Executive Summary:

Epilepsy is a condition of the brain characterized by the unpredictable occurrence of seizures, and despite medication about one third of patients have poor seizure control and become medically refractory. Many antiepileptic drugs (AEDs) must be taken chronically for seizure suppression and produce unwanted side effects. Thus, there is an urgent need for the development of more specific AEDs. Recent critical reviews state the little progress AED development has made over the past years, and call for new concepts to identify new targets for improved treatments. Still, epilepsy research is neuron-centered, which might be part of the dilemma because many of the alterations in neurons seem to be secondary rather than causative to the epileptic condition. The NeuroGLIA consortium is convinced that the interaction of neurons with glial cells is crucial for the development of epilepsy and that disturbed neuron-glia interaction is causative for epileptogenesis. In order to fully understand neuron-glia interactions in the normal and epileptic brain, we investigate brain function in animal models and in tissue obtained from epilepsy patients.

The projects major S & T results can be summarized as follows:

- We have performed a thorough and integrated characterisation of astroglial cell subtypes with respect to neuron-glia interactions.
- We have identified many molecular and signaling pathways from neuron-to-astroglia and astroglia-to-neuron in situ and in vivo.
- We investigated the temporal and spatial dynamics of astroglia-neuron interaction in the normal and epileptic brain in situ and in vivo.
- We have investigated the role of astroglia in neurovascular coupling in the normal and epileptic brain.
- We characterized inflammation-related deregulation of astroglia-neuron signalling in epilepsy.
- We investigated neuron-astroglia signalling in living human brain tissue.
- We have raised evidence that glial dysfunction causes epilepsy.

The results of the NeuroGLIA project have a great potential for European epilepsy research, revealing glial cells as new therapeutic targets for the treatment of epilepsy patients.

Project Context and Objectives:

Recent work on neuroglial cell physiology has revealed that these cells are much more actively involved in brain information processing than previously anticipated. This finding has stimulated the novel view that the brain should no longer be regarded solely as a neuronal network, but instead as a circuit of interactive neuron and glial cell networks. In particular, astroglial cells, the numerically predominant cell type in the mammalian central nervous system, have been shown to be highly polarized cells with some processes contacting the vasculature, i.e. cerebral blood vessels, while others dynamically enwrap thousands of synapses. At their membrane, astroglial cells carry a plethora of ion channels, transporters and receptors, i.e. the machinery necessary to sense neuronal activity. Intriguingly, upon stimulation astroglial cells even release neuroactive compounds, termed „gliotransmitters, and feed back to neurons, other glial cells and endothelial cells, thereby modulating neural population dynamics. A clear involvement of astroglial cells in neurological disorders is now gradually emerging. In particular, epilepsy is quite unique among brain disorders because it is based on increased neuron-glial activity. Evidence available so far indicates that molecular changes occurring in neuron-glia interactions of epileptic tissue may resemble the dysregulation of molecules and pathways common to other disorders. New insights into glia-related mechanisms underlying seizure generation and seizure spread will also promote understanding of the pathogenesis of other neurological diseases. By bringing together leading European research groups, NeuroGLIA uses a sophisticated, multi-disciplinary and functional approach to deliver new knowledge on some of the fundamental mechanisms of astroglia-neuron signalling in health and disease.

NeuroGLIA's focus on few but crucial objectives of current international interest, the selection of appropriate inbred and transgenic animal models, the access to living human brain tissue and the expertise in in vivo imaging and electrical recording technology in anaesthetized and awake animals ensures that the novel insight delivered by NeuroGLIA may serve as the basis for the development of new therapeutic strategies for the treatment of brain disorders, including epilepsy.

The following specific objectives are pursued with this project:

- Integrated characterisation of astroglial cell subtypes with respect to neuron-gliainteractions
- Identification of molecular and signalling pathways from neuron-to-astroglia and astrogliato-neuron in situ and in vivo
- Temporal and spatial dynamics of astroglia-neuron interaction in the normal and epileptic brain in situ and in vivo
- Identification of the role of astroglia in neurovascular coupling in the normal and epileptic brain
- Characterization of inflammation-related deregulation of astroglia-neuron signalling in epilepsy
- Neuron-astroglia signalling in living human brain tissue.

Project Results:

Astroglial heterogeneity

We have functionally characterized NG2 positive cells in the mouse hippocampus (Karram et al., 2008) with the NG2ki-EYFP mouse and have further investigated heterogeneity among hippocampal NG2+ cells by determining their transcript profile (S100beta, CNPase, GFAP, NG2) with single cell RT-PCR.

Using combined electrophysiological recordings and [Ca2+]i imaging in brain slices from postnatal rats, we have characterized the response of thalamic glial cells in the ventrobasal thalamus (VB) to stimulation of the two excitatory afferents to this nucleus, i.e. the glutamatergic sensory and cortical inputs. We have found that VB thalamus glial cells can be divided into two groups based on their [Ca2+]i and synaptic responses to these two afferents. One group consists of astrocytes, which stain positively for S100B and preferentially load with SR101, have linear current-voltage relations and low input resistance, show no voltage-dependent [Ca2+]i responses, but express mGluR5-dependent [Ca2+]i transients following stimulation of the excitatory afferent pathways. Interestingly, the astrocytic thalamic population can be divided in three groups consisting of cells that either do respond to neither pathways, only to sensory inputs or only to cortical inputs. Cells of the other glial group, instead, stain positively for NG2, and are characterized by high input resistance, the presence of voltage-dependent [Ca2+]i elevations and voltage-gated inward currents. These cells also display a heterogeneity in their responsiveness to afferent stimulation, but do not show synaptically induced [Ca2+]i elevations. These results show that thalamic astroglial cell responses to synaptic inputs exhibit a different organisation to that of thalamocortical neurons which all respond to stimulation of both afferents. Since VB astrocytes can respond to synaptic stimulation and signal to neighbouring neurons, this glial cell organisation may have functional implications for the processing of somatosensory information, the modulation of behavioural state-dependent thalamocortical network activities and the expression of epileptic paroxysms.

By characterizing physiological properties of NG2 glia in acutely isolated cerebellar slices of young, postnatal mice, we could demonstrate the existence of two classes of NG2 glia. One NG2 glia population expresses voltage-gated sodium and potassium channels and receives synaptic inputs while the other population possesses passive membrane properties and is devoid of synaptic inputs. This latter population exhibits properties which are very comparable to bona fide protoplasmic-type astrocytes. So far, in none of the NG2 glia populations action potentials could be observed or evoked, neither in the white matter nor in the other regions of the cerebellum. In addition to synaptic inputs, intimate structural contacts between NG2 glia and neighbouring interneurons were observed. Currently, we are studying in more detail whether the second type of NG2 cells (with the passive membrane conductance) expresses more properties known from protoplasmic astrocytes. In particular, we are studying their gap junctional connectivity. In addition, we are analyzing whether this type of NG2 glia is also present in cortical brain areas.

We have investigated GFAP in radial glia and neural progenitors in the human developing cortex. By interacting with other proteins involved in development and cell signalling, GFAP could play a role in the process of neurogenesis in human brain development. In contrast, GFAP+1 immunoreacitivity was undetectable in developing human brain. We also investigated the expression patterns of GFAP in epilepsy-associated lesional pathology: GFAP expression in HS overall appears to mirror regional reactive gliosis. It is a useful marker for the demonstration of balloon cells in FCD and TSC which may be relevant to their abnormal size and within heterotopia supports its composition/localization. Lack of GFAP within heterotopia supports its composition of cells destined for deeper cortical layers. Furthermore, GFAP+1 expression in focal lesions associated with chronic epilepsy was examined. GFAP+1 immunoreactivity in epilepsy-associated pathologies detects a subpopulation of astrocytes in regions of astrogliosis. Further studies are important to understand whether the expression of this isoform may affect the cytoskeletal integrity and the shape and function of glial cells under pathological conditions.

We have further investigated astroglial heterogeneity with respect to connexin expression: With comparative reporter gene assays, immunodetection and fate mapping approaches we demonstrate that Cx30 is more abundant in the hippocampus than previously thought. Interastrocytic tracer coupling analyses in acute slices of Cx30 deficient mice reveals that Cx30 accounts for 20% of interastrocytic gap junctional communication in the mouse hippocampus (Gosejacob et al., 2011). We have characterized the new Cx43ki-ECFP mouse line and have functionally analyzed ECFP positive astroglia in the hippocampus. Here, Cx43ki-ECFP labelled the majority of gap junction coupled astrocytes (Degen et al., 2012). In mice double transgenic for Cx43ki-ECFP and NG2ki-EYFP, we could demonstrate that in both mice, mutually exclusive cell populations are labelled (Degen et al., 2012). With the Cx43ki-ECFP mouse and Cx43/Cx30 DKO mice we further investigated astroglial heterogeneity across brain areas: Only about 70% of biocytin coupled cells were CFP positive in the thalamus, in contrast to the majority of coupled cells being CFP positive in hippocampus, and that comparative semiquantitative RT-PCR analyses with tissue specimens from the hippocampus and thalamus confirmed that Cx43 predominates in the hippocampus while in the thalamus, Cx30 mRNA levels significantly exceed Cx43 (Degen et al., 2012). Consistently, Western blot analysis also showed a twofold higher Cx30 expression in thalamic compared to hippocampal tissue in the juvenile and the adult brain. Cell coupling between thalamic astrocytes was scored, following patch clamp analysis in freshly prepared brain slices, by biocytin filling of astrocytes in GFAP-EGFP mice. Experiments employing the astrocyte-specific dye sulforhodamine 101 (SR101) revealed a Cx43 negative population in Cx43ki-ECFP mice in the thalamus. Furthermore, lack of one Cx43 allele in these mice did not lead to significant reduction of coupling. In contrast, in Cx30 KO animals we observed strongly decreased (-73%) astrocytic cell coupling compared to wild type littermates. We are currently analyzing the extent to which Cx43 and Cx30 are coexpressed in the thalamus (in Cx43ki-ECFP/Cx30ki-LacZ mice).

In freshly isolated, whole cell patch-clamped astrocytes, kainate induced small responses in a subset of the cells, indicating that astrocytes in the thalamus express ionotropic glutamate receptors, which are lacking in the hippocampus. Much larger currents were evoked by GABAA receptor activation. These findings further

corroborate the emerging view that astrocytes in different brain regions display variable physiological properties.

Changes in the expression of Kir channels in astrocytes during postnatal development have been quantitatively characterised, and we could demonstrate that TREK channels contribute to the 'passive' current pattern of astrocytes (Seifert et al., 2009). Distinct properties of radial glia-like cells, a population of astroglial stem cells in the postnatal brain, have been identified. These data corroborate the concept of astroglial heterogeneity across brain areas and identify Cx43 as a key player in the regulation of postnatal neurogenesis (Kunze et al., 2009). Strong expression of PBXIP1/HPIP was observed in radial glia and migrating astrocytic precursor cells during human embryonal development, with strong co-localisation of nestin, vimentin and GFAP, which suggests PBXIP1/HPIP to be a stem cell / glial progenitor marker.

We have established an animal model of TLE in the lab, employing unilateral intracortical kainate injection (Theofilas et al., 2009) and have finished the histological and functional characterization of this new model which reproduces many typical features of human hippocampal sclerosis such as granule cell dispersion, selective neurodegeneration in CA1/CA3 and loss of astrocytic gap junctional coupling. In this model we observed a decrease of gap junction coupling in the latent phase following status epilepticus and a complete loss of gap junction coupling as well as a complete loss of cells with astrocyte current pattern in the late chronic phase of TLE. To further investigate mechanisms underlying astrocyte dysfunction in sclerosis in the kainate model of epilepsy, we performed a fate mapping analysis. We crossed Cx43kiCreER(T) mice with ROSA26-LoxP-Stop-LoxP-EYFP (ROSY) reporter mice. Cre-mediated recombination of the EYFP reporter gene was induced in the double-transgenic mice by tamoxifen injection, resulting in the permanent labelling of astrocytes (Gosejacob et al., 2011). To get a hint at the fate of astrocytes in the course of epilepsy, we performed TUNEL staining 4h, 6h, 1d, 5d, 3 months and 6 months after intracortical kainate injection. While 6 h after kainate injection neuronal cell death was already detected, we never observed cell death in astrocytes at any time point, excluding apoptosis as the cause of astrocyte loss in epilepsy. We have likewise observed subtle changes in the phenotype of astrocytes, i.e. the emergence of small S100beta-positive astrocytes with atypical current pattern.

We have raised evidence for a differential role of Cx43 versus Cx30 in the hippocampus, cortex and thalamus (Gosejacob et al., 2011). In the hippocampus, Cx30 plays a minor role for interastrocytic communication (Gosejacob et al., 2011). Interestingly, we observed on decrease of Cx43 expression a compensatory up-regulation of Cx30, the second astrocytic connexin preferentially in the vicinity of nearby neurons (Degen et al., 2012). In mice with decreased Cx43 expression, we likewise observed a (compensatory) upregulation of the glutamate transporters GLAST and GLT-1 (Unger et al., 2012).

Aquaporin4 (AQP4) is the main water channel in the brain and is primarily localized in astrocytes where the channels are thought to contribute to water and K+ homeostasis during neuronal activity. The close apposition of AQP4 and inward rectifier K+ channels (Kir4.1) gave rise to the hypothesis of direct functional interactions between both transmembrane channels. Comparative analyses were performed with wild-type and AQP4 knock out (KO) mice. Retrograde fiber

stimulation in the stratum oriens provoked smaller increases and slower recovery of [K+]o in the stratum pyramidale of AQP4-/- mice. Presumably, neuronal activity in the absence of AQP4 entails reduced glial swelling and a larger extracellular space as compared to controls. In a next step, we investigated the laminar profile of [K+]o by moving the recording electrode from the stratum pyramidale towards the hippocampal fissure. At distances beyond 300 µm from the stratum pyramidale, the stimulation-induced, normalized [K+]o in AQP4 KO mice significantly exceeded the corresponding values of controls. Astrocytes in AQP4-/- mice displayed enhanced tracer-coupling which might underlie the improved spatial re-distribution of [K+]o in the hippocampus. These findings suggest that Kir4.1 and AQP4 channels are operating independently, and do not support the concept of direct functional interactions (Strohschein et al., 2011).

Together with the groups of K. Willecke and H. Kettenmann, we have investigated gap junctional coupling between astrocytes and oligodendrocytes in the corpus callosum (Tress et al., 2012). In this study, we have examined the contribution of oligodendrocytic connexin47 (Cx47) and astrocytic Cx30 to panglial gap junctional networks as well as myelin maintenance and function by deletion of both connexin coding DNAs in mice. Biocytin injections revealed complete disruption of oligodendrocyte-to-astrocyte coupling in the white matter of 10- to 15-d-old Cx30/Cx47 double-deficient mice, while oligodendrocyte-to-oligodendrocyte coupling was maintained. There were no quantitative differences regarding cellular networks in acute brain slices obtained from Cx30/Cx47 double-null mice and control littermates, probably caused by the upregulation of oligodendrocytic Cx32 in Cx30/Cx47 double-deficient mice. We observed early onset myelin pathology, and 40% of Cx30/Cx47 double deficient animals died within p42-90, accompanied by severe motor impairments. Histological and ultrastructural analyses revealed severe vacuolization and myelination defects in all white matter tracts of the CNS. Furthermore, Cx30/Cx47 double-deficient mice exhibited a decreased number of oligodendrocytes, severe astrogliosis, and microglial activation in white matter tracts. Although less affected concerning motor impairment, surviving double-KO mice showed behavioural alterations in the open field and in the rotarod task. Vacuole formation and thinner myelin sheaths were evident also with adult surviving double-KO mice. Since interastrocytic coupling due to Cx43 expression and interoligodendrocytic coupling because of Cx32 expression are still maintained, Cx30/Cx47 double-KO mice demonstrate the functional role of both connexins for interastrocytic, interoligodendrocytic, and panglial coupling, and show that both connexins are required for maintenance of myelin.

Using NG2ki-EYFP mice, have shown that activation of voltage-gated Ca2+-channels, Ca2+-permeable AMPA-receptors, and metabotropic glutamate-receptors in NG2+-cells increase [Ca2+]i. Furthermore, we have identified Ca2+-release from internal stores that was induced by Ca2+-influx through voltage-gated Ca2+-channels. Additionally, we have evoked action potentials in presynaptic fibres of GABAergic interneurons and glutamatergic CA3 neurons by minimal electrical stimulation. Although stimulation of both cell types evoked postsynaptic currents in NG2+-cells, no changes in somatic [Ca2+]i were detected. We suppose that synaptic input onto NG2+-cells evokes local changes in [Ca2+]i at glial processes rather than at the soma. Besides other possible physiological consequences, this might provoke the release of neuroactive substances from NG2+-cells. Unexpectedly, our data also demonstrate

high motility of NG2 cell processes on a minutes scale (Haberlandt et al., 2011). Together with Christine Rose (Düsseldorf), we have found that Cx43 and Cx30 in hippocampal astrocytes are required for the propagation of intracellular sodium waves (Langer et al., 2012). These sodium waves might be implicated in metabolic supply of neurons, since Na+/K+-ATPase activity leads to ATP consumption, which in turn enhances glycolysis and lactate production in astrocytes. The lactate then serves as metabolic fuel for neurons.

In order to differentiate specific mechanisms of connexin function (intercellular coupling, adhesion, hemichannel activity etc.), we have raised mice expressing the Cx43G138R point mutation and the Cx43K258Stop truncation in astrocytes. During the generation of astrocytic Cx43K258Stop/Cx30KO mice, we observed that the widely used hGFAP-Cre transgene exhibits ectopic activity in early development, leading to deletion of floxed alleles in all cells of the body. We have observed ectopic activity using the nestic-cre transgene to induce the point mutation in astrocytes as well. As previously described for the hGFAP-Cre transgene (Requardt et al., 2009), we have also observed lack of nestin-cre activity, giving rise to 'pseudo-knock in mice'. We have established methods to control for ectopic activity and lack of activity for both the hGFAP-Cre and the nestin-cre transgenes. These methods allow us to select mice with faithful cre activity for phenotypical analyses both with respect to adult neurogenesis and temporal lobe epilepsy.

In another study, we have investigated functional consequences of blood brain barrier dysfunction and albumin extravasation, which both are suggested to play a role in the aetiology of human epilepsy, in glial cells of the hippocampus. We show that fluorescently labelled albumin is taken up by astrocytes, NG2 cells and neurons, with NG2 cells standing out in terms of the quantity of uptake. Within 5 days post injection (dpi), intracellular albumin accumulation was largely reduced suggesting rapid degradation. Electrophysiological analysis of astrocytes and NG2 cells revealed no changes in their membrane properties at either time point. However, astrocytic gap junction coupling was significantly decreased at 1 dpi but returned to control levels within 5 dpi. We found no changes in hippocampal Cx43 transcript expression, suggesting that other mechanisms account for the observed changes in coupling. Kir4.1 mRNA was regulated oppositely in the CA1 stratum radiatum, with a strong increase at 1 dpi followed by a decrease at 5 dpi. Our data demonstrate that extravasation of albumin induces early transient changes of astrocyte function within the hippocampus which can be expected to impair ion and transmitter homeostasis and contribute to hyperactivity and epileptogenesis (Braganza et al., in press).

In contrast to the hippocampus, astrocytes of the thalamus display functional AMPA receptors. No differences exist in the spontaneous Ca2+ oscillation frequency of astrocytes among the different cortical layers and between cortex and hippocampus.

To temporally target gene recombination in NG2 glia in vivo, we generated a mouse line in which the open reading frame of the tamoxifen-inducible form of Cre recombinase (CreERT2) was inserted into the NG2 locus by homologous recombination. In order to characterize this novel TgH(NG2-CreERT2) mouse line, we cross-bred it to the Rosa26-EYFP reporter line. The first analysis (injection of tamoxifen for 5 days and analysis 3 days later) of recombination in adult mice revealed that the Cre recombinase activity was mainly restricted to NG2 glia.

However, with increasing time span after initial tamoxifen injection more and more EYFP-expressing, differentiated oligodendrocytes were detected. These data show that NG2 glial cells are a constant source for new oligodendrocytes even in the adult mouse. Interestingly, also few EYFP+ cells in the cortex were detected that expressed neuronal marker, thereby indicating that at least few NG2 cells possess neurogenic potential. Using another reporter mouse (Rosa26-flox-tdtomato) we could detect even more neurons generated in the cortex from NG2 cells. The identification of the recombined cells was further substantiated by electrophysiology revealing bona fide spontaneous and evoked action potentials. Interestingly, we could further show that NG2 glia gives rise to astrocytes when acute brain injuries were applied. These data demonstrate the wide differentiation potential of NG2 glia in vivo.

Our most significant result is the identification of a context-dependent differentiation potential of NG2 glial cells. During development (during the active phase of myelination) NG2 cells of the white matter differentiate mainly into oligodendrocytes. In grey matter, they mainly persist as NG2 glia. However, we observe the generation of neurons that we might be able to link to enhanced neural activity. After a pathological insult such as acute brain jury, NG2 cells start to differentiate into a third cell type, i.e. into astrocytes. From these observation, we postulate that NG2 glia remain highly plastic in the brain and constitute a cellular source for novel cells according to the brain's requirements.

Spatial and temporal dynamics of neuron-astroglia interactions

A novel communication pathway between neurons and astrocytes has been identified at the molecular level (Navarrete and Araque, 2008). Endocannabinoids are important modulators of neuron-glia communication in the hippocampus. They are directly involved in the neuron-astrocyte communication. Using electrophysiological and Ca2+ imaging techniques, CB1 receptors were detected on hippocampal astrocytes. Activation of these CB1 receptors leads to a phospholipase C-dependent Ca2+ mobilization from internal stores. Activated by endocannabinoids released by neighboring neurons, the increase of Ca2+ levels in the astrocytes, in turn, stimulates glutamate release that subsequently activates NMDA receptors in pyramidal neurons. These results demonstrated the existence of endocannabinoid-mediated neuron-astrocyte communication, revealing that astrocytes are targets of cannabinoids and might therefore participate in the physiology of cannabinoid-related addiction. These findings revealed the existence of a novel neuron-glia signaling pathway where astrocytes serve as a bridge for non-synaptic interneuronal communication. We now show that they potentiate synaptic transmission through stimulation of astroglial glutamate release.

Floxed GABAB receptor mice were obtained from the group of B. Bettler (Haller et al., 2004) and crossbred with Cx43ki-CreRT mice (Eckardt et al., 2004) to achieve inducible, astrocyte-specific deletion of GABAB receptors in the hippocampus. Single cell RT-PCR experiments show that GABAB1 receptors are expressed by hippocampal astrocytes. We have bred floxed GABAB1 receptor mice with Cx43ki-CreRT mice (Gosejacob et al., 2011) for inducible, astrocyte-specific deletion of GABAB receptors in the hippocampus. The mouse colony will be large enough to allow physiological and behavioural studies on a routine basis.

NG2 cells are a glial cell population with astroglial properties. In acutely isolated hippocampal slices obtained from NG2ki-EYFP mice and applying 2P-LSM, calcium signals could be recorded in the somata of NG2 cells after neuronal stimulation of Schaffer collaterals or upon direct depolarization of NG2 cells. Pharmacological analysis and single cell RT-PCR revealed the expression of different types of voltage gated Ca2+ channels in the glial cells (Haberlandt et al., 2011). This represents an important characterization for our understanding of Ca2+ signalling in this cell type. In addition, it paves the way for the future analysis of the impact of distinct voltage gated Ca2+ channels in appropriate genetically modified mouse models. Preliminary results indicate that NG2 cells in the hippocampus show short-term (paired-pulse facilitation) and long-term plasticity (theta-burst induced LTP) similar to neurons, irrespective of the presence of the NG2 gene (NG2ki-EYFP mice). These data are not in line with the prevalent hypothesis that AMPA receptor clustering in NG2 cells critically depends on the presence of the NG2 proteoglycan.

More specifically, we asked whether the NG2 protein is crucial for the formation of functional NG2 cell synapses by influencing clustering of postsynaptic receptors or neuroligin interactions. To address this issue, we investigated synaptic transmission between glutamatergic neurons and NG2 cells in NG2-EYFP-knockin (+/ and /) and wildtype mice. We recorded whole-cell membrane currents from hippocampal NG2 cells during electrical stimulation of Schaffer collaterals and analysed the evoked excitatory postsynaptic currents (eEPSCs). Comparison of the kinetics and paired-pulse ratios of NG2 cell eEPSCs revealed no significant differences among the tested genotypes. We conclude that the lack of the NG2 protein does not cause a general failure of synaptic signaling between glutamatergic neurons and NG2 cells in the hippocampus. It remains to be tested whether miniature EPSCs, which are not synchronised by presynaptic action potentials, are affected in NG2-deficient mice. In vivo recordings by two-photon laser-scanning microscopy (2P-LSM) show dynamic morphological changes of NG2 glia in anesthetized NG2-EYFP mice. In addition to calcium recordings by 2P-LSM, we are establishing the imaging of calcium changes with a camera system that allows the recording of up to 10 kHz (80 by 80 pixels). Furthermore we are habituating mice for image recording in the awake animal. The first recordings have been obtained by two-photon (2P) excitation while they were awake.

In acute hippocampal slices obtained from NG2ki-EYFP mice, two photon laser scanning microscopy detected calcium signals in the somata of NG2 cells after stimulation of Schaffer collaterals or upon direct depolarization of NG2 cells. Pharmacological analysis and single cell RT-PCR revealed the expression of different types of voltage gated Ca2+ channels and metabotropic glutamate receptors in these cells. Moreover we could show that NG2 cell processes display rapid morphological alterations. These data extend our knowledge of Ca2+ signalling pathways in this cell type (Haberlandt et al., 2011).

We have used electrophysiological and calcium imaging techniques in hippocampal slices to investigate the cellular processes and molecular mechanisms underlying astrocyte-neuron signalling and their effects on synaptic plasticity. We performed paired recordings of adjacent CA1 pyramidal neurons, monitored astrocyte calcium levels in stratum radiatum and stimulated Schaffer collaterals, using the minimal

stimulation technique that activate single or very few synapses. We depolarized one neuron to stimulate the release of ECBs while monitoring synaptic transmission properties in the adjacent neuron. Pairing these neuronal depolarizations induced the long-term potentiation of synaptic transmitter release, which was mediated by CB1R activation because it was abolished in the presence of selective antagonist of these receptors and was absent in mice lacking these receptors (CB1R-/- mice). Astrocyte calcium elevations were necessary to induce this synaptic plasticity because it was absent in mice lacking the IP3R2 (IP3R2-/- mice) that is necessary for G protein-mediated calcium signal in astrocytes. The pharmacological analysis of the ECB-induced LTP indicate that it is dependent on NO production in the depolarized postsynaptic neuron that serve as retrograde messenger that activates protein kinase C. In addition, ECB-induced astrocyte calcium elevations stimulate glutamate release that activates presynaptic metabotropic glutamate receptors. When these signaling pathways are temporally coincident, they lead to the long-term potentiation of hippocampal synaptic transmission.

Genetic ablation of ionotropic glutamate receptors in astrocytes resulted in a series of structural, physiological and behavioural events: glial processes were retracted from synapses, subsequently neuronal excitatory postsynaptic currents showed increased amplitude and prolonged decay kinetics indicating reduced glial glutamate clearance. The perturbed neuronal network activity subsequently resulted in impaired fine motor coordination tested on a sophisticated horizontal ladder running paradigm. The impaired motor behaviour in mutant mice highlighted the importance of transmitter and its receptor interaction for bidirectional communication between neurons and astrocytes in the grey matter of the central nervous system (Saab et al., 2012).

Using in vivo experimental approaches, we have found that cholinergic activity evoked by sensory stimulation or electrical stimulation of the septal nucleus, the main cholinergic input to the hippocampus, elevated calcium in hippocampal astrocytes and induced LTP in CA3-CA1 synapses. Using hippocampal slices to investigate the underlying cellular mechanisms, we have found that stimulation of cholinergic axons evoked astrocyte calcium elevations, depolarization of CA1 pyramidal neurons, and LTP in CA3-CA1 synapses. Like in vivo, astrocyte calcium elevations and LTP required mAChR activation, and LTP also required mGluR activation. Cholinergic-induced astrocyte calcium elevations and LTP were absent both in IP3R2 knock-out mice and in wildtype mice after loading astrocytes with BAPTA or GDP?S (which prevented astrocyte calcium signalling). Notably, LTP was rescued by simultaneous astrocyte calcium uncaging and postsynaptic depolarization. Taken together, these results indicate that astrocyte calcium signal is necessary for cholinergic-induced hippocampal synaptic plasticity. Furthermore, these results show that cholinergic LTP requires the astrocyte calcium signal, which stimulates the release of glutamate from astrocytes that activates mGluRs on neurons. Then, cholinergic-induced hippocampal LTP results from the coincidence of astrocyte and postsynaptic activities simultaneously evoked by cholinergic signalling (Navarrete et al., 2012).

To visualize calcium signals, fluorescent calcium indicator dyes such as OregonGreenBAPTA or Fluo4 are injected as a bolus into the respective brain area. Bolus injections of indicator dyes provide the advantage of not-perturbing intracellular signalling cascades as they are often observed on patch-clamp experiments. In addition, a viral approach of indicator delivery has been established.

For that purpose adeno-associated viruses have been constructed which allow the expression of conventional fluorescent proteins but also of genetically encoded calcium-indicators such as TNC-XXL (based on FRET signal between CFP and YFP) or even cGMP and cAMP indicators. Viral infection of mice and subsequent recording of fluorescent signals in the cortex of anesthetized mice have been performed. Image recording could also be achieved repetitively over a period of two weeks after infection.

Furthermore, we analyzed the expression of the genetically encoded calcium indicator TnXXL and the light-gated ion channel channelrhodopsin II. So far, we can successfully induce the expression of TnXXl and channelrhodopsin II in a cell-specific manner. However, the expression level is not as high as expected. It has to be investigated how severely this will impair future functional studies. We corrected for these technical problems by using a novel Rosa26-floxed GCAMP3 mouse with optimized properties for the visualization of Ca2+ signals. We also established EEG recordings of slow wave discharges (SWD) that were evoked by intraperitoneal injection of gamma-hydroxy-butyric acid (GHB). We could detect SWDs in awake mice, but also in anesthetized mice after GHB injection that displayed temporal characteristics as described.

For further investigations of molecular signalling cascades in hippocampal astrocytes, floxed GABAB receptor mice have been crossbred with Cx43ki-CreERT mice. For advanced imaging in slices a fast Ca2+ recording system with kHz acquisition rate has been established. A mouse behavioural model has been established that allows visualization of neural activity in awake mice in vivo.

Astroglia-neuron signalling in AE and TLE

Abnormality in a GABA transporter (GAT-1) present in thalamic astrocytes has been identified in genetic models of absence epilepsy (AE) which leads to an increased neuronal tonic GABAA inhibition (Cope et al., 2009). An increased tonic GABAA current could also be detected in well-established pharmacological models of AE, i.e. the GHB and THIP models. This abnormality in GAT-1 is restricted to brain areas that are involved in the generation and expression of absence seizures (i.e. thalamus and cortex) but absent in others (e.g. cerebellum, hippocampus), is not due to either altered astrocytic expression or genetic mutations of this GABA transporter, but might be the result of altered phosphorylation processes.

In different in vitro models of TLE, astrocytic Ca2+ elevations occur only during ictal, but not interictal events. At the site of ictal discharge the generation Ca2+ elevations occur in the vast majority of astrocytes before ictal discharge onset, suggesting a crucial role for astrocytes in the initiation of focal ictal discharges (Gomez-Gonzalo et al., 2010). The properties of SICs recorded from thalamocortical neurons of Genetic Absence Epilepsy rats from Strasbourg (GAERS) and non-epileptic control rats are similar, whereas amplitude, rise and decay times and total charge of SOCs are different between the two strains. There is no GAT-1 mediated transporter current in VB astrocytes from GAERS, confirming previous indirect evidence that this GABA transporter is not functioning in this polygenic rat model of AE. IL-1 is induced in reactive astrocytes of the somatosensory cortex of GAERS when spike and wave

discharges initiate to occur. Since IL-1 induction is pro-ictogenic in this model, it may play a role in absence seizures. A paper describing the expression and significance of the IL-1 system in GAERS, a model of absence epilepsy, has been published (Akin et al., 2011). The GAT-1, but not the GAT-3, transporter that is present in thalamic astrocytes is malfunctioning in GAERS. This provides the conclusive proof that an abnormality in the GAT-1 transporter underlies the gain-of-function of the extrasynaptic in GABAA receptors that we reported previously in the thalamus of this and other genetic models of absence epilepsy. Glutamatergic astrocyte-to-neuron signalling shows no differences between epileptic and non-epileptic rats, while the GABAergic astrocyte-to-neuron signalling is slightly affected in GAERS compared to non-epileptic control (NEC) rats. The inhibitory barrages opposing focal Interictal Discharge (FID) propagation are largely generated by local parvalbumin fast-spiking interneurons (using Pv-FS-INs - G42 mice) and not by somatostatin interneurons (using GIN mice). A large number of astrocytes in the entorhinal cortex (EC) exhibit a drastic Ca2+ increase upon GABA application. This response appears to be mediated by GABAB-receptors. Astrocytes, activated by either GABA or the GABAB-receptor agonist baclofen, can release glutamate that triggers SICs in Pv-FS-INs. High frequency activity of GABAergic interneurons elevates intracellular calcium in astrocytes and transiently enhances synaptic efficacy of excitatory CA3-CA1 synapses. In contrast, low frequency activity of interneurons inhibits synaptic transmission and fails to elevate astrocyte calcium. This GABAergic-induced synaptic potentiation requires astrocyte calcium elevations and is mediated by GABAB and metabotropic glutamate receptors. Thus, astrocytes decode the firing frequency of inhibitory interneurons, transforming an inhibitory signal into a potentiating signal of excitatory transmission, which may be relevant during epileptic activity.

Activation of astrocytes lowers the threshold for generation of focal epileptiform activity. In the enthorhinal cortex, astrocytes generate Ca2+ increases upon interneuron stimulation which might contribute to focal seizure propagation. We have found evidence for a role of CB1 receptors in early human corticogenesis (Zurolo et al., 2010) and investigated the expression of Kv4.2 channels in neurons and glial cells of human epilepsy-associated focal lesions (Aronica et al., 2009).

We have completed our analysis of SICs and SOCs in the thalamocortical neurons (TC) neurons of the ventrobasal thalamus of the GAERS model of absence epilepsy, and compared these results with those from the non-epileptic control (NEC) rat strain. No differences were observed in the properties (i.e. frequency, amplitude, rise and decay times, total area) of SICs or SOCs between the two strains under normal conditions.

By recording with a standard internal solution, however, the reversal potential of GABAA receptor-mediated effects is close to the testing membrane potential, and therefore small events generated by activation of these receptors could go undetected. In order to overcome this problem, we also measured SOCs using an internal patch electrode solution containing a high [Cl-]. As expected many more SOCs could be recorded under this condition, but their frequency was again not different between GAERS and NEC. However, all other parameters (i.e. amplitude, rise and decay times, total area) showed marked differences between GAERS and NEC.

These results indicate that our previously discovered malfunction of the GABA transporter GAT-1 in the astrocytes of the GAERS ventrobasal thalamus (Cope et al., 2009) not only results in an increased tonic GABAA current in TC neurons of this epileptic rat strain but also leads to larger and longer SOCs. The functional significance of this finding with respect to the pathophysiological mechanism of absence seizure generation remains to be determined.

Our previous work has shown that the GABA transporter GAT-1 in the astrocytes of the ventrobasal thalamus of the epileptic GAERS rats is malfunctioning (Cope et al., 2009). Having overcome some major technical difficulties, we are now in the position to directly measure the GABA transporter current in astrocytes of the ventrobasal thalamus. Our preliminary data show that while NO-711 (a selective GAT-1 blocker) decreases the GABA transporter current elicited in NEC astrocytes by puff application of GABA it has no effect on that of GAERS astrocytes. On-going experiments are now trying to assess the effect of SNAP (a selective blocker of GAT-3) alone or in combination with NO-711 in GAERS and NEC astrocytes. We will also try to compare in the two strains the GABA transporter current elicited in the astrocytes of the ventrobasal thalamus by physiological release of GABA, i.e by stimulation of the afferent GABAergic fibers of the reticular thalamic neurons.

We investigated whether the activation of the IL-1 signalling occurs in (GAERS) during the development of spike-and-wave discharges (SWDs), and whether inhibition of IL-1? biosynthesis could affect SWD activity. IL-1 expression and glia activation were studied by immunocytochemistry in the forebrain of non-epileptic control Wistar rats and in GAERS at postnatal days (PN14, PN20, and PN90). In Wistar rats of all ages, no detectable IL-1 immunoreactivity was observed in any of the areas studied, and glial cells showed a resting morphology. In PN14 GAERS, when no SWDs have developed yet, IL-1 immunostaining was undetectable, and astrocytes were not activated. At PN20, when immature SWDs can be observed, 2 out of 5 GAERS showed IL-1 expression in activated astrocytes, while no IL-1 staining was observed in the remaining 3 rats. These changes were restricted to the somatosensory cortex. In adult PN90 GAERS, when mature SWDs are established, IL-1 immunostaining was observed in numerous reactive astrocytes in the somatosensory cortex. Microglial activation and IL-1 expression in microglia were not observed in Wistar rats or GAERS at any age. The changes in IL-1 induction were concomitant with increased c-fos signaling, which denotes neuronal network activation in the areas of brain inflammation. Inhibition of IL-1beta biosynthesis using a specific IL-1converting enzyme (ICE)/Caspase-1 blocker, significantly reduced both SWDs number and duration in PN90 GAERS over 4 days of systemic administration. These results show that IL-1? is induced in reactive astrocytes of the somatosensory cortex of GAERS when SWDs initiate to occur. The IL-1 induction has proictogenic properties in this model, thus it may play a role in the mechanisms underlying the occurrence of absence seizures.

Using the picrotoxin/zero-Mg2+ model of TLE in entorhinal cortex slices, we have demonstrated that the vast majority of astrocytes are activated by ictal discharges and that in many of these astrocytes Ca2+ elevations occur after the ictal discharge onset. Interictal discharges fail to trigger such a large response and increase only the frequency of independent Ca2+ transients in single astrocytes. A similar distinct

activation of astrocytes during the ictal event is also observed in slices from pGFAP-EGFP mice and in other models (4-AP/picrotoxin and high-K+).

The activation of astrocytes by the ictal discharge is mainly mediated by glutamate and ATP, since it is inhibited by MPEP or PPADS, antagonists of mGlu receptors and purinergic P2Y receptors, respectively. The duration and frequency of ictal episodes in neurons are also significantly reduced by these antagonists, while the rate of interictal discharges is either unaffected (by PPADS) or increased (by MPEP). The ictal discharges generated in picrotoxin/zero-Mg2+ model arise spontaneously and at unpredictable foci, hampering the possibility to study the cellular mechanism of seizure onset accurately. Thus, to investigate whether astrocyte Ca2+ elevations have a specific role in ictal discharge generation, we have developed a new seizure model where ictal discharges are generated at a restricted site in the EC by a localized application of NMDA in the presence of 4-AP and low Mg2+. This new model allowed us to study the early events that occur at this focal site before the ictal onset, and has shown that a massive Ca2+ elevation occur in the astrocytes before the onset of an ictal discharge. Finally, selective inhibition of the Ca2+ signalling in the astrocyte syncytium by BAPTA introduced in single astrocytes blocks ictal discharges, while the activation of astrocytes by TFLLR, an agonist of PAR-1 receptors that are highly expressed in EC astrocytes, enhanced the generation of ictal discharges.

In summary, at the site of ictal discharge generation a Ca2+ elevation occurs in the vast majority of astrocytes before ictal discharge onset. Thus, rather than being a mere consequence of neuronal hyperactivity this abnormal activation of astrocytes bring neurons towards seizure threshold, suggesting the possibility that it plays a crucial role in the initiation of focal ictal discharges. We have begun to investigate the involvement of endocannabinoid signaling in epileptiform activity in a hippocampal slice model of TLE. Preliminary data indicate that the properties of epileptiform discharges induced in these slices by 4-AP/zero-Mg2+ solution are modified by CB1 receptor antagonists and in CB1 receptor ko mice.

The role of astroglial intercellular coupling in epileptogenesis is being studied in our newly established model of unilateral intracortical kainate injection in mice lacking the major astrocytic connexins in astrocytes (Cx43/Cx30 DKO mice). These mice exhibit a much weaker status epilepticus (SE) compared to control littermates but a significantly decreased duration of the latency period until the first spontaneous seizures in the chronic phase. This indicated an ambiguous role of astrocytic connexins depending on the phase of our TLE model. During SE, connexins might mediate metabolite supply to neurons in order to sustain their hyperactivity (a pro-epileptic function), while in the chronic phase of spontaneous seizures they might primarily mediate extracellular potassium homeostasis (an anti-epileptic function). We have also looked for changes in Cx43 expression by immunoblotting 5 days post SE, when gap junctional coupling was decreased. Instead of decreased Cx43 expression we observed a shift in the phosphorylation status of Cx43, indicating that post-translational modifications are responsible for loss of interastrocytic coupling. With mass spectrometry, we are currently analyzing which residues are phosphorylated. In order to differentiate specific mechanisms of connexin function (intercellular coupling, adhesion, hemichannel acticity etc.), we have raised mice expressing the Cx43G138R point mutation in astrocytes. We expected such mice to show loss of Cx43 mediated coupling but preserved hemichannel and adhesive

activity. These mice show loss of interastrocytic coupling in the hippocampus (in the Cx30 KO background) and strongly decreased neurogenesis similar to DKO mice. We have also raised mice expressing a truncated form of Cx43 lacking the C-terminus (Cx43K258Stop). This truncated form of Cx43 was reported to allow normal intercellular coupling at least in Hela cells. Adhesive interactions mediated by the C-terminus of Cx43 were expected to be abolished selectively. Unexpectedly, we found that in the Cx30 KO background, interastrocytic coupling was abolished even in the presence of the Cx43truncated protein. In addition, we observed a strong decrease in adult neurogenesis. Our results so far indicate that lack of gap junction coupling is leading in both Cx43 mutant mice to the observed phenotypical alterations. We will now investigate both mutants in our TLE model.

Neuron-astroglia signalling in the control of neurovascular coupling

After our finding that astrocytes favour epileptiform activities in the entorhinal cortex (EC) by lowering the threshold of focal ictal discharge (FID) generation (Gomez-Gonzalo et al., 2010), we investigated a possible role of astrocytes in the propagation of epileptiform activities. In our new model of focal seizures (see Losi et al., 2010), we performed paired recordings from pyramidal neurons and different interneurons (in different configurations) and simultaneous fast-laser scanning microscope imaging of Ca2+ signals. In these experiments, we started to analyze the recruitment process that underlies FID propagation to neurons distant from the focus. At the time of seizure initiation at the focus, hyperpolarizing events were activated onto distant pyramidal EC neurons. These events always preceded FID in pyramidal neurons, and possibly represent an inhibitory barrier that delays FID propagation to these neurons. Indeed, its inhibitory GABAergic nature was demonstrated by bicuculline applications that abolished both these events and the delay in FID propagation. Similar inhibitory events were also observed to delay spontaneous ID propagation. The inhibitory events were generated mainly by fast spiking interneurons (FS-INs) that in the preictal phase exhibited intense spiking discharges correlated with the inhibitory currents in nearby pyramidal neurons. These results demonstrate that an inhibitory GABAergic transmission to pyramidal neurons effectively opposes the spread of epileptic activity across the cortex and indicate that during the preictal phase a powerful release of GABA occurs in regions distant from the focus.

We have investigated whether astrocytes in these regions are activated by GABA prior to FID propagation, by investigating whether EC astrocytes are responsive to a GABA challenge. We found that about 50% of these cells exhibited large amplitude oscillatory Ca2+ elevations after bath applications of GABA or Baclofen, a response that was abolished by the GABAB receptor antagonist CGP55485. To further clarify the role of GABAB-mediated activation of astrocytes in focal seizure generation, we will use also use floxed GABAB receptor mice (Haller et al., 2004) crossed with Cx43ki-CreRT mice (Eckardt et al., 2004) to delete selectively GABAB receptors in astrocytes. Our most recent findings regarding a contribution of astrocytes to seizure propagation add a new, possibly relevant pathophysiological role of astrocytes in the control of propagating focal seizures, opening perspectives for a new therapeutic approach that could prevent the spread of epileptiform activity.

We used different approaches to trigger focal ictal discharges in in vivo experiments. While interictal-like discharges were regularly observed, none of these experimental procedures, however, elicited a reproducible ictal activity in C57/BL6j mice anaesthetized with urethane. The systemic injection of a general anaesthetic may prevent epileptiform activities by decreasing neuronal excitability. The lack of significant activation of astrocyte endfeet by interictal discharges was further validated by results obtained from in vivo experiments. After bulk loading of Ca2+ dyes in the living brain, the Ca2+ signal from astrocyte endfeet is difficult to analyze due to their small size and to the intensity of the neuropile fluorescence that contaminates the signal from endfeet with a strong component of neuronal origin. To increase the contrast of the tiny endfeet, OGB-1 was slowly injected into the subarachnoid space. Following these procedures, due to the astrocyte intercellular coupling, endfeet enwrapping parenchymal arterioles were well-loaded with the dye, while neurons and neuropile were much less loaded although they were still visible. The contrast of the astrocyte processes was thus greatly improved. To unambiguously mark astrocyte endfeet, the astrocyte-specific dye SR101 was co-loaded with OGB1-AM. As revealed by field potential recordings, a bicuculline application to the cortical surface evoked isolated, short-lasting interictal events that were highly synchronous with Ca2+ signal elevations in the neuropil. These events, however, evoked only rare Ca2+ responses in the astrocyte cell body and the endfoot. Quantitative analysis of interictal events indicates that less than 5% of the endfeet showed a Ca2+ transient associated with this type of epileptic discharge, confirming that interictal events evoked only a weak response in astrocyte endfeet, similarly to results obtained in cortical slices (Gomez-Gonzalo et al., 2011).

In the picrotoxin/low Mg2+ cortical slice model of TLE in young rats we found that the duration of the ictal discharge was significantly decreased during a prolonged epileptiform activity. As illustrated by the Ca2+ change in astrocytes during repetitive ictal discharges, the duration of the second and the third ictal event was decreased with respect to the first episode. A decreased activation of the astrocytes was also observed to accompany the ictal discharge of decreased durations. The relative value of each ictal discharge duration (with respect to that of the first ictal event) is expressed as a function of an index of astrocyte activation. This index is obtained by the product of the relative change in the number of activated astrocytes and the amplitude of the Ca2+ elevation in the ictal discharge successive to the first ictal episode. In both CA3 hippocampal region and EC the correlation coefficient fitting the data demonstrates that the astrocyte activation index and the duration of the ictal discharge are significantly correlated. Imaging of Ca2+ signals in endfeet while recording epileptic activities in the picrotoxin/zero-Mg2+ slice model of epilepsy in young rats revealed that interictal and ictal events had strikingly different effects on astrocytes and their endfeet. While interictal events evoked a weak response, ictal events evoked in both astrocytes and endfeet a massive Ca2+ elevation. Quantitative analysis indicates that less than 10 % of the endfeet showed a Ca2+ transient following interictal events, while more than 80 % of the endfeet were activated by the ictal event. In the same experiments, simultaneous monitoring of arterioles revealed that the Ca2+ increase in endfeet evoked by the ictal discharge was followed by vasoconstriction in 9 of 12 arterioles. Vasoconstrictions initiated with delay with respect to the Ca2+ peak in endfeet. The interictal discharge failed to trigger significant arteriole diameter changes, as expected by the weak activation of endfeet by this epileptic discharge. The type of the arteriole response, i.e., a constriction or a

dilation, is determined mainly by the arteriole resting state. Indeed, in arterioles preconstricted with the tromboxane A2 analogue U46619, the arteriole response to the ictal discharge, that was dominated by constriction in the absence of U46619, became significantly dominated by dilation. A vasodilation was observed in 17 of 26 preconstricted arterioles examined. Thus, the myogenic tone of arterioles contributes to determine the nature of the response. The vasodilating response to the ictal discharge initiated with a delay with respect to the Ca2+ elevation in endfeet. However, in 4 of 17 arterioles it initiated with a prolonged delay. Such a loose association between the Ca2+ increase in endfeet and the vasodilation suggests that the delayed response of the arterioles to the ictal discharge may not be mediated by astrocyte endfeet. To investigate whether delayed vasodilations could be, indeed, fully independent on Ca2+ elevations in endfeet, slices were incubated with cyclopiazonic acid (CPA), that depletes Ca2+ from the intracellular stores. In CPA-incubated slices, the ictal discharge failed to trigger Ca2+ elevations in endfeet and it also failed to trigger the fast vasodilation. The ictal discharge evoked either no changes or a delayed dilation after the ictal discharge onset. This timing is comparable to the delayed vasodilation that we observed in the control arterioles that failed to respond rapidly to ictal-induced Ca2+ elevations in endfeet. These results suggest that only the rapid response of arterioles is mediated by Ca2+ elevations in endfeet, while the delayed vasodilation is likely mediated by factors, not necessarily of astrocytic origin, that develop along with the ictal discharge, such as a perivascular acidosis and an increase in extracellular adenosine. In summary, the ictal discharge triggered a vasomotor response in 76.3% of the monitored arterioles. In contrast, only 11.8 % of arterioles showed a vasomotor response after an interictal discharge. A subset of endfeet were activated rapidly in a subsecond scale, while others showed a Ca2+ elevation within the successive 3 s after the ictal discharge onset (Gomez-Gonzalez et al., 2011).

The cyclooxygenase (COX) inhibitor indomethacin, which in the normal brain significantly reduced the blood vessel response to neuronal activity had no effects on the arteriole response to the ictal discharge. When indomethacin was applied, it blocked ictal discharge generation hampering the possibility to clarify the role of COX products in the blood vessel response to ictal discharges. Similarly, inhibition by the CYP inhibitor miconazole of the synthesis of EETs (these agents are also involved in the control by astrocytes of the neurovascular coupling in the normal brain) had no effects. We also addressed the contribution of adenosine by using a general A2 receptor inhibitor, 3,7-Dimethyl-1-propargylxanthine (DMPX), and a selective A2A receptor antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385). We found that either DMPX alone and its co-application with ZM failed to inhibit rapid vasodilations. To analyze the role of 20-HETE in vasoconstrictions induced by the ictal discharge, arterioles were not preconstricted while 20-HETE production was inhibited with HET0016. Under these conditions, ictal discharge-mediated vasoconstrictions were reduced.

In the close-to-in vivo whole guinea pig brain preparation, we obtained results that are comparable with those obtained in brain slice preparations. In this epilepsy model that uses a brief arterial application of bicuculline to trigger epileptiform activities, the interictal event activated weakly astrocyte endfeet, while the ictal event evoked in both astrocytes and endfeet a diffuse Ca2+ elevation (Gomez-Gonzalez et al., 2011). In an initial series of in vivo experiments in the mouse somatosensory cortex, we

confirmed that the interictal discharge triggered by local application of 4-AP failed to activate significant Ca2+ elevations in astrocyte endfeet.

In rat brain slices, we found that in response to repetitive seizure-like events the activation of astroglia endfeet decreased progressively. This appears to be less evident in the guinea pig brain preparation. In contrast, in both preparations repetitive interictal events regularly failed to activate significant blood vessels responses. Interictal discharges can be very frequent in patients. Our study thus supports the possibility that the fMRI negative BOLD signal, frequently recorded in patients during interictal events, is due to a mismatch between CBF (that does not change significantly during interictal spike activity) and oxygen metabolic rate (that is highly increased under these conditions). A possible pathological consequence of this unbalance is that during interictal activities the CBF can be inadequate to meet the high metabolic demand of neurons leading to cerebral hypoxia and brain damage. A defective neurovascular coupling during interictal activities may thus lead to a progressive functional impairment of the epileptogenic region. Our observations can be relevant for a full understanding of the events that are at the basis of the fMRI signal in the normal and epileptic brain. Notably, an accurate interpretation of fMRI data is also essential for a precise localization of the epileptogenic focus in neurosurgery of untreatable epilepsies.

In Cx43ki-ECFP mice (Degen et al., 2012), we observed labelling of nearly all gap junction-coupled astrocytes in the hippocampus. This would help measurements of Ca2+ responses in the astrocytes. At the same time the ECFP reporter strongly labels the astrocytic endfeet (at least with 2P-LSM) and this will allow us to simultaneously measure endfeet Ca2+ responses.

To address the role of gap junctional communication in neurovascular coupling using mice lacking the major astrocytic connexins Cx43 and Cx30, we established quality control standards to verify faithful hGFAPCre mediated deletion (Requardt et al., 2009; Zhang et al., in revision). We have shown that neuron-to-astrocyte signalling is central to the control of neurovascular coupling in the epileptic brain (Gomez-Gonzalo et al., 2012). Ca2+ elevations in astrocyte endfeet that in brain slices mediate the vasomotor response of arterioles to epileptic discharges, have now also been observed in the whole guinea pig brain preparation. Interictal discharges failed to trigger vasomotor responses in blood vessels in the somatosensory cortex in vivo.

We have also begun to investigate the possible role of the inhibition of the mammalian target of rapamycin (mTOR) pathway in the regulation of blood-brain barrier permeability in epileptic rats using rapamycin. Rapamycin treatment did not alter status epilepticus (SE) severity and duration compared to vehicle treatment in rats. Rapamycin-treated rats developed hardly some or no seizures during the 6-week treatment, whereas vehicle-treated rats showed progressive increases of seizures starting 1 week after SE. Cell loss and sprouting that normally occur after SE were prominent but on average significantly less in rapamycin-treated rats versus vehicle treated rats. Nevertheless, various inflammation markers were dramatically upregulated and not significantly different between post-SE groups. Interestingly, blood–brain barrier leakage was barely detected in the rapamycin-treated group, whereas it was prominent in the vehicle-treated group. Whether the effects on blood–brain barrier leakage in rapamycin-treated rats are a consequence of seizure

suppressing properties of the drug, or contribute to a real antiepileptogenic effect still needs to be determined. This work is now published (van Vliet et al., 2012). We further investigated the relationship between blood–brain barrier leakage in glioneuronal tumors and focal cortical dysplasia (FCD). Albumin extravasation, with uptake in astrocytes, was observed in GG and in FCD type IIB cases and was associated with upregulation of major histocompatibility complex class I (MHC-I).

Inflammation and epilepsy

We demonstrated prominent expression of VEGFs and their signalling receptors in astroglial and microglial cells in epilepsy associated developmental lesions (Boer et al., 2008). We confirmed in specimens of patients with hippocampal sclerosis the upregulation of cyclooxygenase 2 (Cox2) reported in experimental models of chronic epilepsy expression and studied the effects of Cox2 inhibition on epileptogenesis and spontaneous seizures in a rat model of TLE (Holtman et al., 2009). We studied the effects of Cox2 inhibition on pharmacoresistance to anti-epileptic drugs (Holtman et al., 2009). We detected a novel proconvulsant pathway involving high mobility group box (HMGB1) release from neurons and glia and its interaction with TLR4, a key receptor of innate immunity (Maroso et al., 2010).

We investigated the effects of IL-1 in the NMDA focal seizure model (Losi et al., 2010). In Ca2+ imaging experiments AMC found that a single NMDA pulse, per se ineffective in evoking a focal ictal discharge, stimulated an intense neuronal response that evolved into a propagating focal ictal discharge (FID) after IL-1 was applied for 5-10 min either locally or in bath perfusion. This effect was confirmed in patch-clamp recording experiments. Quantitative evaluation indicate that after IL-1? applications an ictal discharge could be evoked in 45 of a total of 90 single NMDA pulses. In control slices only 4 of 53 single NMDA pulses were effective. In a few IL-1 experiments, in which an ictal discharge was induced by a single NMDA pulse, it has been observed that the ictal discharge originated at the site of IL-1 applications. Notably, before IL-1 applications as well as in all control experiments, the ictal discharge always arose at the site of the NMDA applications. It appears that the amplitude of the NMDA receptor response to a local NMDA challenge is increased after IL-1 applications.

We provide evidence for a role of plasminogen activator (PA) system components in different human focal epileptogenic pathologies (Iyer et al., 2010a). Our data demonstrate prominent activation of both innate and adaptive immunity, with involvement of different inflammatory pathways in focal cortical dysplasia type IIB (Iyer et al., 2010b). We provide evidence of activation of TLR4 receptor signalling in human malformations of cortical development and show prominent upregulation of miR-146a in experimental and human TLE (Aronica et al., 2010). Our recent data suggest a slower development of generalized seizures in complement component C6-deficient kindled rats, suggesting that seizure spread is hampered by the absence of MAC. A significant decrease in astrocyte coupling was observed after exposure of acute hippocampal brain slices to pro-inflammatory molecules (such as IL-1?, TNF-alpha, LPS). IL-1 and HMGB1 have also been shown to lower the threshold for focal ictal discharge generation in experimental models of epilepsy.

mTOR inhibition led to strong reduction of seizure development despite the presence of microglia activation, suggesting that effects of rapamycin on seizure development are not due to a control of inflammation. Whether the effects on blood–brain barrier leakage in rapamycin-treated rats are a consequence of seizure suppressing properties of the drug, or contribute to a real antiepileptogenic effect still needs to be determined (van Vliet et al., 2012).

We studied the action of IL-1, TNF and LPS on gap junction coupling of astrocytes in the hippocampus. Incubation of acute brain slices in IL-1 or IL-1 + TNF leads to a significant decrease in astrocyte coupling as revealed by whole cell recording and biocytin filling. To confirm the uncoupling effect of cytokines in vivo, intraperitoneal injections of LPS was performed. Five days after injection, acute brain slices were prepared and the gap junction mediated coupling was checked as mentioned before. Under these conditions, astrocyte coupling in the hippocampus was significantly reduced. We recently started to investigate the effects of HMGB1 in the focal seizure model. Similarly to IL-1, HMGB1 lowered the threshold of FID generation. A single NMDA pulse, per se ineffective, could evoke a fID after HMGB1 local applications. Both the amplitude of the neuronal Ca2+ signal and the number of neurons to a single NMDA pulse were significantly increased after HMGB1. A conditio sine qua non for the efficacy of HMGB1 is its application to a neuronal network that already experienced a sustained seizure activity. When HMGB1 local applications were performed onto a virgin territory, a successive single NMDA pulse evoked a neuronal response that was not different from that observed by the same stimulation applied before HMGB1. These results suggest that a sustained seizure activity renders the neuronal network sensitive to HMGB1 action. The underlying mechanism is currently under investigation.

We studied whether cannabinoid receptor 1 (CB1) activity modifies the expression level of inflammatory pathways in slices from wild type and CB1 knockout mice that were pharmacologically-treated to induce epileptiform activity. These experimental studies are complemented with other studies, in which the characteristics of pharmacologically-induced epileptiform discharges (e.g. induction threshold, burst duration, etc.) are analyzed, in order to establish a correlation between the properties of the epileptiform activity and the expression level of molecular inflammatory pathways. The current studies using the 4-aminopyridine, Mg2+-free model of epilepsy in hippocampal slices will be extended using different models of pharmacologically-induced epilepsy in vitro and in vivo.

We investigated whether nitric oxide (NO) plays a role in the molecular mechanism of microglia activation. We studied acute CNS lesions evoked by high-power laser pulses and applied in vivo two-photon laser-scanning microscopy to mice with fluorescently labeled microglia and neurons. We found that such local tissue damages provoked an immediate attraction of microglial processes. Tissue superfusion with NO synthase and guanylate cyclase inhibitors blocked these extensions. Furthermore, local injection of the NO-donor SPNO or the NO-dependent second messenger cGMP induced efficient migration of microglial cells towards the injection site. High tissue levels of NO, achieved by uniform superfusion with SPNO and mimicking extended tissue damage, resulted in a fast conversion of the microglial shape from ramified to ameboid indicating cellular activation. After preconditioning of the tissue by increased, ambient ATP (known as microglial chemoattractant) levels, the attraction

of microglial processes to local NO release was augmented, while it was abolished at low levels of tissue ATP. Since both signaling molecules, NO and ATP, mediate acute microglial reactions, coordinated pharmacological targeting of NO and purinergic pathways will be an effective mean to influence the innate immune response in the diseased CNS.

We investigated major histocompatibility complex class I in the developmental glioneuronal lesions. Our findings indicate a prominent upregulation of MHC-I as part of immune response occurring in epileptogenic glioneuronal lesions. In particular the induction of MHC-I in neuronal cells appear to be a feature of type II FCD, TSC and GG and may represent an important accompanying event of the immune response observed in these developmental lesions.

MicroRNA-146a is a key regulator of astrocyte-mediated inflammatory response. In response to inflammatory cues miR-146a is induced as a negative-feedback regulator of the astrocyte-mediated inflammatory response. This supports an important role of miR-146a in human neurological disorders associated with chronic inflammation and suggests that this miR may represent a novel target for therapeutic strategies. Our data on the regulation of Kir4.1 expression in astrocytes and astrocytic tumors indicate a role for IL-1. The alterations in expression of Kir4.1 occurring in epilepsy-associated lesions are possibly influenced by the local inflammatory environment and in particular by the inflammatory cytokine IL-1.

Analysis of neuron-astroglia interactions in living human brain tissue

We have performed analyses of excised patches and extracellular glutamate uncaging in hippocampal slices from human hippocampus to determine the glutamate sensitivity of astrocytes and NG2 cells. The data suggest that some of the NG2 cells in sclerotic hippocampal specimens co-express AMPA receptors and glutamate transporters, which was never observed in ‘control-like’ tissue from patients with lesion-associated epilepsy. Patch clamp analyses revealed an almost complete loss of cell coupling and astrocytes displaying a passive current phenotype. We see dramatic changes in astrocyte properties in sclerotic epileptic specimens. Careful re-evaluation of human specimens revealed abundant co-localization of S100ß and GFAP in both non-sclerotic (non-HS) and HS. In contrast to control mouse tissue, all PDGFRalpha-positive cells (putative NG2 cells) in the non-sclerotic human hippocampus co-express S100ß. In HS, all S100ß-positive cells display a dramatically changed morphology (smaller somata, less branching with thicker proximal processes). These data add to our functional findings and suggest that in the course of epilepsy astrocytes undergo a significant transformation of their phenotype.

We have characterized properties of spontaneous astrocyte Ca2+ oscillations and astrocyte responsiveness to synaptically-released neurotransmitters. We have obtained evidence indicating that astrocyte Ca2+ elevations evoke glutamate-induced NMDAR-mediated SICs in neurons. We have found that human astrocytes respond with calcium elevations to synaptically released neurotransmitters, indicating that human astrocytes detect synaptic activity and suggesting the existence of neuron-to-astrocyte communication in human brain tissue. We have also characterized the properties of NMDA receptor-mediated SICs in neurons. Electrophysiological

recordings from neurons revealed the presence of SICs mediated by NMDA receptor activation. The frequency of SICs increased after local application of ATP that elevated astrocyte calcium. These results indicate that human astrocytes are able to release the gliotransmitter glutamate, and suggest that glutamate release from human astrocytes is a calcium-dependent process that may affect neuronal excitability through activation of NMDA receptors in neurons (Navarrete et al., 2012). There is a bidirectional communication between astrocytes and neurons in human hippocampal and cortical tissue, involving ATP, NMDA and endocannabinoid receptors.

GFAP is a protein that might have important functions in radial glia and neural progenitors in the human developing cortex. The interaction of GFAP? with proteins involved in development and cell signalling implies that this specific GFAP isoform has a function in human brain development (Middeldorp et al., 2010). In hippocampal sclerosis, GFAP expression patterns mirror regional reactive gliosis. It is a useful marker for the demonstration of balloon cells in FCD and TSC which may be relevant to their abnormal size and localization. The lack of GFAP within heterotopia supports its composition of cells destined for deeper cortical layers (Martinian et al., 2009). GFAP 164/exon 6, GFAP+1 is specifically expressed by a human astrocyte subtype as revealed by an affinity purified GFAP antibody. These large astrocytes recognized by the antibody are present throughout the brain, e.g., along the subventricular zone, in the hippocampus, in the striatum and in the spinal cord of controls, Alzheimer and Parkinson patients. The presence of a specific GFAP-isoform suggests a specialized function of these astrocytes (Middeldorp et al., 2009). We have immunohistochemically characterized the out-of frame splice variants GFAP 164/exon 6 in focal lesions associated with chronic epilepsy. GFAP+1 immunoreactivity in epilepsy-associated pathologies reveals a specific subpopulation of astrocytes in regions of astrogliosis. Further studies on GFAP+1 positive astrocytes are important to understand whether the expression of this isoform may affect the cytoskeletal integrity and the shape and function of glial cells under pathological conditions. However, while the staining is increased in epilepsy associated pathologies, GFAP+1 is expressed in a small percentage of astrocytes. Thus, the possible role of this subpopulation of astrocytes in epilepsy is likely minor, compared to astrocytes expressing other GFAP isoforms (Boer et al., Epilepsy Res., 2010). We have also characterized the expression patterns of GFAP isoforms (GFAPd , GFAP+1) in glioneuronal and glial tumors.

Adhesion molecule on glia (AMOG) was first described to mediate neuron–glia adhesion and migration during development, as well as ion homeostasis. In addition, AMOG has been identified as a regulator of the Pi3K-mTOR signaling pathway. We characterized the expression pattern of AMOG in the development of the human cerebral cortex. Abundance of perivascular AMOG, with staining of glial endfeet, suggests a possible role of AMOG in the regulation of vascular integrity. MAC has found an altered cellular expression pattern of AMOG in FCD and cortical tubers in patients with TSC compared to histologically normal cortex. In both focal developmental lesions, AMOG immunoreactivity pattern in grey matter was reduced with a diffuse pattern and AMOG was detected in reactive astrocytes and dysplastic or atypically differentiated neural precursors (Boer et al., 2010).

In addition, we studied the expression of SCN7A/Na(x) in the epileptic rat and human hippocampus. The SCN7A gene encodes an atypical sodium channel Na(x), which is

involved in osmoregulation via a sensing mechanism for the extracellular sodium concentration. In both epileptic rat and human hippocampus, increased Na(x) expression was observed in neurons and reactive astrocytes compared to control tissue. Our data support the possible involvement of this channel in the complex reorganization occurring within the hippocampus during the epileptogenic process in temporal lobe epilepsy.

In line with previous findings in experimental models of epilepsy, expression of astrocytic adenosine kinase (ADK) was found to be increased in the hippocampus and temporal cortex of patients with temporal lobe epilepsy and in focal cortical dysplasia, as well as in tumor-associated epilepsy. These results together with the observation that a ketogenic diet reduced ADK expression indicate that alteration of adenosine signalling is relevant to human epilepsy (de Groot et al., 2012; Masino et al., 2012)

Potential Impact:

Impact on understanding brain function

The comparative analysis of basic neuron-glia interaction mechanisms in living tissue of animal models and of patients provided novel insights into our understanding of brain function. The success of the NeuroGLIA consortium results in novel insights into human brain function at the molecular, cellular and systems level.

Impact for our understanding of epilepsy and the development of new therapies

Results from the NeuroGLIA project improve our understanding of the genesis and progression of human epilepsy. The approach taken by the NeuroGLIA consortium was particularly designed to combine the latest developments of electrophysiological and imaging techniques with advanced genetic animal models. We learned more about long-range neuron-astroglia interaction mechanisms and novel contributions of glutamate, GABA, ATP and other transmitters to seizure generation and seizure spread. For the first time, we gained information on the bidirectional interaction of neuronal and astroglial network activities in the forebrain of anaesthetized and awake mice by in vivo imaging at submicrometer and subsecond resolution. The definition of the temporal relationship between the Ca2+ signals in astroglia and the generation of interictal and ictal discharges in epilepsy models not only provides novel insights into mechanisms underlying the epileptic condition, but also outlines areas for new pharmacological interventions.

On the other hand, new insights into glia-related mechanisms underlying seizure generation and seizure spread also promote understanding of the pathogenesis of other neurological diseases. Evidence available so far indicates that molecular changes occurring in neuron-glia interactions in epileptic tissue resembles dysregulation of molecules and pathways seen in other disorders, i.e. amyotrophic lateral sclerosis, stroke, hepatic encephalopathy, schizophrenia, Huntington's disease and Alzheimer's disease. For example, the evaluation of the temporal relationship of Ca2+ signals in astroglial endfeet and dilation and/or constriction of cerebral blood vessels during ictal and interictal epileptic discharges helps to better understand detrimental processes also occurring in stroke or migraine patients. Indeed, a distinct percentage of stroke patients are confronted with seizure attacks. Understanding glia-vasculature interactions leads to the development of new drugs against epilepsy, stroke and migraine. The NeuroGLIA consortium also investigated inflammatory reactions induced in human epileptic disorders. The distinct involvement of astroglial and microglial cells in pro- as well as anti-inflammatory processes allows for the development of medication interfering with specific pathways such as the inflammatory modulation of gap junctional communication or the induction of multidrug transporters which might be the cause for therapy-resistance of epilepsy cases.

The NeuroGLIA project outlines new routes for defined drug therapies of epilepsy patients. In addition, it is expected that the identification and characterization of processes leading to epilepsy will be relevant also to other brain disorders.

Throughout the project's duration, the scientific results of the project were published in more than 80 peer-reviewed journals like Science, Nature Neuroscience, Neuron, Plos Biology, Brain Research Reviews, Glia, Neuroscience, Journal of Neuroscience, Epilepsia, Epilepsy Research, and Experimental Biology, and in the proceedings of more than 170 conferences and workshops during the last four years, including the FENS Forum of European Neurosciences (The Netherlands), European Congress of Epileptology (Germany), Gordon Research Conference on Mechanisms of Epilepsy & Neuronal Synchronization (USA), the International Astrocyte School (Italy), International Epilepsy Congress (Hungary) and the Dutch Endo-Neuro-Psycho conference (The Netherlands). Several additional original publications of project results are under way.

In conjunction with the 41st Annual American Society for Neurochemistry (ASN) Meeting in March 2010 the first NeuroGLIA symposium "Neuron-astroglia interactions in brain function and dysfunction" took place in Santa Fe (New Mexico) and was chaired by the consortium members Christian Steinhäuser (UKB) and Giorgio Carmignoto (CNR) (please see http://asneurochem.org/2010Meeting/ASN2010.htm online).

In the same year, NeuroGLIA consortium members Eleonora Aronica (AMC) and Christian Steinhäuser (UKB) organized the NeuroGLIA satellite symposium "Neuron-astrocyte signalling in the epileptic brain" at the FENS conference in Amsterdam, The Netherlands (July 7, 2010). As an official satellite, this meeting provided large visibility among the scientific community and widely promoted the NeuroGLIA project (please see http://fens2010.neurosciences.asso.fr/pages/index2.php?sub=11&left=11&head=11 online).

One important event for NeuroGLIA was the International Astrocyte School (IAS 2011) which took place in Bertinoro (Italy) from 27 March to 3 April 2011 and addressed the most intriguing hypotheses on the role of astrocytes in brain function. The school, with its strategy of disseminating state-of-the-art knowledge, was especially targeted at young scientists beginning their careers in this field, but was also open to senior scientists wishing to refresh their glial background (please see http://ias2011.azuleon.org/welcome.php online).

As one major final public dissemination activity the NeuroGLIA project was presented at the Astroglia-Neuron Signalling and Neuronal Network Dynamics in Seizures and Epilepsy conference at the Neuroscience and Mental Health Research Institute at Cardiff University, United Kingdom in November 2011. The conference programme mainly focused on reports of NeuroGLIA partners and a number of invited speakers presenting their background in this field (please see http://www.cardiff.ac.uk/research/neuroscience/news/events.html online).

Further dissemination took place continuously via the project website which is accessible at http://www.neuroglia.eu. Here, a list of all peer-reviewed and project-related publications produced by the project partners as well as additional important publications of the project consortium are available.

List of websites:
http://www.neuroglia.eu