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DOCTORAL THESIS

Whole cell patch clamp analysis of diverse synaptic mechanisms in rodent and human neocortical slice preparations

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1 Introduction

The neocortex is the evolutionarily youngest part of the cerebral cortex and at the same time considered to be one of the most complex biological structures. Besides its crucial role in sensory perception and motor execution, this organ makes humans capable of performing sophisticated cognitive tasks. These cognitive abilities make neuroscientists so eager –and able– to decipher the workings of the human brain. Although the ultimate goal of neuroscience is to solve the human brain, most of the research is currently devoted to understanding the nervous system of simpler and more available model organisms, such as rodents'. Despite the rich availability of data from these model organisms, it still proves to be quite ambitious a task. Thus, overall, there are at least two straightforward ways to propel current neuroscience research: either to unveil the fine details of the rodent brain or to use this knowledge and collide these results with research done in the human brain. In my doctorate study, we went down on each path: first, we discovered a novel communication pathway from a unique GABAergic interneuron to the most extensively studied glia cell, and second, we demonstrated a species-specific difference in a well known synaptic pathway between the human and rodent neocortex.

The cortical neurons can be divided in two main groups: the excitatory projecting pyramidal cells and the inhibitory local interneurons. The majority of neocortical neurons, approximately 80–90%, are the pyramidal cells. Their common characteristic features are the spiny dendrites, a triangular cell body, a set of basal dendrites and a thick, radially oriented apical dendrite which usually forms a terminal tuft in the most superficial cortical layer. Using glutamate as neurotransmitter, they are responsible for the majority of excitatory postsynaptic potentials detected in the neocortex. GABAergic interneurons constitute the minor fraction of neurons in the neocortex (10–20%) but are instrumental for normal brain function. Despite their small numbers, these interneurons display extremely diverse morphological, electrophysiological and molecular properties and, therefore, they can be classified them on the basis of these characteristics. In a part of our work we considered

two types of cortical interneurons: the ubiquitous basket and rare axo-axonic cells which are known for being heavily innervated by local pyramidal cells and for their membranes with fast time constant. Due to these features excitatory inputs can efficiently and reliably trigger action potentials in them. These so called fast spiking interneurons in turn can effectively regulate the action potential generation of surrounding pyramidal cells with their GABAergic axon terminals targeting the somatic region or axon initial segment of nearby pyramidal cells. Another unique type of inhibitory interneuron, neurogliaform interneurons (NGFCs), were suggested to specialize in acting on GABA receptors on compartments of the neuronal surface which do not receive synaptic junctions, through a unitary form of volume transmission. The GABA released via volume transmission can effectively reach the extrasynaptic $GABA_A$ and $GABA_B$ receptors on virtually all neuronal processes within the axonal cloud of the NGFC. In the same vein, we hypothesized that neurogliaform interneurons might act on non-neuronal elements of the surrounding cortical tissue without establishing synaptic contacts.

Glial cells are the major class of non-excitable cells in the brain. Their diversity and complex role was overlooked in the dawn of neuroscience –they were regarded as connenctive tissue, hence their name– but glial cells have become the focus of scientific attention in the last decades. According to modern textbooks, glial cells comprise two main groups: microglia with mesodermal origin and the diverse group of macroglia with ectodermal origin. Astrocytes of the macroglia group are extensively studied due to the fact that they comprise the most abundant and diverse glia class. Despite their diversity, their functions are shared: they are crucial for the extracellular ionic homeostasis, neurotransmitter uptake, synapse formation, regulation of blood–brain-barrier, and the development of the nervous system. During the course of development astrocytes have changing functions. Before they acquire their mature form and functions, astrocytes participate in the developmental synaptogenesis, during which the number of synapses dramatic increases in the brain. The major waves of rodent CNS synaptogenesis occur roughly around p15 to p25. This is the time when astrocytes are dynamically growing and differentiating. This change in the astrocytic morphology goes hand-in-hand with a change in their electrophysiological properties, receptor and gene expression profiles. By p30 (after the major wave of synapse formation), astrocytes have taken on their mature morphology, electrophysiology, gene and receptor expression profile.

Direct synaptic junctions from neurons to non-neuronal cells appear to be restricted to connections linking neurons and NG2-expressing glial cells in the cerebral cortex. In spite of the apparent absence of synapses on other glial cells, functional, depolarizing GABAergic responses are characteristic of glia , and the presence of various GABA receptors and transporters is widely established. Accordingly, potentially non-synaptic GABAergic interactions between neurons and several types of glia were suggested by exogenously applied agonists or prolonged, high-frequency stimulation of GABAergic interneurons, however, physiological activation of GABA receptors on astrocytes is not clearly shown.

In the last decades the human neocortex have been investigated and compared to those of other mammals such as primates, carnivores, and rodents. Although, there are species-specific differences in the layering, long-range connections and intrinsic electrical and morphological properties of nerve cells, the cellular elements and local synaptic circuits were found to be rather stereotyped. For example, similar types of cortical GABAergic interneurons, pyramidal cells and comparable local synaptic connectivity patterns were reported in rodent and human neocortices. However, classic theories suggest that synaptic properties also contribute to cognitive abilities and recent studies revealed differences in synaptic communication between cell types that are highly conserved among mammals. Human cortical pyramidal cell to pyramidal cell connections display spike timing-dependent long-term plasticity similar to that found in rodents, but with altered plasticity-inducing activity patterns. It has also been demonstrated that the outputs of human pyramidal cells can be so powerful that individual presynaptic action potentials result in suprathreshold postsynaptic responses in GABAergic neurons and polysynaptic series of events downstream in the network. These powerful pyramidal cell to interneuron connections are not characteristic to nonhuman tissue and were speculated to be important in reliable propagation of information in the human neocortex.

2 Aims

This thesis has two aims: 1) to elucidate the effect of GABA released by unitary volume transmission from a neocortical neurogliaform interneurons to astrocytes. 2) to study specific differences of a highly conserved synaptic pathway, the excitatory connections from pyramidal cells to basket and axo-axonic interneurons in the rat and the human neocortex. Our specific questions were the following:

1. Does physiological spiking activity (e.g. single action potential) of an interneuron elicit the GABA-mediated currents in astrocytes? In addition, are there differences between interneuron types (neurogliaform cell versus non-neurogliaform cell) in their capacity to elicit the currents/potentials?

2. If so, what cellular level mechanisms are involved in the GABAergic astrocytic currents?

3. Is the physiologically released GABAergic response in astrocytes associated with cytoplasmic calcium transients?

4. How biophysical and structural features differ between excitatory glutamatergic synapses from a pyramidal cell to fast spiking interneuron in the rodent and human neocortex?

3 Materials and Methods

3.1 Slice preparation

3.1.1 Rodent brain slice preparation

Experiments were conducted according to the guidelines of the University of Szeged Animal Care and Use Committee. Astrocytes are considered mature after postnatal day 20 (p20); thus, for the experiments including astrocytes we used the somatosensory cortex of young adult (p25–46, p37.4 $\pm$ 4.5, n = 137) male Wistar rats. In the experiments testing local excitatory input to fast spiking interneurons, we used the somatosensory cortex of male Wistar rats (p18–28, n = 9) and medial prefrontal cortex of adult male Wistar rats (p53–65, n = 17).

Animals were anaesthetized by inhalation of halothane, and following decapitation, the brain was removed and immediately put in an ice-cold cutting solution composed of (in mM) 85 NaCl, 3 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 0.5 $CaCl_2$, 4 $MgSO_4$, 25 D(+)-glucose, 75 sucrose gassed with 95 % O_2, and 5 % CO_2. After the removal of the brain (350 µm thick) coronal slices were cut with a vibrating blade microtome (Microm HM 650 V) perpendicular to cortical layers either from the somatosensory or medial prefrontal cortex. Slices were incubated at room temperature for 1 h in a solution composed of (in mM) 130 NaCl, 4.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$, 10 D(+)-glucose, gassed with 95 % O_2, and 5 % CO_2.

3.1.2 Human brain slice preparation

All procedures were performed according to the Declaration of Helsinki with the approval of the University of Szeged Ethical Committee. Human slices were derived from material that had to be removed to gain access for the surgical treatment of deep-brain tumors from the left and right frontal (n = 22), temporal (n = 14), and parietal (n = 3) regions with written informed consent of female (n = 15, aged 53 $\pm$ 13 years) and male (n = 11, aged 43 $\pm$ 24 years) patients prior to surgery.

Anesthesia was induced with intravenous midazolam and fentanyl (0.03 mg/kg, 1–2 mg/kg, respectively). A bolus dose of propofol (1–2 mg/kg) was administered intravenously. To facilitate endotracheal intubation, the patient received 0.5 mg/kg rocuronium. After 120 s, the trachea was intubated and the patient was ventilated with a mixture of O_2 and N_2O at a ratio of 1:2. Anaesthesia was maintained with sevoflurane at monitored anaesthesia care volume of 1.2–1.5. After surgical removing blocks of tissue were immersed immediately in ice-cold cutting solution and were resected and incubated in the same way as the rodent tissue.

3.2 Electrophysiology

The solution used for recordings had the same composition as the incubating solution except that the concentrations of $CaCl_2$ and $MgSO_4$ were 3 mM and 1.5 mM, respectively. Given that neurons and mature astrocytes have similar intracellular ionic milieux, we used the same intracellular solution for both. The micropipettes (3–6 MΩ) were filled with (in mM) 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na_2, 10 HEPES, 10 kreatin phosphate (pH 7.25; 300 mOsm). For presynaptic PCs, the intracellular solution contained also 10 mM L-glutamic acid and thus only 116 mM K-gluconate to prevent the rundown of postsynaptic responses. To reveal the anatomy of the recorded cells we added 8 mM biocytin for neurons, whereas for astrocytes in order to avoid extensive dye coupling, we used 0.3 mM biotinylated dextran (BDA). For some experiments, 1 mM GDP-β-S was also added as described in the text.

Somatic whole-cell recordings were obtained at 36 °C from simultaneously recorded doublets of interneurons and astrocytes or doublets pyramidal cells and fast spiking interneurons visualized by infrared differential interference contrast videomicroscopy (Olympus BX microscopes equipped with oblique illumination, Luigs & Neumann Infrapatch setup and HEKA EPC 10 USB patch-clamp amplifier). Signals were filtered at 7 kHz, digitized at 20-100kHz, and analyzed with Patchmaster (HEKA) and MATLAB software (The MathWorks). Presynaptic interneurons and pyramidal cells were stimulated to elicit action potentials with brief (1–20 ms) suprathreshold pulses in current clamp mode at intervals >90 s and 5 s for interneurons and pyramidal cells, respectively.

3.3 Two-photon calcium imaging

Astrocytes were filled with two fluorescent dyes: a Ca^{2+}-insensitive fluorophore (Alexa Fluor 594, 20–50 µM, Invitrogen), and a Ca^{2+}-sensitive fluorophore (Oregon Green BAPTA-1, 120 µM, Invitrogen). Red fluorescence was used to identify glial processes and cancel movement artefacts. Neurons were occasionally filled with Alexa Fluor 594 (20–50 µM). Imaging was performed with a Femto3D-AO (Femtonics Ltd.) acousto-optic laser-scanning microscope driven by a MaiTai femtosecond pulsing laser (MaiTai, SpectraPhysics) tuned to 850 nm. We used a 60× Olympus (NA = 0.9) objective in order to resolve small processes. Both reflected and transmitted fluorescent lights were collected (through an oil-immersion condenser, Olympus; NA = 1.4). 150–500 ROIs were automatically selected in an approximately 100 × 100 × 50 µm volume based on previously obtained Z-stacks. Image acquisition was controlled by custom-made software written in MATLAB (MES, Femtonics Ltd.). In the 60–120 second-long imaging sessions, the sampling frequency on each ROI ranged from 40 to 120 Hz, and 3–8 mW laser power reached the slice during imaging.

3.4 Post hoc anatomical analysis

3.4.1 Histology and light microscopy

The electrophysiologically recorded cells were filled with biocytin or biotinylated dextran, which allowed post hoc anatomical analysis of the tested connections. After electrophysiological recordings slices were fixed in a fixative containing 4 % paraformaldehyde, 15 % picric acid and 1.25 % glutaraldehyde in 0.1 M phosphate buffer (PB; pH = 7.4) at 4 °C for at least 12 h. After several washes in 0.1 M PB, slices were cryoprotected in 10 % then 20 % sucrose solution in 0.1 M PB. Slices were frozen in liquid nitrogen then thawed in PB, embedded in 10 % gelatin and further sectioned into slices of 60 µm in thickness. Sections were incubated in a solution of conjugated avidin-biotin horseradish peroxidase (ABC; 1:100; Vector Labs) in Tris-buffered saline (TBS, pH = 7.4) at 4 °C overnight. The enzyme reaction was revealed by 3′3-diaminobenzidine tetrahydrochloride (0.05 % , DAB) as chromogen and 0.01 % H_2O_2 as oxidant. Sections were post fixed with 1 % OsO_4 in 0.1 MPB. Three dimensional light microscopic reconstructions were carried out using Neurolucida

system with 100$\times$ objective. Anatomical recovery rates were lower than expected in the case of interneuron-astrocyte pairs.

3.4.2 Statistics

Data were statistically tested by parametric tests (paired t test or two-sample t test) if they passed the Lilliefors test for normal distribution, and nonparametric tests (Wilcoxon signed-rank test (W SR test) or Mann–Whitney U test) were applied in all other cases. Linear correlations were tested using Pearson's linear correlation coefficient. Error bars and shaded areas show standard deviation.

4 Results

4.1 GABAergic volume transmission from interneurons to astrocytes in the rodent cerebral cortex

4.1.1 Cell-type-specific coupling from interneurons to astrocytes

Following reports indicating that certain neocortical interneurons might specialize in non-synaptic volume transmission, we performed dual whole-cell patch-clamp recordings of closely spaced (<130 µm) interneuron–astrocyte cell pairs (n = 209), testing the output of n = 164 neurons in layer 1 of the rat somatosensory cortex. All the glial cells recorded had features typical of mature astrocytes having highly negative membrane potential (–90.2 ± 3.9 mV) and low input resistance (estimated value: 1.05 ± 3.94 MΩ).

The analysis of the responses of astrocytes to single action potentials triggered in interneurons revealed that non-NGFCs induced no detectable current. In contrast, single action potentials in NGFCs evoked measurable ($p < 0.05$, W SR-test) inward currents in 63.1 % of simultaneously recorded astrocytes with amplitudes of 2.48 ± 1.78 pA (n = 82 of the 130 pairs tested). These data suggest a cell-type-specific coupling from interneurons to astrocytes. The NGFC-elicited inward currents consisted of two components, an early component with short (1.6 ms) latency, and a late component with an onset around 35 ms after the action potential. The early component had an average amplitude of 0.68 ± 0.45 pA and average 10–90 % rise time of 12.9 ± 6.9 ms. The late component had an average amplitude of 1.96 ± 1.89 pA and average 10–90 % rise time of 176.6 ± 87.8 ms (n = 82).

All interneurons with anatomical recovery triggering a detectable effect showed the very dense axonal arborization typical of NGFCs. By contrast, non-NGFCs, which –following single spikes– had no effect on astrocytes, displayed relatively

sparse axonal arborizations. We reconstructed the interacting NGFCs and the spatial boundary of astrocytes filled with biotinylated dextran (n = 6) in three dimensions. When counting the number of neurogliaform axonal collaterals crossing the field of the astrocyte, we found linear correlation with the amplitude of the inward current measured on the astrocyte (r = 0.818, p = 0.046, n = 6), which suggested that the density of the interneuron output contributes to the effectiveness of connections.

4.1.2 The astrocytic inward current consists of an early direct and a late indirect component

The biphasic time course of astrocytic responses to NGFC activation detailed above indicates that potentially complex mechanisms form the basis of these cell-type-specific pathways. Astrocytes are known to participate in potassium clearance from the extracellular space; thus, we applied $BaCl_2$, a concentration-dependent blocker of GIRK and KIR channels in the bath in search for potential K^+ currents. These experiments revealed an early, barium-insensitive and a late, barium-sensitive component of the astrocytic inward current induced by single action potentials in NGFCs. Applied at low concentrations (5 µM–10 µM), $BaCl_2$ significantly reduced the late component probably by acting on GIRK channels (from 4.35 ± 1.71 pA to 1.59 ± 0.82 pA, p = 0.004, n = 5) without affecting the early component. High concentration of $BaCl_2$ (100 µM) abolished the late component (from 1.80 ± 1.32 pA to 0.08 ± 0.18 pA, p = 0.0031, n = 7) and increased the early component (from 1.17 ± 0.57 pA to 2.71 ± 1.26 pA, p = 0.009, n = 7) most likely by broadening action potentials (from 0.77 ± 0.12 ms to 0.85 ± 0.13 ms, p = 0.023, n = 7) and increasing the presynaptic neurotransmitter release or by increasing the input resistance and decreasing the attenuation of the signal due to KIR channel blockade.

Following pioneering reports showing that $GABA_A$ receptors depolarize astrocytes and given that NGFCs release GABA, we tested whether GABAergic transmission contributes to single-cell-evoked currents. Bath application of gabazine (5 µM), a $GABA_A$ receptor blocker, significantly reduced both the early potassium-independent and the late potassium-dependent inward current (from 1.28 ± 0.62 pA to 1.01 ± 0.51 pA, p = 0.024, n = 5 for the early component and from 1.76 ± 1.52 pA to 0.88 ± 0.61 pA, p = 0.032, n = 5 for the late component). The biphasic effect of $GABA_A$ receptor blockade reflects the direct $GABA_A$ receptor activation in the early component and the subsequent increase in extracellular K^+ ions due to the neuronal

potassium-dependent chloride extrusion in the late component. Besides $GABA_A$ receptors, astrocytes also express GABA transporters to remove the neurotransmitter from the extracellular space, which is accompanied by an inward Na^+ current. Bath application of the GABA reuptake inhibitor (GAT1 inhibitor) NO711 (100 µM) alone reduced the astrocytic early component (from 1.21 ± 0.51 pA to 0.68 ± 0.29 pA, $p = 0.023$, $n = 6$). These results show that GABA released from single neurogliaform axons non-synaptically acts on astrocytic $GABA_A$ receptors, inducing chloride and probably bicarbonate ion efflux and, furthermore, confirm the contribution of potassium-dependent Cl^- and sodium-dependent GABA transport to neurogliaform-to-astrocyte signaling.

The astrocytic late component was also sensitive to the selective $GABA_B$ receptor antagonist CGP35348 (40 µM), which reduced the late component (from 1.77 ± 0.99 pA to 0.45 ± 0.42 pA, $p < 0.01$, $n = 6$) reminiscent of the $BaCl_2$ application. Thus, the late component appears to be mediated by $GABA_B$ receptors coupled to K^+ channels, and, as such, it is amplified by GABA transporter blockade. However, given the high-resolution immunohistochemical and in situ hybridization results of independent reports, which failed to detect $GABA_B$ receptors on mature astrocytes, the $GABA_B$ receptors responsible for the recorded current might not reside on the astrocytes. So, we tried to block $GABA_B$ receptors in the astrocytes intracellularly, using the G-protein blocker GDP-β-S (1 mM), but this resulted in no significant changes. As a control, the slow $GABA_B$ component of unitary neurogliaform-to-pyramidal cell IPSPs was abolished with intracellular application of GDP-β-S in pyramidal cells under the same conditions ($n = 3$). These results suggest that the activation of neuronal $GABA_B$ receptors leads to potassium efflux from neurons and a subsequent passive inward potassium current in astrocytes.

4.1.3 Physiological release of GABA does not elicit detectable calcium events in surrounding astrocytes

Astrocytes exhibit intense local Ca^{2+} events, the dynamics of which were suggested to be modulated by GABAergic signaling through several mechanisms. The activation of $GABA_A$ receptors was reported to elicit Ca^{2+} signals in astrocytes through membrane depolarization and subsequent voltage-dependent Ca^{2+} channel recruitment, but the effectiveness of endogenously released GABA in astroglial Ca^{2+} dynamics is yet to be established. Increased Ca^{2+} dynamics following astrocytic GABA

receptor activation has repeatedly been observed; however, $GABA_B$ receptor-mediated Ca^{2+} events could only be detected in cultured astrocytes, which are known to be distinct from mature cells, or in young animals consistent with the transient expression of $GABA_B$ receptors in astrocytes. To reveal the diverse Ca^{2+} signals in both astrocytic somata and processes, we intracellularly filled single astrocytes in the close vicinity (<70 µm) of identified NGFCs with the calcium indicator OGB-1 (120 µM) and a calcium-insensitive structure dye Alexa Fluor 594 (40 µM). Then, we randomly selected 100–300 regions of interest on the soma and processes of the intracellularly filled as well as of the neighboring astrocytes dye coupled through gap junctions. We readily detected spontaneous Ca^{2+} events of variable amplitude and duration. However, we could not detect any increase in the amplitude or frequency of these events following single action potentials in NGFCs, although it might have been anticipated on the basis of previous reports. This suggests that the magnitude of unitary evoked measurable inward currents is not sufficient to depolarize the membrane for the recruitment of voltage-gated Ca^{2+} channels, and furthermore, it also indicates the absence of $GABA_B$ receptor activation-dependent Ca^{2+} signaling.

4.2 Species specific differences in supragranular pyramidal to interneuron synapses

4.2.1 Quantal parameters of pyramidal cell to fast spiking interneuron EPSCs in the human and rat cerebral cortex

We compared the functional properties of synaptic connections made by pyramidal cells onto fast-spiking interneurons in the layer 2/3 of human (n = 39) and rat (n = 26) neocortex. Acute brain slices were prepared from small blocks of non-pathological samples of human cortical tissue resected in surgery from female (n = 15, aged 53 ± 13 years) and male (n = 11, aged 43 ± 24 years) patients, from somatosensory cortex of juvenile (p18–28, n = 9) and prefrontal cortex of adult (p53–65, n = 17) male rats using standard acute slice preparation procedures. Both presynaptic pyramidal cells and postsynaptic interneurons were whole-cell recorded with biocytin-containing intracellular solutions, allowing post hoc identification of the recorded cell types and the number of synaptic contacts between them. Neurons were chosen based on their typical membrane and firing properties. Postsynaptic

interneurons were identified as either axo-axonic (AACs, human: n = 6) or basket (BCs, human: n = 18; rat: n = 8) cells based on their characteristic axonal cartridges or axonal branches forming perisomatic baskets, respectively

To determine quantal parameters of the unitary EPSCs, we performed multiple probability fluctuation analysis (MPFA; human: n = 10, rat: n = 12). Different release probability conditions were imposed by altering Ca^{2+} and Mg^{2+} ion concentrations in the extracellular solution during recordings and measured the means and variances of EPSC charge. These recordings were performed in the presence of an NMDA and a cannabinoid receptor antagonist (20 µM D-AP-5 and 10 µmol AM251, respectively) to prevent NMDA channel openings, which might reduce variances and induce long-term plasticity and to exclude undesirable retrograde short- or long-term modification of glutamatergic transmission during MPFA. The low affinity competitive non-NMDA receptor antagonist γDGG (0.5 mM) was included to prevent AMPA receptor saturation.

To avoid rundown of EPSCs during long-lasting recordings, 10 mM L-glutamate was added to the internal solution of the presynaptic cell. As expected, the amplitudes of human and rat unitary EPSCs were effectively modulated by altering extracellular Ca^{2+} and Mg^{2+} ion concentrations, consistent with changes in the probability of release (Pr) of a vesicle at a functional release site. EPSC failure rates were significantly lower in human neurons ($p < 0.012$, MW U-test) in each condition compared to rats (1 mM $[Ca^{2+}]_o$: 15.8 ± 18.7 % vs. 62.8 ± 34.2 %, 1.5 mM $[Ca^{2+}]_o$: 2.0 ± 4.0 % vs. 39.1 ± 24.7 %, 2 mM $[Ca^{2+}]_o$: 0 vs. 26.6 ± 21.7 %, 4 mM $[Ca^{2+}]_o$: 0 vs. 8.4 ± 10.2 %), indicating either different vesicle Pr or number of functional release sites (N_{FRS}). This approach resulted in a large, 10- and 5.5-fold increase in the mean charge transferred by human and rat unitary EPSCs, respectively from the lowest to the highest $[Ca^{2+}]_o$, which is a prerequisite for reliable determination of the quantal parameters. By fitting the mean vs. variance relationship with a parabola, we obtained an estimate of quantal size, Pr and N_{FRS} for each cell pair. Statistical comparisons of data from all cells revealed no significant difference in quantal size (p = 0.11, MW U-test). We found significant difference in Pr (at 1.5 mM $[Ca^{2+}]_o$: human: 0.33 ± 0.10, range: 0.18–0.48, n = 7; rat: 0.17 ± 0.13, range: 0.05–0.53, n = 12; p = 0.01, MW U-test). Furthermore, our analysis revealed a 4.4-times larger N_{FRS} in human connections (20.5 ± 15.4, range 5.1–45.2) compared to rats (4.7 ± 2.2, range 1.3–9.5; $p < 0.001$, MW U-test).

4.2.2 Structural properties of pyramidal cell to fast spiking interneuron connections in the human and rat cerebral cortex

A potential explanation for the differences in EPSC amplitudes and N_{FRS} between human and rat is a larger number of synaptic contacts connecting the PC axons to the postsynaptic IN dendrites in the human cortex. To assess the number of putative synaptic contacts, team members without access to the N_{FRS} searched for close appositions of presynaptic axon terminals and postsynaptic dendrites under light microscopy (LM). The presynaptic axon from the parent pyramidal cell soma to the target interneuron dendrites were reconstructed in nine human (n = 2 PC to AAC and n = 7 PC to BC pairs) and fifteen rat (n = 15 PC to BC pairs) pairs that partially overlapped with MPFA analyzed cell pairs. Comparison of the number of LM detected synaptic contacts (N_{LM}) between human (3.3 ± 1.5; range: 1–6) and rat (2.9 ± 1.5, range: 1–6) revealed no significant difference (p = 0.35, MW U-test). Consequently, dividing the mean N_{FRS} with the mean N_{LM} indicated that each presynaptic bouton contains on average 6.2 and 1.6 functional release sites in human and rat, respectively. This ratio was also larger for human connections when calculated from those pairs for which both MPFA and LM reconstructions could be performed (human: N_{FRS}/N_{LM} = 5.7 ± 5.1, range 1.6–13.3, n = 4; rat: N_{FRS}/N_{LM} = 2.6, n = 1). These results demonstrate that differences in the number of synaptic junctions between PCs and fast-spiking interneurons is not responsible for the observed 4.4-fold larger N_{FRS}, therefore each presynaptic PC axon terminal must contain a larger N_{FRS} in human.

Finally we assessed potential ultrastructural correlates of the above described differences in the N_{FRS} in individual axon terminals. We performed three-dimensional reconstructions of presynaptic axon terminals from 20 nm thick serial EM sections. Biocytin-filled, post hoc recovered human (n = 3) and rat (n = 3) INs were chosen and axon terminals that established asymmetrical synaptic contacts on selected dendrites were fully reconstructed . First we compared the size of presynaptic active zones (AZs) and found that both human and rat active zones have variable sizes (human: 0.02–0.26 mm^2; rat: 0.02–0.08 mm^2), but the human AZs were on average twice as large. Each bouton contained only a single AZ in both species. When we counted docked vesicles in these AZs using the 20 nm thin serial section approach we found a significantly larger number in human (4.2 ± 2.2 / AZ) compared to rats (1.3 ± 0.8 / AZ; $p < 0.001$, MW U-test), resulting in a twice as large docked vesicle

density in human. However, as the precise identification of docked vesicles is challenging in conventional EM even if very thin (20 nm) sections are used, we repeated these measurements using EM tomography; the best currently known method for this application. Analysis of EM tomograms in 3 human (n = 33 synapses) and 3 rat (n = 31 synapses) samples revealed a similar two-fold difference in the mean docked vesicle density.

5 Discussion

5.1 GABAergic volume transmission from interneurons to astrocytes in the rodent cerebral cortex

Non-synaptic or volume transmission acts ubiquitously on all target elements expressing receptors for the released transmitter. Neurogliaform cells were suggested to operate by flooding the axonal arborization with GABA, resulting in a single-cell-driven volume transmission. The results presented here extend this concept and identify a cell-type-specific route for interneuron to astrocyte signaling in addition to the conventional GABAergic output toward neurons. Neurogliaform cell to astrocyte communication has several GABAergic elements. The early component has a contribution of currents mediated by $GABA_A$ receptors and GABA transporters, and its short latency indicates a direct communication pathway from NGFCs to astrocytes. The second, indirect GABAergic pathway involves the activity of chloride transporters and $GABA_B$ receptors presumably located on neuronal elements of the circuit, and the resulting extracellular accumulation of K^+ is taken up by astrocytes as the late component. The net result of this cascade of events is the transport of K^+ through the extracellular space and a transient rise in Cl^- and/or bicarbonate in the extracellular space. Both of these factors contribute to the suppression of neuronal excitability by membrane hyperpolarization and by maintaining the driving force of GABAergic synapses, respectively.

Astrocytes were repeatedly shown to actively participate in neurotransmission with propagating intracellular calcium waves and gliotransmitter release. However, our results demonstrate a passive role of astrocytes in a unitary GABAegic communication pathway. The source of this controversy might be the different experimental procedures used. Our experiments were performed in brain slices prepared from young adult rats (p25–46, p37.4 $\pm$ 4.5) where the electrophysiological, anatomical and molecular properties of astrocytes are considered mature. In contrast, previous

studies showing astrocytic calcium signalling due to $GABA_B$ receptor activation or gliotransmission, were shown mainly in cell cultures or in slice preparations from young rodents (p12-p25), in the timeframe where synaptogenesis takes place.

5.2 Species specific differences in supragranular pyramidal cell to interneuron synapses

Paired recordings, MPFA and post hoc anatomical reconstructions revealed that presynaptic AZs contain on average 6 functional release sites in human, but only 1.6 in rats. High resolution EM analysis identified corresponding species-specific differences in the AZ area and the number of docked vesicles. Our data allowed us to provide the first estimate of the size of Katz's functional release site of cortical synapses; approximately $0.012\,\mu m^2$ AZ membrane area in human and $0.025\,\mu m^2$ in rats. Thus, the space that harbors a functional release site or a docking site seems to be substantially smaller in human AZs. This raises an interesting question: why the molecular machinery necessary for the assembly of a functional release site needs less space in human? Answering this question requires quantitative proteomic analysis of these AZs at nanometer resolutions. Furthermore, it will be also interesting to see whether this species-specific difference is valid for all central synapses or it is a unique feature of the cortical microcircuit.

Our data demonstrating that an AZ in rat PC axons contains on average a single functional release site is consistent with the 'one site-one vesicle' hypothesis and with data previously published for rodent cortical and hippocampal excitatory synapses. In contrast, the same type of axon terminals in human contain on average 6 functional release sites, demonstrating that here multivesicular release (MVR) is the main mode of operation. However, it is important to note that our data clearly demonstrate large variability in the AZ area in both species and that the number of docked vesicles and docking sites correlates with the AZ area. Thus, MVR is not a human-specific feature of cortical synapses, but it is expected to occur at large AZs of this connection in rats, as it has been shown to take place in many other rat glutamatergic synapses. Comparison of the N_{FRS} and the number of docked vesicles allowed the calculation of the average docking site occupancy that is 0.8 in rats and ≈ 0.7 in human synapses, similar to that calculated for the Calyx of Held, and somewhat higher than that found in cerebellar interneuron synapses. Similarly

large docking site occupancy and vesicle Pr between human and rodents predict similar short-term plasticity of transmission, a key feature of synapses of dynamic neuronal networks, with an additional tuning range for human connection strength due to higher N_{FRS}.

Our research had two main conclusions. First, we discovered of a novel interneuron-to-astrocyte signalling pathway, the function of which is probably to stabilize the widespread inhibitory action of neurogliaform interneurons through unitary GABAergic volume transmission. And second, we compared a ubiquitous synaptic connection in the human and rodent neocortex and found the biophysical differences in their workings that make human synapses more efficient.