Homeostasis at brain synapses - options for drug targets

Proceedings of a symposium organized in conjunction with the EU sponsored research project “Dynamics of Extracellular Glutamate” DECG and the RCN sponsored CoE “Centre for Molecular Biology and Neuroscience” CMBN

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Basic research is a fundamental part of culture. It arises from the human drive to explore and understand. It is also a necessary requirement for advances in medical diagnosis and treatment. - “Almost everything we come in contact with in our daily lives has been developed or improved through basic science” (Fred Kavli).

The plan for the meeting at Losby Gods started as a statement in the exploitation and implementation plan of the EU RTD project “Dynamics of Extra-cellular Glutamate (DECG, QLG3-CT-2001-02004)”. The purpose was to discuss the results of the project and share them with the biomedical community. This purpose was enhanced by the contribution from other research environments studying synaptic homeostasis in conjunction with the Centre for Molecular Biology and Neuroscience (CMBN).

Sometimes people meet and make contacts that will last forever. At the Losby Gods meeting we wanted to make excellent scientist meet to sum up their collaboration and to present and discuss important data, doing this in a conducive atmosphere of beautiful surroundings and gastronomic and cultural entertainment. In spite of a tight program schedule, it was our impression that people managed to relax and interact in-between the scientific sessions. Thus we had a “symposion” in the real sense. This was the intention of the organizers.

Linda Hildegard Bergersen       Jon Storm-Mathisen

Cover: Fridtjof Nansen (1861-1930) in his lab at the Bergen Museum (photograph 1887 by Johan von der Fehr; reproduced by permission of the Norwegian Library, http://www.nb.no/baser/nansen/). Nansen, later renowned polar explorer, professor of oceanography, politician (Norway’s independence 1905), and humanitarian (Nobel peace prize 1922), was the first Norwegian neuroscientist and a founder of the neuron doctrine (Edwards JS & Huntford R 1998a F. Nansen and the origins of the neuron doctrine. Soc Neurosi Abstr 24:238; 1998b Fridtjof Nansen: from the neuron to the North Polar Sea. Endeavour 22:76-80). Using the Golgi method (which he went to Pavia to learn from the master) he discovered the “interlacing (not reticulation) of nervous fibrillae” and considered the neuropil as “principal seat of the nervous activity” (Nansen F 1887 The structure and combination of the histolgical elements of the central nervous system. Bergen Museums Aarsberetning for 1886, pp 25-214, XI plates [engraved on stone by Nansen], John Grieg, Bergen, 1887).
Aquaporins in the brain in health and disease

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The realization that water flux across cell membranes depends on membrane proteins rather than mere diffusion forces us to rethink the pathophysiology – and the potential for treatment - of the large number of clinical conditions that reflect perturbed water and salt homeostasis. Notably, brain edema formation cannot any longer be reduced to an issue of osmotic forces acting across a lipid bilayer but must be explained with reference to the properties and differential distribution of membrane proteins that admit water flux through their hydrophilic interior.

Aquaporin-4 is the predominant water channel in brain neuropil (1). There is now solid evidence that this protein is physiologically relevant and that it contributes to the water homeostasis in brain. Immunogold analyses (2) revealed a distinct subcellular compartmentation of this protein. In the brain, AQP4 was enriched in astrocyte plasma membranes facing capillaries and pia. Similarly, quantitative analysis of the specialized astrocytes in the retina (Müller cells) indicated that the endfeet membranes abutting on capillaries and corpus vitreum contained a tenfold higher concentration of AQP4 than non-endfeet membranes. AQP4 was also found in the basolateral membranes of ependymal cells and in the abluminal and adluminal membranes of endothelial cells (3). In contrast, neurons were devoid of AQP4 immunoreactivity. Müller cells show a precise colocalization between AQP4 and Kir4.1, suggesting that the two molecules act in concert in the process of K+ buffering. Physiological evidence for this has been obtained (4). In a pathophysiological context, AQP4 has been shown to modify the development of brain edema (5-7). This is in line with the fact that AQP4 is enriched at the interface between brain and blood.


Luc Pellerin

Provision of energy for synaptic transmission, importance of monocarboxylates

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For a long time, it was considered that brain energy metabolism strictly complies to a set of rules established over several years of investigation. These are 1) energy costs in the central nervous system are dominated by neuronal activity (~95%) while glia accounts for only a small fraction (~5%) 2) Glucose is the sole energy substrate used to support function of the adult brain 3) Complete oxidation of glucose provides all the energy necessary to support brain activity 4) Glucose utilization is tightly coupled to synaptic activity. Recently, results obtained by different approaches both in vitro and in vivo have revealed a more intricate picture and shed light on the importance of astrocytes in providing energy substrates, i.e. lactate, to active neurons. Thus it was shown that astrocytes respond metabolically to glutamatergic activity. As glutamate is released in the synaptic cleft, following its action on postsynaptic receptors, it is taken up into the astrocytes where it will be recycled in large part via its conversion into glutamine. Concomitantly to its uptake, an increase in intracellular sodium will take place, causing the activation of a specific subunit of the Na+/K+ ATPase, the alpha2, which colocalizes with the glial glutamate transporters GLAST and GLT1 in astrocytic fine processes surrounding glutamatergic synapses. Increased Na+/K+ ATPase activity in astrocytes is responsible for the enhancement in glucose utilization and lactate production that is observed both in vitro and in vivo.

A direct implication of this process is that lactate formed and released by astrocytes should represent a privileged energy substrate for neurons. Although numerous studies had already suggested that lactate could be an adequate energy substrate for neurons, it was only recently that it could be demonstrated using NMR that lactate is a predominant oxidative substrate for neurons even in presence of substantial amounts of glucose. In addition, because lactate is an hydrophilic substance, the presence of specific transporters allowing lactate to be released by astrocytes but also to be taken up by neurons should be demonstrated. A family of transporters shown to transport lactate has been described and collectively known as monocarboxylate transporters or MCTs. The distribution of three of its members, MCT1, MCT2 and MCT4, has been studied in the central nervous system both at the regional and cellular levels. It was shown that both MCT1 and MCT4 are strongly expressed by astrocytes, consistent with a role in mediating lactate efflux from astrocytes. In contrast, MCT2 which exhibits the highest affinity for lactate is the predominant neuronal monocarboxylate transporter. Interestingly, MCT2 was recently shown to be present in the postsynaptic density area of several glutamatergic synapses in the hippocampus and the cerebellum. Its colocalization with the GluR2/3 subunits forming AMPA receptors, not only in the postsynaptic density but also within the spine constituting an intracellular pool suggests that MCT2, like AMPA receptors, could undergo a process of exo/endocytosis at the postsynaptic membrane. Such a mechanism might allow to adjust energy supply to the level of synaptic activity.

Recent data obtained by two-photon microscopy monitoring NADH fluorescence with a subcellular resolution have provided support for the concept known as the astrocyte-neuron lactate shuttle. Thus, it was demonstrated in hippocampal slices that upon stimulation, an initial dip in NADH fluorescent signal was observed and corresponded to a mitochondrial response in dendrites. Such a change is consistent with early lactate utilization by neurons, lactate being taken from a preexisting extracellular pool. Then, a strong NADH signal emanating from astrocytes was identified, representing an important activation of glycolysis in this cell type in response to enhanced synaptic activity. The consequence would be a massive production of lactate that would contribute to replenish the extracellular pool. The overall result would be a net transfer of lactate from astrocytes to neurons in order to fulfill the fluctuating energy needs of active neurons. Although the concept that glucose represents the major energy substrate for the adult brain still remains valid under most circumstances, it appears quite likely now that neurons do not rely solely on glucose but that lactate provided by astrocytes, via the formation of an extracellular pool, most likely contribute to satisfy a large part of increasing neuronal energy consumption as they undergo enhanced activity.
Oleg Shupliakov

Recruitment of endocytic proteins to the periactive zone in the giant glutamate synapse in lamprey

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Synapses are specialized signaling units composed of a pre- and a postsynaptic element. The postsynaptic element contains neurotransmitter receptors and protein machinery involved in signaling and receptor trafficking. The presynaptic nerve terminal contains neurotransmitter filled synaptic vesicles aggregated at active zones. Recent studies have led to the definition of a novel compartment in the presynaptic nerve terminal, periactive zone. This region surrounds the active zone and has been known to be the site, or hot spot, at which clathrin-coated vesicles bud during synaptic vesicle endocytosis. More recently, this compartment has also been implicated in signaling during regulation of synaptic development.

We used the giant reticulospinal synapse in lamprey to study the function of the periactive zone during neurotransmitter release. The synaptic vesicle cluster in this glutamatergic synapse is separated by a large region of axoplasm that makes this preparation particularly well suited for the subcellular localization of synaptic proteins. Immunogold and microinjection techniques in combination with electron microscopy were employed in our studies.

It was found that several proteins such as synapsin, amphiphysin, dynamin, and intersectin were trafficking to the endocytic zone during synaptic activity. These proteins appeared to be involved at specific stages of endocytosis. In addition a reorganization of cytoskeleton occurred in the periactive zone. Compounds interfering with actin functions, including phallolidin, catalytic subunit of Clostridium botulinum C2 toxin, and N-ethylmaleimide-treated myosin S1 fragments were microinjected into the axon. Severe defects in synaptic vesicle trafficking and the clathrin-coated pit formation were observed. Thus, suggesting a critical role of actin both in endocytosis and transport of recycling vesicles from the site of endocytosis to the synaptic vesicle cluster.

Our results show that synaptic periactive zone is a dynamic compartment, which controls proper recycling of vesicles during synaptic activity.

Øyvind Hvalby

Bidirectional regulation of transmitter release induced by synapsins I and II

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The mechanisms responsible for short-term synaptic plasticity appear predominantly to reside presynaptically, where they partly depend on the efficacy of the exo/endocytotic recycling of synaptic vesicles. Both structural and functional studies support a prominent role for the synapsins, a family of neuronal phosphoproteins tightly associated with synaptic vesicles and the neuronal cytoskeleton, in regulating the size of the reserve vesicle pool. In preparations devoid of synapsins this effect has been described experimentally as a prominent short-term synaptic depression, presumably due to insufficient recruitment of vesicles from the reserve pool. In contrast, a possible role of synapsins in regulating release originating from the immediately releasable vesicle pool (RRP) remains unclear. In invertebrate, both exocytosis and dynamics of RRP recycling may be regulated by synapsin homologs. In mammals, synapsins are encoded by at least three genes, two of which give rise to synapsins I and II, both of which are strongly expressed in adult brain. Studies on transgenic mice where the genes encoding synapsins I and/or II had been inactivated have found unchanged synaptic transmission during low frequency stimulation of identified excitatory synapses.
Employing hippocampal slices from mice where the genes encoding synapsins I and/or II had been inactivated, we here show that synapsins I+II affect synaptic plasticity by modulating transmission deriving from the immediately releasable vesicle pool in the CA3-CA1 excitatory glutamatergic synapse. Moreover, our data indicate that, depending on stimulation frequency and levels of Ca²⁺, synapsin expression can both enhance and decrease this kind of plastic response.

We thank Dr. Paul Greengard, The Rockefeller University, for the kind gift of transgenic animals.

Inger Lise Bogen

Down-regulation of vesicular transport in transgenic mice devoid of synapsins I and II

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The synapsins are a family of vesicle-associated proteins involved in presynaptic development, synaptic vesicle recruitment and vesicle exocytosis. Mice lacking synapsins I and II are viable and fertile, but develop grand mal seizures after approximately 1 month of age. The neurochemical consequences of inactivation of synapsin genes are still not elucidated. We have examined the ability to transport amino acid neurotransmitters into synaptic vesicles in mice devoid of synapsins I and II. The vesicular uptake of glutamate was decreased to 59% of uptake in wild type (WT) littermates, while the uptake of GABA was decreased to 77% of uptake in WT. Moreover, investigation of the levels of vesicular transporters for glutamate (VGLUT-1,-2 and -3) and GABA (VGAT) in mice forebrain indicated that mice devoid of synapsins I and II showed major decreases (37 - 43% of WT) in VGLUT-1, VGLUT-2 and VGAT levels, while the level of VGLUT-3 was unaffected by the lack of synapsins (99% of WT). Our results indicate that, in mouse brain, a general absence of synapsins I and II decreases the concentrations of some, but not all, vesicular transporter proteins.

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Anders Kielland

Synapsin involvement differs among functional classes of thalamic synapses in mice

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The synapsin proteins, believed to be ubiquitously expressed in nerve terminals, may modulate the efficacy of synaptic transmission. We studied the involvement of synapsin I and synapsin II in the synaptic transmission at the two types of glutamatergic synapses on principal cells in the dorsal lateral geniculate nucleus. Synaptic transmission in mice with double knockout (DKO) mutations of synapsins I and II was compared with the transmission in the wild type (WT) mouse. At the retinogeniculate synapses, which transmit the primary afferent input to the cells, no effect of the synapsin inactivation was found, suggesting that the synapsins are not involved at this type of synapses. In contrast, at the corticogeniculate synapses,
which provide modulatory input to the principal cells, the DKO animals showed increased paired-pulse facilitation and increased depression of response during train stimulation compared to the WT. Thus, synapsins are involved in the transmission in these synapses. Immunohistological studies confirmed a differential involvement of synapsins in the two types of synapses. Retinogeniculate terminals were unlabeled when stained with antibodies against synapsin I and with antibodies against synapsin II, whereas both types of antibodies labeled corticogeniculate terminals. Previous work, have shown that synapsins are neither involved in the transmission between photoreceptors and bipolar cells, nor in the transmission between bipolar cells and retinal ganglion cells. Together with our data, this suggests that the synapsins are not involved in transmission of primary afferent input to cells in the vertical visual pathway up to the thalamic level. At least in this system, the synapsins seems to be involved only in synapses related to modulatory functions.

Simen Gylterud

Structural alterations in glutamatergic terminals in hippocampus of knock-out mice lacking synapsin I+II

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Synapsin I/II knockout mice develop epilepsy and hence alterations in the transduction of signals in the hippocampus are suspected. The synaptic vesicle-associated proteins synapsin I and II have been shown to take part in organizing synaptic vesicles at release sites. Possible roles are in the maintenance of what is referred to as a reserve pool of vesicles. We used gene knock-out mice lacking both synapsin I and synapsin II to examine the effect on the ultrastructure of nerve terminals in hippocampus. In the knock-out mice, glutamatergic synapses, such as those of the mossy fibers from granule cells of the dentate gyrus, which make synapses onto the proximal parts of the apical dendrites of CA3 pyramidal neurons, have only about half the normal density of synaptic vesicles in an area 100 to 500 nm away from the synapse. In other glutamatergic terminals, such as the Schaffer collateral/commissural synapses in the stratum radiatum of CA1, the density is down to two thirds at 100 nm away from the synapse. We proposed that the changes would impair the capacity to release glutamate and that the synapse might adapt by changing other factors in the signalling cascade. Preliminary findings with the immunogold method suggest a reduced labeling with antibodies against the calcium permeable AMPA subunit GluR1, both in mossy fiber terminals and in Schaffer collateral terminals. On the other hand, immunoreactivity to the AMPA subunit GluR2/3 was increased, but only in mossy fiber terminals. Our results confirm previous findings in other species/tissues that the density of vesicles is reduced in the absence of synapsins and suggest that there are alterations in the densities of receptor subtypes that might perturb the normal function of the synapse in certain conditions.

We thank Dr. P. Greengard, The Rockefeller University, New York, USA and Dr. H. T. Kao, Nathan Kline Institute, Orangeburg, New York, USA, for the gift of knock-out mice.
Immunogold detection of VGLUTs in hippocampal astrocytes

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Glutamate release from cultured astrocytes has been shown to be Ca2+ and clostridium toxin sensitive. However, no evidence for a vesicular compartment capable of glutamate release has been detected in astrocytes in the intact brain. Here we show, by use of electron microscopic immunogold techniques, that astrocytes (identified by labelling for the astrocytic glutamate transporters GLT and GLAST or by filaments) in the dentate gyrus contain synaptic-like vesicles resembling synaptic vesicles, inasmuch as they are small (about 30 nm in diameter), round and clear. In addition, like synaptic vesicles, the astrocytic vesicles express the vesicle-associated membrane protein cellubrevin together with VGLUT1 and VGLUT2. We also found that VGLUT positive astocytic vesicles were located close to neuronal membranes, of which some carry NMDA receptors. Our findings strongly suggest that astrocytes in the intact hippocampus contain a vesicular compartment competent of glutamate exocytosis and that the VGLUT-containing vesicles in the astrocytes could form sites for point-to-point transmission between astrocytes and neurons.

Andrea Volterra

Imaging vesicular exytosis in astrocytes with TIRFM: the role of prostaglandins

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We have recently shown that astrocytes possess synaptic-like microvesicles expressing vesicular glutamate transporters and the v-SNARE, cellubrevin. Such vesicles release glutamate via a Ca2+-dependent exocytosis process in response to physiological stimuli (Bezzi, Gundersen et al., Nat. Neurosci., 2004). Total internal reflection fluorescence (TIRF) imaging of vesicles tagged with VGLUT-EGFP and filled with the dye Acridine Orange enabled us to monitor individual vesicle fusion events in living astrocytes and to collect information on the kinetic properties of the secretory process evoked by activation of metabotropic glutamate receptors (mGluRs). Application of the group I mGluR agonist (DHPG, 100 M, 2 sec pulse) induced in the TIRFM field a single burst of Ca2+-dependent exocytosis of about 100 vesicles, which reached the maximal fusion rate within 100-200 ms and lasted for about 600 ms.

Prostaglandins (PGs), cyclooxygenase (COX) metabolites of arachidonic acid, are known to be produced in response to mGluR stimulation and to participate in the Ca2+-dependent process of glutamate release from astrocytes, both in cultured cells and in hippocampal slices (Bezzi et al., Nature, 1998; Bezzi et al., Nat. Neurosci., 2001; Pasti et al., J. Neurosci., 2001; Zonta et al., J. Physiol., 2003). However, it remains undefined whether PGs regulate specifically the glutamate exocytosis process and, if so, by which mechanism. To clarify these issues, here we use TIRF microscopy and study which consequences has the suppression of PGs formation with indomethacin (INDO, 5 µM), a broad-spectrum COX inhibitor, on the exocytosis process evoked by mGluR stimulation. In the presence of INDO, astrocytes respond to DHPG stimulation (as above) with: (a) a drastic reduction in the total number of fusion events; (b) a significant change in the time distribution of the residual fusions.

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Pascal Jourdain

An astrocyte control of excitatory transmission in hippocampus via P2Y1 Purinoreceptors

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A recent study has shown that astrocytes are able to release glutamate contained in vesicles via a mechanism similar to that described for synaptic release (Bezzi et al., Nature Neurosci., 7: 613-20, 2004). The question is to know if this glial glutamate is involved in synaptic activity. Here, we identify a modulatory role of astrocyte glutamate release in hippocampus via the activation of the P2Y1 purinoreceptors.

Thus, light and electron microscopy immunochemistry show that in the molecular layer of the dentate gyrus P2Y1 is localized preferentially in astrocytic processes directly apposed to asymmetric synapses. The specific P2Y1 receptor agonist 2MeSADP (10 µM) triggers glutamate release from acute hippocampal slices but not from hippocampal synaptosomes, suggesting that the release is of non-neuronal origin. The mechanism of release is blocked by a Ca²⁺ chelator, BAPTA/AM (50 µM) and by the cyclooxygenase blocker, indomethacin (1-5 µM). Ca²⁺- and prostaglandin-dependent glutamate release in hippocampus was shown to occur specifically from astrocytes (Bezzi et al., Nature, 391: 281-5, 1998). Importantly, this glial release is sensitive to bafilomycin A1, a blocker of exocytosis. Functionally, P2Y1 activation with 2MeSADP rapidly and reversibly increases mEPSC frequency (not amplitude) in granule cells, suggesting a modulatory role on synaptic transmission via a pre-synaptic control. This control is mediated by an NMDA-dependent pathway because AP5, an NMDA receptor antagonist, prevented the facilitatory effect of 2MeSADP. More specifically, it should involve NMDA receptors containing the NR2B sub-unit, because the effect of 2MeSADP is blocked also by the specific antagonist, ifenprodil (3-10 µM).

To confirm the impact of glial glutamate on synaptic activity, we have studied a direct connection between astrocytes and neurons by a double patch approach (Kang et al., Nat Neurosci., 1: 683-92, 1998). Preliminary data show that electrical stimulation of astrocytes triggers an increase of mEPSC frequency, similar to the one produced by 2MeSADP, in 20-30% of the recorded granule cells. This effect is blocked by ifenprodil, suggesting that glial glutamate controls mEPSCs via presynaptic NMDA receptors.

In conclusion, astrocyte glutamate release plays modulatory roles at granule cell synapses, revealing the active participation of astrocytes to the functioning of these synapses.

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Ragnhildur Káradóttir

Glutamate signalling to oligodendrocytes in brain slices

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Glutamate is reported to play a role in modulating oligodendrocyte development and also in causing damage to these cells in conditions like perinatal asphyxia/cerebral palsy, stroke, spinal cord injury and multiple sclerosis. We recorded from oligodendrocytes in the white matter of cerebellar slices at different developmental stages. Cell identity was confirmed by labelling with antibodies to NG2, O4 and MBP. Cells at all stages responded to glutamate by opening receptor-gated channels; the currents were potentiated by the glutamate transport blocker TBOA, indicating that the current was not produced mainly by transporters. In brain ischaemia glutamate release contributed in some cells to an increase in inward current which developed...
Pauline Cavelier

Tonic release of glutamate in hippocampal slices

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Tonic glutamate release may regulate the baseline excitability of neurons. It has been suggested to be non-vesicular and by cystine/glutamate exchange. We assessed tonic glutamate release by measuring the inward NMDA receptor-mediated current produced when glutamate transporters were blocked by TBOA. (Control experiments assessed the effects of the manipulation used on the NMDA receptors used to sense glutamate). This release was unaffected by reducing vesicular release with cadmium, but was potentiated by blocking glial glutamine synthetase. It was unaffected by blocking swelling-activated ion channels, prostaglandin-mediated release from astrocytes, P2X7 receptors or gap junction hemichannels. Superfused cystine increased glutamate release, but block of cystine/glutamate exchange had no effect on release in the absence of superfused cystine. DIDS reduced the release, and this was not mimicked by propionate, implying that this suppression did not simply result from the acidification of internal pH that DIDS produces.

David Attwell

Energy stores preventing anoxic depolarization and glutamate release in hippocampal ischaemia

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During brain ischaemia a fall of ATP level leads to a sudden run-down of transmembrane ion gradients (the anoxic depolarization or AD), which releases glutamate. By whole-cell clamping CA1 pyramidal cells, we investigated the energy stores which delay the AD in hippocampal slices when O2 and glucose are removed. With glycolytic and mitochondrial ATP production blocked, in P12 slices the AD occurred in ~7 mins at 33°C. Allowing glycolysis fuelled by glycogen, in the absence of external glucose, delayed the AD by 5.5 mins, while superfused glucose prevented the AD for >1 hour, even though glycolysis normally provides only 6% of the brain’s ATP. With glycolysis blocked, the latency to the AD was 6.5 mins longer when mitochondria were allowed to function, demonstrating that metabolites downstream of glycolysis (pyruvate and citric acid cycle intermediates) provide a significant energy store for oxidative phosphorylation. With glycolysis blocked but mitochondria functioning, superfusing lactate did not significantly delay the AD, showing that production of ATP from lactate is much less than that from endogenous metabolites. These data demonstrate a preferential role for glycolysis in preventing the AD.

Paikan Marcaggi

Purkinje cell mGluRs as spillover detectors at the parallel fibre synapse

Paikan Marcaggi and David Attwell
We have previously shown, by recording the AMPA receptor mediated fast EPSC, that activating many nearby parallel fibres leads to interactions between nearby synapses as a result of glutamate diffusing through the extracellular space (Marcaggi et al., 2003). We have now extended this analysis to the slow EPSC mediated by Purkinje cell mGluRs. High frequency trains of stimuli to the parallel fibres evoked slow EPSCs which increased in amplitude, when the stimulus strength was increased, more rapidly than did the fast EPSC, indicating an interaction between synapses in producing the slow EPSC. For a given size of fast EPSC, stimulating the granule cell axons in the granular layer evoked a much smaller slow EPSC than when stimulating in the molecular layer. Granular layer stimulation is expected to activate synapses that are more spatially dispersed on the dendritic tree than for molecular layer stimulation. Similarly, molecular layer stimulation evoked more short term, endocannabinoid-mediated, synaptic plasticity than did granular layer stimulation. These data indicate that the magnitude of activation of mGluRs is crucially dependent on the number of nearby synapses activated.

Marko Kreft

Properties of Ca^{2+}-dependent exocytosis in photoreceptors and cultured astrocytes

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Synaptic transmission at the photoreceptor synapse is characterized by continuous release of glutamate in darkness. Release is regulated by the intracellular calcium concentration ([Ca^{2+}]). We here examined the physiological properties of exocytosis in tiger salamander (Ambystoma tigrinum) retinal rods and cones. Patch clamp capacitance measurements were used to monitor exocytosis elicited by a rapid and uniform increase in [Ca^{2+}], by photolysis of the caged Ca^{2+} compound NP-EGTA. The amplitude of flash-induced increases in membrane capacitance (C_m) varied monotonically with [Ca^{2+}], beyond ~ 15 µM. Average rate constants of rapid and slow exocytotic responses were 420±168 s⁻¹ and 7.85±5.02 s⁻¹ respectively. Our results confirm that photoreceptors posses a large rapidly releasable pool activated by a low affinity Ca^{2+} sensor whose kinetic and calcium-dependent properties are similar to those reported in retinal bipolar cells and cochlear hair cells (Kreft et al., 2003; J. Neurophysiol. 90: 218-225.)

Astrocytes, a subtype of glial cells, apparently release the chemical transmitter glutamate and atrial natriuretic peptide via Ca^{2+}-dependent exocytosis. We here studied the properties of regulated exocytosis by electrophysiological measurements. Experiments show that astrocytes exhibit Ca^{2+}-dependent increases in membrane capacitance, with a Kd of ~ 20 µM [Ca^{2+}]. The delay between the flash delivery and the peak rate in membrane capacitance increase is in the range of tens to hundreds of milliseconds. The pretreatment of astrocytes by the tetanus neurotoxin, which specifically cleaves the neuronal/neuroendocrine type of SNARE protein synaptobrevin, abolished flash-induced membrane capacitance increases, suggesting that Ca^{2+}-dependent membrane capacitance changes involve tetanus neurotoxin-sensitive SNARE-mediated vesicular exocytosis. Immunocytochemical experiments show distinct populations of vesicles containing glutamate and atrial natriuretic peptide in astrocytes. We conclude that the recorded Ca^{2+}-dependent changes in membrane capacitance represent regulated exocytosis from multiple types of vesicles, which is around 100-times slower than the exocytotic response in photoreceptors and neurons (Zhang et al., 2004; J. Biol. Chem. 26;279(13):12724-33; Kreft et al., 2004; Glia 46: 437-445).
Matjaz Stenovec

The EAAT2 density in astrocyte surface membrane and Ca^{2+} regulated exocytosis

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Astrocytes are non-neuronal cells in the central nervous system, which like neurons are capable of releasing neuroactive molecules, such as glutamate, possibly via regulated exocytosis. In addition astrocytes represent a major cellular element for the removal of glutamate from the extracellular space. To study whether astrocytes posses the mechanism of regulated exocytosis we investigated the release of atrial natriuretic peptide (ANP) from cultured cortical astrocytes by confocal microscopy (Kržan et al., J. Neurosci. 23(5): 1580-1583, 2003). Briefly, astrocytes were transfected with a construct to express pro-atrial natriuretic peptide fused with the emerald green fluorescent protein (ANP.emd). As shown previously in other cell types, the transfection of astrocytes with ANP.emd resulted in numerous fluorescent puncta in the cytoplasm that represent secretory organelles. If atrial natriuretic peptide is released by exocytosis, in which the vesicle fuses with the plasma membrane, then the total intensity of the green fluorescing probe should decrease, while the vesicle membrane is incorporated into the plasma membrane. To monitor surface area changes we labeled the membrane with the FM 4-64 fluorescence probe. The application of ionomycin to elevate cytoplasmic [Ca^{2+}], increased the fluorescence intensity of FM 4-64, while that of ANP.emd decreased. These effects were not observed in the absence of extracellular Ca^{2+}, suggesting that ANP is released by regulated Ca^{2+}-dependent exocytosis from astrocytes.

These results prompted us to ask the next question: does activated exocytosis affect the density of glutamate transporters EAAT2? Astrocytes also play a major role in removal of glutamate that is cleared from cleft during synaptic transmission, mainly through glutamate transporters, such as EAAT2. The removal of glutamate depends among other factors also on the density of glutamate transporters. We investigated the putative involvement of exocytosis in glutamate transporter density regulation in the astrocyte plasma membrane by transfecting cells with a construct to express excitatory amino acid transporter 2-tagged with enhanced green fluorescent protein (EAAT2-EGFP). This construct predominantly labeled the astrocyte surface membrane. To monitor exocytosis we labeled the cell membrane with the FM 4-64. Addition of ionomycin to increase cytosolic Ca^{2+} strongly increased the FM 4-64 intensity of the plasma membrane, indicating the presence of regulated exocytosis in astrocytes. However, concomitant with Ca^{2+}-dependent FM 4-64 intensity increase, ionomycin-induced increase in EAAT2-EGFP intensity could not be detected. These results suggest that rapid translocation of EAAT2-EGFP to the plasma membrane is unlikely in these cells. Interestingly, while the fluorescence intensity of EAAT2-EGFP decreases in stimulated astrocytes and the surface area of the plasma membrane increases as monitored by FM 4 64, these data suggest that the density of EAAT2-EGFP molecules in the surface membrane diminishes due to Ca^{2+}-dependent exocytosis. A reduction of glutamate density in astrocyte membrane may affect the time course of synaptic transmission.

Maja Potokar

Fluorescently labeled vesicles show different types of mobility in astrocytes

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Astrocytes are glial cells of the central nervous system, which play an important role in the modulation of synaptic transmission and also in metabolic coupling between neurons and blood vessels. These cells also release many neuroactive substances, which are stored in membrane-bound vesicles and may play a role in synapse modulation and in the coupling between nervous activity and the local blood flow. However, the mobility of vesicles containing neuroactive substances has not been studied yet. Here we used green fluorescent protein-tagged preproatrial natriuretic peptide (ANP) to label single vesicles. Vesicles containing pro-ANP fused with the emerald green fluorescent protein (ANP.emd) were recently found to be connected with Ca<sup>2+</sup>-dependent regulated exocytosis (Kržan et al., J. Neurosci. 23(5): 1580-1583, 2003). To accurately track labeled vesicles we developed a custom-made software tool to analyse movements of single vesicles in images recorded every 300 ms. We examined the length and velocity of vesicle movements. The average length vesicles travelled in a 15 s interval was 5.8 ± 0.1 µm and the average velocity 0.4 ± 0.007 µm/s (n = 300). We found two different types of vesicle mobility. In one group, vesicles remained close to the origin of tracking, while in the other group vesicles displayed apparent directional mobility from the origin of tracking. The average diffusion constant of these two groups of vesicles differed by one order of magnitude between rapid (6.8 x 10<sup>-10</sup> cm<sup>2</sup>/s) and slow (1.4 x 10<sup>-11</sup> cm<sup>2</sup>/s) vesicles. The average velocity of rapid vesicles resembles the velocity of anterograde transport in neurons. Since the mean square displacement (MSD) of slow vesicles is a linear function of observation time, we conclude that they move by free diffusion process. In contrast, in rapid vesicles the MSD relationship with time more closely resembles a quadratic function, which indicates that in addition to free diffusion other mechanisms of vesicle transport may contribute to rapid vesicle mobility in astrocytes. We currently investigate the role of cytoskeleton in vesicle mobility in astrocytes.

Niels Christian Danbolt

The enigma of the glutamate transporter in glutamatergic nerve endings


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The relative contribution of synaptic terminals to total brain glutamate uptake is still debated. By incubating hippocampal slices in D-aspartate (a poorly metabolizable glutamate transporter substrate) and studying the distribution of D-aspartate-like immunoreactivity electron microscopically, we show that around half of the total uptake of externally added substrate is into nerve terminals, the ones making asymmetrical synaptic contacts in particular. The uptake into terminals displays the substrate selectivity typical of EAAT2 and is absent in EAAT2-knockout mice. Dendrites and neuronal cell bodies had low uptake activity. The proportion of uptake into nerve terminals was even higher in “synaptosomal” preparations (which also contain glia). Approximately 10% of the total EAAT2 protein in rat slices (after incubation in vitro) is distributed in nerve terminal membranes. Using a reconstituted assay, we show that heteroexchange is faster than net uptake. This difference in translocation rates partly explains the mismatch between EAAT2 distribution and activity. Thus, astrocytes represent the major site of net uptake, but terminals with higher internal levels of glutamate mediate more rapid heteroexchange of transporter substrates. These data reconcile previously conflicting data and suggest that most synaptically released glutamate will be taken up by glia.
Silvia Holmseth

The determination of the concentrations of the glutamate transporter EAAT3 (EAAC) in brain tissue and unexpected cross-reactivities of antibodies directed to EAAT3

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The central nervous system (CNS) expresses five different glutamate transporter proteins (EAAT1-5). EAAT1 and EAAT2 are predominantly astroglial proteins and are essential for maintaining low resting levels of extracellular glutamate and for protecting neurons against excitotoxicity. The roles of the other three transporters remain elusive. EAAT4 and EAAT5 are predominantly expressed in the cerebellar Purkinje cells and in the retina, respectively, while EAAT3 is expressed in neurons throughout the CNS. Here we have produced a variety of antibodies to EAAT3 and subjected them to extensive specificity testing. Several of them recognized non-EAAT3 proteins in addition to or in stead of EAAT3. One particular stretch of the EAAT3-sequence gave rise to antibodies recognizing tubulin in spite of the absence of primary sequence homology. This illustrates the importance of proper specificity testing of antibodies. The best antibodies were used to immunoisolate EAAT3 protein and to determine the concentrations of EAAT3 protein in absolute terms (µg/mg tissue) in the major CNS regions of young adult rats by quantitative immunoblotting using known amounts of the pure protein as standards. The total concentration of EAAT3 is about 100-fold lower than that of EAAT2.

Dimitri Kullmannn

Relevance of glutamate dynamics to epilepsy

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Agonists of ionotrophic glutamate receptors are powerful chemoconvulsants, and antagonists have anticonvulsant activity. This, together with the assumption that seizures result from an imbalance of excitation and inhibition, has led to the view that glutamate is intimately related to epilepsy. What is the evidence that altered glutamate dynamics play a role in epilepsy?

In support of such a link, deletion of the mouse glial glutamate transporter GLT (although not GLAST or EAAC1) is associated with severe seizures. Observations from anti-sense experiments have led to a different conclusion: knock-down of EAAC1, but not GLT, has been reported to cause seizures, which may result from impaired GABA synthesis in inhibitory terminals. Pharmacological blockade of glutamate transporters has not been reported to cause seizures, although the specific blocker TBOA causes spontaneous oscillations in immature cortex, both in vitro and in vivo.

In order to reconcile these apparently conflicting observations, it is important to bear in mind that impaired glutamate clearance potentially has multiple effects, including desensitisation of synaptic receptors, enhanced activation of presynaptic metabotropic receptors, and potentiated activation of extra- and hetero-receptors. Many of these effects are likely to reduce, and not increase, excitatory signalling in the cortex.

In vivo experiments suggest that seizures can be preceded and accompanied by elevations in glutamate. Is glutamate clearance impaired in epilepsy? Conflicting evidence exists about the effect of human or experimental temporal lobe epilepsy on the density of glutamate transporters. We have recently adapted a
quantitative method to detect inter-synaptic crosstalk mediated by NMDA receptors to attempt to detect spillover of glutamate between lateral and medial perforant path synapses on dentate granule cells in the rat. Significant cross-talk was not be detected in control tissue. We repeated the experiments on tissue obtained from rats that had spontaneous seizures three weeks after pilocarpine-evoked status epilepticus. In this sample, significant cross-talk between lateral and medial perforant path was detected. Although this result is consistent with impaired glutamate uptake, alternative explanations, including changes in the distribution of glutamate receptors, or in the anatomical relationships between synapses and astrocyte membranes, cannot be excluded.

Glutamate dynamics thus play an important although complex role in epilepsy.

Vidar Gundersen

Does manipulation of glutamate pathways have a role in the treatment of parkinson’s disease?

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Parkinson’s disease is a chronic neurodegenerative disorder. The treatment with levo-dopa is effective at early stages of the disease. However, advanced stages show reduced effect of levo-dopa. In addition side effects such as hyperkinesias develop. This urges the need of alternative treatment strategies that can bypass the dopamine system. One such strategy is to play with glutamatergic signaling systems. Drugs acting as NMDA receptor inhibitors have so far showed moderate efficiencies, although some positive effects in the treatment of dyskinesias have been reported. Drugs acting at metabotropic glutamate receptors look promising in animal models of parkinsonism, but no clinical trials have been published. Another strategy is invasive treatment with deep brain stimulation of the subthalamic nucleus (that consists mainly of glutamatergic neurons), which show robust and long lasting effects on tremor, rigidity and bradykinesia.

Dimitri Rusakov

Strategic positioning of glutamate transporters and rapid glutamate signaling

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The time course of neurotransmitter in the synaptic cleft determines the kinetics of rapid synaptic signals. Hippocampal mossy fibres (MFs), a well-characterised excitatory pathway from dentate granule cells to the hippocampus proper, form large glutamatergic synapses at branched spiny structures (thorny excrescences, TE) on CA3 pyramidal cell dendrites. We apply multi-photon microscopy to image rapid, action potential driven Ca²⁺ signals in large MF boutons that occur in stratum lucidum. We find that these boutons are electrically isolated from the granule cell soma (which is 500-1000 µm away) suggesting an important role for the intracleft glutamate in both post- and presynaptic ionotropic receptor signalling. To assess the kinetics of intracleft glutamate at MF synapses, we use a simple geometrical approximation representing the characteristic architecture of the TE-MF interface and use Monte Carlo simulations to monitor movements of individual glutamate molecules released within the cleft. Simulation results predict that, in the absence of neuronal glutamate transporters, it should take approximately 10 ms for 50% and 60-70 ms for 90% of glutamate molecules to escape the MF synapse. We then show that adding neuronal glutamate transporters that occupy only 10-15% of the cleft volume would reduce the glutamate transient many-fold. Glutamate transients at MF synapses, therefore, could be tightly controlled by neuronal (pre- or postsynaptic) glutamate transporters.
Knut Petter Lehre

Quantitative distribution of glutamate and GABA transporters

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In order to investigate the roles of transporters in the regulation of neurotransmission, we are producing models of the synaptic environment. The aim is to have ultrastructural 3-dimensional models describing transmitter diffusion pathways and the distribution and absolute amounts of transporters in the cell membranes. These structural models will be the basis for development of mathematical models, allowing computer simulations of transmitter diffusion and receptor activation. The amount of transporters in tissue subregions is measured by quantitative immunoblotting, using pure transporter proteins as standards. Surface areas of membranes expressing the proteins are measured in serial electron micrographs. In collaboration with Dmitri Rusakov, we have previously published a model describing the average glutamate transporter distribution at central synapses. Computer simulations based on that model show that glutamate is more rapidly removed at the postsynaptic than the presynaptic part of the synapse. To provide the experimental data required for our future models, we have recently determined the tissue concentrations of the EAAC glutamate transporter and are currently working on quantification of GABA transporters.

Fabio Grohovaz

PKCγ as a coincidence detector of glutamate-induced signals in hippocampal neurons

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Activation of protein kinase C (PKC) is a key step in synaptic plasticity and neurodegenerative processes. The main actor appears to be PKCγ, the neuronal specific isoform that belongs to the conventional PKC family (cPKCs). Although it is widely recognized the role of calcium and diacylglycerol in cPKC activation, little is known about the intracellular signalling pathways responsible for the production of these two second messengers in neurons.

We focused on the characterization of the signaling steps leading to translocation of PKCγ at post-synaptic level in hippocampal pyramidal neurons, upon glutamate receptor stimulation. For this purpose, cultured neurons were transfected with GFP-tagged PKCγ and with GFP-tagged diacylglycerol binding domain (C1 domain), used as fluorescent biosensor for this second messenger.

The plasma membrane translocation of the fluorescent constructs was explored by total internal reflection microscopy (also known as evanescent wave microscopy), an imaging technique that allows appreciating plasma membrane translocation of GFP-tagged constructs even in the thinnest neuronal processes, thanks to a resolution on the z axis higher than 100 nm. This approach was combined with fura-2-videoimaging, in order to simultaneously monitor variations in intracellular calcium concentration.

We show that the DAG necessary to sustain PKCγ translocation can be produced by either stimulation of metabotropic glutamate receptors, or activation of PLCδ by strong calcium influx via N-methyl-d-aspartate (NMDA) glutamate receptors or voltage-operated calcium channels (VOCCs). These findings might explain
why mildly stimulated neurons, but not those exposed to strong activity, require the participation of metabotropic glutamate receptors to produce long term potentiation.

Franca Codazzi

Sphingosylphosphocholine-induced glutamate release: a role in neuronopathic Niemann-Pick type A disease

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Niemann-Pick type A is a genetic disease characterized by the absence of a functional ASM gene and the abnormal accumulation of sphingomyelin. Under these conditions, also sphingosylphosphocholine (SPC, a sphingomyelin metabolite) accumulates in various tissues, including brain, where it is thought to act as a toxic stimulus, contributing to the appearance of the neurological symptoms. We have studied the effects of sphingosylphosphocholine onto astrocytes and neurons, the two main cellular targets in the central nervous system. In particular, we have investigated the possibility that SPC principally acts on astrocytes and that this represents the first step leading to neurodegeneration. Our results show that acute administration of SPC to astrocytes in culture induces activation of a plasma membrane receptor with ensuing release of Ca$^{2+}$ from intracellular stores. In addition, we demonstrate that SPC-stimulated astrocytes release glutamate which, in turn, promotes [Ca$^{2+}$] elevations in adjacent neurons. Finally, we observed that chronic exposure to SPC can drive astrocytes to a reactive state, leading them to proliferate and to release inflammatory mediators such as TNFalfa.

In conclusion, we present data in favour of a direct effect of SPC on astrocytes, the most abundant cells in the CNS. Acute and chronic stimulation of glial cells by SPC might then represent the trigger for the neurodegenerative process typical of Niemann-Pick type A disease.

Announced in the program but not presented:

Yvette Dehnes

Conformationally sensitive cysteine mutants in intracellular loop 3 of the dopamine transporter

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The dopamine transporter (DAT) is responsible for the inactivation of released dopamine through its reuptake at the plasma membrane. DAT is also the major molecular target of several psychoactive drugs, including cocaine. We have previously shown that Cys342 of the intracellular loop 3 (IL3) is conformationally sensitive (Ferrer et al., 1998; Chen et al., 2000). In an effort to investigate the properties and putative roles of the residues throughout IL3, we made a background DAT construct in which we mutated all intra- and extracellular cysteines (X7C). Twenty cysteine mutants in IL3 from Phe332 to Ser351 in the X7C background were stably expressed in EM4 cells. The affinity of most of the mutants for binding the tritiated cocaine analog [3H]MFZ 2-12 was similar to that of X7C. However, the binding properties of several mutants (F332C, S334C, Y335C and D345C) were severely altered, with affinities 100-1000 fold lower than X7C. A similar pattern of apparent affinity changes was seen for cocaine- and mazindol binding, as well as for tyramine uptake. Interestingly, in S334C the presence of 10 µM Zn$^{2+}$ stimulated uptake and
dramatically lowered the apparent $K_m$ for tyramine. Similar effects of Zn$^{2+}$ were observed with Y335A, K264A, D345A, and D436A (Loland CJ et al., 2002; Loland CJ et al., 2004). Further, the methanethiosulfonate (MTS) derivatives (-EA, -ET and -ES), dramatically inhibited [$^3$H]MFZ 2-12 binding in membrane preparations of a number of the IL3 Cys mutants (F332C, S333C, S334C, Y335C, N336C, N340C, M342C, D345C and T349C). Cocaine and dopamine protected S333C, N336C, M342C and T349C from reaction with the MTS reagents. The protection was seen at 4 °C as well as RT. In summary, many positions in IL3 appear to be conformationally sensitive, as illustrated by the change in MTS-reactivity upon cocaine and dopamine binding, and the Zn$^{2+}$ dependent reversal of a mutation-induced shift of the conformational equilibrium in the transporters.

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Recycling and packaging of transmitter glutamate: glutamine transporters and vesicular glutamate transporters
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Sustained synaptic transmission requires replenishment of the transmitters in the synaptic vesicles. It is generally believed that the amino acid glutamine is the precursor for the fast transmitters glutamate and GABA. We have characterized a family of glutamine transporters involved in the recycling of glutamine. The Na$^+$ dependent translocation of glutamine by SN1 is coupled to countertransport of H+. Thus, the electroneutral transport generates shallow gradients enabling the protein to work in both directions depending on the concentration gradients. The homologues SAT1/ATA1/SA2 and SAT2/ATA2/SA1 have lost such coupling to H+, and are thus able to generate much higher concentration gradients energized by the electrochemical gradient of Na$^+$. SAT1 and SAT2 are enriched on a subset of nerve terminals. We find specific targeting of SN1 to glial processes surrounding some of these synapses. Thus, our data suggest that these transporters are involved in the glutamate/GABA-glutamine recycling, and that due to differentiated expression of these transporters a functional diversity exists among synapses. A different family of proteins, VGLUT1-3, transporting glutamate into the synaptic vesicles, has been identified on the vesicular membranes. We detect high levels of VGLUT2 expression during early development. Developmentally transient expression of VGLUT3 has been detected in a variety of cells, including non-glutamatergic ones. VGLUT3 co-expresses with the vesicular transporter proteins for monoamines (VMAT2), acetylcholine (VACHT) and GABA (VGAT) in select neuronal populations at specific developmental stages. Our data suggest complex modulatory roles of glutamate.

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