouse, or anti-goat IgG (1:10,000) secondary antiserum. Each incubation step was of 1 h duration, following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with the ECL-kit instructions and apposed to photosensitive X-OMAT film. Signal intensities were subsequently measured by densitometry using Adobe Photoshop (Toronto, ON, Canada). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves.

2.4. Immunocytochemistry

Astrocytes were fixed for 10 min with 10% neutral buffered formalin and then incubated for 10 min in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Cells were washed in PBS (3×10 min), blocked for 20 min with 5% donkey serum in PBS, and then incubated with 5% donkey serum and polyclonal rabbit antisera directed against the excitatory amino acid transporter 1 (EAAT1) {A522 (Ab #314); 0.25 μg/ml} and mouse-derived Cpx II antibody (1:250) at 4 °C for 24 h. Sections were then washed (3×10 min) and incubated for 1 h with Alexa Fluor-488 (green) and Alexa Fluor-596 (red) secondary antibodies (1:200), then mounted in Prolong Gold AntiFade reagent and examined using an Olympus BX51 microscope and attached Spot RT digital camera. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity. Images were processed using *Image-Pro Plus* 6.2 image analysis software (Media Cybernetics, Inc., Bethesda, MD, USA).

3. Results

Fig. 1 shows immunoblots for Cpx I and Cpx II. A band corresponding to Cpx II was detected in cultured astrocytes while no band for Cpx I was detected, indicating the presence of Cpx II protein but not Cpx I in these cells. Cpx II was identified both in cells treated with dcAMP and in its absence. While cultures showed high levels of the astrocyte-specific protein GFAP, since Cpx II is found in synaptic terminals, we examined the possibility that our cultures may contain significant amounts of neurons as a contaminating cell type by performing immunoblotting for the neurofilament protein α-internexin and synaptic terminal protein synaptophysin. In both cases, a band for these proteins was not observed, indicating an absence of this cell type in these cultures (Fig. 1). Further examination of the purity of these astrocyte cultures by immunoblotting for MOG and the microglial protein CD11b/c (OX-42) revealed an absence of both cell type markers (Fig. 1), indicating a lack of oligodendrocytes and microglia in our cultures.

Double label immunocytochemical staining of astrocyte cultures revealed the presence and co-localization of both Cpx II and the astrocytic glutamate transporter EAAT1 (Fig. 2), confirming our immunoblotting findings of Cpx II protein in these cells. In order to examine the sensitivity of Cpx II to changes in the external environment in astrocytes, cells were exposed to DMEM lacking horse serum for either 30 min or 24 h. While removal of serum from media for 30 min had no effect on levels of Cpx II, absence of the serum for 24 h resulted in a 2.6-fold increase in levels of Cpx II (Fig. 3).

4. Discussion

Astrocytes make up the majority of the cells in the mammalian brain and are responsible for many important processes including buffering of K^+, inactivation of released neurotransmitters, including glutamate, and water homeostasis. Studies have identified a calcium-dependent astrocytic glutamate release process capable of modulating both spontaneous [23,24] and evoked [25] synaptic activity in either an excitatory or inhibitory manner, which has important implications in terms of our understanding of the control of synaptic transmission, and the role of astrocytes in this process. Since these cells have been shown to possess protein

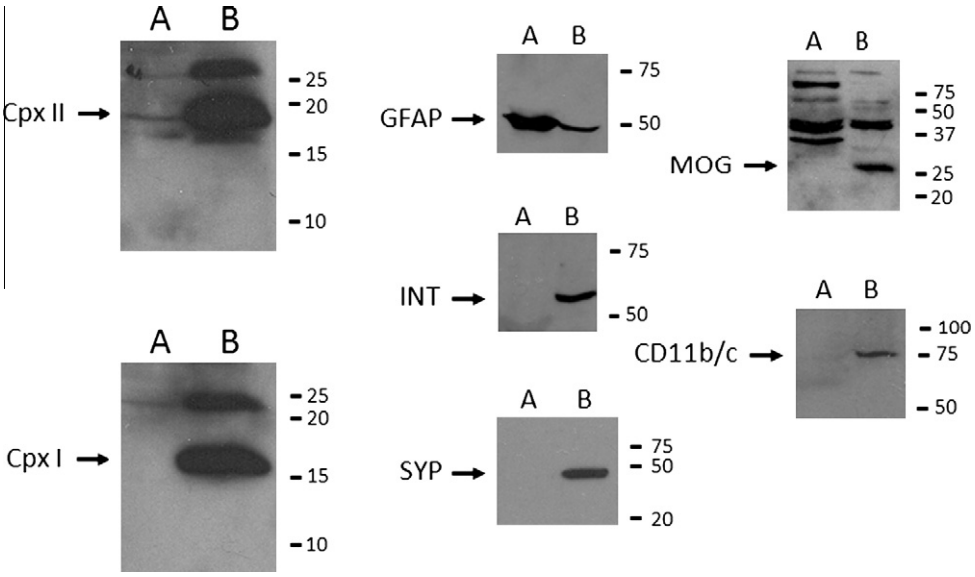

Fig. 1. Complexin II (Cpx II) in cultured astrocytes. Immunoblots showing the presence of Cpx II (but not Cpx I) in cultured astrocytes (A) and in brain (B). Cultures contain the astrocyte-specific glial fibrillary acidic protein (GFAP) but lack neuronal contamination, a potential non-astrocytic source of Cpx II, as evidenced by the absence of the axonal neurofilament protein α-internexin (INT) and synaptic terminal protein synaptophysin (SYP). In addition, cultures display no evidence of myelin oligodendrocyte glycoprotein (MOG) or CD11b/c marker proteins for oligodendrocytes and microglia respectively, thus confirming the high purity of the astrocyte preparation.

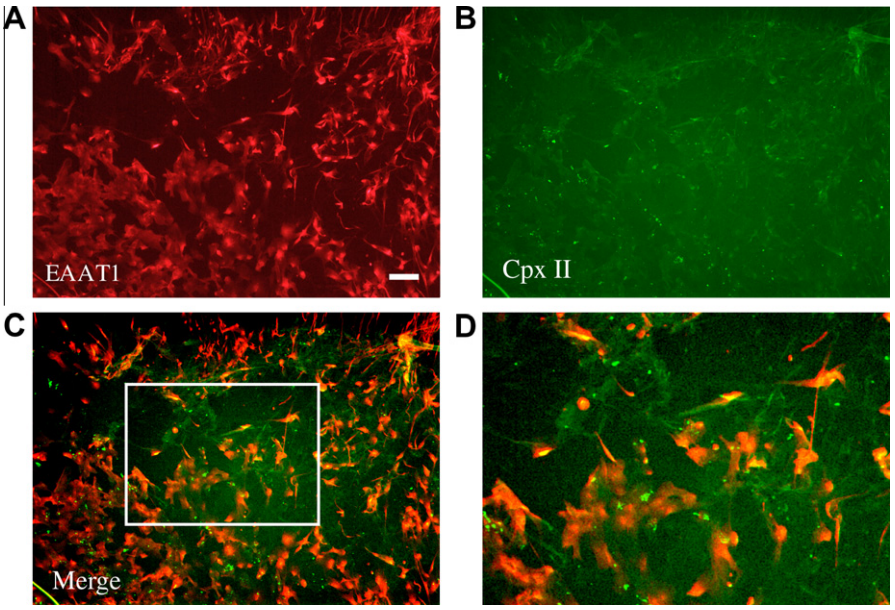

Fig. 2. Photomicrographs show double labeling of cultures for the astrocytic glutamate transporter EAAT1 (A, Alexa Fluor 596) and Cpx II (B, Alexa Fluor 488) indicating the presence of Cpx II in these cells (C and D). Panel D represents magnified area of box shown in (C). Bar, 40 μm (A–C), 10 μm (D).

machinery for release of glutamate that include SNARE proteins and vesicular glutamate transporters [16,17], it is important to determine if complexins such as Cpx I and Cpx II, proteins considered to play a crucial role in the release of neurotransmitters such as glutamate, are also involved in this process in these glial cells. Structural analysis have indicated that complexins bind to a groove in the central part of the SNARE bundle, formed by syntaxin-1 and synaptobrevin [26,27], suggesting that complexins may bind to the SNARE complex immediately before fusion, thus making these proteins late-acting and thus key regulatory proteins in the vesicular release process.

In the present study, we have identified the presence of Cpx II (but not Cpx I) in primary cultures of astrocytes. This finding provides important evidence supporting previous findings that a similar mechanism to that operating at synaptic terminals exists in these cells. In addition, Cpx II is associated with excitatory synapses [7,8], suggesting it may play an important role in vesicular glutamate release in astrocytes. Since release of glutamate by astrocytes can lead to excitotoxic consequences in which extracellular glutamate concentration is increased, and can result in neuronal cell death, manipulation of Cpx II levels in these cells may provide an important future therapeutic strategy for the treatment of disorders of mental health such as schizophrenia, bipolar disease, major depression and neurological illness in which an involvement of complexins has been demonstrated [28–31].

Exactly what is the role of complexins in the vesicular release process is unclear. Although they appear to act as positive regulators at or following the Ca^{2+}-triggering step of synaptic vesicle

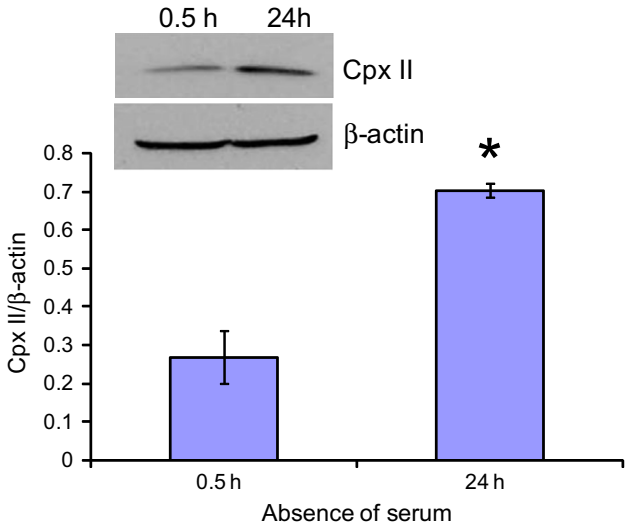

Fig. 3. Effect of serum withdrawal on complexin II (Cpx II) in cultured astrocytes. Immunoblot showing upregulation of Cpx II following serum deprivation for 24 h. Graph shows densitometric results for Cpx II protein following normalization to β-actin.*$p < 0.01$ compared with control group (Mann–Whitney *U*-test).

fusion [32–35], other findings are more compatible with complexins having an inhibitory role by maintaining SNARE complexes in a highly fusogenic state but preventing them from actually executing fusion until triggered by Ca^{2+} [12,36–38]. Most recently, however, Xue and colleagues [39] have reported that complexins contain distinct domains that are highly conserved, and which facilitate or inhibit vesicle fusion. This feature may explain in large part these differences in the apparent role of complexins reported previously.

Given the importance of complexins in neurotransmitter vesicular release, and that Cpx II is particularly associated with excitatory release at the synaptic terminal, the present finding that Cpx II but not Cpx I is present in primary cultures of astrocytes adds credence to a significant role for this complexin in the vesicular release of glutamate in these cells. Furthermore, the ability of 24 h serum deprivation to increase Cpx II above basal values in these cells suggests that levels of this protein can be altered relatively easily, thus supporting the idea that it may be a useful therapeutic target using approaches that aim to reduce the extent of excitotoxic insults, modulate synaptic activity, and treat psychiatric illness.

Acknowledgments

We thank Drs. K. Sawada and S. Takahashi (Kochi Medical School, Japan) for their generous gift of the complexin antibodies. We are also grateful to Dr. Niels C. Danbolt (University of Oslo, Norway) for providing the EAAT1 antibody used in this investigation. This study was funded by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada.

References

[1] J. Drejer, O.M. Larsson, A. Schousboe, Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells, Neurochem. Res. 8 (1983) 231–243.
[2] D. Nicholls, D. Attwell, The release and uptake of excitatory amino acids, Trends Pharmacol. Sci. 11 (1990) 462–468.
[3] S. Takahashi, H. Yamamoto, Z. Matsuda, M. Ogawa, K. Yagyu, T. Taniguchi, T. Miyata, H. Kaba, T. Higuchi, F. Okutani, Identification of two highly homologous presynaptic proteins distinctly localized at the dendritic and somatic synapses, FEBS Lett. 368 (1995) 455–460.
[4] T. Ishizuka, H. Saisu, S. Odani, T. Abe, Synaphin: a protein associated with the docking/fusion complex in presynaptic terminals, Biochem. Biophys. Res. Commun. 213 (1995) 1107–1114.
[5] H.T. McMahon, M. Missler, C. Li, T.C. Südhof, Complexins: cytosolic proteins that regulate SNAP receptor function, Cell 83 (1995) 111–119.
[6] K. Reim, H. Wegmeyer, J.H. Brandstätter, M. Xue, C. Rosenmund, T. Dresbach, K. Hofmann, N. Brose, Structurally and functionally unique complexins at retinal ribbon synapses, J. Cell Biol. 169 (2005) 669–680.
[7] P.J. Harrison, S.L. Eastwood, Preferential involvement of excitatory neurons in medial temporal lobe in schizophrenia, Lancet 352 (1998) 1669–1673.
[8] M. Yamada, H. Saisu, T. Ishizuka, H. Takahashi, T. Abe, Immunohistochemical distribution of the two isoforms of synaphin/complexin involved in neurotransmitter release: localization at the distinct central nervous system regions and synaptic types, Neuroscience 93 (1999) 7–18.
[9] S. Pabst, J.W. Hazzard, W. Antonin, T.C. Südhof, R. Jahn, J. Rizo, D. Fasshauer, Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions, J. Biol. Chem. 275 (2000) 19808–19818.
[10] W. Wickner, R. Schekman, Membrane fusion, Nat. Struct. Mol. Biol. 15 (2008) 658–664.
[11] K. Hu, J. Carroll, C. Rickman, B. Davletov, Action of complexin on SNARE complex, J. Biol. Chem. 277 (2002) 41652–41656.
[12] J. Tang, A. Maximov, O.H. Shin, H. Dai, J. Rizo, T.C. Südhof TC, A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis, Cell 126 (2006) 1175–1187.
[13] V. Parpura, T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija, P.G. Haydon, Glutamate-mediated astrocyte-neuron signalling, Nature 369 (1994) 744–747.
[14] P. Bezzi, G. Carmignoto, L. Pasti, S. Vesce, D. Rossi, B.L. Rizzini, T. Pozzan, A. Volterra, Prostaglandins stimulate calcium-dependent glutamate release in astrocytes, Nature 391 (1998) 281–285.
[15] A. Araque, N. Li, R.T. Doyle, P.G. Haydon, SNARE protein-dependent glutamate release from astrocytes, J. Neurosci. 20 (2000) 666–673.
[16] P. Bezzi, V. Gundersen, J.L. Galbete, G. Seifert, C. Steinhäuser, E. Pilati, A. Volterra, Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate, Nat. Neurosci. 7 (2004) 613–620.
[17] V. Montana, E.B. Malarkey, C. Verderio, M. Matteoli, V. Parpura, Vesicular transmitter release from astrocytes, Glia 54 (2006) 700–715.
[18] Q. Zhang, T. Pangrsic, M. Kreft, M. Krzan, N. Li, J.Y. Sul, M. Halassa, E. Van Bockstaele, R. Zorec, P.G. Haydon, Fusion-related release of glutamate from astrocytes, J. Biol. Chem. 279 (2004) 12724–12733.
[19] V. Montana, Y. Ni, V. Sunjara, X. Hua, V. Parpura, Vesicular glutamate transporter-dependent glutamate release from astrocytes, J. Neurosci. 24 (2004) 2633–2642.
[20] J. Booher, M. Sensenbrenner, Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures, Neurobiology 2 (1972) 97–105.
[21] A.S. Hazell, P. Pannunzio, K.V. Rama Rao, D.V. Pow, A. Rambaldi, Thiamine deficiency results in downregulation of the GLAST glutamate transporter in cultured astrocytes, Glia 43 (2003) 175–184.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 263–275.
[23] A. Araque, R.P. Sanzgiri, V. Parpura, P.G. Haydon, Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons, J. Neurosci. 18 (1998) 6822–6829.
[24] M.C. Angulo, A.S. Kozlov, S. Charpak, E. Audinat, Glutamate released from glial cells synchronizes neuronal activity in the hippocampus, J. Neurosci. 24 (2004) 6920–6927.
[25] A. Araque, V. Parpura, R.P. Sanzgiri, P.G. Haydon, Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons, Eur. J. NeuroSci. 10 (1998) 2129–2142.
[26] A. Bracher, J. Kadlec, H. Betz, W. Weissenhorn, X-ray structure of a neuronal complexin-SNARE complex from squid, J. Biol. Chem. 277 (2002) 26517–26523.
[27] X. Chen, D.R. Tomchick, E. Kovrigin, D. Araç, M. Machius, T.C. Südhof, J. Rizo, Three-dimensional structure of the complexin/SNARE complex, Neuron 33 (2002) 397–409.
[28] S.L. Eastwood, P.J. Harrison, Synaptic pathology in the anterior cingulated cortex in schizophrenia and mood disorders. A review and a western blot study of synaptophysin, GAP-43 and the complexions, Brain Res. Bull. 55 (2001) 569–578.
[29] K. Sawada, C.E. Young, A.M. Barr, K. Longworth, S. Takahashi, V. Arango, J.J. Mann, A.J. Dwork, P. Falkai, A.G. Phillips, W.G. Honer, Altered immunoreactivity of complexin protein in prefrontal cortex in severe mental illness, Mol. Psychiatry 7 (2002) 484–492.
[30] A.S. Hazell, C. Wang, Downregulation of complexin I and complexin II in the medial thalamus is blocked by *N*-acetylcysteine in experimental Wernicke's encephalopathy, J. Neurosci. Res. 79 (2005) 200–207.
[31] J.H. Yi, R. Hoover, T.K. McIntosh, A.S. Hazell, Early, Transient increase in complexin I and complexin II in the cerebral cortex following traumatic brain injury is attenuated by *N*-acetylcysteine, J. Neurotrauma 23 (2006) 86–96.
[32] K. Reim, M. Mansour, F. Varoqueaux, H.T. McMahon, T.C. Südhof, N. Brose, C. Rosenmund, Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release, Cell 104 (2001) 71–81.
[33] M. Xue, K. Reim, X. Chen, H.T. Chao, H. Deng, J. Rizo, N. Brose, C. Rosenmund, Distinct domains of complexin I differentially regulate neurotransmitter release, Nat. Struct. Mol. Biol. 14 (2007) 949–958.
[34] H. Cai, K. Reim, F. Varoqueaux, S. Tapechum, K. Hill, J.B. Sørensen, N. Brose, R.H. Chow, Complexin II plays a positive role in Ca^{2+}-triggered exocytosis by facilitating vesicle priming, Proc. Natl Acad. Sci. USA 105 (2008) 19538–19543.

[35] M. Xue, A. Stradomska, H. Chen, N. Brose, W. Zhang, C. Rosenmund, K. Reim, Complexins facilitate neurotransmitter release at excitatory and inhibitory synapses in mammalian central nervous system, Proc. Natl. Acad. Sci. USA 105 (2008) 7875–7880.
[36] C.G. Giraudo, W.S. Eng, T.J. Melia, J.E. Rothman, A clamping mechanism involved in SNARE-dependent exocytosis, Science 313 (2006) 676–680.
[37] J.R. Schaub, X. Lu, B. Doneske, Y.K. Shin, J.A. McNew, Hemifusion arrest by complexin is relieved by Ca^{2+}-synaptotagmin I, Nat. Struct. Mol. Biol. 13 (2006) 748–750.
[38] S. Huntwork, J.T. Littleton, A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth, Nat. Neurosci. 10 (2007) 1235–1237.
[39] M. Xue, Y.Q. Lin, H. Pan, K. Reim, H. Deng, H.J. Bellen, C. Rosenmund, Tilting the balance between facilitatory and inhibitory functions of mammalian and Drosophila complexins orchestrates synaptic vesicle exocytosis, Neuron 64 (2009) 367–380.