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α-Latrotoxin stimulates glutamate release from cortical astrocytes in cell culture

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Abstract The mechanism responsible for the ability of bradykinin to cause calcium-dependent release of glutamate from astrocytes in vitro was investigated. The glutamate transport inhibitor, dihydrokainate, did not block bradykinin-induced glutamate release, and bradykinin did not cause cell swelling. These data exclude the involvement of glutamate transporters or swelling mechansims as mediating glutamate release in response to bradykinin. α-Latrotoxin (3 nM), a component of black widow spider venom, stimulated calcium-independent glutamate release from astrocytes. Since α-latrotoxin induces vesicle fusion and calcium-independent neuronal neurotransmitter release, our data suggest that astrocytes may release neurotransmitter using a mechanism similar to the neuronal secretory process.

Key words: Astroglia; Secretion; Endocytosis; Exocytosis; Glutamate; Calcium

1. Introduction

Astroglia are now thought to play many roles in the nervous system. While initially they were thought to be a 'nerve glue' [1], it is clear that they also have the potential to play dynamic roles. Neurons have been shown to signal to adjacent glia [2–4]. Waves of calcium can propagate amongst electrically coupled glia [2,5–9], and glia can signal back to adjacent neurons [10,11]. Stimuli that specifically elevate astrocyte calcium levels cause a subsequent elevation of neuronal calcium [10–12]. Glia–neuron signaling may be mediated by multiple signaling mechanisms including gap junctions [11] and the release of neurotransmitters [10].

Our previous work has demonstrated that bradykinin elevates internal calcium levels of cortical astrocytes [10] which evokes the calcium-dependent release of the excitatory amino acids (EAAs) glutamate and aspartate. When astrocytes are co-cultured with neurons, bradykinin stimulates the release of glutamate from astrocytes which causes an elevation of neuronal calcium that is mediated by the *N*-methyl-D-aspartate (NMDA) receptor [10]. The nature of the astrocyte glutamate release mechanism responsible for mediating astrocyte–neuron signaling has not been defined.

There are three prominent mechanisms that could mediate the release of neurotransmitter from astrocytes: (i) the reverse operation of a glutamate transporter [13], (ii) release through a swelling-induced pathway [14], or (iii) a calcium-dependent vesicle release mechanism [15]. In this study we have investigated the mechanism responsible for controlling release of neurotransmitter from astrocytes.

2. Materials and methods

2.1. Cell culture

Enriched astrocyte type-1 cultures were prepared from 1- to 4-day-old Sprague–Dawely rat cortices as previously described [10,16]. Briefly, cortices were freshly dissected and tissue was enzymatically (papain 20 IU/ml; 1 h at 37°C) and mechanically dissociated. The cells were plated into culture flasks and maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. After 7–13 days in culture, cells were shaken twice, first for 1.5 h and then for 18 h on an orbital shaker at 260 rpm. The population of adherent cells was subsequently detached using trypsin (0.1%). Detached cells were spun at $100 \times g$ for 10 min, resuspended and plated either onto poly-L-lysine (1 mg/ml; MW 100,000)-coated glass coverslips (for release studies) inlayed in a 35 mm culture dish or into glass-bottomed dishes (for imaging experiments). Enriched cultures were kept at 37°C in a humidified 5% CO_2/95% air atmosphere. All experiments were performed on cells that have been in culture for 3–5 days after re-plating.

2.2. Immunocytochemistry

Immunocytochemistry was performed using antibodies raised against glial fibrillary acidic protein (GFAP; 1:5000, ICN Immunobiological) and the microtubule associated protein, MAP-2 (1:2000; Boehringer). Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) with nickel-intensification.

2.3. Excitatory amino acid release

Coverslips containing astrocyte cultures were mounted into a 50 μl perfusion chamber. Saline was perfused at a rate of 200 μl/min and was collected to determine the levels of excitatory amino acids in 1 min 200 μl samples. Excitatory amino acid levels were determined by high-performance liquid chromatography (HPLC) with fluorescence detection as previously described [10]. Saline used for perfusion contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, and HEPES 10 (pH 7.4). In calcium-depleted solution, calcium was replaced by 1 mM EGTA, 3.8 mM $MgCl_2$ and 0.2 mM $CaCl_2$ to yield 26 nM free calcium. Max Chelator (version 5.6), written by Chris Patton at Stanford University, was used to estimate free extracellular calcium levels. All amino acid release experiments were performed at 37°C.

2.4. Volume measurement

Cells were incubated with BCECF-AM (5 μM) and 0.02% pluronic acid for 40 min at 37°C. After several washes cells were kept at 37°C for 40 min to permit dye de-esterification. All experiments were performed at room temperature (22–24°C). The content of normal imaging solution was identical to that of saline used for HPLC experiments. The osmolarity of this solution was 286–302 mOsm/kg as determined using a freezing point osmometer (Advanced Instruments Inc). In hyposmotic solution NaCl was reduced to 90 mM (measured osmolarity 202–203 mOsm/kg). BCECF was excited at 440 nm and emission was collected at 510 nm using a SIT camera, a Matrox frame grabbing board and

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Image-1 software (Universal Imaging). Hyposmotic solution and bradykinin (1 μM) were bath applied for 100 s. Images of astrocytes were acquired and the intensity of dye emission was quantified. Previous studies have shown that when cells swell the dye trapped in cells is diluted leading to a reduction in fluorescence emission intensity [17]. We find that BCECF effectively monitors the immediate swelling response of astrocytes to hyposmotic media as well as the subsequent regulatory volume decrease that has been characterized by other methods [18].

2.5. Calcium imaging

Cells were incubated with Fura-2AM (2 μM) and 0.02% pluronic acid for 40 min at 37°C. After several washes cells were kept at 37°C for 40 min to permit dye de-esterification. All imaging experiments were performed at room temperature (22–24°C) according to previously described methodology [10,19]. The contents of normal and calcium-depleted solutions were identical to those of salines used for HPLC experiments. Latrotoxin (3 nM; Alomone labs) was bath applied, while bradykinin (1 μM) was applied by pressure ejection (1 min).

3. Results and discussion

Phase-contrast microscopy indicated that these cultures were devoid of neurons. This was confirmed by immunocytochemistry which demonstrated that cultures lacked MAP2 immunoreactivity but were immunopositive for glial fibrillary acidic protein (GFAP; Fig. 1a,b). In parallel cultures which contained neurons, MAP2 immunoreactivity was reliably detected (Fig. 1c). Therefore, cultures in the current study are neuron-free and contain GFAP-positive polygonal astrocytes.

We have previously shown that bradykinin-induced release of neurotransmitter from astrocytes is not blocked by the glutamate transport inhibitors *p*-chloromercuriphenylsulphonic acid (p-CMPS) and L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) [10]. To further test the potential involvement of glutamate transporters in mediating bradykinin-induced glutamate release we have used dihydrokainate (DHK) which blocks both glutamate uptake and the reverse operation of glutamate transporters [20].

The release of glutamate was monitored from astrocyte cultures by collecting superfusate that was subjected to HPLC [10]. Under resting conditions there was a basal release of glutamate from astrocytes into the saline (Fig. 2a). Bradykinin caused a dose-dependent stimulation of glutamate release (Fig. 2a) as detected by the elevated levels of glutamate in the superfusate. Ten nM bradyknin elevated glutamate levels from 43 ± 7 nM to 87 ± 13 nM (mean ± S.E.M., $n = 6$), while 100 nM elevated glutamate levels from 51 ± 12 nM to 140 ± 18 nM ($n = 6$). Addition of DHK elevated the basal level of glutamate, indicating that DHK effectively reduced the activity of the glutamate transporter (Fig. 2b). Bradykinin stimulated the release of glutamate in the presence of DHK. Therefore, we conclude that bradykinin activates glutamate release from astrocytes through a mechanism distinct from the glutamate transporter since DHK does not reduce bradykinin-induced glutamate release.

Kimelberg and colleagues [14,18] have demonstrated that hyposmotic media cause a cell swelling-dependent, calcium-independent release of EAAs from astrocytes. To ask whether bradykinin might utilize such a mechanism we monitored astrocyte volume using BCECF. At an excitation wavelength of 440 nm BCECF is pH-insensitive. Cell swelling results in a dilution of the dye that leads to a reduction in the fluorescence intensity [17]. Treatment with hyposmotic medium reversibly reduced the BCECF fluorescence signal demonstrating that it is an

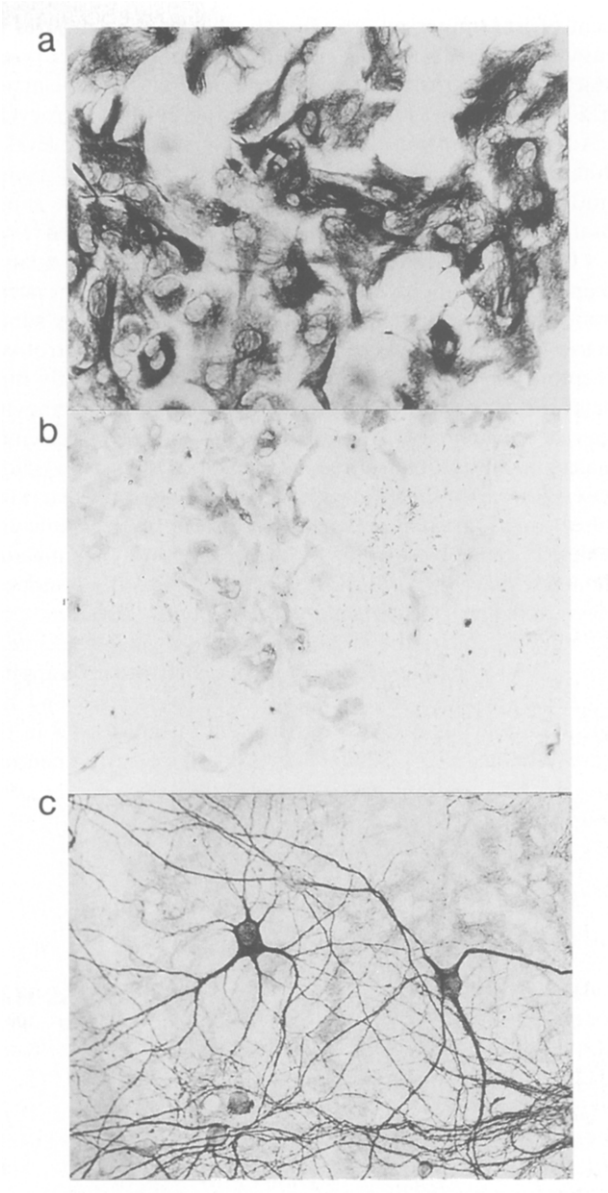

Fig. 1. (A) Astrocyte cultures are immunopositive with anti-GFAP. (B) Astrocyte cultures are devoid of immunoreactivity for MAP2, demonstrating that the cultures are neuron-free. (C) Co-cultures of neurons and astrocytes were treated with anti-MAP2. Immunopositive neurons act as a positive control for B.

effective method monitoring cell volume (Fig. 2c) [17]. Addition of bradykinin, however, did not significantly modify the intensity of the fluorescence signal (Fig. 2c). Since bradykinin does not change cell volume it must utilize a release mechanism distinct from the cell swelling pathway to stimulate the release of glutamate from astrocytes.

An active component of black widow spider venom, α-latrotoxin, binds to a receptor on nerve terminals which induces the release of neurotransmitter by directly stimulating the secretory machinery [21]. At least one of the receptors is a member of a distinct protein family, the neurexins, which are transmembrane proteins that bind intracellularly to the neuronal synaptic vesicle protein, synaptotagmin [22]. By directly interacting with synaptic proteins α-latrotoxin bypasses the calcium-require-

ment of transmitter release. To ask whether the astrocyte release machinery has fundamentally similar properties to the vesicular apparatus of the nerve terminal we tested the action of α-latrotoxin on glutamate release from purified astrocytes.

Addition of 3 nM α-latrotoxin rapidly increased the level of glutamate in the superfusate (Fig. 3a). α-Latrotoxin significantly increased the concentration of glutamate in the superfusate from 7 ± 2 nM to 18 ± 3 nM ($n = 6$; paired t-test $P < 0.02$; Fig. 3a). Previous studies have shown that α-latrotoxin has two actions, it forms channels in the plasma membrane that cause a calcium elevation, and it can directly stimulate secretory machinery through interactions with α-latrotoxin receptors [23–26]. We asked whether α-latrotoxin directly stimulates the astrocyte glutamate release mechanism by removing external calcium ions. Application of α-latrotoxin in calcium-depleted saline (estimated free calcium 26 nM) stimulated glutamate release to a similar magnitude as that induced when external calcium ions were present (Fig. 3a). However, in calcium-depleted saline α-latrotoxin only transiently stimulated glutamate release. Following this initial period of induced release, the levels of glutamate declined until this amino acid was undetectable in the saline. In neurons a similar decline in transmitter release has been shown when α-latrotoxin is applied in calcium-depleted saline. Parallel electron microscopy has indicated that this is due to a depletion of the vesicles in the nerve terminal [25,26]. Similar effects of α-latrotoxin in neurons and astrocytes raises the possibility that the transmitter release mechanisms of these two cell types share some fundamental properties.

To further investigate the calcium-dependence of α-latrotoxin action on astrocytes we monitored intracellular free calcium levels. Astrocytes were loaded with the calcium-sensitive indicator Fura-2. Bradykinin (1 μM), which stimulates glutamate release through receptor-mediated calcium elevation, raised the internal calcium level of astrocytes from 83 ± 8 to 444 ± 52 nM (mean ± S.E.M.; $n = 26$; Fig. 2d,e). By contrast, α-latrotoxin (3 nM) which significantly stimulated glutamate release, only elevated internal calcium from 69 ± 4 to 87 ± 5 nM ($P < 0.01$, paired t-test; $n = 34$; Fig. 3b–d) when calcium ions were present in external saline. In calcium-depleted saline, α-latrotoxin (3 nM) did not significantly elevate internal calcium levels ($P > 0.33$, paired t-test; $n = 29$; Fig. 3b).

When taken together with the HPLC glutamate release data shown in Fig. 3a, these calcium imaging experiments demonstrate that α-latrotoxin exerts effects on astrocytes that are similar to the toxin's actions on neurons. α-latrotoxin stimulates an increase in basal release from neurons in the presence and absence of external calcium ions [25,26]. In the absence of external calcium ions transmitter release is stimulated through a direct induction of exocytosis. Similar to these neuronal data, α-latrotoxin stimulated glutamate release from astrocytes in the absence external calcium (Fig. 3a) and without a detectable

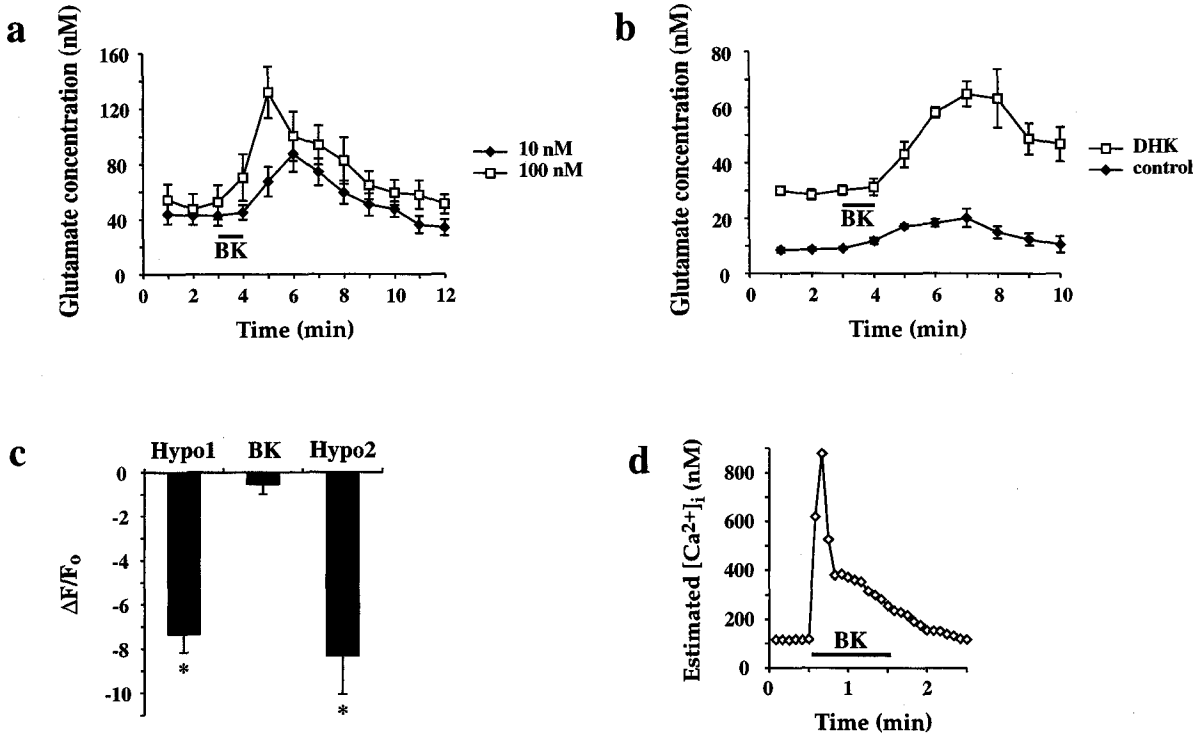

Fig. 2. (A) Bradykinin ($n = 8$ at 10 nM, $n = 4$ at 100 nM) causes a dose-dependent release of glutamate from astrocytes as measured by HPLC [10] of superfusate. (B) The glutamate transport inhibitor DHK (50 μM) elevates the basal level of glutamate in the superfusate, indicating that it effectively blocks transporter activity, but does not block bradykinin induced glutamate release. (C) Astrocytes were loaded with BCECF to monitor cell volume. Dye was excited at 440 nm and emission was collected at 510 nm. Addition of hyposmotic medium (hypo1) caused cell swelling, detected as a reduction in intensity of the fluorescence signal ($P < 0.01$). Changes in cell volume are represented as the change in fluorescence intensity/original intensity ($\Delta F/F_0$). Addition of bradykinin (1 μM) did not change the fluorescence intensity ($P > 0.25$). Subsequent addition of hyposmotic medium (hypo 2) significantly reduced fluorescence intensity ($P < 0.01$). Thus, bradykinin does not cause cell swelling. (D) Bradykinin (1 μM) elevates calcium levels in astrocytes.

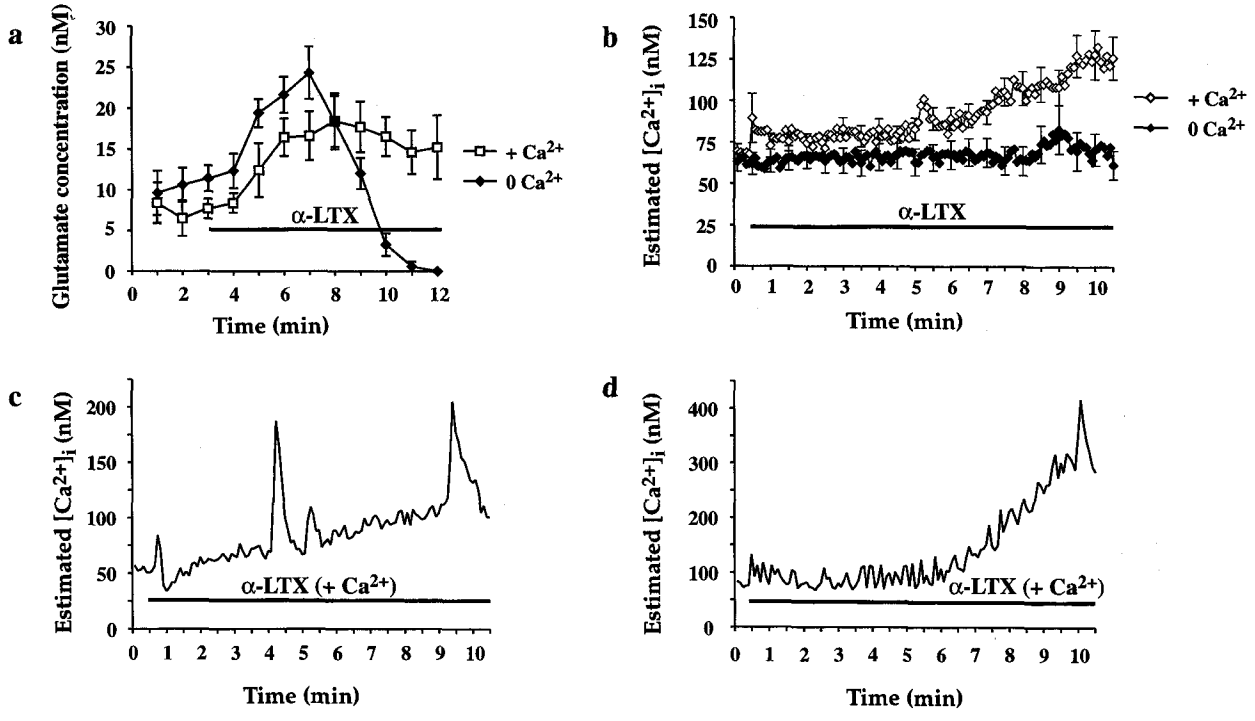

Fig. 3. (A) α-Latrotoxin (3 nM) stimulates the release of glutamate from astrocytes. In the presence of external calcium, α-latrotoxin causes a sustained release of glutamate. However, in calcium-depleted saline, α-latrotoxin causes a transient stimulation of glutamate release. Subsequently, all glutamate release is inhibited. (B) Summary data demonstrating the effect of α-latrotoxin on internal calcium levels of astrocytes. In the presence of external calcium, α-latrotoxin, causes a small elevation of internal calcium level (n = 34). In calcium-depleted saline α-latrotoxin does not elevate internal calcium levels (n = 29). (C and D) When viewed on a cell by cell basis in the presence of external calcium, α-latrotoxin, can cause transient elevations of calcium (C) as well as a gradual increase in internal calcium level (D).

calcium elevation (Fig. 3b). These data are consistent with α-latrotoxin directly stimulating transmitter release from astrocytes.

Previous studies in neurons and our studies in astrocytes (Fig. 2a and 3a) demonstrate that there is an external calcium-dependence for sustained transmitter release in response to α-latrotoxin. In neurons the requirement for external calcium has been identified. Correlated electron microscopy and electrophysiology has demonstrated that in the absence of external calcium, α-latrotoxin stimulates transmitter release and a loss of synaptic vesicles [25,26]. α-Latrotoxin stimulates transmitter release transiently because external calcium is needed for endocytosis and the replenishment of the pool of transmitter filled vesicles. Since astrocytes have a similar calcium-dependence for sustained transmitter release this data provides the first indication that astrocytes have a transmitter release mechanism similar to that of neurons. However, the identification of the exact nature of this secretory process and whether it represents a vestigial expression of the neuronal vesicular release mechanism requires further structural and biochemical study.

Three potential glutamate release mechanisms have been suggested as having the potential to mediate bradykinin-induced glutamate release [10,27]. Our data using PDC, pCMPS and DHK demonstrate that a glutamate transporter does not mediate bradykinin-induced glutamate release. In addition our data do not support a swelling-induced release mechanism. We have previously shown that the anion transport inhibitor, furosemide, blocks bradykinin-induced glutamate release [10]. This suggested that a furosemide-sensitive swelling-induced mechanism [14] might mediate glutamate release. However, the astrocyte swelling release mechanism is not calcium-sensitive [14,18]. In this study we have investigated the relationship between swelling and bradykinin-induced glutamate release further. Since we demonstrate that bradykinin does not cause cell swelling, but does cause glutamate release, we conclude that a mechanism distinct from the swelling-induced release mechanism must mediate glutamate release. The ability of α-latrotoxin to stimulate transmitter release from astrocytes suggests that they may contain a transmitter release mechanism similar to that of neurons. This raises the hypothesis that bradykinin causes glutamate release using such a secretory process.

It is perhaps not surprising that astrocytes contain a secretory system that has similarities to the neuronal release process given that muscle and fibroblasts are capable of quantal transmitter release under appropriate conditions [28,29]. Because of limitations with our HPLC assay for transmitter release it has not been possible to compare the kinetics of transmitter release from neurons and astrocytes nor to determine whether astrocyte release is quantal. However, the conservation of proteins regulating membrane traffic from yeast to man [30] suggests that similar building blocks will be used for calcium-regulated transmitter release in neurons and astrocytes of the same species. The particular molecular details and structural organization of this astrocyte release machinery remain to be identified.

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