Glutamatergic and GABAergic gliotransmission

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LIST OF PAPERS

Paper I

Ormel, L., Stensrud, M. J., Bergersen, L. H., & Gundersen, V. (2012). VGLUT1 is localized in astrocytic processes in several brain regions. Glia, 60(2), 229-238.

Paper II

Ormel, L.*, Stensrud, M. J.*, Chaudhry, F. A., & Gundersen, V. (2012). A distinct set of synaptic‐like microvesicles in atroglial cells contain VGLUT3. Glia, 60(9), 1289-1300.

Paper III (manuscript)

Ormel, L., Lauritzen, K. H., & Gundersen, V. GABA, but not bestrophin 1, is localised in astrocytic processes in the hippocampus and the cerebellum.

*these authors contributed equally to the work

AIMS OF THE THESIS

1. Are astrocytes competent of releasing glutamate by exocytosis?
   Do astrocytes contain vesicular glutamate transporters (VGLUTs)?
   Do astrocytes in different brain regions contain VGLUTs?
   Do single astrocytes contain more than one VGLUT?

2. Are astrocytes competent of releasing GABA by non-exocytotic routs?
   Do astrocytes contain the Ca^{2+} activated Cl^- channel bestrophin 1?

INTRODUCTION

The glutamatergic synapse

An axon projecting from a nerve cell ends in a convex structure called the terminal or the bouton (Kristen and Harris, 2012). The terminal opposes a spine, a convex structure projecting from the dendrite belonging to another nerve cell (Kristen and Harris, 2012). These two structures are divided by a 20 nm synaptic cleft consisting of extracellular matrix (Kristen and Harris, 2012).

Synaptic vesicles of 40 nm in diameter are located within the terminal (Kristen and Harris, 2012). The vesicles express vesicular glutamate transporter 1 (VGLUT1) or vesicular glutamate transporter 2 (VGLUT2) in their membrane, depending of the brain area (Fremeau et al, 2001). VGLUT1 (described in more detail later) and VGLUT2 (not discussed here) transport glutamate into the vesicles by an electroctrochemical gradient made by an ATP driven proton pump (Fremeau et al, 2001). Also located in the vesicle membrane is synaptobrevin and synaptotagmin (Südhof, 2008). Together with SNAP-25 and syntaxin, which are both located on the presynaptic membrane, these four proteins are essential for attaching synaptic vesicles to the presynaptic membrane and exocytosis of glutamate (Südhof, 2008; Südhof, 2012).

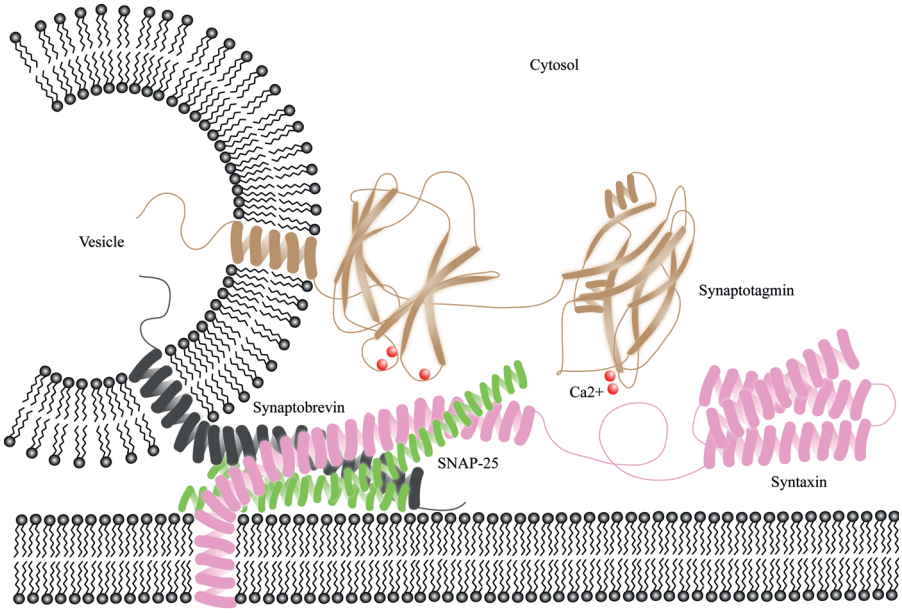

Figure 1: Exocytotic machinery-synaptotagmin, synaptobrevin , syntaxin and SNAP-25

Exocytosis of glutamate is initiated by arrival of an action potential in the terminal. The action potential activates voltage-dependent calcium channels (VDCC) located in the presynaptic membrane (Dunlap et al, 1995). Opening of VDCC leads to an influx of calcium that binds to the C2 region of synaptotagmin, which will then interact with the phospholipids in the presynaptic membrane and induce assembly of the SNARE proteins (Südhof, 2008). In turn, this will accomplish fast and synchronous vesicle exocytosis of glutamate into the synaptic cleft (Chapman, 2008; Pang and Südhof, 2010; Südhof, 2004). Neurons lacking synaptotagmin will still be able to exocytose neurotransmitters, although it will be slow and asynchronous (Chapman, 2008; Pang and Südhof, 2010; Südhof, 2004).

Exocytosed glutamate will diffuse over the synaptic cleft and bind to glutamate receptors on the membrane of the dendritic spine facing the terminal (the postsynaptic membrane). The glutamate receptors located on the postsynaptic membrane are mainly of the AMPA and the NMDA receptor types (Dingledine et al, 1999). The AMPA receptor is a fast executing potassium and sodium channel (Dingledine et al, 1999). The influx of sodium leads to a fast excitatory postsynaptic potential (EPSP). The NMDA receptor is a sodium and calcium channel (Dingledine et al, 1999). In

contrast to the AMPA receptor, the NMDA receptor only opens when the membrane is depolarized and need binding of a coagonist on the glycin binding site for maximum effect (Dingledine et al, 1999). This preconditioning of the membrane to open the NMDA receptor channel and the fact that opening of the channel increase the intracellular level of calcium, which also can function as a second messenger, indicates that activation of NMDA receptor have other consequences than just induce EPSP (Dingledine et al, 1999).

The EPSP causes a depolarization of the membrane of the spine, but one EPSP is rarely enough to mediate an action potential. However, if several EPSP reach the same dendrite within the time its membrane reach the resting potential, either through high frequent exocytosis from one terminal (temporal summation) or single exocytosis from many terminal facing different spines on the same dendrite (spatial summation) the dendritic membrane may be depolarized above the threshold (Yuste, 2013; Takagi, 2000). This will open voltage gated sodium channels and give an action potential.

At a membrane potential of 55 mV the sodium equilibrium is reached and sodium channels inactivates (Bean, 2007). The reduced permeability to sodium and the prolonged opening of voltage dependent potassium channels due to the raised voltage will repolarize the membrane and bring it back to resting potential (Bean, 2007).

If only an EPSP is induced the duration of the EPSP is dependent of desensitization of the receptor channels. AMPA receptors are desensitized more rapidly than NMDA receptors, but within the two groups of receptor there are variations due to the compositions of subunits (Dingeldine et al, 1999).

The tripartite synapse

A concave astrocytic process enclaves the terminal and the adjacent spine. Together this unit is called the tripartite synapse (Araque et al, 1999).

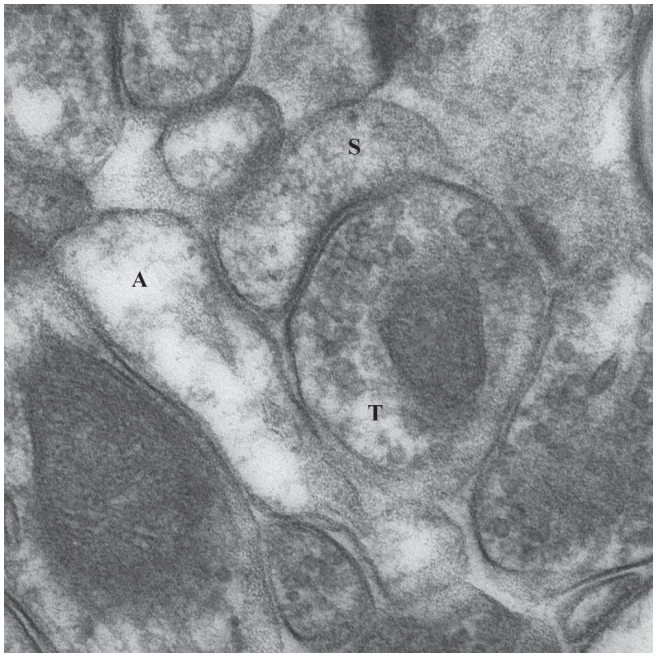

Figure 2: Electromicrograph of the tripartate synapse-terminal (T), spine (S) and astrocyte (A)

Astrocytes have traditionally been regarded to play a "passive" role in synaptic transmission. However, during the last years many important functions for neuron-astrocyte signaling have been discovered. After synaptic release glutamate diffuses passively away from the synaptic cleft to the extrasynaptic space (Danbolt, 2001). To maintain an efficient diffusion of glutamate it is important to keep a low concentration of glutamate in the extrasynaptic space (Danbolt, 2001). The only way to do this is by an active glutamate uptake (Danbolt, 2001). This is mainly accomplished by glutamate transporters located in the membrane of astrocyte processes surrounding the synapse, the perisynaptic astrocyte processes (Danbolt, 2001). It is important to keep the intrasynaptic and extrasynaptic concentration of glutamate low both to avoid sustained glutamate receptor activation (keeping "signal/noise ratio" high) and toxic effects of high glutamate concentrations (Danbolt, 2001). The most abundant plasma membrane glutamate transporter in astroglial cells in the hippocampus is GLT (EAAT2) and in the cerebellum GLAST (EAAT1) (Danbolt, 2001). Within the astrocyte the glutamate is either converted to glutamine through glutamine synthetase or enter the Krebs cycle via conversion to α-ketoglutarat by glutamate dehydroxylase (Danbolt, 2001). Glutamine is transported out of the astroglial cell, taken up by the terminal and either converted to glutamate or used as an energy substrate (Danbolt, 2001).

During the repolarization of the neuronal elements of the synapse there will be an increase in extracellular potassium concentration. In order to keep a constant potassium concentration astroglial cells take up the excess of potassium ions (Walz, 2000).

The astroglial glutamate uptake and the potassium uptake are established properties of these cells. Although these properties are vital to the synaptic function, they only have an indirect impact on synaptic signaling. Subsequently the astroglial cell was regarded as a non-signaling cell for many years. The last 20 years it has been revealed that astroglial cells both respond to neuronal signaling and release transmitter substances referred to as gliotransmittors. Thereby the name: the tripartite synapse. Many release mechanisms have been suggested, only a few will be mentioned here, with the emphasis on astroglial exocytosis of glutamate.

Glutamate release from astrocytes

Exocytosis

To initiate a calcium dependent fusion of an astrocytic vesicle with the plasma membrane, the astrocyte is dependent on a calcium sensitive protein. In neurons synaptotamgin I and II are the main proteins that initiate exocytosis of synaptic vesicles (Südhof, 2004). However, different studies show different results concerning the main subtype of synaptotagmin in astrocytes. The conclusion of most studies, both in cultures and in the intact brain is that synaptotagmin IV is expressed by astrocytes (Crippa et al, 2006; Malarkey and Parpura, 2011; Potokar et al, 2008; Zhang et al, 2004; Stenovec et al, 2007). However, other subtyper, eg synaptotagmin I, has also been detected in astrocytes (Maienschein et al, 1999; Colombo and Bentham, 2006). To be noted is that Mittelsteadt et al (2009) showed that different astrocytes express different subtypes of synaptotagmin, which might explain some of the discrepancy in the results. Whether synaptotagmin IV is a calcium sensor that can mediate calcium dependent exocytosis is a matter of debate.

When synaptotagmin binds calcium it initiates vesicular fusion and assembly of the SNARE complex, which is necessary to complete the fusion. Synaptobrevin, which is attached to vesicles, is one of the units in the SNARE complex. In cultured astrocytes both synaptobrevin 2 and 3 have been found (Mothet et al, 2005; Crippa et al, 2006;

Zhang et al, 2004b), while most in vivo studies have shown synaptobrevin 3, but no synaptobrevin 2 (Chilcote et al, 1995; Bezzi et al, 2004; Schubert et al, 2011).

The proteins of the SNARE complex located in the plasma membrane, syntaxin and SNAPs, has also been detected in astrocytes. Syntaxin 1 is shown to be present in both culture studies (Parpura et al, 1995; Calegari et al, 1999), as well as in one in vivo study (Schubert et al, 2011) of astrocytes.

The last component of the ternary SNARE complex is SNAP. Like in neurons some culture studies have localized SNAP-25 in astrocytes (Jeftinija et al, 1997; Maienschein et al, 1999), while other culture studies and in vivo studies found its homologue SNAP-23, but not SNAP-25 (Crippa et al, 2006; Yasuda et al, 2011; Schubert et al, 2011; Hepp et al, 1999). An explanation to this discrepancy might be that astrocytes express SNAP-25 abundantly when cultured, in contrast to astrocytes in intact tissue, which only express small amount of SNAP-25, but considerable amount of SNAP-23 (Wilhelm et al, 2004).

Like in neurons, exocytosis of glutamate by astrocytes is initiated by a cytosolic rise in calcium. The main mechanism of cytosolic calcium increase in astrocytes is thought to be through binding of glutamate to metabotropic glutamate receptor (mGlurR) type 1 or 5, or to purinergic ATP receptors (eg metabotropic P2Y2 receptors) (Verkhratsky et al, 1998). These metabotropic receptors are G-protein coupled and the G-protein activates phospholipase C, which generates inositol (1,4,5)-triphosphate ($InsP_3$) (Verkhratsky et al, 1998). The $InsP_3$ binds to $InsP_3$ receptors on the endoplasmic reticulum (ER) and subsequently release stored calcium from the ER (Verkhratsky et al, 1998). Although this mechanism is well described there are several other routes for calcium increase in the astrocyte cytosol:

1. Ionotropic receptors

   The AMPA (lacking GluR2) and NMDA glutamate receptors, as well as the purinoceptor 2x (P2x), are shown to be located in the plasma membrane of the astrocyte and by binding of glutamate and ATP, respectively, could lead to an influx of extracellular calcium to the cytosol (Verkhratsky et al, 1998).

2. Voltage gated calcium channels (VGCC)

One studie have indicated the presence of VGCC in the plasma membrane of the astrocyte (Latour et al, 2003). Although astrocytes do not form action potentials, studies have shown that high extracellular potassium concentrations, lead to a depolarization of the astrocyte and opening of VGCCs (Yaguchi and Nishizaki, 2010). It has also been suggested that the influx of extracellular sodium through ionotropic channels may lead to a depolarization of the retinal glial cell and opening of VGCCs (Linnertz et al, 2011). So far the VGCCs are not found in the Bergman glia cells (Verkhratsky et al, 1998).

3. Sodium-calcium exchanger (NCX)
   The NCX is located in the plasma membrane of astrocytes and is working bidirectional (Reyes et al, 2012; Paluzzi et al, 2007). The direction of the NCX is dependent of the trans-membrane sodium gradient and the plasma membrane potential. An increase of cytosolic sodium, like when ionotropic receptors are activated, will lead to an inward calcium current and transport of sodium to the extracellular space (Reyes et al, 2012; Paluzzi et al, 2007).

4. Mitochondrial regulation of cytosolic calcium
   Mitochondria have the ability both to take up cytosolic calcium through uniporters and to release calcium through sodium-calcium exchangers and permeability transient pores (Demaurex et al, 2009). Glial mitochondria have been shown to both limit and enhance the calcium level in astrocytes (Boitier et al, 1999; Reyes and Parpura 2008**)**. Whether glial mitochondria release calcium and thereby initiate glutamate release or take up calcium to limit glutamate release is unclear.

VGLUTs

When I started my PhD project it was known that vesicles, ranging from about 30 nm (synaptic-like microvesicles (SLMV)) (Bezzi et al, 2004; Jourdain et al, 2007; Stigliani et al, 2006), 30 nm-110nm (Crippa et al, 2006), 50-170 nm (Stenovec et al, 2007; Xu et al, 2007) to over 300 nm (Chenet al, 2005), and large dense core vesicles (Calegari et al, 1999; Coco et al, 2003) could be located in astrocytes. Such vesicles represent the glial organelles thought to exocytose gliotransmitters. In addition,

lysosomes were shown to undergo exocytosis glutamate (Jaiswal et al, 2007; Li et al, 2008).

Moreover, the three vesicular glutamate transporters, VGLUT1, VGLUT2 and VGLUT3 were all located in astrocytes (Stenovec et al, 2007; Montana et al, 2004; Kreft et al, 2004; Anlauf and Derouiche 2004, Bezzi et al 2004; Zhang et al 2004b, Stigliani et al 2006; Potokar et al, 2009; Fremeau et al, 2002; Crippa et al, 2006; Ni et al 2009; Liu et al, 2011; Martineau et al, 2013; Cali et al, 2014). Of these, one electron microscopical study has revealed VGLUTs in association with astrocytic vesicles in intact brain tissue (Bezzi et al, 2004). The latter study showed that VGLUT1 and VGLUT2 positive SLMVs were located in glial processes enclosing glutamatergic synapses (Bezzi et al, 2004). Further corroborating the idea that VGLUTs are localized in astrocytes was that functional studies using the VGLUT inhibitors trypan blue and rose bengal, as well as the proton pump inhibitor bafilomycin A reduced glutamate release from astrocytes (Liu et al, 2011; Montana et al, 2004; Zhang et al, 2004b). However studies on cultured astrocytes from cortex and hippocampus have shown colocalization of VGLUT1 with lysosomal markers (Li et al, 2008).

Common for all VGLUTs are that they have channel like properties connected to glutamate transport (Tabb et al, 1992). The driving force for transporting glutamate into vesicles by VGLUTs is the high intravesicular proton level. Although it is important to emphasize that it is not the pH gradient itself, but the electrical gradient across the vesicle membrane, which is made by the high proton level, that initiate glutamate transport into to the vesicle. The pH gradient is accomplished by a H+-ATPase which create a proton influx to the interior of the vesicle (Naito and Ueda, 1985). Besides being dependent upon ATP, the efficiency of VGLUT1 seems to be affected by the cytosolic chloride level (Tabb et al, 1992).

VGLUT1

The VGLUT1 protein consists of 560 amino acids and has 6-8 membrane spanning segments (Ni et al, 1994). In adults VGLUT1 is found in neuronal terminals mainly in the cerebral cortex, cerebellar cortex and hippocampus. VGLUT1 is also present in more central nuclei, such as thalamus, hypothalamus and striatum, whereVGLUT2 is the dominant VGLUT, as well as amygdala where VGLUT1 and VGLUT2 is equally

present (Fremeau et al, 2001). At birth neuronal VGLUT1 is absent or at very low levels in rats and mice (Boulland et al, 2004; Fremeau et al, 2004). During postnatal development VGLUT1 increases progressively until adulthood (Boulland et al, 2004; Fremeau et al, 2004).

Among the VGLUTs, VGLUT1 is the least glutamate sensitive with a Km of 3,4mM (Herzog et al, 2001). However, VGLUT1 has the fastest kinetics with a Vmax of 500 pmol/mg (Herzog et al, 2001). An increase in expression of VGLUT1 in neurons has shown increased release of glutamate from each vesicle (Wojcik et al, 2004). Overexpressing VGLUT1 in astrocytes has no effect on their exocytotic glutamate release, which might be due to the rather high Km value of VGLUT1 and the rather low glutamate level in the astrocytes (Ni amd Parpura, 2009). However, overexpression of VGLUT3 in astrocytes, as well as increasing cytosolic glutamate levels, caused increased glutamate release (Ni and Parpura, 2009).

Mice lacking VGLUT1 show no phenotypic differences from their littermates the first 2 weeks after birth (Wojick et al, 2004; Fremeau et al, 2004). After two weeks of age the VGLUT1 knock out mice fed poorly and they started to die after 3 weeks of age, unless they were separated from their littermates and supplied with food and water (Fremeau et al, 2004). If they survived the critical period between 3-4 weeks after birth they could live for months, but showed a phenotype of blindness, incoordination and enhanced startle response (Fremeau et al, 2004).

VGLUT3

VGLUT3 is structurally highly similar to VGLUT1 (72,5%). It consists of 589 amino acids and has eight membrane spanning segments (Takamori et al, 2002). At the protein level VGLUT3 is localized in the hippocampus (pyramidal cell layer, dentate gyrus, granule cell layers, between stratum radiatum and lacunosum moleculare), cerebral cortex, striatum, amygdala, cerebellum, nucleus accumbens, thalamus, hypothalamus, substantia nigra, brain stem and inner hair cells (Herzog et al, 2004; Fremeau et al, 2002; Boulland et al, 2004; Seal et al, 2008). Unlike VGLUT1, VGLUT3 is frequently colocalized with other vesicular transporters such as the vesicular GABA transporter (VGAT), vesicular monoamine transporter (VMAT) and vesicular acetylcholine transporter (VAChT). For instance, in the hippocampus (pyramidal cell layer and granular cell layer) VGLUT3 colocalizes with a

subpopulation of GABAergic terminals, although not all VGLUT3 positive structures were GABAergic (Herzog et al, 2004; Fremeau et al, 2002; Boulland et al, 2004). In the striatum VGLUT3 has been found in cholinergic neurons (Herzog et al, 2004; Boulland et al, 2004). VGLUT3 has also been found at non synaptic sites such as Bergman glia cells in the cerebellum, neuronal axons from the raphe nuclei and dendrites in the striatum (Fremeau et al, 2002; Boulland et al, 2004). The level of VGLUT3 increases after birth, but no regional changes are seen (Boulland et al, 2004), although it is observed transient expression of VGLUT3 in cell bodies of the paraventricular zone, terminal-like structures of the cerebellar nuclei and terminal-like structures in the paraolivary nucleus (Boulland et al, 2004).

VGLUT3 has similar properties considering glutamate transport as VGLUT1 (Takamori et al, 2002; Schäfer et al, 2002; Gras et al, 2002). VGLUT3 has a Vmax of 20,3pmol/mg and a Km ranging from 0,52mM to 1,5mM making VGLUT3 the slowest and most glutamate sensitive of the VGLUTs (Gras et al, 2002; Fremeau et al, 2002). In contrast to VGLUT1, increased expression of VGLUT3 in astrocytes elevates glutamate release from astrocytes (Ni and Parpura, 2009). This might be due to the rather low Km and the low Vmax of VGLUT3. Since VGLUT3 has low Km it could be sensitive to even the low concentrations of glutamate in the astrocytes. Since VGLUT3 has a low Vmax the existing level of VGLUT3 in astrocytes is probably not sufficient to fill the vesicles with glutamate.

Knockout VGLUT3 mice suffer from total hearing loss (Seal et al, 2008; Ruel et al, 2008). The hearing loss is due to the loss of vesicle filling with glutamate in the inner hair cells (Seal et al, 2008; Ruel et al, 2008), leading to an impairment of neurotransmission at the inner hair cell afferent terminals, where VGLUT3 is the only VGLUT present (Seal et al, 2008; Ruel et al, 2008). In coherence with this, one study has revealed gene mutations in the VGLUT3 gene in humans suffering from deafness (Ruel et al, 2008).

Non-vesicular release of glutamate from astrocytes

Volume regulated anion channels (VRAC) are, among other types of cell, localized in the cell membrane of astrocytes and were initially found to release anions due to cell swelling under pathological conditions (Kimelberg et al, 2006). VRACs have also been shown to release anions, like glutamate, following calcium rise in astrocytes

under physiological conditions (Takano et al, 2005; Liu et al, 2009). In these two studies the calcium rise was induced by P2Y ATP receptors and bradykinin receptors, respectively.

As mentioned P2X receptors create a calcium influx upon ATP binding to the receptors. In addition one study have shown that the P2X receptor also creates an efflux of anions like glutamate (Duan et al, 2003).

Under pathological conditions, like ischemia, astrocytic glutamate transporters located in the plasma membrane (excitatory amino acid transporters (EAATs) can be reversed and thereby release glutamate (Rossi et al, 2000). Unlike other physiological mechanisms to release glutamate the reversion of astrocytic glutamate transporters are calcium independent.

Hemichannels in astrocytes have been shown to release gliotransmitters. Among the most studied astrocytic hemichannels are connexin 43 and pannexin 1. Connexin 43 has so far only shown to release glutamate under pathological conditions, i.e. independent of intracellular calcium levels (Ye et al, 2003; Spray et al, 2006; Jiang et al, 2011). However, pannexin 1 seems to release ATP under physiological conditions (Iglesias et al, 2009). In xenopus oocytes pannexin 1 has shown to open in response to increased concentrations of cytoplasmic calcium (Locovei et al, 2005). So far pannexin 1 in astrocytes have only been studied considering ATP release, although it is likely that pannexin 1 also release glutamate (Malarkey and Parpura 2008).

GABA release from astroglia

GABA has been found in Bergman glial cells and hippocampal astrocytes under physiological conditions (Le Meur et al, 2012; Gundersen et al, 2001; Blomqvist and Broman, 1988). Astroglial GABA can stem from cellular uptake through the GABA transporters (GATs). GAT1 is found in neurons and asroglial cells in many structures of the brain, among them hippocampal astrocytes and cerebellar Bergman glial cells (Borden 1996; Morara et al, 1996; Yan et al, 1997). GAT3 is also found in the brain, although whether GAT3 is found in glial cells in hippocampus and cerebellum is disputed (Borden 1996; Ribak et al, 1996). GATs contribute to an increased intracellular level of GABA. This, however, will depend on the distance from a GABAergic source, for instance a GABAergic terminal, and the efficiency of the

GABA metabolizing enzyme GABA-α-ketoglutaric acid aminotransferase (GABA-T) within the astroglial cell. An alternative source for intraglial GABA is glial production through glutamic acid decarboxylase (GAD). GAD is mainly found in GABAergic terminals, but has also been found in hippocampal astrocytes and cerebellar Bergman glia cells (Lee et al, 2011; Benagiano et al, 2000).

Unlike the EAATs, GATs can be reversed also under physiological conditions (Richerson and Wu, 2003). Thus, glial cells containing GATs can potentially release GABA. One culture study has shown release of GABA through GATs upon AMPA stimulation, while a slice study have shown GABA release through GATs induced by changes in extracellular GABA concentration (Gallo et al, 1991; Barakat and Bordey, 2002). Both studies have been done on cerebellar glial cells. GABA can also be released through volume sensitive anion channels when P2X7 receptors are activated (Wang et al, 2002). Based on the presence of hemichannels in astrocytes and that GABA is small enough to pass through a hemichannel, hemichannels could have a potential role in the release of GABA, even this is not shown in any study.

Finally, studies have shown that astrocytes in the stratum radiatum of CA1 hippocampus and Bergman glia cells in the cerebellum release GABA through the calcium sensitive anion channel bestrophin 1 (Lee et al, 2010; Yoon et al 2011). The glial release of GABA from these channels lead to tonic inhibition (Lee et al, 2010; Yoon et al 2011). The authors found that there was a higher density of GABA positive cerebellar astroglial cells than hippocampal astrocytes, proposing that this could explain the observed higher level of tonic currents in the cerebellum compared with the stratum radiatum in the CA1 hippocampus (Yoon et al 2011).

Bestrophin 1

Bestrophin 1 consists of 585 amino acids and forms 4 transmembrane helices, a transmembrane reentrant loop and the N- and C-termini within the cytoplasma (Tsunenari et al, 2003). Milenkovic et al represented an alternative topology without the transmembrane reentrant loop (Milenkovic et al, 2007). The presence of the protein in different tissues is sparsely studied. So far the bestrophin 1 protein has been found in retinal pigment epithelium (RPE), colon epithelium, kidney epithelium and epithelial cells of airways in human and mouse (Marmorstein et al, 2000; Bakall et al, 2003; Kunzelmann et al, 2009; Soria et al, 2006). Recently, bestrophin 1 was also

found in the cerebellum and the hippocampus in the mouse brain (Lee et al, 2010; Park et al, 2009). Among other tissues, in both human and mouse, there is high RNA expression of bestrophin 1 in the testis (Krämer et al, 2004; Pifferi et al, 2006; Stöhr et al, 2002). Few developmental studies on Bestrophin 1 have been done, although one study shows that Bestrophin 1 is absent in retinal pigment epithelium cells until the mice are 10 days old (Bakall et al, 2003).

Bestrophin 1 is a chloride channel, where the second transmembrane domain is involved in forming the pore (Sun et al, 2001; Tsunenari et al, 2003). The single channel conductance of drosophilia Bestrophin 1 is ≈ 2pS (Chien et al, 2006). Bestrophin 1 is a calcium activated channel with a K_d of ≈ 210nM calcium (Qu et al, 2003). Although it resembles the classical calcium activated chloride channels there are some important differences. Bestrophin 1 is not voltage dependent and does no exhibit outward rectification (Hartzell et al, 2005). Besides calcium, Bestrophin 1 is also stimulated by hypoosmotic solutions (Fischermeister and Hartzell, 2005). Since hyperosmotic solutions inhibit bestrophin 1, it is proposed that Bestrophin 1 might be a type of VRAC (Fischermeister and Hartzell, 2005). There are, however differences, for instance the kinetics and the rectification in normosmotic solutions (Fischermeister and Hartzell, 2005). Bestrophin 1 is also less sensitive to hyposmolarity than VRAC (Fischermeister and Hartzell, 2005). There are also some studies indicating that bestrophin 1 is regulated by phosphorylation (Duran et al, 2013; Duta et al 2006).

The cellular function of bestrophin 1 is not clear or at least not uniform among cell types. In RPE bestrophin 1 has been localized close to the basolateral plasma membrane (Marmorstein et al, 2000). Initially it was thought that bestrophin 1 was located in the basolateral plasma membrane to mediate chloride release and creating a light peak (Marmorstein et al, 2000). Later studies, however, have shown unchanged chloride release from the RPE cells when bestrophin 1 is either knocked out or mutated (Marmorstein et al, 2006; Zhang et al, 2010). It has therefor been suggested that bestrophin 1 is located in the membrane of endoplasmic reticulum close to the basolateral membrane in RPE cells working as a counter ion channel for calcium release (and reuptake) from the endoplasmic reticulum (Barro-Soria et al, 2010; Strauß et al 2012). The calcium released from endoplasmic reticulum activates other

chloride channels in the basolateral membrane of RPE (Barro-Soria et al, 2010). In epithelial cells of the airways, bestrophin 1 has been shown to be localized in the basolateral membrane and function as chloride channels (Duta et al, 2004).

Mutations in the human bestrophin 1 gene are closely related to Best vitelliform macular dystrophy (Marquardt et al, 1998; Petrukhin et al, 1998). The mechanisms of how mutations in bestrophin 1 cause this disease are still unclear. Bestrophin 1 knockout mice do not present any retinal pathology or any other gross pathology, as well as they breed normally (Marmorstein et al, 2006). Why there is a discrepancy between the bestrophin 1 phenotype of humans and mice is not certain. Mice are, however, nocturnal and do not have macula, and therefore it might be difficult to compare mice with humans. In addition, mice might have other chloride channels, which probably could substitute the functions of bestrophin 1.

METHOD

Immunocytochemistry

In our studies we used antibodies to localize the proteins of interest. Antibodies consist of two heavy and two light chains. At the terminal end of the heavy and light chain pair is the paratope. The paratope recognize specific structures. Which structures varies among different antibodies. The variability is mainly accomplished by loops of β strands refered to as complementarity determining regions (Sela-Culang 2013). The ability of antibodies to be designed to recognize specific structures make them ideal to visualize cellular proteins. However, some antibodies show cross-reaction with other epitopes. To reveal any cross-reaction (unspecific binding) we controlled our experiments with knockout animals and western blots. Typically, cross-reacting antibodies label knockout and wild type equally and show more than one band on western blot. Other antibodies do not recognize a protein in the tissue even if their paratope should recognize a peptide from the protein (Holmseth 2005). In such cases negative tissue labelling needs to be controlled by labelling tissue known to contain the proteins or by methods independent of the antibody.

All experiments included in this thesis were performed on in vivo tissues. This was done to avoid exogenous factors that could manipulate the expression of astrocytic proteins. Several concerns have been admitted to in vitro studies since they not always seem to capture the nature of in vivo tissue (Kimelberg 2000).

Confocal microscopy

Crosstalk is a phenomenon where the same laser excites two fluorophores of different wavelength and the emitted light are captured in the same picture. To avoid crosstalk we chose fluorophores distant in wavelength. Although there are only small amounts of VGLUTs in astrocytes we chose fluorophores with wavelength within visible range. We also chose appropriate emission filters to make shore we only obtained light from one fluorophore. Finally, the signals from the two fluorophores were captured sequentially.

For optimal resolution one should use a pinhole of 1 Airy unit. However, with such a narrow pinhole some information will be lost. In our studies we therefor used a pinhole of 1,2 Airy unit. Since optics tens to convolute image information this would

create out of focus blurres light. To optimize the resolution we deconvolved the confocal pictures. Deconvolution would reassure the borders of astrocytes and terminals by removing the out of focus blurred light. Image J or Autoquant accomplished the deconvolution.

The confocal pictures are captured by a digital camera producing a picture composed of pixels. The size of the pixels applied is crucial for how well the details of the structures are preserved in the picture. We therefor applied the pixel size according to Nyquist theorem, both in x, y and z axis.

Immunogold

Mainly epitopes on the tissue surface are labelled by the immunogold method. That is because the Lowicryl HM20 resin is hydrophobic and does not allow antibody penetration into the section (Bergersen et al 2008). This will lead to a lower labelling density than found in for instance confocal microscopy. However, it will result in a more reliable quantification since all epitopes are equally available for binding to the antibodies.

The measurements of VGLUT gold particles are only an indirect value of the actual amounts of VGLUT epitopes found in astrocytes. There are two main reasons for this: 1) The VGLUT antibodies will not attach to all VGLUT epitopes in the tissue. 2) Many epitopes will be “hidden” in the section and not reached by any antibodies. The fact that we only obtain two-dimensional electron micrographs will not disturb the immunogold results. According to Delesse principle the proportion of a compound in an area is the same as the proportion in a volume. Since the astrocytic perisynaptic processes are cut at different locations more or less of vesicle clusters would be present and though more or less epitopes. To avoid that this could give a false mean labelling density we obtained many micrographs in each study. However, the fact that more or less epitopes are represented in the sections would lead to a wide variation.

Due to the complex of the primary and the secondary antibody the distance from the epitope to the centre of the gold particle will be approximately 30 nm (Bergersen et al, 2008). This means that the epitope could be anywhere within this distance from the centre of the gold particle. It is, however, possible to find out what structure the epitope most likely is located on. This is possible by measuring the structure that

appear most frequently in the distance of 30 nm from the centre of the gold particle. In our case we investigated whether the astrocytes have VGLUT positive vesicles and therefor only measured the amount of gold particles that could represent labelling of epitopes on vesicles.

Real time polymerase chain reaction

In this thesis we used two-step PCR, where cDNA synthesis and the amplification occur in two different tubes. Based on the findings in the confocal experiments of bestrophin 1 we expected to detect little bestrophin 1 RNA and therefore the two-step method was more suitable since it is more sensitive than the one-step method.

The amount of RNA in the samples was quantified by the standard curve method and normalized with the housekeeping gene GAPDH. The standard curve method was chosen since we do not know the amplification efficiency of bestrophin 1. We had too few animals to statistically analyse whether there was a significant difference in the bestrophin 1 RNA signal between bestrophin 1 knockout and wild type. However, in all wild type samples the bestrophin 1 RNA level was equal or lower compared to the level in the knockout samples, strongly suggesting that the bestrophin 1 signal in the wild type brain was at background level. Besides the measurement of the RNA amount was important to control the quality of the bestrophin 1 primer. A high level bestrophin 1 RNA in the positive control indicates that the primer worked. A low level or none bestrophin 1 RNA in the negative control suggests a high selectivity.

SUMMARY OF RESULTS

Paper I

"VGLUT1 is localized in astrocytic processes in several brain regions"

In this article we studied the presence of VGLUT1 in astrocytic processes. Using confocal microscopy we visualized VGLUT1 labelling within large astrocytic processes in the dentate-molecular layers, the stratum radiatum of CA1 hippocampus, the frontal cortex, and the striatum. With immunogold cytochemistry we found a significant level of VGLUT1 positive gold particles in astrocytic perisynaptic processes in the above mentioned brain regions. More than 95% of the VGLUT1 positive gold particles in astrocytic processes were within 25 nm from the membrane of synaptic-like microvesicles. These findings indicate that astrocytes in the studied brain regions can acumulate glutamate into microvesicles through of VGLUT1.

Paper II

"A distinct set of synaptic-like microvesicles in astroglial cells contain VGLUT3"

In this article we studied the presence of VGLUT3 in astrocytic processes. By confocal microscopy we found VGLUT3 labelling within large astrocytic processes of the CA3 hippocampus and the frontal cortex, as well as Bergmann glia processes of the cerebellum. Using imunogold cytochemistry we found a significant level of VGLUT3 positive gold particles in astrocytic perisynaptic processes in these brain regions. The VGLUT3 gold particles were in most cases closer than 30 nm from synaptic-like microvesicles, although it was not the same population of synaptic-like microvesicles that were labelled for VGLUT1. These findings suggest that astrocytic processes in the above mentioned brain region have a subpopulation of synaptic-like microvesicles that can store glutamate due to the presence of VGLUT3.

Paper III

"GABA, but not bestrophin 1, is localised in astrocytic processes in the hippocampus and the cerebellum"

In this article we studied the presence of GABA and bestrophin 1 in astrocytic processes. Both astrocytes from CA1 hippocampus and Bergmann glia from the

cerebellum showed GABA labelling with confocal microscopy. There was a higher density of GABA positive hippocampal astrocytes than cerebellar Bergmann glia in the confocal study. Although immunogold cytochemistry showed a significant level of GABA positive gold particles in hippocampal and cerebellar astroglia, the level of GABA was very low, approximately 0,3mM. The concentration of GABA was similar in hippocampal astrocytes and cerebellar Bergmann glia.

Using confocal microscopy bestrophin 1 showed the same faint labelling of astrocytes and Bergmann glia in wild type and bestrophin 1 knockout brains. Western blots of brain tissue showed no significant bands neither in wild type nor bestrophin 1 knockout tissue. There was, however, a band in the testis at the appropriate molecular weight. RT-PCR of hippocampus and cerebellum showed very low levels of bestrophin 1. These levels were equal to the level of bestrophin 1 mRNA in bestrophin 1 knockouts and were therefore considered to be background signals. To conclude, we found that astroglia contain low, but significant, levels of GABA and that the level of bestrophin 1 in the brain is very low. This points to a release mechanism for GABA from astrocytes that do not involve bestrophin1.

DISCUSSION

My results suggest that amino acid release from astrocytes can happen through regulated exocytosis. I show that astrocytes contain vesicles, resembling synaptic vesicles, and that they contain vesicular glutamate transporters, the VGLUTs. In search for proteins that can release amino acids by non-exocytotic mechanisms, I studied the localization of bestrophin 1 in the brain, without finding any firm evidence for its presence.

Methodological considerations

Immunocytochemistry is dependent on high sensitivity and selectivity of the antibodies to produce reliable results. The VGLUT1 and VGLUT3 antibodies used in the articles have high sensitivity, i.e. they strongly label glutamatergic asymmetric and GABAergic symmetric terminals, respectively. The selectivity is also high. The antibodies show only one band with appropriate molecular weight on western blots of brain tissue. Even if other proteins have the same molecular weight and the antibodies could theoretically cross-react and label totally different proteins, the selective labelling of "appropriate" structures in the brain tissue support the idea that the antibodies are selective. However, the most important tool to evaluate the selectivity of the astrocyte labelling is the knockout versus wild type comparison. The VGLUT3 antibodies showed a very weak labelling of astrocytes in the VGLUT3 knockout, and a strong VGLUT3 labelling of astrocytes in wild type tissue, indicating that the VGLUT3 labelling of the astrocytes was highly selective. The VGLUT1 antibody did also produce one single band on Western blots of brain tissue. However, there was some immunogold labelling in the VGLUT1 knockout tissue, both in nerve terminals and in astrocytic processes, although the difference in VGLUT1 labelling between wild type and knockout tissue was significant. Concerning the findings in VGLUT1 knockout and wild type they could be explained by two factors. Firstly, probably due to suboptimal perfusion fixation of the young mice, the tissue morphology of the VGLUT1 knockout and the wild type was of suboptimal quality. This gave a lower overall tissue labelling intensity than in rat tissue from the same brain structures and lead to a lower VGLUT1 labelling in astrocytes from wild type tissue. Secondly, we compared only VGLUT1 positive labelled structures in wild type and VGLUT1 knockout. This difference is probably an underestimation of the true difference, as

there are more astrocytic processes devoid of VGLUT1 labelling in the knockout compared to the wild type. The experiment with preembedding immunoperoxidase histochemistry therefore better reflects the specificity of the VGLUT1 antibody. In this experiment the number of VGLUT1 positive astrocytic processes in VGLUT1 knockout and wild type was counted in a comparative area. The experiment showed a high level of VGLUT1 positive astrocytic processes in wild type, while only a very few astrocytic processes contained VGLUT1 staining in the VGLUT1 knockout.

The selectivity of the two bestrophin 1 antibodies from Marmorstein and Schreiber is a bit more intricate to evaluate due to the negative findings in my experiments and the sparse number of studies showing the location of bestrophin 1. However, the above mentioned antibodies both showed similar labelling patterns with dense labelling of astrocytic nuclei and a "spotted" labelling in the grey matter neuropil, with no distinct resemblance to any micro-anatomical structure. Moreover, both types of antibody showed weak and rather similar labelling of astrocytes in bestrophin 1 knockout and wild type brains. These findings indicate that the observed bestrophin 1 staining in the brain is unspesific. In further support of this is the finding on Western blots of brain tissue, which did not reveal any specific bestrophin1 band, but some "unspecific" bands, which were also present in Western blots of betrophin1 knockout brains. These "negative" results could be due to the possibility that the bestrophin 1 antibodies not recognize the bestrophin 1 protein in the brain. However, both types of antibodies showed labelling of the retinal pigment epithelium and labelling that could represent Sertoli cells in the testis (Marmorstein et al, 2006; Petrukhin et al, 1998). They also gave a strong band at appropriate molecular weight on Western blots of testis tissue. Thus, the bestrophin 1antibodies used in the present thesis seem to recognize the bestrophin 1 protein. The sensitivity of the antibodies based on the confocal studies is difficult to interpret since the concentration of bestrophin 1 in retinal pigment epithelium and Sertoli cells have not been evaluated. Is the sensitivity of the antibodies too low to discover bestrophin 1 in the brain and particularly in astrocytes? From the work with the antibody based techniques in this thesis I cannot strictly rule out this possibility. Given a low sensitivity of the antibodies we wanted to detect bestrohin 1 with a method not depending on the use of antibodies. In this case we used real time PCR, which will be discussed later.

Confocal microscopy was used to evaluate qualitatively the VGLUT1, VGLUT3 and bestrophin 1 labelling in large astrocytic processes. The purpose of using confocal microscopy in the VGLUT1 and VGLUT3 study was mainly to support the findings of immunogold electron microscopy. Despite a low frequency of labelled astrocytes the high sensitivity and selectivity of the VGLUT1 and the VGLUT3 antibodies indicates that the fluorescence signals represent genuine astrocytic presence of VGLUT1 and VGLUT3. The lack of VGLUT1 labelling in the VGLUT1 KO mouse with the immunofluorescence method supports this notion. A low level of positive fluorescence labelling should always be interpreted carefully if using quantitative methods. Some quantitative colocalization methods could even be inappropriate, like Pearson's correlation coefficient, depending on the proteins to be correlated. For instance Li et al 2013 used Pearson to evaluate the colocalization of S100β, a cytosolic protein, with VGLUT1, a vesicular protein. Since Pearson achieves the best correlation coefficient when the two fluorophores show the same intensity in the pixels they appear together, it is unlikely that a cytosolic and a vesicular protein will colocalize by this method. S100β and VGLUT1 belong to different compartments of the cell and their signal will not appear in the same pixels and as expected the Pearsons correlation coefficient will be low. There is not so much knowledge about unique astrocytic vesicular proteins and quantitative colocalization studies might seem difficult to accomplish. Besides, in whole tissue studies there always will be some noise. The noise, which is often a weak unspecific (not authentic) labelling, can easily mask the real labelling if the real labelling is rare, resulting in a non-correlation result (Bolte and Cordelieres, 2006). Schubert et al 2011 seemed to solve the abovementioned problems. However, this study had a more frequent labelling of tissue elements than represented in our VGLUT1 and VGLUT3 study. A final problem affecting VGLUT1 confocal experiments is that astrocytes are closely apposed to the abundantly expressed VGLUT1 terminals. The light from a fluorophore is spread concentrically and due to a low lateral resolution VGLUT1 labelling in the terminal can falsely be seen at the border of adjacent small astrocytic processes. Deconvolution can partly solve this problem. However, since deconvolution removes some of the signal, it could be hard to know whether too much, to little or a proper amount of the signal is removed. Together, these were the reasons why we only examined the colocalization of VGLUT1 and GS in large astrocytic processes even after deconvolution. In the case of bestrophin 1, where the

labelling of bestrophin 1 knockout and wild type showed the same pattern and intensity in astrocytes and the brain tissue in general, we did not go any further with quantitative immunofluorescence or immunogold experiments. If there is a quantitative difference in the bestrophin 1 signal between the wild type and bestrophin 1 KO tissue we would expect a clear visual difference between knockout and wild type tissue, like in the VGLUT1 immunoflourescence experiment. This was not the case for bestrophin 1.

The immunogold method is suitable for quantitative measurements of proteins in small astrocytic processes. Small astrocytic processes are easily recognized due to their localization and their morphologic features. In rare occasions some spines can mimic small astrocytic processes. To avoid inclusion of spines, the tissue was labelled with GLT1/GLAST or glutamine synthetase to ensure that we included astrocytic processes in the analyses. The centre of a 10 nm gold particle could be approximately 30 nm away from an antigen. Thus, gold particles found within perisynaptic astrocytic processes represent labelling of antigens within the astrocyte. The problem is to interpret in which type of astrocytic organelle the antigen is present. It could be located anywhere within the radius of about 30 nm in the horizontal or vertical plane. Finally, on the electron micrographs, although they are few, we find small vesicular like structures with approximately 30 nm in diameter. Over 90% of the VGLUT1 and VGLUT3 gold particles are below 30 nm away from the outline these vesicular structures, a strong indication that VGUT1 and VGLUT3 in astrocytes are located in the membrane of vesicular structures. These vesicular structures could be mistaken for transversely cut endoplasmatic reticulum, but we do not find VGLUT1 or VGLUT3 in relation to any allantoid structure. In the rat immunogold study we analysed only astrocytic processes positive for VGLUT1 and VGLUT3. This selection was made because a substantial part of the processes probably lack VGLUTs (Bezzi et al, 2004) and we wanted to examine the level of VGLUTs in the VGLUT containing processes to that in e.g. excitatory nerve terminals. Inclusion of negative labelled astrocytic processes would falsely disturb this analysis. We also compared the positive labelled astrocytes with background labelling over mitochomdria and found that the density of VGLUT labelling in astrocytic processes was lower than in nerve terminals, but significantly higher than background labelling. Comparing only positive labelled astrocytes with a mixture of negative and positive labelled background mitochondrial

structures could seemingly give a false positive result. However, the true background labelling is given by both labelled and unlabelled profiles. Including only positive labelled background would give a falsely high background and not represent the actual background. Based on the present findings we do regard that the results in the rat brain represent a significant level of VGLUT1 and VGLUT3 and not false positive labelling.

Quantitative real time PCR (qRT-PCR) is a highly sensitive method for detecting the RNA level from a specific gene (Wong and Medrano, 2005). As discussed above the bestrophin 1 antibodies showed low selectivity and their sensitivity was difficult to interpret. Due to its high sensitivity and selectivity qRT-PCR was a suitable method to test the uncertainty in the confocal experiments. As expected we found no evidence for bestrophin 1 RNA in the bestrophin 1 knockout tissue. In consistence with the confocal experiments this was also the case for the wild type tissue. The tissue used for the qRT-PCR was whole tissue from the cerebellar cortex, which consists of several cell types. If there are low levels of bestrophin 1 RNA in cerebellum the bestrophin 1 RNA could have been diluted and given a false negative result. However, in testis where bestrophin 1 is only present in Sertoli cells qRT-PCR showed a clear expression of bestrophin 1 RNA even if the number of Sertoli cells is low in comparison with for instance spermatocytes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the target gene. This gene has shown to be affected by treatments, biological processes and different tissues or cell types (Wong and Medrano, 2005). In this study GAPDH showed stable and nearly equal results in the cerebellum from bestrophin 1 knockout and wild type and testis wild type. A high level of GAPDH could therefore not explain the lack of bestrophin 1 RNA in cerebellum. However, with the use of multiple housekeeping genes we could have been even more certain.

Despite our many attempts we failed to reveal any significant signal for bestrophin 1 RNA on qRT-PCR nor any bestrophin 1 protein on Western blots in brain. Confocal immunofluorescence experiments showed the same faint astrocytic staining in both wild type and bestrophin 1 knockout. Even if the selectivity of the antibodies was not optimal, the lack of bestrophin 1 RNA and protein and the lack of a clear cellular staining pattern truly support our final notion: there is no, or very low concentrations

of bestrophin 1 in astrocytes. Previous studies showing bestrophin 1 in astrocytes also showed release of GABA through bestrophin 1 (Lee et al, 2010). In our study we showed a very low level of astrocytic GABA. Some of the GABA is of course lost due to the fixation (up to 50%), but even if we make correction for this loss the level of GABA is still very low (Storm-Mathisen and Ottersen, 1990). GABA is both a weak acid and a weak base with an isoelectric point of 7.3 (Roberts and Sherman, 1993). The cytosolic pH of astrocytes is somewhere between 7,0 and 7,2 (Brookes, 1997). This indicates that GABA is slightly positive in astrocytic cytosol under physiological conditions. Bestrophin 1 however is an anion channel (Hartzell et al, 2008). So the permeation of GABA through the bestrophin 1 channel will probably be very low.

Is enough glutamate released from astrocytes to activate neuronal receptors?

Even though it is estimated that a single synaptic vesicle contains about 10 VGLUT1 or 2 transporters, and that the VGLUT copy number determines the vesicular glutamate content and release probability of glutamate at synapses, it has been shown that even a single VGLUT is enough to fill a vesicle with glutamate (Takamori et al, 2006; Herman et al, 2014; Daniels et al, 2006). So, even the low levels of VGLUTs in astrocytes should be sufficient to fill astrocytic vesicles with glutamate. In astrocytes the vesicular accumulation of glutamate seems not to be dependent on the vesicular content of VGLUT1, but on that of VGLUT3. Overexpression of VGLUT3 led to increased astrocytic release of glutamate (Ni and Parpura, 2009). In addition, as is the case for synapses the intravesicular glutamate content in astrocytes can also be altered by the available concentration of glutamate in the cytosol (Ni and Parpura, 2009; Tabb et al, 1992). Moreover, the number and size of vesicles completing exocytosis and full fusion versus kiss and run is important for the glutamate level released into the gap between astrocytes and neurons (Harata et al, 2006; Chen et al, 2005). Neurons are equipped with a large cluster of synaptic vesicles in their nerve endings, while astrocytic processes only have scattered vesicles.

This leaves us with the question: do astrocytes contain sufficient density of vesicles to release enough glutamate by exocytsis to have an impact on neuronal presynaptic NMDA receptors?

Theoretical glutamate level in astrocytic vesicles

The driving force of glutamate uptake in vesicles are ΔΨ and ΔpH, together formulated ΔH^+ (Tabb et al, 1992; Juge et al, 2006). To estimate the intravesicular glutamate level in astrocytes one can use Nernst equation:

$$\Delta\mu H^+ = F\Delta\psi + RTln\frac{[H^+{}_{in}]}{[H^+{}_{out}]}$$

F is the Faraday constant 96485,3399 C/mol. R is the gas constant 8,314 F is the Faraday constant 96485,3399 C/mol. R is the gas constant 8,3145 J/Kmol. T is the absolute temperature, here at 37^0 Celsius 310,15 K. $[H^+{}_{in}]$ is the intra vesicular consentration of H^+. $[H^+{}_{out}]$ is the cytosolic concentration of H^+.

At chloride levels of 0 mM ΔΨ is the major driving force (Tabb et al, 1992). By using Nernst equation one can use the following formula to calculate the intra vesicular level of glutamate:

$$RTln\frac{[Glu^-{}_{in}]}{[Glu^-{}_{out}]} = F\Delta\psi$$

In astrocytes the $[Glu^-{}_{out}]$ (cytosolic concentration of glutamate) is approximately 2 mM (Gundersen et al 1998; Bergersen et al, 2012). Measurements of vesicular ΔΨ have varied between 0,05V and 0,125V (Russell 1984; Johnson and Scarpa, 1979; Shioi et al, 1989). Thus an estimation of intravesicular glutamate concentration can be done. What slightly complicates this is the presence of chloride. The level of intravesicular glutamate concentration increases with increasing level of cytosolic chloride until 4mM, where the vesicular glutamate concentration reaches its maximum (Tabb et al, 1992). Increasing the chloride level further will decrease the level of intra vesicular glutamate (Tabb et al, 1992). A second effect of increasing the

level of cytosolic chloride is a decrease of ΔΨ and an increase of ΔpH (Tabb et al, 1992). Thereby a gradual changes in the driving force of vesicular glutamate uptake from ΔΨ to ΔpH (Tabb et al, 1992; Juge et al, 2006). Adding ΔpH in the Nernst equation gives us this formula:

$$RTln\frac{[Glu^{-}{}_{in}]}{[Glu^{-}{}_{out}]} = F\Delta\psi + RTln\frac{[H^{+}{}_{in}]}{[H^{+}{}_{out}]}$$

Although the glutamate uptake seems not to correlate with the changes in ΔΨ and ΔpH according to the data presented by Tabb et al 1992 and Juge et al 2006. Nernst equation is not suitable for chloride levels other than 0 mM. Astrocytes have a chloride level about 40 mM (Walz, 2002). According to Tabb et al 1992 and Juge et al 2006 vesicular glutamate uptake is 2-3 times higher at 40mM chloride than 0mM. Of course one has to be precocious to use the measurements in Tabb et al and Juge et al to make an estimation of intra vesicular glutamate concentration since there is a divergence between the two articles in the measurements of glutamate uptake not to ignore, especially at 4mM chloride. Although at 40mM chloride they seem relatively comparable.

With ΔΨ=0,05V, $[Glu^{-}{}_{out}]$=2mM in the Nernst equation and doubling the result (40mM chloride), the lowest intra vesicular level of glutamate estimated is 26 mM. This fits with the estimated value of approximately 50 mM glutamate in astrocytic vesicles in the hippocampus (Bergersen et al, 2012).

Activation of NMDA receptors

With a vesicle diameter of 28 nm one glial vesicle can release 180 glutamate molecules (Bezzi et al, 2004):

$$\frac{4\pi r^3}{3} * L * x$$

L is Avogadros number (molecules pr mole), r is the vesicle radius and x is mole/L glutamate in vesicles.

The study of Clements 1996 showed that one neuronal vesicle with 5000 glutamate molecules is enough to saturate the postsynaptic NMDA receptors. Holmes 1995 estimated that 1000 to 4000 glutamate molecules saturate the NMDA receptors with 16% to 81% respectively. More than 5500 glutamate molecules are necessary to saturate the NMDA receptors more than 90% (Holmes, 1995). Holmes 1995 concludes that more than one neuronal vesicle is necessary to saturate the postsynaptic NMDA receptors. Thus astrocytes need to exocytose at least 31 vesicles to saturate an equal amount of NMDA receptors as found postsynaptic. It has been shown that one astrocytic process may contain clusters of 2-15 small synaptic-like microvesicles and that some processes can contain more than one cluster (Bergersen et al, 2012). In the study by Marchaland et al 2008 they report roughly 20 fusion events per 50 ms in an area of 6 μm^2 when stimulated with 3,5-dihydrophenylglycine (an agonist of mGluR5). As 6 μm^2 covers a bigger area than a perisynaptic astrocytic process it is not straightforward to estimate the number of fusion events in a perisynaptic process. However, when joining functional and structural findings it is likely that a perisynaptic astrocytic process can exocytose more than 31 vesicles (Marchaland et al, 2008; Bergersen et al, 2012). Full fusion of SLMVs from astrocytes will be able to saturate presynaptic NMDA receptors. Marchaland et al 2008 found that astrocytic vesicles fuse partly by “kiss and run” (63%) and partly by full fusion (37%), while Chen et al 2005 claim they only fuse by “kiss and run” under physiological conditions. According to Staal et al 2004 small synaptic vesicles doing “kiss and run” release 25-30% of their contents. Thus astrocytic processes need to exocytose somewhere between 58 and122 vesicles in the kiss and run mode to saturate synaptic glutamate receptors. 58 vesicles are within range, but whether 122 vesicular fusions from one astrocytic process can take place within a short time window (msec) is more doubtful, unless the vesicles can release their full content under “kiss and run” (like claimed by Stevens and Williams, 2000).

Physiological role of glutamate release from astrocytes

Many studies have found that gliotransmitters can modulate long term potentiation (LTP) and synaptic plasticity. The mechanisms are diverse, and only the effect of astrocytic glutamate will be discussed here. One study showed that inhibition of calcium induced release of glutamate by astrocytes decreased the probability of neurotransmitter release and to an inhibition of spike-timing-dependent LTP (Bonasco et al, 2011). Perea and Araque 2007 and Jourdain et al 2007 also find that astrocytic glutamate release increases the probability of glutamate release in adjacent synapses. These studies indicate that astrocytic derived glutamate can trigger a short-term increase in synaptic plasticity. In addition, the former study discovered that when astrocytes released glutamate during postsynaptic depolarization the probability of neurotransmitter release where persistently increased and thereby induced a form of non-classical Hebbian LTP (Perea and Araque, 2007). Both presynaptic mGLuRs (Perea and Araque, 2007) and NMDA receptor 2B (Jourdain et al, 2007) seem to be involved in the potentiating effect of astrocytic glutamate release. Such a release has been shown to be induced by activity in pathways releasing ATP, endocannaboids or acetylcholine (Jourdain et al, 2007; Navarrete and Araque, 2010; Navarrete et al, 2012). These differences could partly be explained by the fact that different brain regions display different mechanisms of astrocytic glutamate release. However, when LTP was discovered in experiments by Bliss and Lømo, 1973 they found that a cessation of tetanic stimulation of hippocampal pyramidal cells would make the cells capable of discharge on a single volley short time after the tetanic stimulation. Also taking the more general Hebbian LTP into account, the postsynaptic neuron need to fire immediately after the presynaptic neuron (spike-timing-dependent plasticity). This seems not to be a direct consequence of glutamate release from astrocytes. Hence, from classical LTP studies it is difficult to explain that glutamate release from astrocytes alone can induce LTP. However, the studies by the group of Araque describe that glutamate from astrocytes can trigger a long lasting increase in the probability of neurotransmitter release, and thereby a long lasting increase of the amplitude of the postsynaptic potentials, which seem to be dependent on the temporal coincidence of postsynaptic activity and the release of glutamate from astrocytes.

Extrasynaptic glutamate has been shown to activate presynaptic calmodulin dependent protein kinase II (CaMKII) and to have a possible role in long term

plasticity since it increases the frequency of spontaneous glutamate release and the number of presynaptic functional boutons for over 30 minutes (Ninan and Arancio, 2004). Increased frequency of synaptic glutamate release has also shown to be the implication of astrocytic glutamate release and activation of presynaptic NMDA receptors (Jourdain et al, 2007). The phenomenon observed by Ninan and Arancio 2004 could therefore be a consequence of astrocytic glutamate release.

Probability of neurotransmitter release differs widely between terminals within CNS, even between terminals of the same axon and it is also adjustable (Branco and Staras, 2009). Adjustment in the probability of neurotransmitter release could either be a short-term or a long-term event (Branco and Staras, 2009). In general, an adjustable probability of neurotransmitter release will contribute to regulate which synaptic inputs that would lead to a change in the neuronal output (Branco and Staras, 2009). One implication of this regulation could be to increase the signal to noise ratio. Since recovery from postsynaptic stimulation is energy-demanding, signal to noise ratio should be high to use as little energy as possible (Branco and Staras, 2009). Another implication of astrocyte regulation of activity at the level of a single synapse is that it allows terminals from one axon to be regulated individually, instead of a global regulation (Branco and Staras, 2009). Finally, the different probabilities of neurotransmitter release could be useful to regulate the importance of signals from different fibers (Branco and Staras, 2009). However, since the postsynaptic site also has a regulatory role on the probability of presynaptic neurotransmitter release, the contribution of astrocytic glutamate release is still rather unclear.

Astrocytic exocytosis is also found to be involved in spike-timing dependent long-term depression (t-LTD) in sensory neocortex (Min and Nevian, 2012). Endocannabinoids, which are thought to be released postsynaptically, can bind to cannabinoid receptors on perisynaptic astrocytes (Min and Nevian, 2012). This will induce a calcium elevation in the astrocyte and thereby exocytosis of astrocytic glutamate, which in turn will bind to presynaptic NMDA receptors and lead to t-LTP (Min and Nevian, 2012). The induced t-LTD was expressed as a presynaptic decrease in probability of transmitter release (Min and Nevian, 2012). However, the astrocytic induced t-LTD seems not to be a general principle for triggering LTD. In one study by Chevaleyre and Castillo 2003 they found that endocannaboids released from the

postsynaptic site activated endocannaboid receptors on the presynaptic terminal and thereby induced LTD in a NMDA receptor independent manner. The differences described above could be due to the possibility that different brain regions and synapse types use different mechanisms of inducing t-LTD (excitatory cerebral cortical synapses vs. hippocampal inhibitory synapses).

As mentioned above glutamate release from astrocytes has a possible role in short term plasticity. Activation of astrocytic NMDA receptors will induce a presynaptic calcium influx (Jourdain et al, 2007; Zito and Scheuss, 2009; Bezzi et al, 2004). The increase in NMDA receptor mediated presynaptic calcium levels is not enough to induce fast and synchronous exocytosis of vesicles, but might be enough to have an impact on calcium sensitive proteins and thereby for example activate CaMKII or calcineurin. These proteins seem to modulate the function of several presynaptic proteins important for transmitter release.

Vesicles and VGLUTs

My finding of VGLUT containing vesicles in astrocytes from intact brain tissue is in agreement with previous electron microscopic findings (Bezzi et al, 2004; Stigliani et al, 2006). Cultured astrocytes contain small (about 30-40 nm) and clear vesicles, which are positive for VGLUT (Stenovec et al, 2007; Martineau et al, 2013; Crippa et al, 2006). Although functional live imaging studies have not directly visualized vesicles, they have suggested that glutamatergic vesicles are present in astrocytes. From such studies in cultured astrocytes the size of the vesicles is estimated to vary between about 30-50 nm (Bowser and Khakh, 2007) and above 300 nm (Malarkey and Parpura, 2011; Chen et al, 2005). Even vesicles of several µm have been found in brain slice astrocytes, but these are produced by stimulating the astrocytes either mechanically or with high glutamate concentrations (Kang et al 2005; Xu et al 2007; Kang et al 2013; Peng et al 2013), suggesting that these vesicles are involved in release of gliotransmitters in pathological conditions (see below).

Moreover, the results of the present thesis are in line with previous localization studies suggesting that the three vesicular glutamate transporters, VGLUT1, VGLUT2 and VGLUT3, are all present in astrocytes. In cultured astrocytes VGLUT1, 2 and/or 3 have been detected by immunohistochemistry (Bezzi et al, 2004; Montana et al, 2004; Kreft et al, 2004; Zhang et al, 2004b; Anlauf and Derouiche, 2005; Crippa et al,

2006; Bowser and Khakh, 2007; Stenovec et al, 2007; Marchaland et al, 2008; Potokar et al, 2009; Ni et al, 2009; Liu et al, 2011; Cali et al, 2014), and by reverse transcription polymerase chain reaction (rt-PCR) (Montana et al, 2004). It has been advocated that cultured cells can express proteins that normally are not expressed in cells in situ. This is probably not the case for VGLUTs in astrocytes, as they have all been found to be localized in acutely isolated astrocytes both at the protein (Zhang et al, 2004b; Montana et al, 2004; Liu et al, 2011) and the mRNA (Zhang et al 2004b) level. In line with this are the present and previous immunohistochemical results, showing that VGLUT1, VGLUT2 and VGLUT3 are indeed located within delicate perisynaptic astrocytic processes in the intact brain (Paper 1 and 2; Fremeau et al, 2002; Bezzi et al, 2004, Zhang et al, 2004b; Platel et al, 2010; Bergersen et al, 2012). In particular, the present thesis supports the findings of VGLUT3 in astrocytes. VGLUT3 have been shown to be upregulated in astrocytes after focal cerebral ischemia (Sánchez-Mendoza et al, 2010). In addition to astrocytes, retinal Muller cells have been shown to release glutamate by exocytosis of VGLUT3 containing secretory vesicles (Linnertz et al, 2011).

In discordance to the above mentioned studies some studies do not find VGLUTs in astrocytes. By use of knock out controls for VGLUT1-3 one confocal study on cultured astrocytes and astrocytes in vivo shows that none of the VGLUTs are present in astrocytes (Li et al, 2013). In the present thesis the VGLUT1 and VGLUT3 labelling in astrocytes were also controlled for by using VGLUT1 and VGLUT3 knockout tissue. The discrepancies between the results of Li et al 2013 and mine are somewhat obscure. The main difference between the studies is that the first study used another type of VGLUT1 antibodies than used by me (while both studies used the same type of VGLUT3 antibodies). Li et al 2013 detected VGLUT localization in astrocytes by analyzing the intensity of the co-localization signal from VGLUTs and the astrocyte marker, while in the present thesis I directly detected VGLUT signals within astrocytic processes by analyzing several confocal planes, which spanned through entire processes of astrocytes. For a more thorough discussion of these findings, see above (Methodological considerations).

Using microarray analysis two studies did not detect RNA transcripts for VGLUTs in isolated astrocytes from the mice brain (Cahoy et al, 2008). This is in contrast with

three previous studies detecting mRNA for VGLUTs in acutely isolated astrocytes by RT-PCR (Zhang et al, 2004b; Bezzi et al, 2004; Danik et al, 2005).

Glutamate is released from astrocytes

The presence of VGLUT containing small synaptic-like vesicles observed in my thesis is in line with the findings that astrocytes are competent of releasing glutamate from a vesicular compartment by regulated exocytosis. It should be noted that, besides the vesicular release of glutamate, release through anion channels is Ca^{2+} dependent (bestrohin 1:Sun et al, 2002; Kuo et al, 2014; VRACs: Mongin and Kimelberg, 2005). Thus, demonstrating Ca^{2+} dependent release of glutamate from astrocytes is probably not sufficient to conclude that astrocytes can release glutamate by exocytosis.

Direct detection of glutamate released from astrocytes has mostly been performed in cultured astrocytes. Many such studies are consistent with a vesicular release mechanism of glutamate (Jeftinja et al, 1997; Bezzi et al, 1998; Bezzi et al, 2001; Pascual et al, 2001; Pasti et al, 2001; Bal-Price et al, 2002; Coco et al, 2003; Montana et al, 2004; Bezzi et al, 2004; Zhang et al, 2004b; Zhang et al, 2004; Domercq et al, 2006; Cali et al, 2008; Höltje et al, 2008; Marchaland et al, 2008; Görg et al, 2010; Yaguchi and Nishizaki, 2010; Liu et al, 2011; Kanno and Nishizaki, 2012; Trkov et al, 2012; Cali et al, 2014). These reports do not only show that astrocytic glutamate release is Ca^{2+} dependent, but demonstrate that the glutamate release is sensitive to inhibition of various parts of the exocytotic release machinery in astrocytes. E.g. glutamate release was inhibited by the VGLUT inhibitors rose bengal/trypan blue, the vesicular H^+-ATPase inhibitor bafilomycin A1 and SNARE protein cleavage by clostridium toxins. However, it has been suggested, that because cleavage of SNARE proteins could interfere with insertion of plasma membrane proteins, through which glutamate can be released (see below), the effect of clostridium toxins on glutamate release does not necessarily reflect vesicular release (Hamilton and Attwell, 2010). Therefore, the effect of blocking VGLUTs and the vesicular H^+-ATPase is particularly important for concluding that the astrocytic release of glutamate is exocytotic.

Astrocytic VGLUTs and glutamate release under pathological conditions

An excess of extracellular glutamate induces neuronal damage (Choi, 1988). VGLUTs in astrocytes have shown to be able to increase the vesicular uptake of glutamate when the amount of cytosolic glutamate increases (Ni and Parpura, 2009). Could VGLUTs play a protective role in cases with an excess of glutamate? Astrocytes scavenge extracellular glutamate via the excitatory amino acid transporters. Will then the VGLUTs contribute to further scavenging by storing glutamate in vesicles? One study showed that application of extracellular glutamate or increasing cytosolic glutamate by blocking glutamate synthetase stimulated to formation of large vesicles (2-7μm) in astrocytes (Xu et al, 2007). The formation of the vesicles was due to uptake of glutamate by VGLUTs (Xu et al, 2007). However after 4-5 minutes the large vesicles fused with the astrocytic membrane and released their content (Xu et al, 2007). This could happen while another vesicle was filled with glutamate (Xu et al, 2007). This suggests that VGLUTs in astrocytes do not contribute to protect the neuron under conditions when the extracellular glutamate concentration is increased.glutamate.

Epilepsy is a condition with periods of intensive bursts from a group of neurons. Astrocytes in CA1 hippocampus are in contact with approximate 140 000 synapses and may potentially have an impact on several synapses at the same time (Bushong et al, 2002). Due to this and the astrocytic influence on neurotransmission several studies have investigated whether astrocytes have a possible role in synchronized intensive bursts. One study found that in hippocampal slices, bathed in 4-aminopyridine (giving action potential like membrane depolarizations), calcium oscillations in astrocytes mediated astrocytic glutamate release and thereby neuronal paroxysmal depolarization shifts (PDS) within 200μm (Tian et al, 2005). PDSs are described as: “abnormal prolonged depolarization with repetitive spiking characteristic of neurons in epileptic cortical zones that are reflected as interictal discharges in the electroencephalogram” (Rogawski and Löscher, 2004). PDS has been observed in several models of epileptogenesis (Curtis and Avanzini, 2001). Another study revealed that inositol 1,4,5-trisphosphate (IP3) injected into astrocytes induced glutamate release, which in turn triggered transient depolarizations in hippocampal pyramidal cells due to slowly decaying inward currents (Kang et al, 2005). The transient depolarization reminded of PDS (Kang et al, 2005). In addition,

astrocytic astrocytic glutamate release also induced negative shift in field potential and possible synchronized activity, although these results relied on pathological high levels of astrocytic derived glutamate (Kang et al, 2005). Gómez-Gonzalo et al 2010 found that stimulating neurons with N-methyl-D-aspartic acid (NMDA) in entorhinal cortex slices, perfused with the proconvulsant 4-aminopyridine, evoked synchronized calcium elevation in a high number of astrocytes which contributed to ictal discharges. They proposed that the astrocytes recruited neurons into epileptic discharges (Gómez-Gonzalo et al, 2010). Interestingly, the calcium elevation of astrocytes had no role in propagation of ictal discharges outside the focal area and did not occur during interictal discharges (Gómez-Gonzalo et al, 2010). Epileptiform activity induced by picrotoxin and removal of external magnesium have been shown to increase the astrocytic glutamate release and thereby the frequency of neuronal slow inward currents (SICs) (Fellin et al, 2006). Astrocytes inducing SICs may lead to the generation of action potentials, however, they are not necessary for the generation epileptiform activity, although increased frequency of SICs might enhance the duration of epileptiform events (Fellin et al, 2006). These disparate findings make it unclear whether astrocytes actually can induce PDS.

Besides, PDS is initiated by other mechanisms than by astrocytic release of glutamate, as for instance by dendritic activation (Altrup and Wiemann, 2003). Even if PDS is associated with epileptogenesis it seems not to generate seizures (Curtis and Avanzini, 2001). A more active role for astrocytic glutamate release in propagating seizures seems unlikely, since astrocytes have a short range (μm) and there is a relative long latency from activation to glutamate release. In contrast, ictal activity is fast synchronized bursts with mm range.

Studies have shown that NMDA receptors are important for survival of neuroblast and new neurons, mGLUR5 receptors have an impact on proliferation of neural precursor cells, while AMPA receptors may regulate the migratory process of newborn neuronal cells (Tashiro et al, 2006; Giorgi-Gerevini et al, 2005; Jansson et al, 2013; Platel et al, 2010). The findings indicate a role for glutamate in neurogenesis. One study found an increased level of VGLUT2 and 3 in reactive astrocytes in ischemic corpus callosum and the perilesional cortex after brain ischemia (Sánchez-Mendoza et al, 2010). Thus astrocytic glutamate release might have a role in neurogenesis after brain ischemia.

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