

Vesicular Release of L- and D-Aspartate from Hippocampal Nerve Terminals: Immunogold Evidence

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Abstract: Glutamate is established as the most important excitatory transmitter in the brain. The transmitter status of aspartate is debated. There is evidence that aspartate is released from nerve terminals by exocytosis. However, release through excitatory amino acid transporters (EAATs) could be an alternative mechanism. We further investigated this by use of light and quantitative electron microscopic immunocytochemistry. The nerve terminal localisation of aspartate was compared to that of glutamate using antibodies specifically recognising the amino acids. Rat hippocampal slices were incubated under normal (3 mM) and depolarising (55 mM) concentrations of K⁺ with and without the excitatory amino acid transporter inhibitor threo-beta-benzyloxyaspartate (TBOA). If aspartate is released either through reversal of the EAATs or through exchange with synaptically released glutamate, we would expect that TBOA would block the depolarisation induced release of aspartate. We found, however, that there was a substantial depletion of aspartate, as well as of glutamate, from hippocampal nerve terminals during K⁺ induced depolarisation in the presence of TBOA. The possibility that aspartate is released through exocytosis from synaptic vesicles was further investigated by the use of a D-aspartate uptake assay, including exposure of the slices to exogenous D-aspartate and the use of D-aspartate immunogold cytochemistry to localise D-aspartate in the fixed tissue. We found that D-aspartate taken up into the terminals was concentrated in synaptic vesicles as opposed to in the cytoplasmic matrix. This is in line with the presence in synaptic vesicles of the recently identified vesicular transporter for aspartate.

Keywords: Synaptic vesicles, immunocytochemistry, amino acids, electron microscopy, transport.

The release of glutamate at synapses in the brain is well studied. Glutamate is released upon depolarisation of the presynaptic nerve terminal, leading to opening of voltage gated Ca²⁺ channels and Ca²⁺-dependent fusion of glutamate filled synaptic vesicles with the plasma membrane. The mechanism of release of L-aspartate at central synapses remains less defined. L-aspartate has been shown to be released in a Ca²⁺ and clostridium toxin sensitive manner from various *in vitro* brain preparations (e.g. [1-7]). Strongly in favour of an exocytotic release mechanism are our previous immunocytochemical results showing that K⁺ induced depolarisation elicited depletion of L-aspartate from nerve endings, which could be inhibited by low extracellular Ca²⁺-concentrations and tetanus toxin [8-10]. In addition, D-aspartate has been shown to be subject to exocytotic release both from cultured neurons [11] synaptosomes [12] and intact brain tissue [13]. Taken together, these results indicate that synaptic vesicles harbour a transporter for L- and/or D-aspartate (see [14]). This is in good agreement with a very recent study showing that hippocampal synaptic vesicles contain a vesicular excitatory amino acid transporter (VEAT) for aspartate and glutamate [15]. It was shown that sialin, a H⁺/sialic acid co-transporter belonging to the SLC17A5 group of Na⁺/phosphate transporters, could carry also aspartate and glutamate into synaptic vesicles [15].

There is ample evidence that excitatory nerve endings in the brain contain functional plasma membrane excitatory amino acid transporters (EAATs) [16-20]. The previous aspartate release data do not clearly distinguish between L-aspartate release *via* exocytosis and *via* these EAATs, which could release aspartate either through reversal of the transporter or through heteroexchange with glutamate released from synaptic vesicles. Although some biochemical studies have given evidence that release through the EAATs does not play a pivotal role in the release of aspartate [12, 21], this has not been directly studied with high resolution at the morphological level. In order to investigate the proportion of L-aspartate release which is mediated through the EAATs, we incubated hippocampal slices with the non-transportable glutamate transporter inhibitor (2S,3S)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate (PMB-TBOA) during K⁺-induced depolarisation. By using light- and electron microscopic immunocytochemistry we compared the depletion of L-aspartate from excitatory nerve terminals with the depletion of glutamate. To shed additional light on the vesicular release of aspartate we exposed the slices to exogenous D-aspartate and used a quantitative D-aspartate immunogold method [16-18, 22] to analyse the synaptic vesicle content of D-aspartate that had been taken up into excitatory nerve terminals.

MATERIALS AND METHODOLOGY

Hippocampal Slice Incubation

Hippocampal slice experiments were performed as previously described [8, 9, 18]. In brief, in each experiment we

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harvested a brain from a decapitated Wistar rat (~200 g). Then the hippocampi were dissected out and cut in 0.3 mm thick slices. The slices were preincubated in oxygenated Krebs' solution (126 mM NaCl, 3 mM KCl, 10 mM sodium phosphate buffer (pH 7.4), 1.2 mM CaCl₂, 1.2 mM MgSO₄ and 10 mM glucose) for 1 h at 30 °C. The slices, which were subjected to EAAT inhibition, were transferred to a Krebs' solution containing 30 µM PMB-TBOA [18] during the last 15 minutes of the preincubation. Then the slices were incubated for 1 h at 30 °C in Krebs' solution under physiological (3 mM) and depolarising (55 mM) concentrations of K⁺ with and without the EAAT-blocker PMB-TBOA (30 µM) (6 slice experiments). To characterise the localization of D-aspartate taken up in the nerve terminals, after preincubation some slices were incubated for 15 minutes in Krebs' solution at 3 mM K⁺ with and without 50 µM D-aspartate (3 slice experiments). After incubation the slices were fixed in 2.5% glutaraldehyde and 1% formaldehyde for 1 h in room temperature (about 20°C). The slices were stored in the fixative at 4°C until sectioning and embedding.

Light Microscopic Immunocytochemistry

The hippocampal slices were cryo-protected in sucrose (30%) before 20 µm thick sections were cut on a freezing microtome. Then the sections were subjected to immunocytochemistry according to a three layer immunoperoxidase method. After incubation with primary antibodies (no 435 L-aspartate (1:5000) and no 607 L-glutamate (1:5000)) overnight at room temperature (about 20°C), the sections were treated with biotinylated donkey anti-rabbit Ig (Amersham, Little Chalfont Buckinghamshire, UK) and with streptavidin-biotinylated horseradish peroxidase (Amersham, Little Chalfont Buckinghamshire, UK), before the antigen-antibody binding was visualized with hydrogen peroxide/di-amino-benzidine.

Electron Microscopic Immunocytochemistry

The slices were embedded in Lowicryl HM20 as previously described [18, 22-24]. After the embedding ultrathin sections (80-100 nm) were cut perpendicular to the surface of the slices (so that immunogold analysis could be made at definite distances from the slice surface) and labelled with the 482 D-aspartate (1:300), 607 L-glutamate (1:5000) or 435 L-aspartate (1:100) antisera. The primary antibodies were visualised with 15 nm gold particles coupled to secondary anti-rabbit antibodies. The sections were studied in a Tecnai 12 or a Philips CM10 electron microscope. Electron micrographs were randomly taken from the CA1 stratum radiatum and the inner dentate molecular layer. The number of L-aspartate, D-aspartate and glutamate gold particles were counted in nerve terminals making asymmetric synapses on dendritic spines (excitatory synapses). The area of the nerve terminals (the area of mitochondria was not included) was estimated by point counting using an overlay screen [23] and the gold particle densities were calculated. The results were statistically evaluated by a non-parametric Mann-Whitney U test (Statistica).

For the analysis of D-aspartate in synaptic vesicles gold particles signalling D-aspartate and grid points for area determination were recorded over synaptic vesicles and cytoplasmic matrix (areas free of vesicles and mitochondria) in excitatory nerve terminals. Synaptic vesicles were identified

as round, clear membrane bounded structures with a diameter ranging from 20 to 80 nm [9]. As the lateral resolution of the immunogold method is about 30-40 nm [25, 26], which is similar to the diameter of the synaptic vesicles and to the section thickness, the method cannot localize a gold particle to a single vesicle. To by pass this problem, gold particles were ascribed to synaptic vesicles if the particle centres were within a 30 nm distance from the outer border of the vesicles. To correct for the contamination of the vesicle labelling with the cytoplasmic matrix labelling, the gold particle densities recorded over cytoplasmic matrix were subtracted from the gold particle densities recorded for the vesicles. The areas of the profiles included in the analysis were determined by point counting using an overlay screen [23]. The densities of D-aspartate immunogold particles (average number of gold particles/µm²) in the different tissue profiles were calculated and the results statistically evaluated by a non-parametric test (Mann-Whitney-U, Statistica).

Specificity of the Antibodies

The rabbit no 607 L-glutamate, no 435 L-aspartate and no 482 D-aspartate antisera have been previously characterized [9, 10, 16, 27]. To avoid possible cross-reactivities the L-glutamate, L-aspartate and D-aspartate antisera were used with the addition of 0.2 mM complexes of glutaraldehyde/formaldehyde treated L-aspartate plus glutamine and L-asparagine, L-glutamate plus GABA and L-glutamate plus L-aspartate, respectively. As a specificity control for each immunoperoxidase incubation, spots of conjugates of different amino acids linked to brain macromolecules by glutaraldehyde/formaldehyde were placed on cellulose nitrate-acetate filters and incubated along with the tissue sections [8, 9]. For the immunogold experiments conjugates were made according to [28]. The concentration of the fixed amino acids in the embedded conjugate clumps is about 100 mM [28]. These test systems showed that the primary antibodies only labelled the conjugate containing the amino acid against which the antibodies were raised. Furthermore, the glutamate, L-aspartate and D-aspartate immunoreactivities of tissue and test sections were blocked by adding 0.3 mM of aldehyde treated glutamate, L-aspartate or D-aspartate, respectively, to the antisera. The relationship between the density of L-aspartate, D-aspartate and glutamate immunogold particles and the concentration of fixed glutamate in the tissue was linear, as assessed by simultaneously processed test sections with conjugates containing known concentrations of glutamate, L-aspartate and D-aspartate [16, 29].

RESULTS

Effects of Membrane Depolarisation and TBOA

We have previously shown that K⁺ induced membrane depolarisation causes aspartate, in the same manner as glutamate, to be depleted from excitatory nerve endings in a Ca²⁺-dependent and tetanus toxin sensitive manner [8,9]. The question is whether such a depletion of aspartate could be caused by heteroexchange through the EAATs with exocytosed glutamate. An ideal tool for investigating this would be to use a non-transportable blocker of the EAATs during membrane stimulation, such as threo-beta-benzyloxy-aspartate (TBOA) [30]. In a recent study we showed that 30 µM PMB-TBOA, which is a potent blocker of EAAT1-3 [31], totally inhibits D-aspartate transport in exci-tatory

nerve endings (but not in astrocytes, see below) in hippocampal slices [18].

As observed previously [8, 9] we here show that in slices incubated with 3 mM K^+ the aspartate and glutamate antibodies produced a zonal labelling pattern corresponding to

the nerve terminal field of the excitatory afferents in hippocampus (Fig. 1A, 1B). The aspartate and glutamate staining were mostly located in small dots (insets in Fig. 1A and B) along weakly labelled dendritic structures. Electron microscopic immunogold cytochemistry [9] confirmed that the

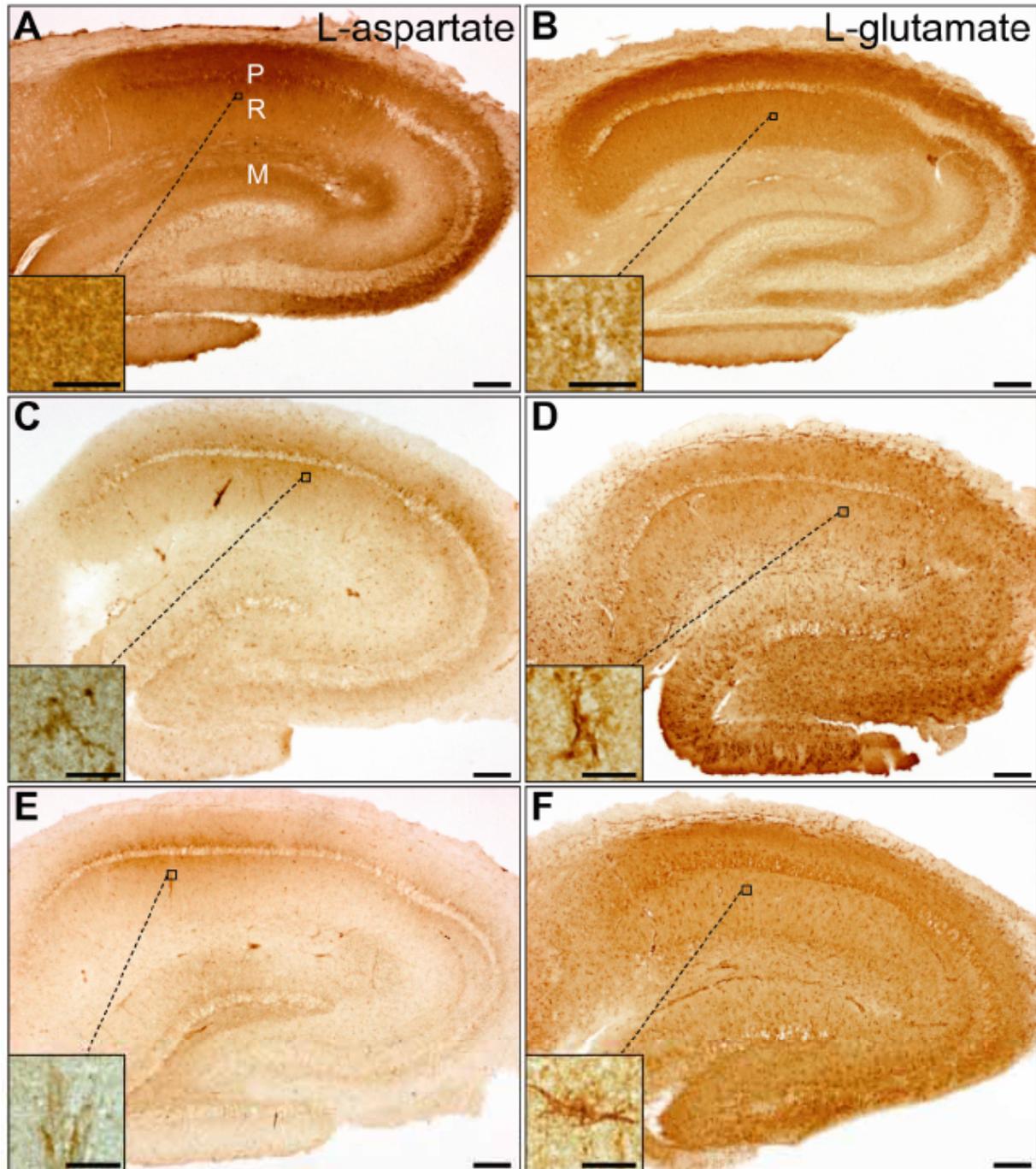


Fig. (1). The K^+ -induced depletion of aspartate and glutamate from nerve ending-like dots was not substantially inhibited by PMB-TBOA. At physiological K^+ -concentrations (3 mM) aspartate (A) and glutamate (B) immunoreactivities were located in dots (insets in A and B) corresponding to the excitatory nerve terminals in the hippocampus. At 55 mM K^+ the aspartate (C) and glutamate (D) staining mostly disappeared from the nerve terminal-like dots and accumulated in astroglial cells (insets in C and D), including in those around blood vessels (perivascular astroglia). At 55 mM K^+ in the presence of PMB-TBOA the aspartate (E) and glutamate (F) staining pattern was approximately unchanged, still showing that the most prominent staining was located in astroglial cells (insets in E and F). Similar aspartate and glutamate labelling patterns were observed in 6 slice experiments. Symbols: P, str. pyramidale; R, str. radiatum; M, dentate molecular layers. Bars: 200 μ m, 20 μ m (insets).

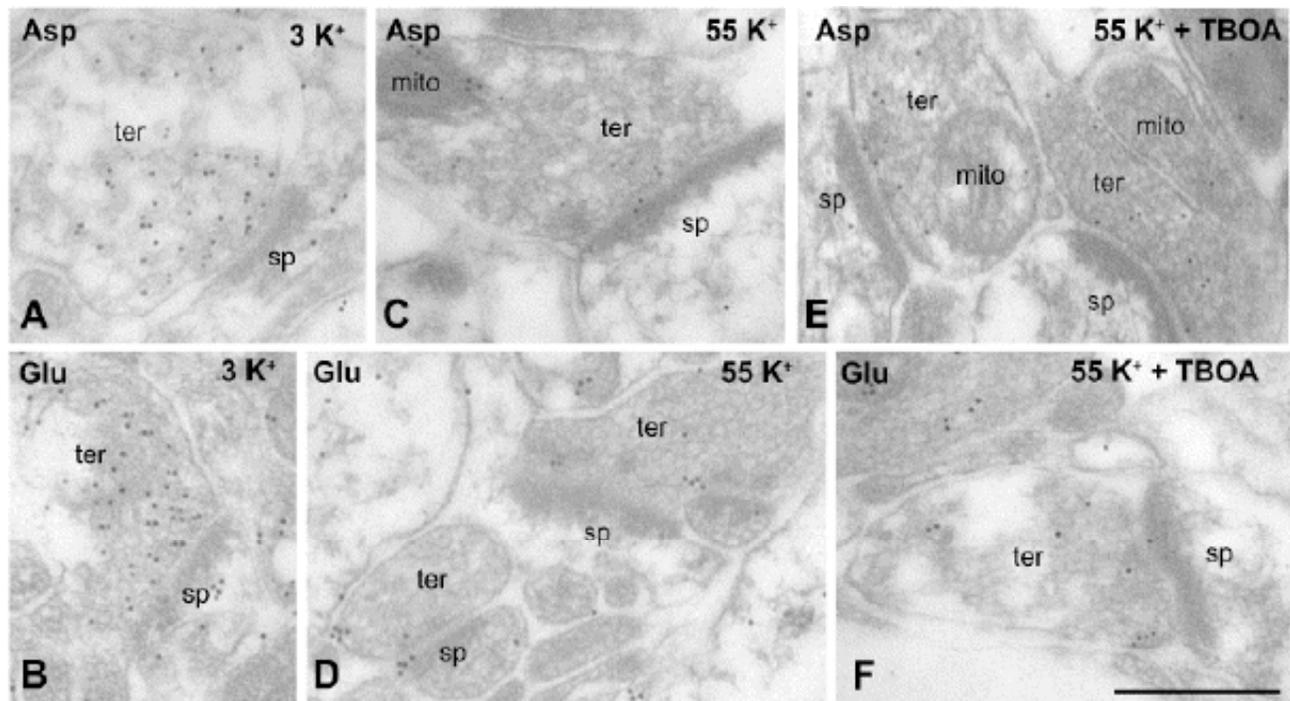


Fig. (2). Electron micrographs showing gold particles signalling aspartate (A, C, E) and glutamate (B, D, F) in excitatory synapses between nerve terminals (ter) and postsynaptic spines (sp) in hippocampal slices incubated at 3 mM K^+ (A and B), 55 mM K^+ (C and D) and 55 mM K^+ plus PMB-TBOA (E and F). Note that both aspartate and glutamate are depleted from the terminals upon stimulation with an elevated concentration of K^+ and that the treatment with PMB-TBOA does not block this depletion. Symbol: mito, mitochondria. Scale bar: 200 nm.

dotted staining represents labelling of nerve terminals making asymmetric synapses with dendritic spines (i.e. synapses with an “excitatory appearance”) (Fig. 2A, 2B). Thus, we call the labelled dots nerve ending-like dots. When the slices were depolarised with a high K^+ concentration (50 mM) the aspartate and glutamate immunoreactivities were depleted from the nerve ending like dots. Instead, the staining appeared in astrocytes (Fig. 1C, 1D). Immunoelectron microscopy of slices exposed to membrane depolarisation showed a robust reduction in the content of gold particles signalling aspartate and glutamate in excitatory nerve terminals (Fig. 2C, 2D). This was substantiated by quantitative immunogold analysis, which showed that K^+ -induced depolarisation caused the nerve terminal densities of aspartate and glutamate immunogold particles to be significantly reduced (Fig. 3). Taken together, this suggests that aspartate and glutamate is released from nerve terminals and taken up into astrocytes when the neurons are depolarised.

At 3 mM K^+ PMB-TBOA did not have any effect on the aspartate and glutamate labelling patterns (not shown). During depolarisation with 55 mM K^+ , the slices which were treated with PMB-TBOA showed a redistribution from a nerve ending like staining pattern to an astrocyte like staining pattern (Fig. 1E, 1F), similar to that observed at 55 mM K^+ without PMB-TBOA (Fig. 1C and 1D). The uptake of aspartate and glutamate in astrocytes during K^+ stimulation in the presence of TBOA probably occurs through a sodium dicarboxylate transporter [18]. The immunogold labellings revealed that the densities of gold particles signalling aspartate and glutamate were somewhat higher in the depolarised terminals exposed to PMB-TBOA than in the terminals not exposed to PMB-TBOA, but lower than in the terminals

from slices incubated at 3 mM K^+ (Figs. 2E, 2F and 3). The immunogold quantifications (Fig. 3) showed that about 30% and 36% of the L-aspartate and glutamate immunogold particles depleted from the terminals during depolarisation could be blocked by PMB-TBOA. This indicates that about 64-70 % of immunogold particles signalling L-aspartate and glutamate are depleted from the terminals due to exocytosis.

Localisation of D-Aspartate Immunogold Particles

As we found that the contribution from the EAATs for the total release of aspartate from nerve terminals was relatively minor, and several lines of evidence suggest that both L- and D-aspartate could be released by exocytosis (see Introduction), there should be a transporter capable of pumping L- and D-aspartate into synaptic vesicles (see Miyaji *et al.* 2008). To further investigate this uptake process we made use of a D-aspartate uptake assay, in which hippocampal slices were incubated with exogenous D-aspartate (50 μ M), fixed with glutar- and formaldehyde and subjected to electron microscopic immunogold cytochemistry using specific antibodies against D-aspartate [16-18, 22]. As D-aspartate is very slowly metabolised [32], it is “trapped” in the cellular compartment into which it was taken up.

Slices not exposed to D-aspartate did not contain any significant D-aspartate immunogold signal (not shown, see [16-18, 22]). In contrast, slices exposed to 50 μ M D-aspartate showed clear sign of D-aspartate uptake in excitatory nerve terminals (Fig. 4A and C) (as well as in astrocytic processes (not shown, see [16]). A closer examination of the labelled terminals showed that D-aspartate gold particles were located over synaptic vesicles (insets in Fig. 4A and Fig. 4B). Interestingly, when quantifying the density of D-

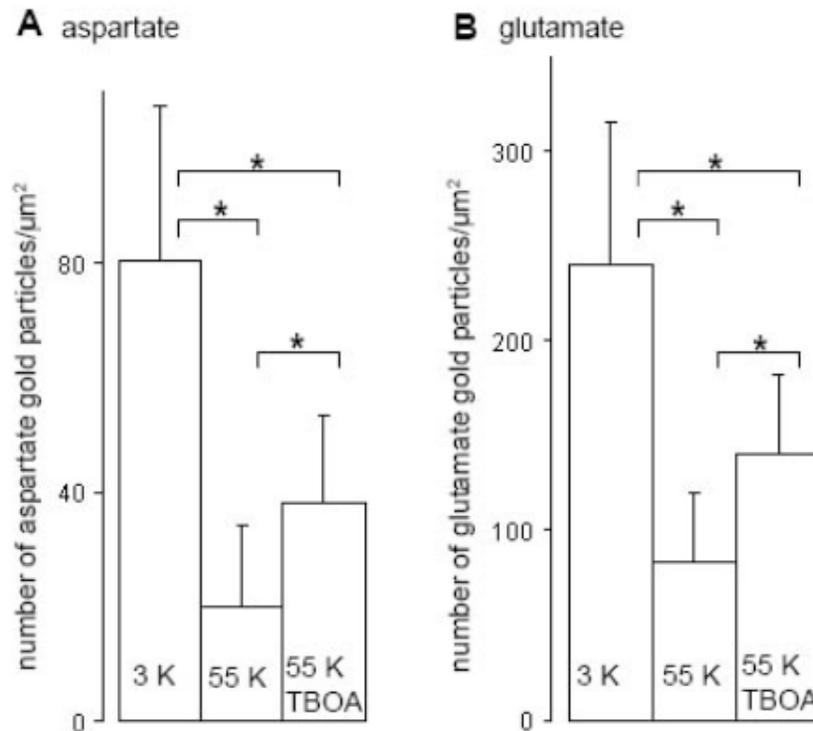


Fig. (3). Quantitative representation of the density of aspartate (**A**) and glutamate (**B**) immunogold particles in excitatory nerve terminals in hippocampal slices incubated with 3 mM K^+ ($n=45$ nerve terminals in **A** and **B**), 55 mM K^+ ($n=40$ terminals in **A** and **B**) and 55 mM K^+ in the presence of PMB-TBOA ($n=42$ nerve terminals in **A** and **B**). Asterisks denote that aspartate and glutamate values at 3 K^+ are significantly different from those at 55 K^+ and 55 K^+ + TBOA and that the values at 55 K^+ are significantly different from those at 55 K^+ + TBOA ($p < 0.01$, Mann-Whitney-U test, two tails). The results are from one slice experiment. The same results were obtained in another experiment.

aspartate immunogold particles over synaptic vesicles and cytoplasmic matrix in the excitatory terminals (see Methods), we found that the gold particle density was much higher over the former than the latter compartment, giving a synaptic vesicle/cytoplasmic matrix ratio of about 9. From test sections with known concentrations of D-aspartate incubated with the D-aspartate antibodies in parallel with the hippocampal sections [22] we could estimate that the fixed D-aspartate concentration in the cytoplasmic matrix was about 2 mM, whereas the D-aspartate concentration in synaptic vesicles was about 18 mM.

DISCUSSION

Previously we have shown that the depletion of aspartate and glutamate from excitatory nerve terminals is sensitive to low Ca^{2+} conditions and tetanus toxin [9]. This result could be explained by the possibility that it is glutamate that is released from synaptic vesicles, whereas aspartate is released from the cytosol through EAAT mediated heteroexchange with synaptically released glutamate. In this way the aspartate release would seemingly appear as exocytotic. However, here we show that there is a substantial depletion of both aspartate and glutamate from excitatory nerve terminals during K^+ induced depolarisation, despite the presence of the EAAT blocker TBOA. This strongly suggests that release through the EAATs cannot account for the overall release of aspartate from the terminals, indicating that at a major part of the released aspartate during K^+ stimulation is derived from exocytosis of synaptic vesicles.

Our previous immunogold results from excitatory nerve terminals show that there is a residual depolarisation induced depletion of aspartate and glutamate, which is not blocked by conditions inhibiting exocytosis [9]. Thus, parts of the aspartate and glutamate release could be mediated by the EAATs. The non-exocytotic aspartate and glutamate depletion is about 30-40% of the total depletion during K^+ triggered depolarisation [9]. This fits with the present data showing that approximately 30-36% of aspartate and glutamate released from the excitatory terminals could be blocked by blocking the EAATs. These data suggest that a minor fraction of aspartate and glutamate released from the terminals escapes through the EAATs, whereas the largest fraction (about 70%) is released through exocytosis.

The present data, showing that the depletion of aspartate and glutamate from the nerve terminals is inhibited to a similar extent by blocking the EAATs, is compatible with the notion that the non-exocytotic release of the excitatory amino acids during K^+ -induced depolarisation is caused by a reversal of the nerve terminal EAATs. This is in line with biochemical data, showing that the K^+ -induced release of newly taken up D-aspartate from hippocampal nerve endings occurs in part by exocytosis (about 65%) and in part by transporter reversal (about 35%) [12].

Our finding that the ratio of D-aspartate labelling between synaptic vesicles and cytoplasmic matrix was about 9 supports the idea that aspartate can be taken up into synaptic vesicles before exocytotic release. This uptake is mediated by other transporters than the vesicular glutamate transport-

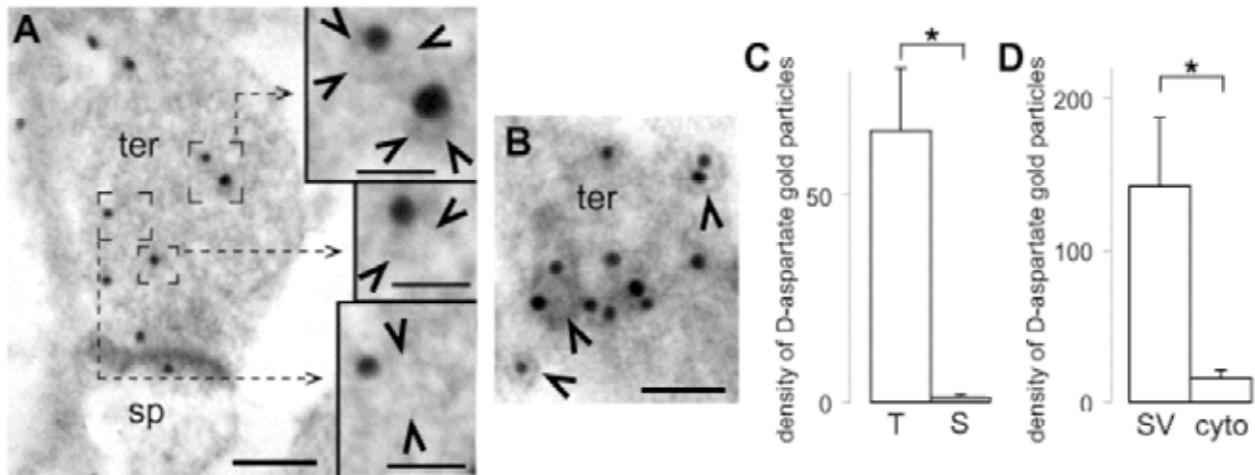


Fig. (4). D-aspartate is taken up into synaptic vesicles in excitatory nerve terminals. The electron micrographs (**A**, **B**) show gold particles representing D-aspartate in nerve terminals (ter) making asymmetric synapses with postsynaptic spines (sp) in hippocampal slices exposed to exogenous D-aspartate at resting conditions (3 mM K^+). Note the clear association between the D-aspartate immunogold particles and synaptic vesicles (arrowheads in the insets in **A** and in **B**). Scale bars: 100 μm in **A**, 50 μm in insets and in **B**. **C** shows the density of D-aspartate gold particles (average number of gold particles/ $\mu\text{m}^2 \pm \text{SD}$) in 20 excitatory synapses between nerve terminals (T) and spines (S). **D** shows the density of D-aspartate gold particles in synaptic vesicles (SV) and cytoplasmic matrix (cyto) in the 20 nerve terminals represented in **C**. Asterisks denote that the D-aspartate value in T is significantly different from that in S and that the value in SV is significantly different from that in cyto ($p < 0.01$, Mann-Whitney-U test, two tails). The quantifications are from one slice experiment. Similar results were obtained in two other experiments.

ers (VGLUTs), which neither transport L-aspartate nor D-aspartate [33-39]. Even though Miyaji *et al.* [15] did not give any information about vesicular D-aspartate uptake, the present data are in good agreement with their identification of a vesicular excitatory amino acid transporter that carries both aspartate and the aspartate analogue threo-hydroxy aspartate [5] into hippocampal synaptic vesicles [15]. Our results also support those of Fleck and co-workers [14], who showed that radiolabelled L- and D-aspartate could be taken up in synaptic vesicles, which were immunisolated with synaptophysin after amino acid preincubation of intact synaptosomes. However, it should be noted that especially the L-aspartate uptake data from this study should be interpreted with caution. During the preincubation of the synaptosomes some of the radiolabel on L-aspartate could have been transferred to L-glutamate through metabolism in the Krebs' cycle. However, as D-aspartate is very slowly metabolised in the brain [32], the possibility that such a mechanism underlies the result of the D-aspartate uptake experiments of [14] is not very likely. Thus, the present results and those of Fleck and co-workers [14] strongly suggest that also D-aspartate is transported into synaptic vesicles, probably by the same transporter as L-aspartate. This is in line with the large body of evidence showing exocytosis of both L-aspartate [1-4, 6-10, 14] and D-aspartate [11, 12, 40-45].

We found that the cytoplasmic concentration of D-aspartate (about 2 mM) after uptake into nerve terminals was in the range of the apparent K_m values for aspartate uptake into synaptic vesicles reported both by Miyaji *et al.* [15] and Fleck *et al.* [14]. The substrate specificity of VEAT indicates that it carries both glutamate and aspartate. This may explain the previous immunogold findings of glutamate and aspartate in the same nerve terminals [9, 27]. There are so far no detailed electron microscopic localisation studies of VEAT in the brain, only previous light microscopic results showing

that the sialin protein (see Introduction) is present at quite high concentrations throughout the forebrain, including in the hippocampus [46, 47]. Whether vesicles containing VEAT is located in the same terminals as those containing VGLUT is one important question that must await further studies.

The conclusion from previous uptake studies using whole brain isolated synaptic vesicles is that the vesicles do not transport aspartate [48-52]. However, most of these studies did not analyse direct uptake of radiolabelled aspartate, but showed that neither L- nor D-aspartate could inhibit uptake of L-glutamate into synaptic vesicles. This indicates that L- and D-aspartate are not transported by the same transporter proteins as glutamate, but does not exclude the possibility that aspartate is transported by another vesicular transporter, such as VEAT [15]. It should, however, be noted that the initial study of Naito and Ueda [48] showed that whole brain synaptic vesicles could indeed accumulate L-aspartate, although the uptake was much lower (7.5%) than that of L-glutamate. In the studies of Miyaji *et al.* [15] threo-hydroxy aspartate could not significantly inhibit glutamate uptake into whole brain synaptic vesicles (only into hippocampal vesicles), indicating that VEAT is much less abundant than the VGLUTs.

CONCLUSION

We here show that excitatory synaptic vesicles in the hippocampus can accumulate D-aspartate and release L-aspartate by exocytosis, further corroborating the evidence for a vesicular aspartate transporter [15] in these vesicles.

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REFERENCES

- [1] Nadler JV, Vaca KW, White WF, Lynch GS, Cotman CW. Aspartate and glutamate as possible transmitters of excitatory hippocampal afferents. *Nature* 1976; 260: 538-40.
- [2] Burke SP, Nadler JV. Regulation of glutamate and aspartate release from slices of the hippocampal CA1 area: effects of adenosine and baclofen. *J Neurochem* 1988; 51: 1541-51.
- [3] Burke SP, Nadler JV. Effects of glucose deficiency on glutamate/aspartate release and excitatory synaptic responses in the hippocampal CA1 area *in vitro*. *Brain Res* 1989; 500: 333-42.
- [4] McMahon HT, Foran P, Dolly JO, Verhage M, Wiegand VM, Nicholls DG. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *J Biol Chem* 1992; 267: 21338-43.
- [5] Fleck MW, Barrionuevo G, Palmer AM. Release of D, L-threo-beta-hydroxyaspartate as a false transmitter from excitatory amino acid-releasing nerve terminals. *Neurochem Int* 2001; 39: 75-81.
- [6] Bradford SE, Nadler JV. Aspartate release from rat hippocampal synaptosomes. *Neuroscience* 2004; 128: 751-65.
- [7] Wang L, Nadler JV. Reduced aspartate release from rat hippocampal synaptosomes loaded with Clostridial toxin light chain by electroporation: evidence for an exocytotic mechanism. *Neurosci Lett* 2007; 412: 239-42.
- [8] Gundersen V, Ottersen OP, Storm-Mathisen J. Aspartate- and Glutamate-like Immunoreactivities in Rat Hippocampal Slices: Depolarization-induced redistribution and effects of precursors. *Eur J Neurosci* 1991; 3: 1281-99.
- [9] Gundersen V, Chaudhry FA, Bjaalie JG, Fonnum F, Ottersen OP, Storm-Mathisen J. Synaptic vesicular localization and exocytosis of L-aspartate in excitatory nerve terminals: A quantitative immunogold analysis in rat hippocampus. *J Neurosci* 1998; 18: 6059-70.
- [10] Gundersen V, Holten AT, Storm-Mathisen J. GABAergic synapses in hippocampus exocytose aspartate on to NMDA receptors: Quantitative immunogold evidence for co-transmission. *Mol Cell Neurosci* 2004; 26: 156-65.
- [11] Cousin MA, Nicholls DG. Synaptic vesicle recycling in cultured cerebellar granule cells: Role of vesicular acidification and refilling. *J Neurochem* 1997; 69: 1927-35.
- [12] Raiteri L, Zappettini S, Milanese M, Fedele E, Raiteri M, Bonanno G. Mechanisms of glutamate release elicited in rat cerebrocortical nerve endings by 'pathologically' elevated extraterminal K⁺ concentrations. *J Neurochem* 2007; 103: 952-61.
- [13] Potashner SJ, Dymczyk L, Deangelis MM. D-aspartate uptake and release in the guinea pig spinal cord after partial ablation of the cerebral cortex. *J Neurochem* 1988; 50: 103-11.
- [14] Fleck MW, Barrionuevo G, Palmer AM. Synaptosomal and vesicular accumulation of L-glutamate, L-aspartate and D-aspartate. *Neurochem Int* 2001; 39: 217-25.
- [15] Miyaji T, Echigo N, Hiasa M, Senoh S, Omote H, Moriyama Y. Identification of a vesicular aspartate transporter. *Proc Natl Acad Sci USA* 2008; 105: 11720-4.
- [16] Gundersen V, Danbolt NC, Ottersen OP, Storm-Mathisen J. Demonstration of glutamate/aspartate uptake activity in nerve endings by use of antibodies recognizing exogenous D-aspartate. *Neuroscience* 1993; 57: 97-111.
- [17] Gundersen V, Ottersen OP, Storm-Mathisen J. Selective excitatory amino acid uptake in glutamatergic nerve terminals and in glia in the rat striatum: quantitative electron microscopic immunocytochemistry of exogenous (D)-aspartate and endogenous glutamate and GABA. *Eur J Neurosci* 1996; 8: 758-65.
- [18] Holten AT, Danbolt NC, Shimamoto K, Gundersen V. Low-affinity excitatory amino acid uptake in hippocampal astrocytes: a possible role of Na⁺/dicarboxylate cotransporters. *Glia* 2008; 56: 990-7.
- [19] Furness DN, Dehnes Y, Akhtar AQ, *et al.* A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for EAAT2. *Neuroscience* 2008, in press.
- [20] Waagepetersen HS, Qu H, Sonnewald U, Shimamoto K, Schousboe A. Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. *Neurochem Int* 2005; 47: 92-102.
- [21] Zhou M, Peterson CL, Lu YB, Nadler JV. Release of glutamate and aspartate from CA1 synaptosomes: selective modulation of aspartate release by ionotropic glutamate receptor ligands. *J Neurochem* 1995; 64: 1556-66.
- [22] Gundersen V, Shupliakov O, Brodin L, Ottersen OP, Storm-Mathisen J. Quantification of excitatory amino acid uptake at intact glutamatergic synapses by immunocytochemistry of exogenous D-aspartate. *J Neurosci* 1995; 15: 4417-28.
- [23] Bergersen LH, Storm-Mathisen J, Gundersen V. Immunogold quantification of amino acids and proteins in complex subcellular compartments. *Nat Protoc* 2008; 3: 144-52.
- [24] Holten AT, Gundersen V. Glutamine as a precursor for transmitter glutamate, aspartate and GABA in the cerebellum: a role for phosphate-activated glutaminase. *J Neurochem* 2008; 104: 1032-42.
- [25] Chaudhry FA, Lehre KP, van Lookeren CM, Ottersen OP, Danbolt NC, Storm-Mathisen J. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* 1995; 15: 711-20.
- [26] Nagelhus EA, Veruki ML, Torp R, *et al.* Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Muller cells and fibrous astrocytes. *J Neurosci* 1998; 18: 2506-19.
- [27] Gundersen V, Fonnum F, Ottersen OP, Storm-Mathisen J. Redistribution of neuroactive amino acids in hippocampus and striatum during hypoglycemia: a quantitative immunogold study. *J Cereb Blood Flow Metab* 2001; 21: 41-51.
- [28] Ottersen OP. Postembedding light- and electron microscopic immunocytochemistry of amino acids: description of a new model system allowing identical conditions for specificity testing and tissue processing. *Exp Brain Res* 1987; 69: 167-74.
- [29] Ottersen OP. Quantitative electron microscopic immunocytochemistry of neuroactive amino acids. *Anat Embryol (Berl)* 1989; 180: 1-15.
- [30] Shimamoto K, Lebrun B, Yasuda-Kamatani Y, *et al.* DL-threo-beta-benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Mol Pharmacol* 1998; 53: 195-201.
- [31] Shimamoto K, Sakai R, Takaoka K, *et al.* Characterization of novel L-threo-beta-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. *Mol Pharmacol* 2004; 65: 1008-15.
- [32] Davies LP, Johnston GA. D-aspartate oxidase activity in extracts of mammalian central nervous tissue. *J Neurochem* 1975; 25: 299-304.
- [33] Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 2000; 407: 189-94.
- [34] Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J Neurosci* 2001; 21: RC182.
- [35] Takamori S, Malherbe P, Broger C, Jahn R. Molecular cloning and functional characterization of human vesicular glutamate transporter 3. *EMBO Rep* 2002; 3: 798-803.
- [36] Bellochio EE, Reimer RJ, Freneau RT, Jr., Edwards RH. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 2000; 289: 957-60.
- [37] Freneau RT Jr, Troyer MD, Pahner I, *et al.* The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* 2001; 31: 247-60.
- [38] Freneau RT Jr, Burman J, Qureshi T, *et al.* The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc Natl Acad Sci USA* 2002; 99: 14488-93.
- [39] Herzog E, Bellenchi GC, Gras C, *et al.* The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J Neurosci* 2001; 21: RC181.
- [40] Malthe-Sorensen D, Skrede KK, Fonnum F. Calcium-dependent release of D-[3H]aspartate evoked by selective electrical stimulation of excitatory afferent fibres to hippocampal pyramidal cells *in vitro*. *Neuroscience* 1979; 4: 1255-63.
- [41] Drejer J, Larsson OM, Schousboe A. Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochem Res* 1983; 8: 231-43.
- [42] Potashner SJ. Uptake and release of D-aspartate in the guinea pig cochlear nucleus. *J Neurochem* 1983; 41: 1094-101.
- [43] Mine-Golomb D, Levy Y, Kleinberger N, Schramm M. D-[3H]aspartate release from hippocampus slices studied in a multi-

- well system: controlling factors and postnatal development of release. *Brain Res* 1987; 402: 255-63.
- [44] Cousin MA, Hurst H, Nicholls DG. Presynaptic calcium channels and field-evoked transmitter exocytosis from cultured cerebellar granule cells. *Neuroscience* 1997; 81: 151-61.
- [45] Rousseau SJ, Jones JW, Pullar IA, Wonnacott S. Presynaptic $\alpha 7$ and non- $\alpha 7$ nicotinic acetylcholine receptors modulate [3H]d-aspartate release from rat frontal cortex *in vitro*. *Neuropharmacology* 2005; 49: 59-72.
- [46] Aula N, Kopra O, Jalanko A, Peltonen L. Sialin expression in the CNS implicates extralysosomal function in neurons. *Neurobiol Dis* 2004; 15: 251-61.
- [47] Yarovaya N, Schot R, Fodero L, *et al.* Sialin, an anion transporter defective in sialic acid storage diseases, shows highly variable expression in adult mouse brain, and is developmentally regulated. *Neurobiol Dis* 2005; 19: 351-65.
- [48] Naito S, Ueda T. Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles. *J Biol Chem* 1983; 258: 696-9.
- [49] Naito S, Ueda T. Characterization of glutamate uptake into synaptic vesicles. *J Neurochem* 1985; 44: 99-109.
- [50] Maycox PR, Deckwerth T, Hell JW, Jahn R. Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. *J Biol Chem* 1988; 263: 15423-8.
- [51] Tabb JS, Ueda T. Phylogenetic studies on the synaptic vesicle glutamate transport system. *J Neurosci* 1991; 11: 1822-8.
- [52] Fykse EM, Iversen EG, Fonnum F. Inhibition of L-glutamate uptake into synaptic vesicles. *Neurosci Lett* 1992; 135: 125-8.

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