# universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Interneurons in the Medial Prefrontal Cortex -
Molecular Expression Profiles, Postsynaptic Targets and
The Qest for Novel Molecular Markers

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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...dedicated to Elie Bahbah, the person I missed the most during my stay in Oxford

and to my parents, who supported me throughout my student career without

questioning what I am doing...
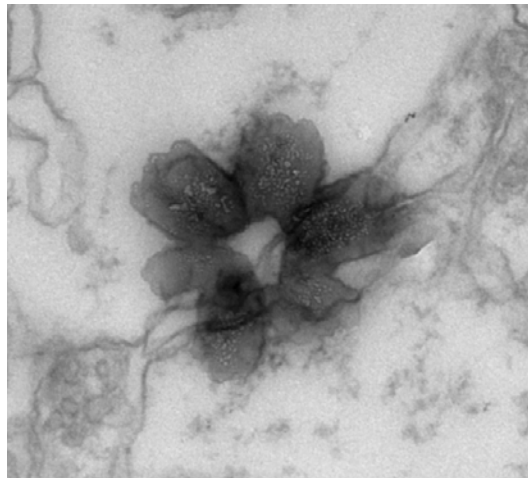
in memoriam of Patricia S. Goldman-Rakic, who dedicated her life to

understanding the circuitry of the prefrontal cortex

Content

Abstract

During working memory/decision making tasks correlated firing between the hippocampus and the medial prefrontal cortex (mPFC) of rats is enhanced and neurons in the mPFC are phase-locked to hippocampal theta oscillations. In order to understand the contribution of GABAergic interneurons of the mPFC to temporal network organisation, the firing patterns of single interneurons in the mPFC were recorded in anaesthetized rats and their soma, dendrites and axons specifically labelled with neurobiotin (done by Katja Hartwich). In this diploma thesis, I have investigated the molecular expression profile and postsynaptic targets of the recorded and labelled interneurons. Most of the cells expressed either parvalbumin (PV), or Calbindin (CB) or both calcium-binding proteins. Cells expressing PV only and cells expressing PV and CB target small and apical dendrites, dendritic spines and somata of pyramidal cells, and could therefore be identified as basket cells. In contrast, CB only expressing cells exclusively terminate on small dendrites and spines. Additionally, an axo-axonic cell was identified, which targets exclusively axon initial segments and does not express PV or CB. In order to support future characterization of interneuron classes using novel molecular markers, I have also tested several antibodies against molecules that could further distinguish between cell classes. In conclusion, in a subset of GABAergic interneurons in the mPFC, molecular expression profile and postsynaptic target-specificity seems to be correlated: PV expressing, but also PV/CB co-expressing interneurons could be identified as basket cells and CB only expressing cells as dendrite targeting interneurons.

Zusammenfassung

In Aufgaben, die eine Entscheidungsfindung unter Mitwirkung des Arbeitsgedächtnisses erfordern, tritt verstärkt korreliertes Feuerungsverhalten von Neuronen im Hippocampus und im medialen, präfrontalen Kortex (mPFC) auf und Neurone im mPFC feuern zu einer bestimmten Phase von Theta-Oszillationen im Hippocampus. Um zu verstehen welche Rolle Interneurone im mPFC bei diesem Phänomen spielen, wurde das Feuerungsmuster von einzelnen Interneuronen im mPFC aufgezeichnet und deren Soma, Dendriten und Axons mit Neurobiotin angefärbt (durchgeführt von Katja Hartwich). In dieser Diplomarbeit habe ich die molekularen Expressionsmuster und postsynaptischen Zielzellen dieser angefärbten Interneurone untersucht. Der Großteil dieser angefärbten Zellen exprimiert entweder Parvalbumin (PV) oder Calbindin (CB) oder beide dieser Calcium-bindenden Proteine. Zellen, die nur PV exprimieren oder PV und CB co-exprimieren bilden Synapsen an kleinen und apikalen Dendriten, dendritischen Dornen und Zellkörpern von Pyramidenzellen und konnten daher als Korbzellen identifiziert werden. Im Gegensatz dazu bilden CB-exprimierende Zellen Synapsen ausschließlich an kleinen Dendriten und dendritischen Dornen von Pyramidenzellen aus. Außerdem wurde noch ein Zelltyp identifiziert, der ausschließlich Synapsen an initialen Axonhügeln ausbildet und weder PV noch CB exprimiert. Um zukünftig die Charakterisierung von Interneuronklassen durch neue molekulare Marker zu unterstützen, habe ich auch diverse Antikörper getestet, die es erlauben zwischen Zelltypen zu unterscheiden.

Als Fazit, in einer Subpopulation von GABAergen Interneuronen im mPFC scheint das molekulare Expressionsmuster mit der Spezifität von postsynaptischen Zielzellen korreliert zu sein: PV-exprimierende aber auch PV und CB co-exprimierende Zellen konnten als Korbzellen und Zellen, die nur CB exprimieren, als Dendriten innervierende Interneurone identifiziert werden.

Introduction

Interaction of hippocampus-dependent short-term memory and neocortex-dependent long-term memory is important for intelligent behaviour (Goldman-Rakic, 1995). György Buzsaki (Buzsaki, 1989) put forward a model of memory consolidation in which the animal is considered to be in a recording mode during exploratory behaviour. This recorded information is played back in temporally compressed form during subsequent periods of inactivity and sleep in the prefrontal cortex (Euston et al., 2007), the hippocampus (Diba and Buzsaki, 2007) and the visual cortex (Ji and Wilson, 2007), whereas the latter might account for vivid images during dreaming. Periodic playback could promote a gradual integration of newly acquired knowledge into the neocortex (Wiltgen et al., 2004). A gradual integration is important, because a rapid incorporation of new information into a pre-existing knowledge system could cause interference and previously acquired information would be erased (McClelland, 1995).

Once the information is consolidated in the neocortex, it becomes independent of the hippocampus. For subsequent retrieval and integration with newly acquired information stored in the hippocampus, the prefrontal cortex (PFC) plays a central role (Wiltgen et al., 2004). This integration process is referred to as working memory and can be described as retaining acquired knowledge for an impending action, which is dependent on that knowledge (Fuster, 2001). In delayed response tasks the functioning of working memory can be elucidated and it was shown that in rats with lesions in the prelimbic/infralimbic medial PFC, working memory was impaired (Gisquet-Verrier and Delatour, 2006).

The rodent PFC is divided into medial, lateral and orbital parts with the medial part (mPFC) consisting of four sub-divisions (Ongur and Price, 2000). The most dorsal divisions are the medial (frontal) agranular and the anterior cingulate cortices, which receive sensorimotor input and are associated with motor behaviour. The prelimbic (PL) and infralimbic (IL) cortices make up the medio-ventral portion of the mPFC, receive limbic input from the hippocampus, subiculum and amygdala and are involved in emotional, cognitive and mnemonic processes (Heidbreder and Groenewegen, 2003). Furthermore, these brain areas are highly interconnected among themselves (Vertes, 2006). The functional analogue to the PL in primates is the dorsolateral PFC (Vertes,

2006) and it was reported that lesions of this brain area disrupt performance on delayed response tasks in monkeys (Goldman and Rosvold, 1970).

The mPFC receives input from various brain regions, including the hippocampus. Retrograde tracing has shown that PL and IL of rats are targeted by glutamatergic afferents of the CA1 / subiculum (Hoover and Vertes, 2007) – the major output areas of the hippocampal formation. This is consistent with anterograde tracing studies (Jay and Witter, 1991) and has also been shown in primates with both retrograde and anterograde tracing (Goldman-Rakic et al., 1984). Not only the mPFC but also this Hippocampo-mPFC pathway seems to be important for working memory as it was shown that rats with a transient inactivation of this pathway were impaired in a delayed choice of a radial-arm maze task (Floresco et al., 1997).

The cerebral cortex contains two main types of neurons, excitatory pyramidal cells (DeFelipe and Farinas, 1992), which are the vast majority (70-80 %) and are relatively uniform, and interneurons, which are mostly inhibitory and have diverse morphological, physiological, molecular and synaptic properties (Kawaguchi and Kubota, 1997). Single pulse stimulation of the hippocampus in anaesthetized rats induced EPSPs in mPFC pyramidal neurons of fixed latency, suggesting a monosynaptic connection, followed by prolonged IPSPs (Degenetais et al., 2003). Afferent projections from CA1 / subiculum not only target pyramidal cells of the mPFC (Carr and Sesack, 1996), but also interneurons (Gabbott et al., 2002). So whilst the IPSP could result from innervation of interneurons by these activated PFC pyramidal cells through a feedback process, it might also result from interneurons being excited directly by CA1 projections. Indeed, a large proportion of recorded mPFC interneurons showed an excitatory response with a latency consistent with the conduction time of the hippocampo-mPFC pathway, suggesting a monosynaptic connection (Tierney et al., 2004). However, recorded and juxtacellularly labelled interneurons were not characterised further in this study. Traditionally neurons have been classified by their morphology (Ramon y Cajal, 1911), but because this is a highly variable feature of neurons, morphology alone cannot reliably define a cell type (Markram et al., 2004).

It has been suggested (Somogyi and Klausberger, 2005) that cortical neurons can be characterized and defined on application of following measures: (I) brain area- and cell domain-specific distribution of presynaptic inputs and postsynaptic targets, (II) expression profiles of signalling molecules and ion channels, and (III) *in vivo* firing patterns.

Interneurons can be classified by investigating their postsynaptic targets and can be functionally divided into axo-axonic cells targeting exclusively axon initial segments, soma- and proximal dendrite-targeting basket cells, proximal dendrite-targeting, and distal dendrite- and spine-targeting interneurons (Somogyi et al., 1998). Axo-axonic cells have their boutons aligned along axon initial segments and can thus be recognized in some cases on the light microscopic level. On the other hand, axons of basket cells, which are soma- and proximal dendrite-targeting cells, show little directivity in their orientation in close proximity to their somata (Somogyi and Klausberger, 2005). Furthermore, there are subpopulations of interneurons that target preferentially other interneurons (Acsady et al., 1996).

Molecules such as calcium binding proteins Parvalbumin (PV), Calbindin (CB), or Calretinin (CR), or neuromodulators like neuropeptide Y (NPY), Somatostatin (SM), Cholecystokinin (CCK), vasoactive intestinal peptide (VIP) are known to be differentially expressed in subpopulations of interneurons in the hippocampus (Somogyi and Klausberger, 2005). However, it has been demonstrated in the hippocampus that the expression of a single molecule cannot define a distinct class of interneurons as defined by synaptic connectivity, because most molecules are expressed by several classes. For example, parvalbumin is expressed by basket, axo-axonic, bistratified and oriens-lacunosum moleculare (O-LM) cells (Somogyi and Klausberger, 2005). In order to further characterize interneuron classes, novel molecular markers were investigated and potential candidates are described below.

The mPFC receives dopaminergic input from the ventral tegmental area (VTA) and it was shown that working memory performance was at its best with moderate stimulation of D1 family dopamine receptors (Muly et al., 1998). Dopamine D1 receptors are expressed by both pyramidal cells and GABAergic interneurons (Muly et al., 1998); most PV-expressing and 10 % of CB-expressing interneurons express D1 as shown by in situ hybridization (Le Moine and Gaspar, 1998). Furthermore, the effect of dopamine on inhibition dominates over that on excitation (Bandyopadhyay and Hablitz, 2007) and the PFC in turn acts through various pathways to excite or inhibit dopaminergic neurons in the VTA (Gao et al., 2007). Because of the importance of dopaminergic input for reinforcement learning mechanisms (Hazy et al., 2006), it would be interesting to investigate, which subpopulations of PV- and/or CB-expressing interneurons also expresses the dopamine D1 receptor.

The mPFC also interacts with the thalamus especially during sleep, which leads to the generation of spindle oscillations (7-14 Hz) (Steriade and Amzica, 1998). Furthermore, afferents from the thalamus express the vesicular glutamate transporter type 2 (vGLUT2), whereas cortical terminals either express vGLUT1 or a minor subpopulation of cortical terminals co-express both types (Kubota et al., 2007). It would be interesting to investigate, whether a subpopulation of PV-expressing cells receives more thalamic input than others.

Neurons in the mPFC are also modulated by Serotonin and in fact, the 2c subunit of the serotonin receptor (5-HT2c) is expressed particularly by neurons in deep layers of the PL area and 50 % of these neurons were immunopositive for glutamate decarboxylase (GAD67) (Liu et al., 2007a), the enzyme that converts glutamate into γ-aminobutyric acid (GABA). Furthermore, this receptor subunit is predominantly expressed by PV-expressing interneurons and to a significantly lesser extant in CB- or Calretinin-expressing cells (Liu et al., 2007a), so it would be interesting to see which subpopulation of PV-expressing cells also expresses 5-HT2c.

A subset of PV-expressing cells (26 %) receives about five times more boutons immunopositive for VIP than the rest of PV-expressing cells (David et al., 2007), but it is not known which subpopulation receives more input.

It was shown that the α1 subunit of a nitric oxide (NO)-sensitive guanylyl cyclase is specifically expressed in interneurons, but only in a subpopulation of PV-positive cells (75%) in the hippocampus (Szabadits et al., 2007). Nitric oxide contributes to synaptic plasticity as a retrograde messenger and this enzyme plays an important role in this pathway. This enzyme could therefore be used as a molecular marker for a subpopulation of PV-expressing cells.

Voltage-gated potassium channels comprise the most divers group of heteromeric ion channels discovered so far. There are more than 20 genes encoding these channels and examples of interneuron specific potassium channels are Kv3.1 (Sekirnjak et al., 1997) and Kv4.3 (Serodio and Rudy, 1998), Kv3.1 is co-expressed with PV, but not found in other subpopulations of interneurons (Chow et al., 1999). For Kv4.3 on the other hand, a highly uneven subcellular distribution of this potassium channel was reported (Kollo et al., 2006). Both of these channels might be usable as a molecular marker for characterization of cell types.

Another type of proteins that are differentially expressed in subsets of neurons are neurofilaments. For example, N200 is expressed in a subpopulation of pyramidal cells in the prefrontal cortex (Law and Harrison, 2003), but it would also be interesting to show whether these neurofilaments are also expressed in certain types of interneurons.

The metabotropic glutamate receptor subtype 1α (mGluR1α) has been associated with a variety of contributions to network activity, including modulation of synaptic transmission and plasticity and is predominantly expressed by interneurons of the hippocampal CA1 area. In particular, only a small subset of PV-expressing cells – mostly those, which also express SM - co-express mGluR1α (Ferraguti et al., 2004). This molecular marker could be employed to differentiate subpopulations of PV-expressing cells.

Network oscillations of various frequency bands in the brain provide a time frame and temporal coordination within and between distinct brain areas. Theta oscillations (4-8 Hz), for example, occur during exploratory behaviour and rapid-eye-movement sleep (Buzsaki, 2002) and are considered to be the online state of the hippocampus. Furthermore, during sleep the thalamus interacts with the mPFC, which leads to the generation of spindle oscillations (7-14 Hz) during slow wave sleep (0.5-4 Hz) (Steriade and Amzica, 1998).

It has been shown (Siapas et al., 2005) that in freely behaving rats mPFC neurons are phase-locked to hippocampal theta oscillations with a delay of approximately 50 ms suggesting that hippocampal activity is leading neural activity in the mPFC. Furthermore, mPFC neurons exhibit phase precession to the hippocampal theta rhythm (Jones and Wilson, 2005a), which indicates that the cell discharges at earlier phases of consecutive theta cycles (O'Keefe and Recce, 1993). Moreover, correlated firing of neurons in the hippocampus and the mPFC is selectively enhanced during behaviour that involves working memory (Jones and Wilson, 2005b).

As it has been shown that interneurons during theta oscillations coordinate the temporal activity of pyramidal cells in the hippocampus (Klausberger et al., 2003), interneurons in the mPFC might therefore play an important role for spike timing and phase-locking of mPFC neurons. Hence, Katja Hartwich in our lab has been investigating interneurons in the PL area of the mPFC in the context of hippocampal theta oscillations in anaesthetized rats. In order to do so, single interneurons were extracellularly recorded simultaneous with the local field potential (LFP) in the mPFC and the LFP in the CA1

region of the hippocampus and subsequently juxtacellularly labelled with neurobiotin. With the juxtacellular labelling method single-cells can be labelled specifically by using a glass electrode filled with Neurobiotin and injecting positive current pulses. The cell modulates its firing to these pulses and takes up the Neurobiotin, which distributes throughout the cell including dendrites and axon (**Figure 1**, (Pinault, 1996).
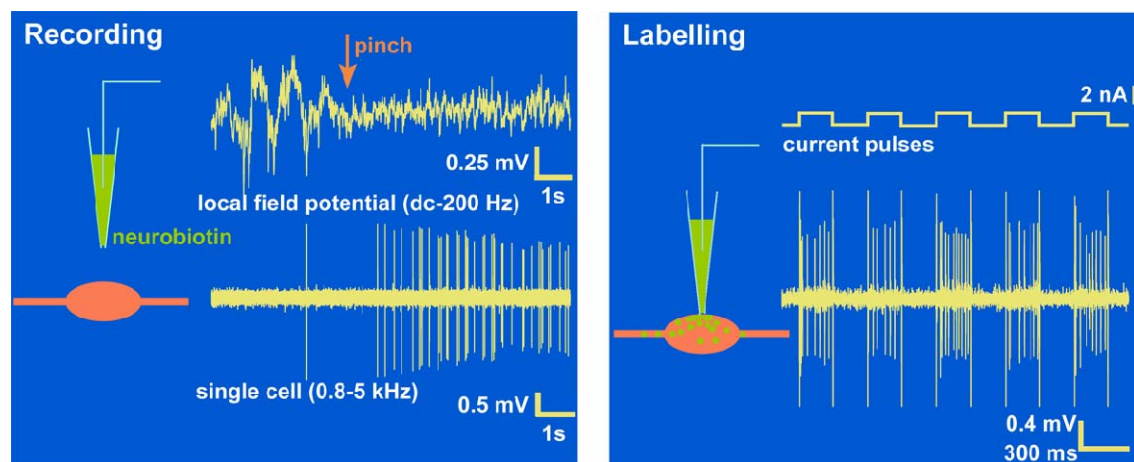

Figure 1: modified from Pinault (1996). The left panel shows extracellular recording from a single cell with a glass electrode filled with neurobiotin from which the local field potential (frequency band up to 200 Hz; top trace) and the in vivo-firing pattern of the cell depicted (high frequency: 0.8-5 kHz; bottom trace) can be extracted. Note that the anaesthetized rat was pinched in the foot (red arrow indicating the time point), which causes a change from slow wave oscillations (0.5-4 Hz) to theta oscillations (4-8 Hz). The right panel illustrates the labelling procedure, where the glass electrode was lowered very closely to the cell and neurobiotin was ejected together with a current pulse. If the cell is modulated to the current pulse, it takes up the ejected neurobiotin.

In order to determine the synaptic connectivity, molecular expression profile and neuronal classes of these labelled cells, I have tested their expression of various molecular markers and identified their postsynaptic targets with electron microscopy.

Methods

Preparing buffers

TBS buffer

For 1 l of buffer (pH 7.4) 6.61 g TRIZMA Hydrochloride (Sigma-Aldrich Inc., St. Louis, USA, T-3253), 0.97 g TRIZMA Base (Sigma-Aldrich Inc., St. Louis, USA, T-1503), 9 g NaCl (BDH Laboratory Supplies, Poole, UK, 44827W) were dissolved in ddH$_2$O and filled up to an end volume of 1 l.

PB buffer

For 5 l of 0.2 M PB buffer 142.39 g Na$_2$HPO$_4$ x 2H$_2$O (BDH Laboratory Supplies, Poole, UK, 103834G) were dissolved in 4 l of dH$_2$0. 27.6 g NaH$_2$PO$_4$ x H$_2$O were dissolved in 1 l of dH$_2$O, mixed with the other solution and the pH was confirmed to be below 7.39.

Vibratome cutting

The rat was perfused through the aorta with 0.9 % saline followed by fixative (4 % Paraformaldehyde + 15 % w/v of saturated Picric acid + 0.05 % Glutaraldehyde in 0.1 M PB, pH 7.3) for approximately 20 minutes. The brain was removed from the skull and was washed over night in 0.1 M PB. Then the frontal part of the right hemisphere was cut on the vibratome (Leica, Wetzlar, Germany, VT 1000S) in 70 µm thick coronal sections. The sections were washed twice in 0.1M PB for 15 minutes on the shaker and stored in 0.1M PB + 0.05 % Sodium Azide (VWR International Ltd., Poole, England, 103692K) at 4ºC (Maccaferri et al., 2000).

Strep-Alexa488 labelling

Sections stored in 0.1 M PB + 0.05 % Sodium Azide (VWR International Ltd., Poole, England, 103692K) were washed twice in 0.1 M PB and once in TBS + 0.3 % Triton X-100 (VWR International Ltd., Poole, England, 306324N) for 20 min on the shaker at room temperature. After that they were incubated with StrepAlexa488 (1:1000) in TBS + 0.3 % Triton X-100 + 2 % normal horse serum for 4 hours on the shaker at room temperature (or alternatively 24 hours at 4ºC, although this was abandoned during the

course of the thesis because it resulted in higher unspecific background labelling). The sections were washed three times with TBS + 0.3 % Triton X-100 for 20 min on the shaker at room temperature and mounted on microscopic slides (VWR International bvba, Leuven, Belgium, 631-0117) with vectashield mounting medium for fluorescence (Vector Laboratories Inc, Burlingame, USA, H-1000) and covered with a cover slip (VWR International bvba, Leuven, Belgium, 631-0133) (Losonczy et al., 2002).

Immunoreaction

Sections stored in 0.1 M PB + 0.05 % Sodium Azide (VWR International Ltd., Poole, England, 103692K) were washed twice in 0.1 M PB and once in TBS + 0.3 % Triton X-100 (VWR International Ltd., Poole, England, 306324N) for 20 min on the shaker at room temperature. The sections were blocked by incubating them in 20 % normal horse serum (Vector Laboratories Inc, Burlingame, USA, S-2000) in TBS + 0.3 % Triton X-100 for one hour on the shaker at room temperature. They were then incubated with the primary antibodies (see

Table 1 for concentration) in TBS + 0.3 % Triton X-100 + 2 % normal horse serum for 48 hours on the shaker at 4ºC.

After washing the section three times with TBS + 0.3 % Triton X-100 for 20 min on the shaker at room temperature they were incubated with the secondary antibodies (**Table 2**) in TBS + 0.3 % Triton X-100 + 2 % normal horse serum for 24 hours on the shaker at 4ºC. The section were washed three times with TBS + 0.3 % Triton X-100 for 20 min on the shaker at room temperature and mounted on microscopic slides (VWR International bvba, Leuven, Belgium, 631-0117) with vectashield mounting medium for fluorescence (Vector Laboratories Inc, Burlingame, USA, H-1000) and covered with a cover slip (VWR International bvba, Leuven, Belgium, 631-0133) (Losonczy et al., 2002).

Negative and positive controls for immunoreaction

Antibody labelling was considered to be specific, if it was comparable with labelling previously published for this molecular marker (see

Table 1 for references). Some antibodies that were tested for the first time (including vGLUT2, 5-HT2c, Kv4.3 and D1) showed unspecific labelling and therefore negative and positive controls were applied to verify the reason for unspecific labelling. First of all, the secondary antibodies used for these reactions were tested without primary antibody, to verify whether the unspecific labelling stems from the secondary antibody. Additionally, all primary antibodies were tested with two different secondary antibodies (Alexa488 and Cy3). Furthermore, each species of secondary antibody was used in combination with primary antibodies, often used in the lab and known to give strong and characteristic labelling as a positive control. In all cases secondary antibodies did not show any labelling after omitting primary antibodies, different secondary antibodies showed the same result in combination with the same primary antibody, and all positive controls showed specific labelling.

In order to avoid shine through – labelling can be observed in a channel with a filter of longer wavelength – the primary and secondary antibodies should not be used too concentrated (ideal concentration of antibodies has to be evaluated for each antibody individually). Furthermore, also the Strep-Alexa488 labelling should not shine through in other channels and therefore after labelling images are captured with all channels as a negative control, before any immunoreaction are performed on the section.

Table 1: List of primary antibodies used in this study.

Antibody to	Host	Dilution	Source	Reference of characterisation	Immunogens
α-actinin	mouse	1:1000	Sigma-Aldrich, St. Louis, Missouri, USA. Code No. A7811	Labelling pattern as published with other antibodies.	sarcomeric α-actinin
	rabbit	1:1000	Dr. A. Beggs, Howard Hughes Medical Institute, Boston, USA.	(Chan et al., 1998)	recombinant α-actinin-2 (N-17)
5-HT2c	goat	1:500	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA. Code No. sc-15081	(Liu et al., 2007b)	SR-2C (N-19)
CB	rabbit	1:10000	Swant, Bellinzona, Switzerland. Code No. CB-38	Labelling pattern as published with other antibodies.	rat CB D-28k
	mouse	1:500	Swant, Bellinzona, Switzerland. Code No. 300	(Celio et al., 1990)	chicken CB D-28k
CCK	rabbit	1:500	DiaSorin, Stillwater, USA. Code No. 20078	Labelling pattern as published with other antibodies.	CCK octapeptide
	mouse	1:5000	Dr. G Ohning, CURE/Digestive Diseases Research Center. Antibody/RIA Core, UCLA. Code No. 9303	(Ohning et al., 1996)	gastrin-17
COUP-TFII	mouse	1:250	Perseus Proteomics Inc., Tokyo, Japan. Code No. PP-H7147-00	(Lee et al., 2004)	human COUP-TF2
CR	goat	1:1000	Swant, Bellinzona, Switzerland. Code No CG1	(Schwaller et al., 1994)	human CR
D1	rat	1:5000	Sigma-Aldrich, St. Louis, Missouri, USA. Code No. D187	(Paspalas and Goldman-Rakic, 2005)	C-terminal 97 aa of human D1 receptor
GAD	mouse	1:100	Dr. D. Gottlieb, Dept. of Anatomy & Neurobiology, Washington University School of Medicine, St. Louis, USA	(Chang and Gottlieb, 1988)	rat brain GAD (65kDa)
GCα1	rabbit	1:10000	Sigma-Aldrich, St. Louis, Missouri, USA. Code No. G4280	(Szabadits et al., 2007)	synthetic peptide (673-690) of rat GC α1
GluR2	mouse	1:100	Antibodies Inc., Davis, CA, USA. Code No. 75-002	Labelling pattern as published with other antibodies. (Fujiyama et al., 2004)	monoclonal, clone L21/32

Kv4.3	rabbit	1:1000	Alomone Labs Ltd. Jerusalem, Israel. Code No. APC-017A	(Yang et al., 2001)	Human Kv4.3 451-467
	goat	1:1000	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA. Code No. sc-11686	(Kollo et al., 2006)	peptide of internal region of human Kv4.3
mGluR1α	Guinea pig	1:1000	Dr. M. Watanabe, Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan	(Tanaka et al., 2000)	rat mGluR1α (945-1127)
MOR	guinea pig	1:1000	Chemicon, Hampshire, United Kingdom. Code No. AB1774	(Rodriguez et al., 2001)	synthetic peptide (384-398) from rat MOR-1
N200	mouse	1:1000	Sigma-Aldrich, St. Louis, Missouri, USA. Code No. 0142 N52	(Law and Harrison, 2003)	Phosphorylated and non-phosphorylated N200
NOS	rabbit	1:1000	Chemicon, Hampshire, United Kingdom. Code No. AB5380	Labelling pattern as published with other antibodies. (Moro et al., 1998)	recombinant human nNOS
	sheep	1:500	Chemicon, Hampshire, United Kingdom. Code No. AB1529	Labelling pattern as published with other antibodies.	rat NOS (1409-1429)
NPY	sheep	1:500	Chemicon, Hampshire, United Kingdom. Code No. 1017	Labelling pattern as published with other antibodies. (Blessing et al., 1986)	
PV	mouse	1:10000	Swant, Bellinzona, Switzerland. Code No. 235	(Celio et al., 1988)	carp muscle PV
Reelin	mouse	1:1000	Chemicon, Hampshire, United Kingdom. Code No. MAB5364	(Demyanenko et al., 2004)	Recombinant reelin amino acids 164-496
SOM	rat	1:500	Chemicon, Hampshire, United Kingdom. Code No. MAB354	Monoclonal, labelling pattern as published with other antibodies, (Vincent et al., 1985)	synthetic 1-14 cyclic SOM
vGLUT2	guinea pig	1:2500	Chemicon, Hampshire, United Kingdom. Code No. AB5907	Labelling pattern as published with other antibodies, (Fremeau et al.,	Synthetic peptide from rat vGLUT2

			2001)	
VIP	rabbit	1:50,000	Dr. TJ Görcs, Dept. Anatomy, Sote, Hungary	(Gulyas et al., 1990)	synthetic porcine VIP

Table 2: List of secondary antibodies used in this study

Antibody/Streptavidin	Fluorophore	A_{max}	Supplier
Donkey Anti-Goat	Alexa 350	346nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Sheep	Alexa 350	346nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Mouse IgG	AMCA	350nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Streptavidin	AMCA	350nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Streptavidin	Alexa488	495nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Goat	Alexa488	495nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Guinea Pig	Alexa488	495nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Mouse	Alexa488	495nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Rabbit	Alexa488	495nm	Molecular Probes, Inc., Eugene, OR, USA
Donkey Anti-Mouse	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Guinea Pig	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Rabbit	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Rat	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-sheep	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Goat	Cy3	550nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Guinea Pig	Cy5	650nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Goat	Cy5	650nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA
Donkey Anti-Mouse	Cy5	650nm	Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA

Image Acquisition and Interpretation of Immunoreactions

The immunoreaction was examined with a Leitz fluorescence microscope (Leica, Wetzlar, Germany, DMRB) with the following filter blocks: A4 (excitation filter band pass (BP), 360/40nm; reflection short-pass (SP) filter, 400nm: suppression filter BP,

470/40nm), L5 (excitation filter BP, 480/40nm; reflection SP, 505nm; suppression filter BP, 527/30nm), Y3 (excitation filter BP, 545/30 nm; reflection SP, 565 nm; suppression filter BP, 610/75 nm), and Y5 (excitation filter BP, 620/60nm; reflection SP, 660 nm; suppression filter BP, 700/75 nm). Images were acquired with a CCD camera (Hamamatsu Photonics, Welwyn Garden City, UK, C4742-80) using the software package openlab 5.0.2 (Improvision, Coventry, UK) (Ferraguti et al., 2004). For each image the exposure time had to be adjusted depending on the reaction quality, the objective used and the channel (A4: 500-2000ms, L5: 200-500 ms, Y3: 20-150 ms, Y5: 1000-3500 ms). If images had to be taken from more than two channels, a macro programmed by Wai-Yee Suen was used.

In order to show whether a neurobiotin-labelled cell expresses a certain marker or not, a co-localisation study with sections containing either soma, dendrites, or axons of the labelled cell was performed. For soma sections, images with at least three different focuses were acquired for each channel. For co-localisation in dendrites, images were taken of at least three individual dendrites of the same cell with as many focuses required for an unequivocal judgment. For co-localisation in axons, images were acquired from at least ten boutons of at least 3 different axons. If there was a co-localisation, the cell was called "immuno-positive" for this marker. If no co-localisation was observed with the tested marker, the cell was called "immuno-negative", although weak expression cannot be excluded with this method. In case of ambiguous results, the cell was called "not tested" for this marker.

Section preparation for Electron Microscopy

Freeze thaw

Sections containing axons of neurobiotin-labelled cells, which have not been used for immunofluorescence and were stored in 0.1 M PB + 0.05 % Sodium Azide (VWR International Ltd., Poole, England, 103692K) were washed four times for 15 minutes in 0.1 M PB on the shaker at room temperature. Then they were incubated for 15 minutes in 0.1 M PB + 10 % Sucrose (Sigma-Aldrich Inc., St. Louis, USA, S7903-1KG) and for at least 2 hour in 0.1 M PB + 20 % Sucrose or until they sank to the bottom of the well without pushing them down. Afterwards, the sections, one by one, were transferred into a well of a TC-plate six well (Greiner bio-one Ltd., Stonehouse, UK, 657 160) and any remaining buffer discarded. The plate was dipped into liquid nitrogen until the section

was frozen as indicated by turning into a white colour. The section was then thawed as quickly as possible by pouring 0.1 M PB (room temperature) into the well and rubbing with a finger underneath the section. Then the sections were washed twice with 0.1 M PB and once with TBS for 15 minutes at room temperature on the shaker. Meanwhile, component A and component B of the Vectastain ABC kit elite (Vector Laboratories Inc, Burlingame, USA, PK-6100) were mixed both 1:100 in TBS and pre-incubated for 30 minutes at room temperature. The sections were incubated in the pre-incubated mixture for 48 hours at 4ºC on the shaker.

DAB-reaction

In order to prepare the DAB solution, 10 mg of DAB (3,3'-Diaminobenzidine tetra-hydrochloride, approx. 97 %, Sigma-Aldrich Inc., St. Louis, USA, D5637-1G) were dissolved in 9.6 ml distilled water and mixed with 10 ml 0.2 M PB, 200 µl of 20 % Glucose (BDH Laboratory Supplies, Poole, UK, 10117) in distilled water + 200 µl of 0.4 % Ammonium Chloride (Sigma-Aldrich Inc., St. Louis, USA, A-4514-100G) in distilled water. The solution was divided in two portions and one was used to pre-incubate the sections in 0.5 ml per well of a TC-plate 24 well (Greiner bio-one Ltd., Stonehouse, UK, 662 160) for 15 minutes at room temperature in the fume hood (all following steps were carried out in the fume hood as well). To the other portion, 15 µl of Glucose-oxidase (0.00542 g / ml, Sigma-Aldrich Inc., St. Louis, USA, G-2133) were added. After pre-incubation, the sections were incubated with the DAB solution containing Glucose-oxidase at room temperature for approximately 45 minutes, although the progress of the reaction had to be monitored carefully and checked regularly under the microscope. This glucose oxidase reaction was used to produce H_2O_2 in situ, which is required for the DAB reaction. To stop the reaction the sections were washed three times in 0.1 M PB at room temperature. The sections were then incubated for 45 minutes in Osmium tetroxide (4 % w/v solution, TAAB Laboratories Equipment Ltd., Reading, UK, O012) with a concentration between 0.5 to 2 % in 0.1 M PB according to the colour and the level of unspecific background labelling of the section after the DAB reaction. The sections were washed twice in 0.1 M PB for ten minutes and three times rinsed with distilled water in order to remove phosphate, which would form a precipitate with uranium in subsequent steps.

Dehydration and Uranyl acetate staining

The sections were dehydrated by incubating them for 10 min each in a series of solutions with increasing ethanol concentration starting with 50 % Ethanol (VWR International Ltd., Poole, UK, 103692K). A solution of 1 % Uranyl acetate (Emscope Laboratories Ltd., Kent, UK, C024) in 70 % Ethanol was prepared. The sections were then incubated in this solution in the dark for 30 - 60 min depending on the colour and level of unspecific background labelling of the section. After that the dehydration continued with 70 %, 90 %, 95 %, twice 100 % ethanol and eventually twice in propylene oxide (VWR International Ltd., Poole, UK, 282904W) each for 10 minutes at room temperature. The propylene oxide was only used in glass ware, so if the reaction was started in a 24 well plate, sections needed to be transferred to glass vials before pursuing dehydration with propylene oxide. Meanwhile, the Durcupan ACM Fluka resin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was prepared by mixing 10 g of single component A (44611), 10 g of B (44612), 0.3 g of C (44613) and 0.3 g of D (44614). The sections were transferred into resin and incubated for 12 to 18 hours at room temperature. Then the sections were mounted on microscopic slides (VWR International bvba, Leuven, Belgium, 631-0117) with the resin as mounting medium and covered with a cover slip (VWR International bvba, Leuven, Belgium, 631-0133); both the slide and the cover slip were greased on the forehead and no pressure was applied onto the cover slip. The slides were incubated at 60ºC for 48 hours to harden (Halasy et al., 1996).

Re-embedding

A decision of which area to re-embed for electron microscopic investigation was made using the following criteria:

- Area should contain a good amount of labelled axon
- There should be no dendrite in this area (these are instead saved for reconstruction of the dendritic tree using light microscopy)
- The size of the area depends on the amount and density of axons (a lot of axon - small area, fewer axons - larger area (excess tissue can be trimmed away under the ultra-microtome (Leica ultracut UCT, Leica AG, Vienna, Austria, 500613))
- If there are more boutons closer to one of the surfaces, this surface should face up on the block to be cut first

Pictures were then taken of the selected area of interest and the cover slip was removed by using a double-edged razor blade (Wilkinson Sword).

A droplet of Durcupan ACM Fluka resin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (usually leftover resin from embedding, which was stored in the frigde and can be reused for a few weeks for this purpose only) was put in the lid of the embedding capsule (TAAB Laboratories equipment limited, Aldermaston, UK, MS9051). Using a surgical blade type 26 (Swann-Morton, Sheffield, UK, 0213) the first cut into the tissue was made in an area, away from the selected area of interest in order to see how the tissue was cutting. In some cases the tissue was very brittle in which case the slide was placed on a heating plate (Agar scientific Ltd., Stansted, UK) at 60°C for a couple of minutes to soften the resin. The area was then cut out and transferred to the droplet of resin with the preferred surface face down. An antistatic gun Zerostat (Discwasher, England, 3997817) was used with all metal equipment, to prevent the loss of the cut piece (usually around 1 mm^2) due to electrostatic forces. Another security measure was to put a finger close to the area of cutting. If the piece would jump, it would often be trapped on the fingertip. The embedding capsule was then labelled with a number, filled up with resin, covered with a cover slip and incubated at 60°C for 48 hours to harden. After removing the cover slip and the embedding capsule, the block could be used for ultra microtome cutting (Halasy et al., 1996).

Ultra microtome cutting

The excess resin around the area of interest of the block was trimmed away using a double bladed razor blade (Wilkinson Sword) to make a trapezoid shaped area on top of a pyramid. A glass knife was then made with the knife maker (LKB-Produkter AB, Stockholm, Sweden, 7801B) and aligned to the surface of the block by following adjustments:

- The base of the trapezoid and the edge of the knife should be parallel
- The left and right side of the surface should be equidistant from the edge of the knife (can be adjusted by aligning the edge of the knife with the reflection of the edge of the knife on the surface of the block)
- The top and bottom of the surface should be equidistant from the edge of the knife (if the distance between edge of the knife and the reflection of the edge of

the knife on the surface of the block doesn't change when moving the block up

and down, then top and bottom are equidistant)

When the block was aligned the resin above the tissue was trimmed away. Then final cuts around the area of interest were made to get a block as small as possible without losing any labelled axon (empty tissue can be trimmed away) by using a double-edged razor blade. After aligning the block again with a glass knife and trimming a few μm of the surface away, the glass knife was replaced by a diamond knife Diatome (TAAB Laboratory equipment limited, Aldermaston, UK, MS9051) and realigned. Then the water bath was filled with ddH$_2$O, such that the water level was slightly lower then the edge of the knife (can be adjusted by watching the reflection of light on the water surface). After setting the cutting window, the thickness (desired thickness was 70nm, which can be judged by the reflection colour of the sections on the water surface after cutting; so after cutting a few sections, settings were adjusted accordingly) and the speed (around 1mm/s, but optimal speed varied between blocks) cutting was started and serial sections were collected on Piloform coated copper grids (3.05mm, slot 2mm x 1mm, Agar scientific Ltd., Stansted, UK, G2500C) (Halasy et al., 1996).

Image Acquisition and Interpretation of Electron micrographs

Images were acquired with either CM10 or CM100 Transmission Electron Microscope (Philips, Eindhoven, The Netherlands) using DigitalMicrograph™ 3.9.3 (Gatan Software Team, Pleasanton, CA, USA). In order to achieve optimal image quality, magnification, focus and beam size were set by eye, the camera was then inserted and the image acquired with auto exposure. To get an optimal image of a synapse where the synaptic cleft is clearly visible, the specimen holder was tilted accordingly. In some cases, the copper grid had to be rotated, since tilting of the specimen holder was only possible in one dimension.

In order to find labelled boutons – the most likely part of an axon to find a synapse – the first section was searched in regions where labelled axon could be seen at the light microscopic level. When an axon was found, it was followed through subsequent sections until it forms a bouton. In order to find a synapse on either side of the bouton, it was necessary to tilt the specimen holder until both plasma membranes – the one of the bouton and the one of the neighbouring structure – became clearly visible. Then the following criteria were used to identify Type II synapses according to Gray (Gray, 1959):

- Widening between the two parallel running membranes (synaptic cleft) containing electron-dense protein (appearing grey in the electron microscope)

- Vesicles on the presynaptic side (sometimes difficult to jugde, since the bouton appears completely black in some cases (because of the DAB labelling) but there should at least not be a mitochondrion in direct apposition to the putative synapse on the presynaptic side)

- A small postsynaptic density (compared to a nearby Gray Type I synapse)

Having identified a synapse, the next step was to identify, whether the target was a dendritic shaft (proximal or distal), spine, soma or axon initial segment of a pyramidal cell or an interneuron.

The criteria for the identification of spines were:

- Gets smaller in one direction (spine neck) and ends in the other when followed through serial sections

- Contains spine apparatus (looks like smooth endoplasmatic reticulum)

- Does not contain mitochondria or microtubuli

- Also receives a single Gray Type I synapse (not observable when synapse parallel to cutting plane)

A dendritic shaft, on the other hand, does contain mitochondria and microtubules. If the diameter exceeds 500 nm, it was considered as an apical dendrite. A soma can usually be identified by the presence of the nucleus, but rough endoplasmatic reticulum and ribosomes are also only found in the soma. Axon initial segments have a characteristic undercoating beneath the plasma membrane, receive a high number of Type II but no Type I synapses, do not emit spines and contain bundles of microtubules (Kosaka, 1980).

In order to distinguish between pyramidal cells and interneurons, the following criteria were applied:

- Dendrites of pyramidal cells have spines, whereas most interneurons have a smooth dendritic shaft

- Pyramidal cells predominantly receive Gray Type I synapses on spines (in the hippocampus exclusively), very rarely on their dendritic shaft and not on soma

- Pyramidal cells contain non-membrane bound protein aggregations (dendrites and soma) (Halasy et al., 1996).

Results

In order to identify prefrontal interneurons that are involved in the temporal coordination of the prefrontal cortex and hippocampus, single cells were extracellularly recorded with glass electrodes in the medial prefrontal cortex together with the local field potential (LFP) in the same region and in the hippocampal CA1 area of anaesthetized rats. The LFP is an electroencephalogram (EEG) recorded extracellularly within certain brain areas and information about brain oscillations of different frequency bands (theta (4-8 Hz; during exploratory behaviour and rapid-eye-movement sleep (Buzsaki, 2002)), gamma (at 30-80 Hz and associated with working memory and attention (Engel et al., 2001)), slow wave or delta (0.5-4 Hz; a certain stage of sleep is referred to as slow wave sleep (SWS) (Steriade and Amzica, 1998)), hippocampal sharp wave-associated ripple oscillations (120-200 Hz; associated with memory consolidation (Ylinen et al., 1995)), spindles (12-16 Hz and occur during SWS (Steriade and Amzica, 1998))) can be extracted from the LFP. After electrophysiological data was acquired, the cell was juxtacellularly labelled with Neurobiotin, by ejecting a small volume of Neurobiotin from the recording electrode and by stimulating the cell with a current pulse. The Neurobiotin is then specifically taken up by the stimulated cell only. The Neurobiotin was allowed to be distributed throughout the cell (including axons and dendrites), and the rat was perfused with fixative via the aorta only after 2-4h; the brain was removed and cut into 70 μm thick sections with a vibratome[1].

For organisation purposes each cell has a code number, for example K125a. K for Katja (the recorder), the rat brains are consecutively numbered and each recording attempt in each brain gets a small letter (not every recorded cell was successfully labelled).

Next, every third section was incubated with Streptavidin-Alexa488 and investigated under the fluorescence microscope. On average, the dendrites of a cell can be observed in 5-10 sections around the soma, the axon of an interneuron usually spans in 10-15 sections.

Depending on the electrophysiological data and the dendritic and axonal arborisation a decision was made on which molecular markers to test first. In most cases the calcium

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[1] All in vivo experiments – from recording to perfusion - were performed by Katja Hartwich.

binding proteins Parvalbumin (PV) and Calbindin (CB) were tested initially, because they are both expressed by many cell types and so the result of this experiment narrows down the possibilities for further investigations[2].

Immunoreaction on labelled cells[3]

Most of the cells that were recorded in the prelimbic medial prefrontal cortex in fact express either PV or CB or both (**Table 3**). In contrast to the hippocampus, where PV and CB are not co-expressed (unpublished observation), most PV cells in layer II/III of the frontal cortex are either weakly (80%) or strongly (11%) immunopositive for CB (Kawaguchi and Kubota, 1997).

Table 3: Molecular expression profile of in vivo recorded and labelled GABAergic interneurons

	PV	CB	SM	CCK	GABAARα1	GAD	CR	mGLUR1α	VIP	NPY	NOS
K19a	+	-	-		+		-			-	-
K48a	+	-		-	-		-				
K64a	+	-									
K79a	+	-			+						
K94e	+	-						-			
K105a	+	-									
K109a	+	-									
K126c	+	-									
K131g	+	-									
K133a	+	-									
K135b	+	-									
K137c	+	-									
K146c	+	-									

[2] Cutting the brain, Strep-Alexa488 labelling, Immunoreactions on labelled cells and image acquisition were performed by both Katja Hartwich and me. In this thesis only figures are shown were I did the reaction, the image acquisition and editing, if not explicitly stated otherwise.

[3] My contribution to this part of the project was about 30%. A lot of the data was already acquired before I joined the project and Katja Hartwich and I did both the immunoreactions and image acquisition in parallel.

K148c	+	-								
K155a	+	-				+				
K35a		+	+							+
K60a	-	+	-	-						
K70b	-	+	-	-					-	
K125a	-	+	-	-		+			-	
K65b	+	+								-
K72a	+	+			+					
K74d	+	+								
K75a	+	+			+					
K101a	+	+						-		
K107b	+	+								
K122c	+	+					-			
K123a	+	+								
K142a	+	+								
K145a	+	+								
K157a	+	+								
K158c	+	+								
K47b	-	-	-	+					-	
K58a	-	-	-	-						
K93d	-	-	-							
K104a	-				-					
K108d	-	-	-							
K110b	-	-	-	-						
K119a		-	+							
K149d	-	-								
K153b	-	-	-							
K154b	-	-	-							

PV only expressing cells

Out of 27 labelled PV expressing cells, 15 were immunonegative for CB. Importantly, immunonegativity cannot distinguish between the total absence of expression of this

protein and a very weak expression that falls below the detection threshold of this method. Therfore we cannot exclude that all PV immuno-positive cells might express low levels of CB. An example of a PV immunopositive cell is shown in Figure 2 (K146a); this cell was tested on a proximal dendrite. Of the PV only expressing cells K19a was additionally tested for somatostatin (SM), calretinin (CR), neuropeptide tyrosin (NPY), nitric oxide-synthase (NOS), and the $\alpha1$ subunit of the $GABA_A$ repector ($GABA_AR\alpha1$) of which only the latter one was immunopositive. Cell K48a was immunonegative for CCK, $GABA_AR\alpha1$ and CR, whereas K79a was immunopositive for $GABA_AR\alpha1$. K94e was tested negative for mGluR1α and K155a positive for GAD.
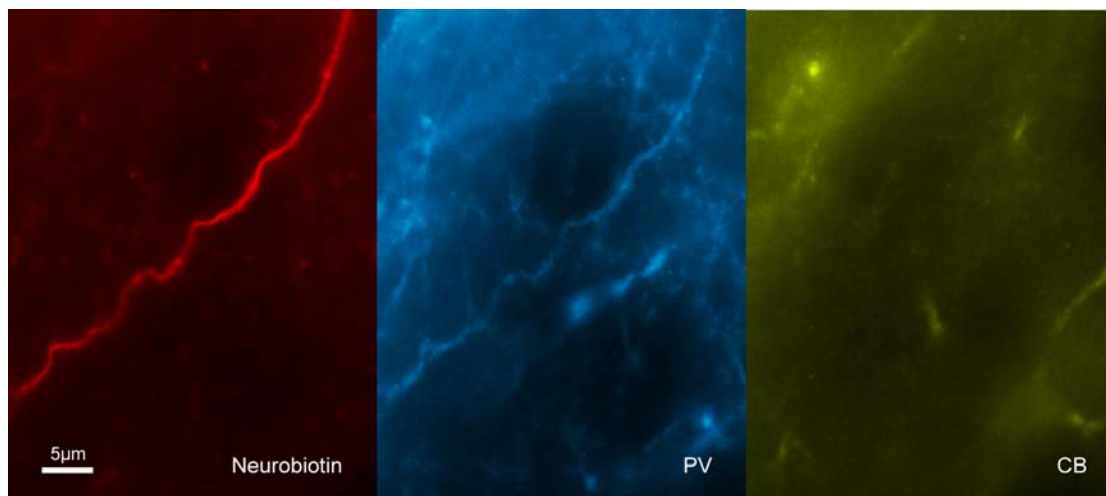
Figure 2: Fluorescence micrograph shows a dendrite of a GABAergic interneuron (K146a) labelled with Neurobiotin. The dendrite is immunopositive for Parvalbumin (PV), but immunonegative for Calbindin (CB).

Almost all PV expressing cells fire rhythmically in relation to local and hippocampal network oscillations and are correlated to the spindle trough and the hippocampal theta peak, as tested by Katja Hartwich. One exception was K19a, which is correlated to the theta trough, but that might be due to problems with the theta detection during the analysis of firing patterns.

CB only expressing cells

Three (K60a, K70b, K125a) CB only positive cells were observed in our sample (**Figure 3**). Two of the cells (K60a and K125a) had remarkably spiny dendrites (shown for K60a in **Figure 4**), whereas K70b had rather smooth dendrites.
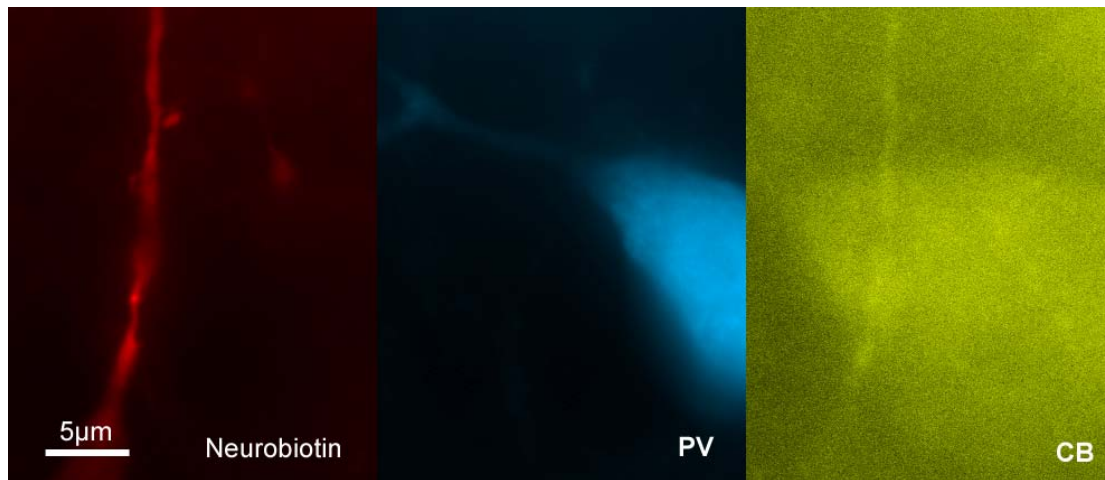
Figure 3: Fluorescence micrograph showing the dendrite of a Neurobiotin-labelled cell (K125a) immunopositive for Calbindin (CB), but immunonegative for Parvalbumin (PV).
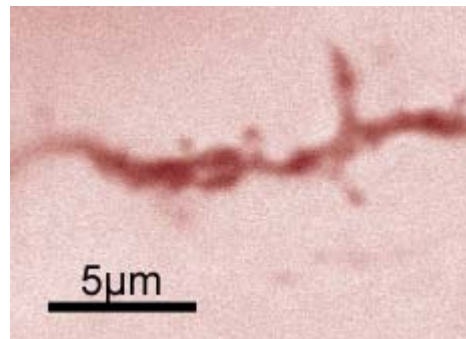

Figure 4: Light micrograph showing a spiny dendrite captured from K60a – a CB only expressing cell.

All of them were tested for PV, SM, and CCK and K70b and K125a additionally for VIP; all reactions tested immunonegative for these cells. The cell K125a was also immunopositive for GAD (**Figure 5**) and all three cells form Gray Type II synapses, as defined by Gray (Gray, 1959) and usually attributed to GABAergic synapses. The most striking feature of these three cells is that they are not correlated to theta oscillations recorded in the hippocampus and spindle oscillations detected locally (recorded and tested by Katja Hartwich), whereas all PV and PV/CB expressing cells are correlated to spindle oscillations and hippocampal theta. One CB expressing cell (K35a), which was immunopositive for SM and NPY, has not been characterized further yet.
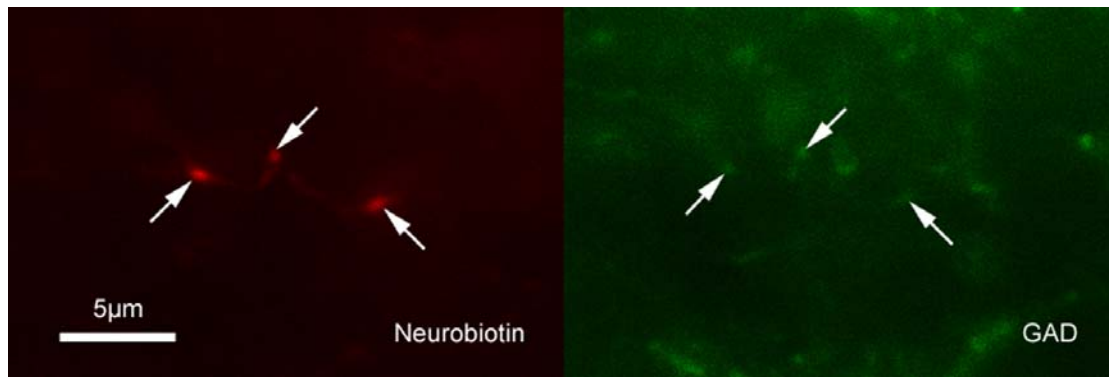
Figure 5: Fluorescence micrograph showing the axon of cell K125a (CB only expressing cell) labelled with Neurobiotin, which expresses L-Glutamic Acid Decarboxylase (GAD).

PV/CB co-expressing cells

Out of twelve labelled PV/CB co-expressing cells K65b was the only cell that was labelled weakly for PV and strongly for CB, whereas most cells exhibit immunoreactivity for PV and CB at comparable levels (as jugded in comparision to nearby immunopositive cells), with three exceptions that were only weakly labelled for CB (K72a, K75a, K123a). An example of a PV/CB co-expressing cell is shown in Figure 6 (K145a).
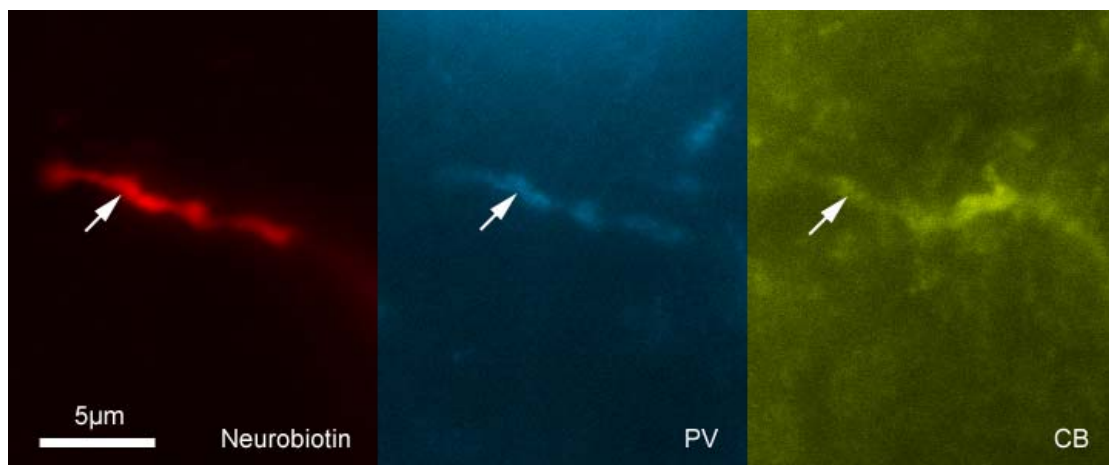
Figure 6: Fluorescence micrograph showing the dendrite of neuron (K145a) labelled with Neurobiotin. The cell is immunopositive for both, Parvalbumin (PV) and Calbindin (CB).

Cell K65b was also tested for NPY and is immunonegative for this marker, whereas two cells (K72a and K75a) were immunopositive for $GABA_AR\alpha 1$. Cell K122c was negative for CR and K101a for mGluR1α.

These co-expressing cells are mostly correlated to network oscillations similar to PV only expressing cells, but there is some heterogeneity within this group. One way to

further characterize these cells would be to find molecular markers that are differentially expressed by these PV/CB co-expressing cells.

Other cells

Additionally, eight interneurons recorded and labelled in vivo tested negative for both PV and CB. One of them expressed SM (K119a), one CCK (K47b) and six other cells do not express any of the markers tested so far. As for the PV/CB co-expressing cells, testing of additional molecular markers will be required in the future.

Identification of postsynaptic targets[4]

The majority of all labelled cells expressed PV, CB or both and in order to test whether these cells have distinct synaptic connectivity, their postsynaptic targets were identified by randomly sampling synapses of the labelled cells using electron microscopy.

PV expressing cells

Labelled axons were traced through serial ultrathin sections using an electron microscope until a synapse was identified and then the postsynaptic target was identified using criteria described in the method section.

The results are described here for each cell in detail and summarized in Figure 16. Of 23 identified synapses originating from cell K79a, which expressed PV and $GABA_A R\alpha 1$, ten synapses targeted small dendritic shafts (including distal and oblique dendrites), seven targeted spines, four made synapses onto apical dendrites, and two synapses innervated the soma of pyramidal cells. Most of the dendrites could be identified to belong to a pyramidal cell, whereas two of them were identified as interneuron dendrites and one dendrite could not be clearly identified (shown in **Figure 7**).

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[4] My contribution to this part of the project was about 95%. Katja Hartwich prepared some of the sections for electron microscopy, but I did the the re-embedding, the cutting and image acquisition with the electron microscope.
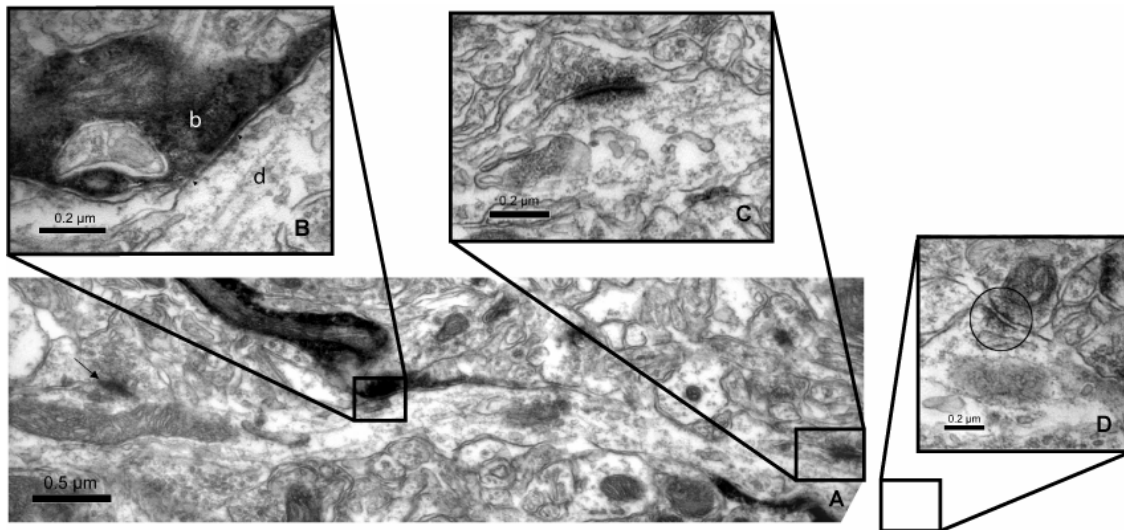
Figure 7: Electron micrographs showing a labelled bouton (b) forming a Typ II synapse (arrow; B) onto the dendritic shaft (d) of a target neuron (A) which receives two Type I synapses (arrowhead) on its shaft (arrow and C, rarely for pyramidal cells), but also has a sessile spine with a synapse (D) unclear if Typ I or II. B is from the neighbouring section of A, whereas C is about 300nm in one direction and D about 550nm in the other direction on a part of the target neuron not depicted in A (figure derived from K79a – a PV-expressing cell).

Furthermore, the postsynaptic targets of PV only expressing cells - K19a, K48a, and K64a - were tested; all cells target apical dendrites and/or somata of predominantly pyramidal cells, additionally to distal and oblique dendrites. In more detail, K19a targeted seven distal dendritic shafts, six apical dendrites (**Figure 8**), three spines, and one soma of 17 identified synapses in total.
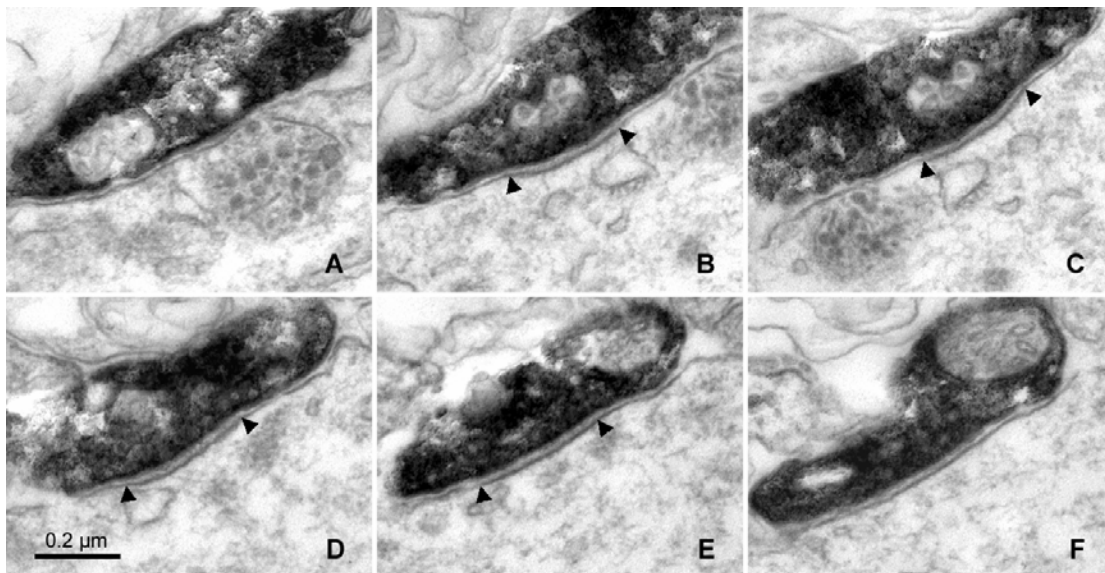
Figure 8: Electron micrographs (A-F) derived from K19a (a PV-expressing cell) showing serial sections (one section about 70nm) of a Type II synapse of a labelled bouton onto an apical dendrite of a pyramidal cell. Synapse (between arrow heads) was observed in 4 sections (arrows) and therefore spanning 280nm perpendicularly.

Boutons of cell K48a formed synapses onto four distal or oblique dendritic shafts, three apical dendrites, nine spines and one soma out of 17 identified synapses. For K64a eleven synapses were identified of which four targeted somata of pyramidal cells and seven targeted small dendritic shafts.

Interneurons of the cerebral cortex which target somata (20-30%), apical and basal dendrites (35-50%) and dendritic spines (20-30%) have previously been classified as basket cells in the neocortex of cats (Kisvarday et al., 1985; Somogyi et al., 1983a). Accordingly, all PV expressing cells analysed so far in this study are identified as basket cells, because they target apical dendrites (15-35%) and somata (5-35%) of pyramidal cells (**Figure 16**), which is also consistent with findings in the rat neocortex (Kubota et al., 2007).

CB only expressing cells

The postsynaptic targets of the CB expressing cell K70b were investigated and of the 14 identified synapses, ten targeted small dendrites on the shaft (**Figure 9**) and four targeted spines of pyramidal cells. Eleven of the targets could be identified as pyramidal cells and three of the dendritic shaft targets could not be verified as originating from pyramidal cells or interneurons.
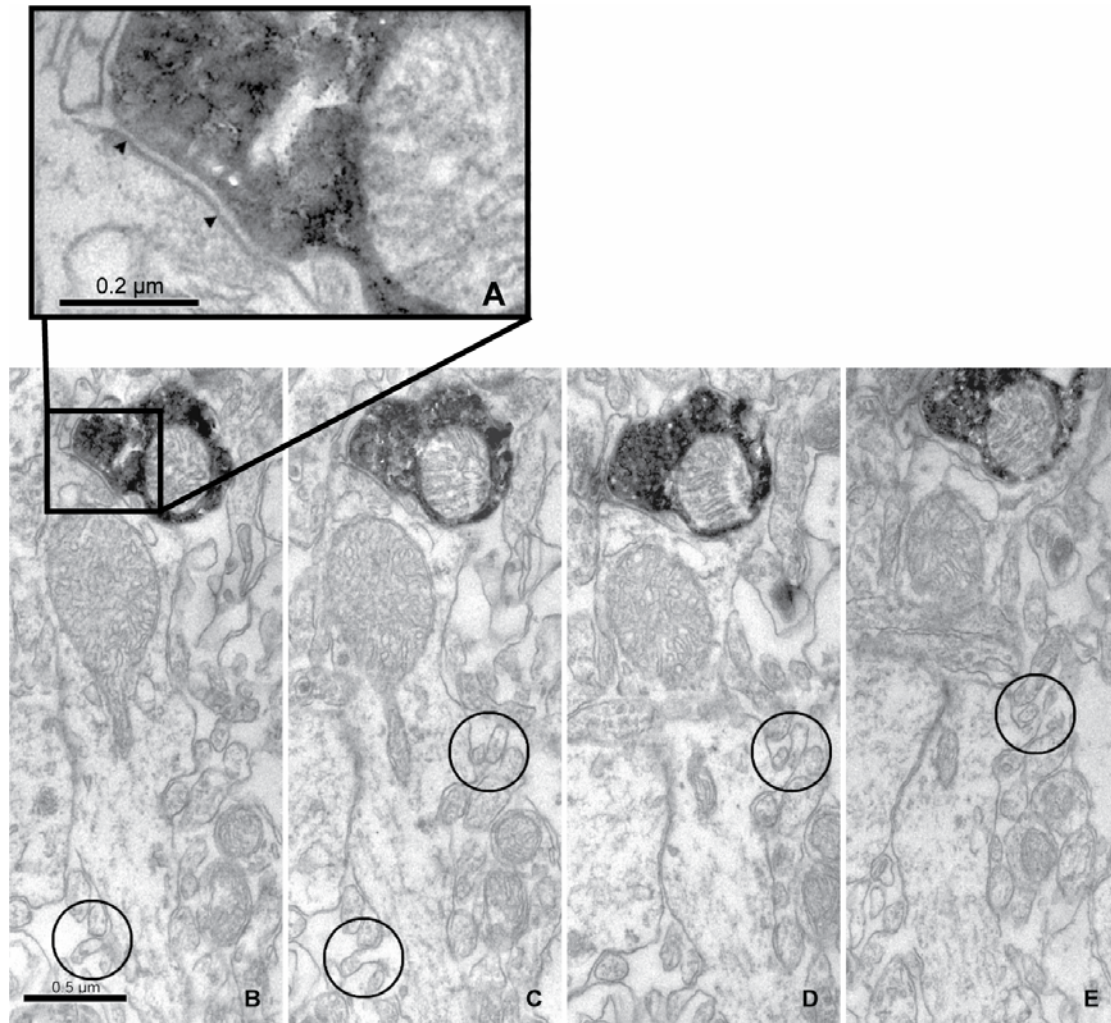
Figure 9: Electron micrograph showing a labelled bouton targeting the dendritic shaft of a pyramidal cell (A). Two spines (circles) emerge from the target dendrite (B-E) (captured from K70b – a CB-expressing cell).

The other two CB expressing cell also targeted exclusively small dendrites (K60a eleven of 15 in total, K125a eight of twelve) and spines (K60a three of 15, K125a four of twelve). One bouton was observed in direct apposition to a soma and another axon in direct proximity to an apical dendrite, but both axons did not form a synapse. This suggests that these cells specifically target distal and oblique dendrites and dendritic spines of predominantly pyramidal cells and avoid somata and apical dendrites. All CB expressing cells form Gray Type II synapses (**Figure 10**). In contrast to PV only expressing basket cells, CB only expressing cells are not correlated to theta and spindle oscillations, differ in molecular expression profile, and exclusively target small dndrites and spines.
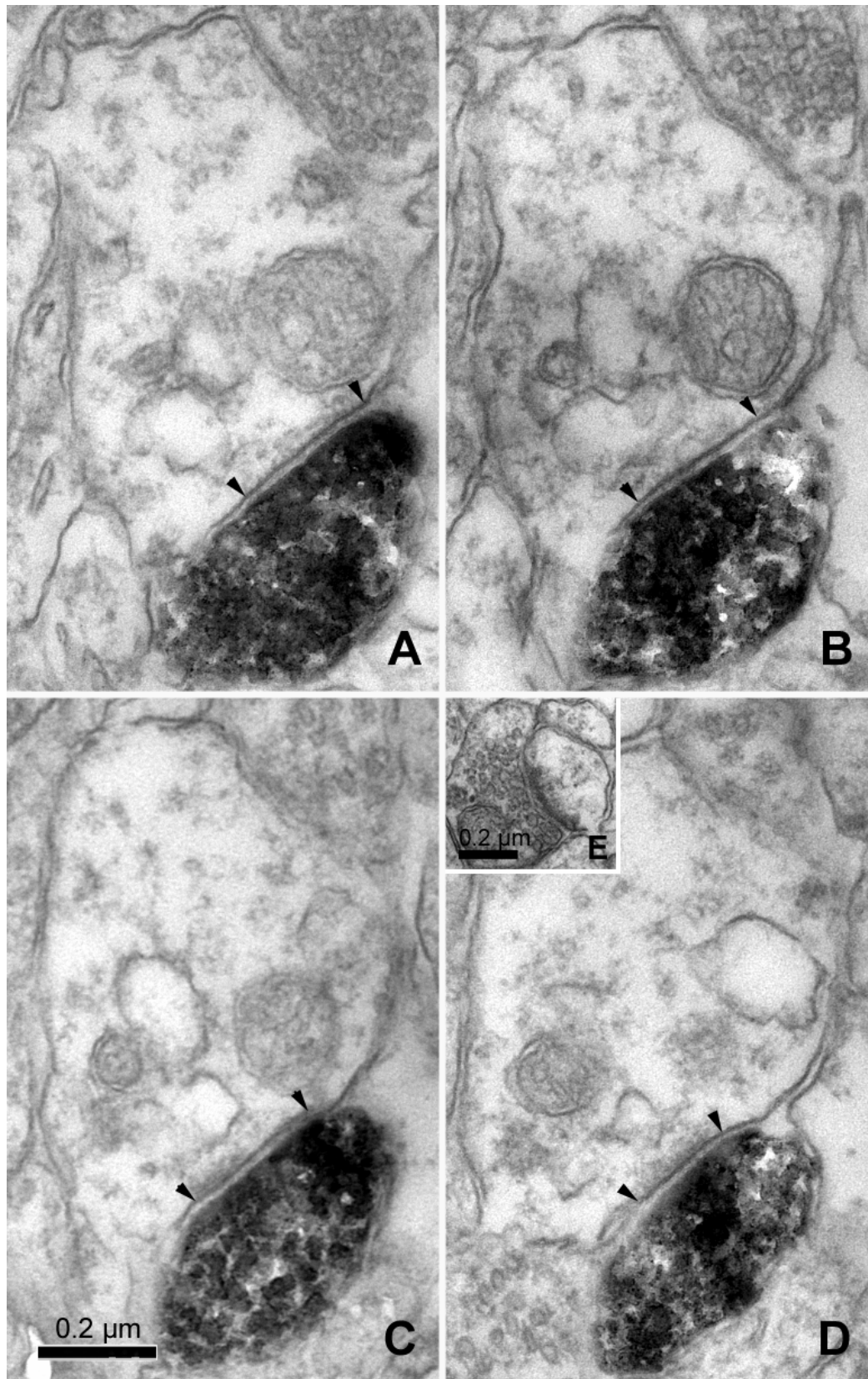
Figure 10: Electron micrographs (A-D) captured from cell K60a (a CB only expressing cell) showing serial sections of a Gray Type II synapse (between arrow heads). As a comparison E shows a Type I synapse from the same area. The target was classified as a pyramidal cell dendrite, because it emits two spines within a few sections (not shown).

PV/CB co-expressing cells

The postsynaptic targets of four PV and CB co-expressing cells were tested. Sixteen synapses of cell K65b, which expresses PV weakly and CB strongly (in contrast to all other co-expressing cells, which show either opposite expression levels or equal expression levels), were identified of which five targeted distal dendritic shafts, one an apical dendrite, six spines and four somata (three boutons targeted the same soma) of mainly pyramidal cells (two distal dendritic shafts remained unidentified). Therefore, this cell was classified as a basket cell.

Boutons sampled of cell K72a formed synapses onto four small dendritic shafts, three apical dendrites, nine spines and one soma (in total 17 identified synapses) of pyramidal cells (only one target remained unidentified). From cell K75a one synapses targeted a distal dendritic shaft, two apical dendrites, three spines and three somata (nine synapses in total) (**Figure 11**).
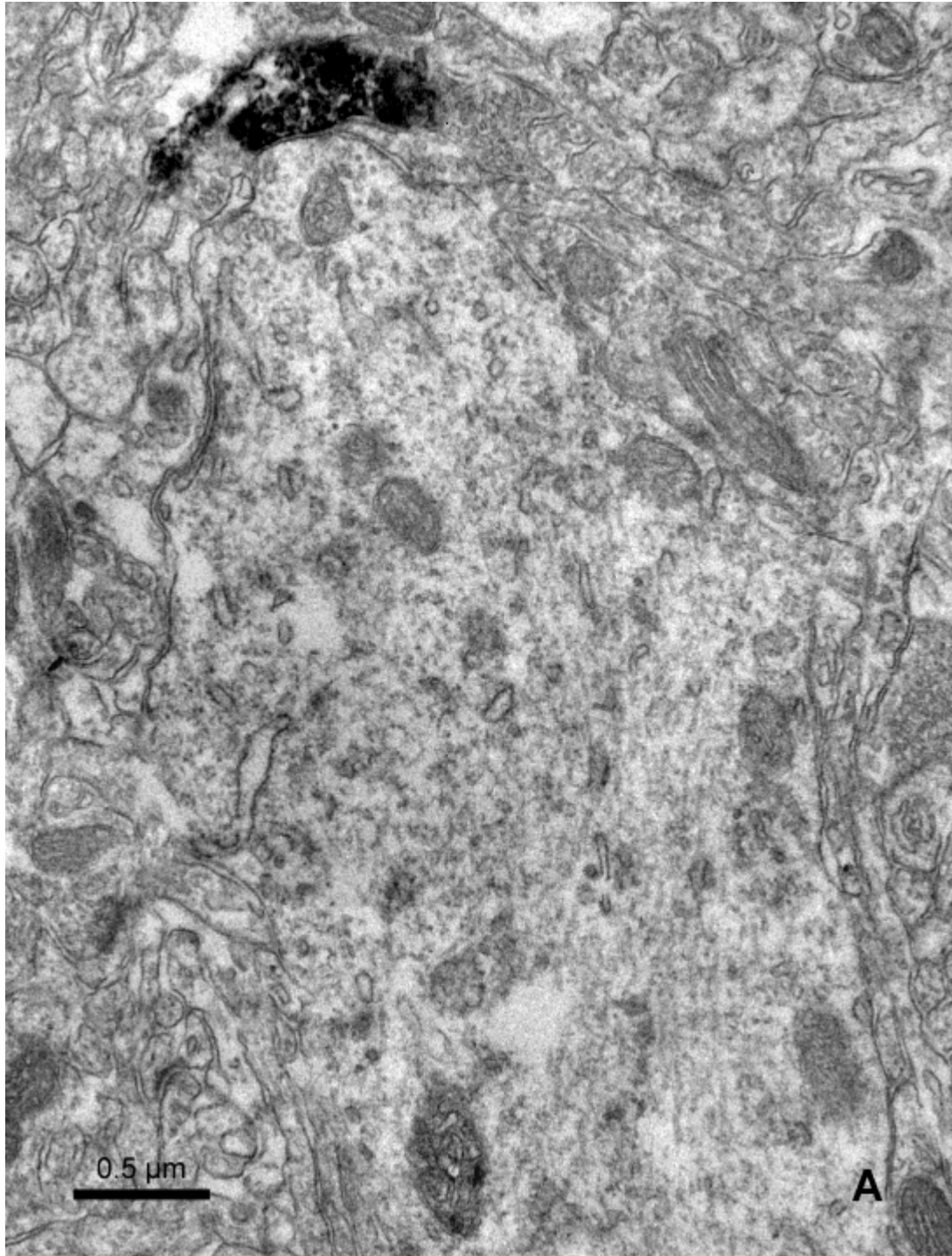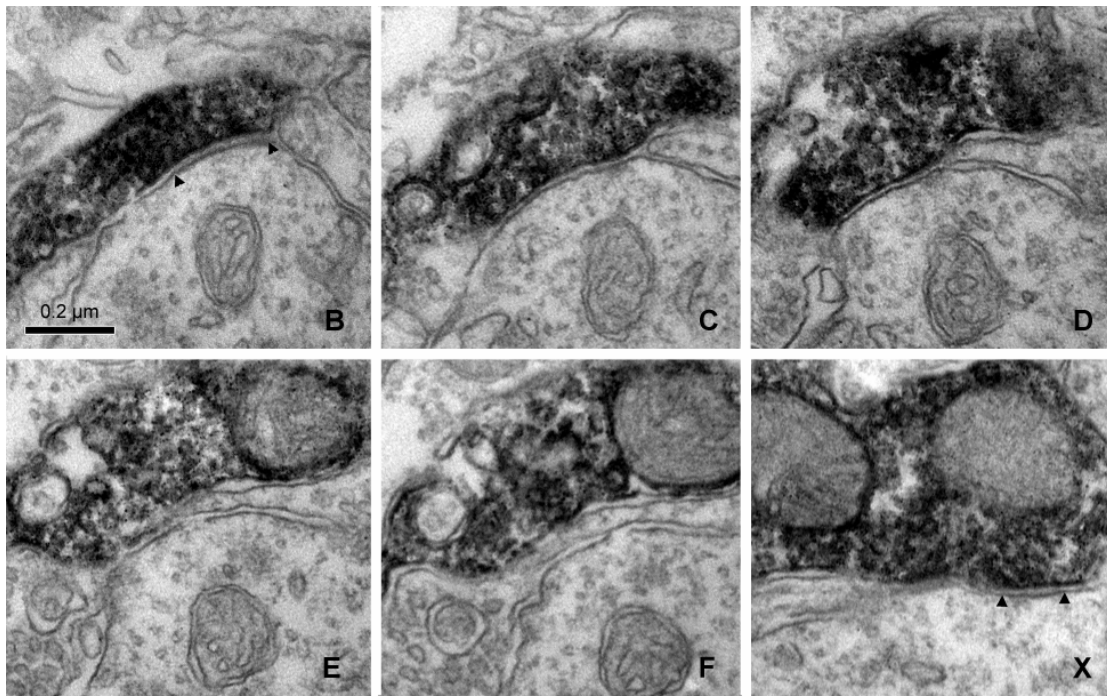
Figure 11: Electron micrograph showing a labelled bouton targeting an apical dendrite of a pyramidal cell (A). B-F serial sections of a Typ II synapse (B) and an axon between the labelled bouton and its target. Approximately 600 nm away, the same bouton forms another synapse onto the same apical dendrite (X) (electron micrographs derived from K75a – a PV and CB-co-expressing cell).

For cell K101a not many synapses could be identified because of poor ultrastructural preservation of the tissue, but an interesting finding with this cell was that one bouton targeted two somata simultaneously (**Figure 12**). Additionally, one synapse onto a spine and two synapses onto distal dendritic shafts of a pyramidal cell or interneuron were identified for this neuron.

In conclusion, the PV/CB co-expressing cells investigated so far could also be identified as basket cells, because they target apical dendrites and somata of pyramidal cells.
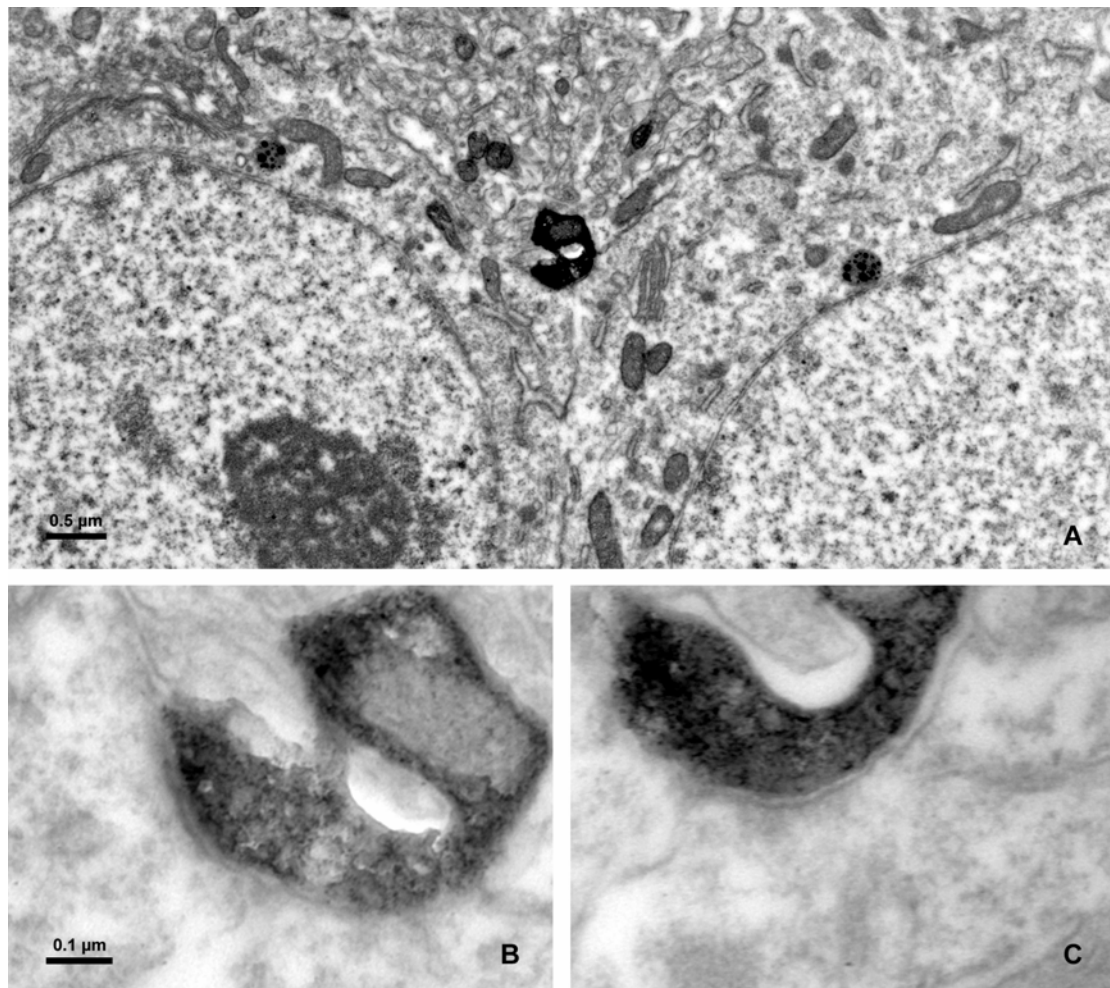
Figure 12: Electron micrograph shows a bouton of a labelled neuron targeting two somata (A). B, the synapse onto the left soma shown in A; C, synapse onto the right soma in panel A (electron micrographs captured from K101a – a PV and CB-co-expressing cell).

Other cells

Cell K104a belongs to a very distinct type of cells, because their axon runs along axon initial segment, which appears in the light microscopic level as chandeliers (**Figure 13**).
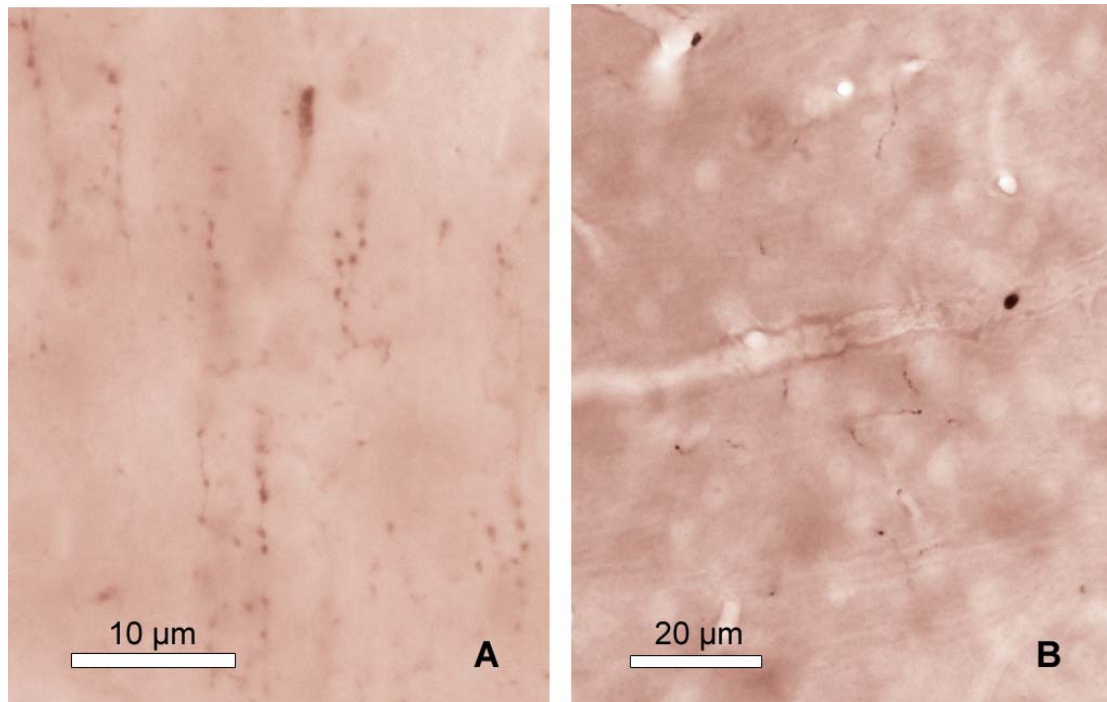
Figure 13: Light micrograph showing boutons of a labelled axo-axonic (K104a); A) and PV/CB expressing basket (K101a; B) cell after DAB reaction. Note in A that the axons form chandeliers - typical for cells targeting axon initial segments – and in B an axonal arborisation typical for basket cells (most of its axon in close proximity to its soma in random orientation).

The boutons of this cell target exclusively axon initial segments of pyramidal cells (**Figure 14** and **Figure 15**) (ten boutons and six synapses sampled) and therefore the interneuron was classified as an axo-axonic cell (Somogyi et al., 1983b).
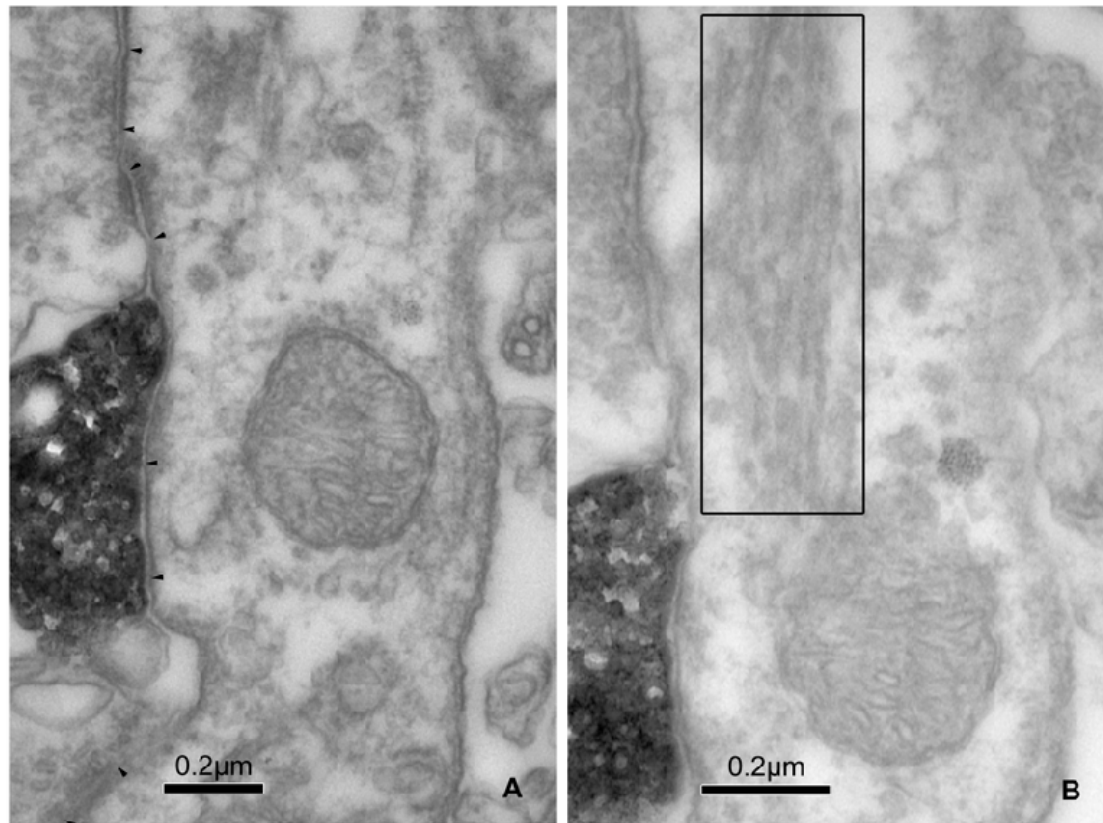
Figure 14: Electron micrographs showing a labelled bouton targeting an axon initial segment. These can be recognized in the electron microscope by their undercoating beneath the plasma membrane (A, opposite of labelled bouton), bundles of microtubules (B in box) and their innervation with several Gray Type II synapses (four synapses in figure A, whereas the top two release specialisations are from the same bouton and might belong to the same synapse).
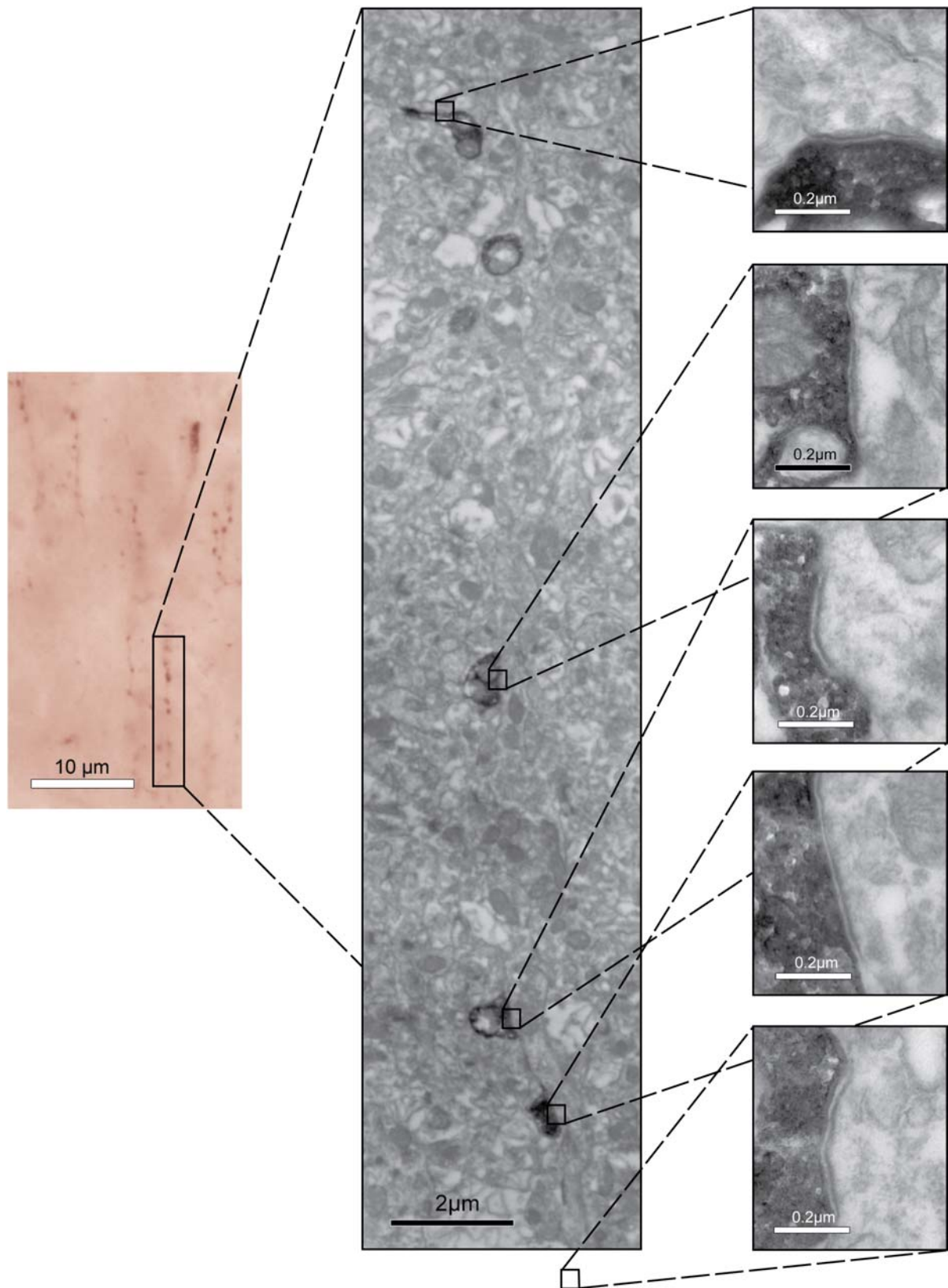
Figure 15: Light and electron micrographs of axo-axonic cell K104a showing labelled axons forming chandeliers along axon initial segments. For each bouton that forms a synapse onto the same axon initial segment, the synapse is shown with high magnification. For one of the boutons no synapse could be observed and one of the boutons is not shown in the low magnification electron micrograph. All synapses are within 20 ultrathin sections of 70 nm thickness.

In conclusion, the cells that were investigated for their postsynaptic targets can be divided into three distinct groups: Axo-axonic cells targeting exclusively axon initial segments, CB-expressing cells targeting small dendrites and spines, and PV expressing and PV/CB co-expressing basket cells, which target also apical dendrites and somata of predominantly pyramidal cells (**Figure 16**). Although there is some variation within the latter group, the postsynaptic target analysis does not distinguish between PV only expressing and PV/CB co-expressing cells.
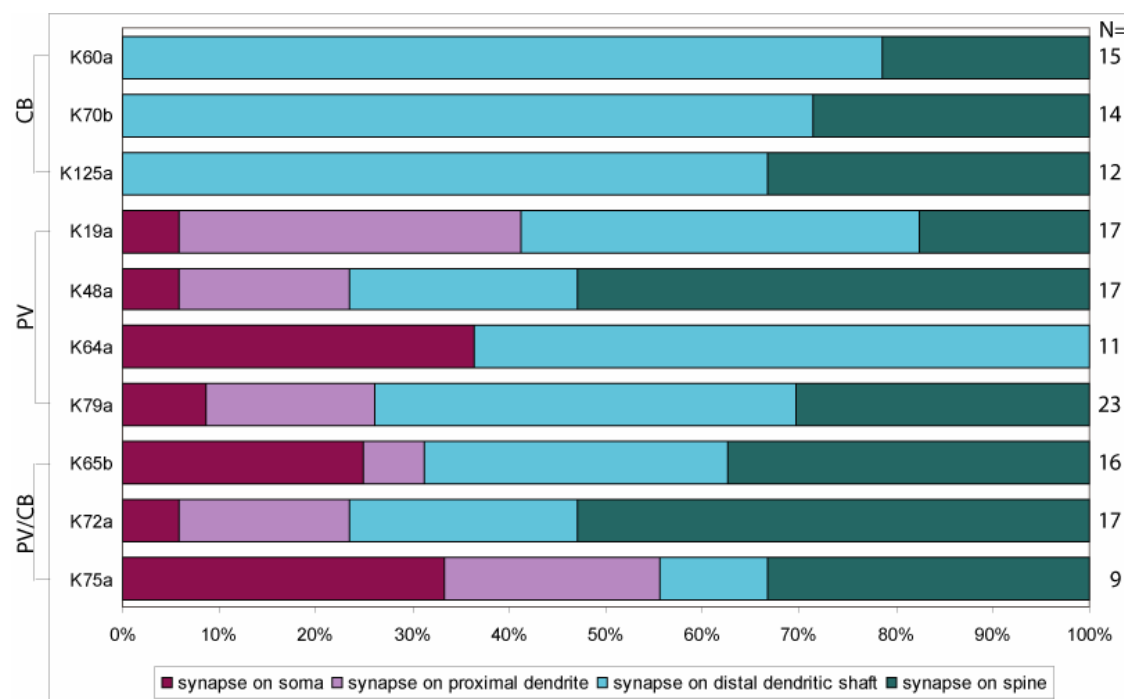

Figure 16: Diagram showing the percentage of the different types of targets for each cell. Cells expressing only CB exclusively target small dendrites and spines, PV-expressing cells and PV/CB co-expressing cells, additionally target apical dendrites and somata and were therefore identified as PV- and PV/CB-expressing basket cells. On the right the number of identified synapses is depicted for each cell.

Quest for novel molecular markers[5]

In order to identify additional moelcular markers that could further distinguish interneurons, antibodies against various molecules, for example, known to be expressed in subsets of PV expressing cells were tested for their suitability in our conditions (for instance the perfusion/fixation cannot be changed retrospectively). Some of these

[5] My contribution to this part of the project was about 95%. Katja Hartwich did two of the immunoreactions (mGluR1α, PV, CB and SM-co-localisation and CB, SM and NPY-co-localisation) before I arrived.

antibodies can be used in the future to further investigate PV/CB co-expressing cells and cells that were not positiv for markers tested so far.

mGluR1α

A co-localisation study was performed using antibodies of the metabotropic glutamate receptor 1α subunit (mGluR1α), PV, SM, and CB raised in different species, respectively. Of 33 cells that were immunopositive for at least one of the markers, 18 were immunopositive for PV and seven of these cells also expressed mGluR1α, five cells expressed PV, mGluR1α and CB and one cell expressed PV and CB, whereas five cells only expressed PV. Additionally, two cells were observed that express both CB and mGluR1α, four cells expressing CB, SM and mGluR1α and three cells which only express CB.

In summary, PV, CB and mGluR1α can be expressed in all sorts of combinations, except PV and SM are never co-expressed. In contrast to this observation in the medial prefrontal cortex (mPFC), bistratified and O-LM (oriens-lacunosum moleculare) cells in the hippocampal CA1 co-express PV and SM (Klausberger et al., 2003; Klausberger et al., 2004). An additional difference between mPFC and hippocampus is indicated by the observation that PV and mGluR1α are much more frequently co-expressed in the mPFC (**Figure 17**) than in the hippocampus (Ferraguti et al., 2004).
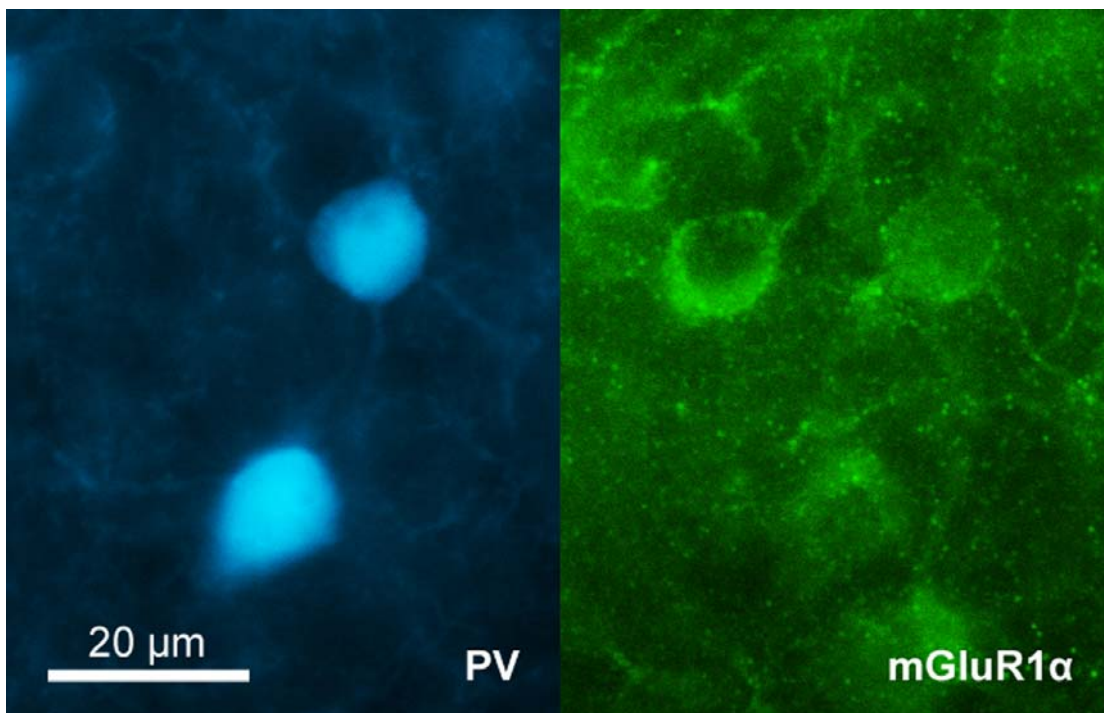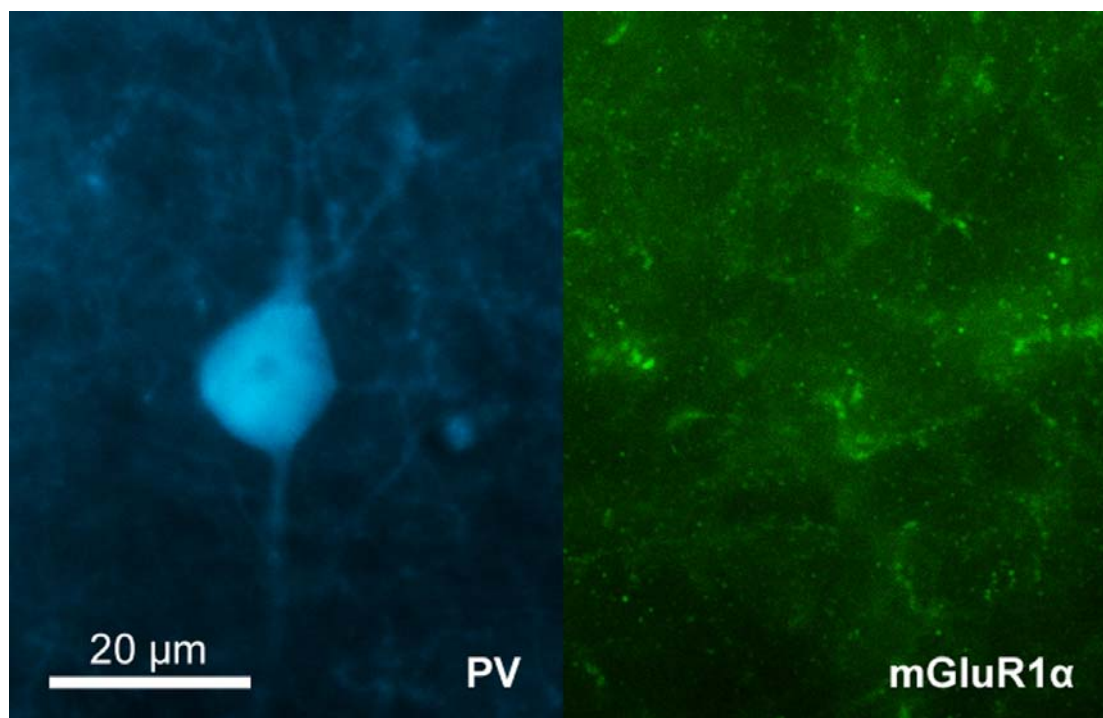
Figure 17: Fluorescence micrographs showing a co-localisation between PV and mGluR1α in the same neurons. In the first row two PV-positive cells are depicted which also express mGluR1α, whereas one cell expresses only mGluR1α, but not PV. In the second row a PV-expressing cell is shown, which is mGluR1α-negative.

vGLUT2

An antibody against the vesicular glutamate transporter type 2 (vGLUT2) raised in guinea pig was tested in dilutions of 1:2500 and 1:5000, which both showed a dotted labelling pattern, supposedly boutons, throughout the CA1 area of the hippocampus, but remarkably dense in the stratum lacunosum-moleculare. The labelling in the prefrontal cortex was much weaker in the 1:5000 dilution, so for the co-localisation with PV and CB a concentration of 1:2500 was used. The high number of vGLUT2-positive dots, presumably representing axonal boutons from the thalamus (Kubota et al., 2007) did not allow to judge at the light microscopic level, whether these boutons form synapses preferentially onto the soma or dendrites of certain PV-expressing cells and not on others. To investigate if terminals of certain PV- and/or CB-expressing cells target spines, which receive a Gray Type I synapse of a terminal immunopositive for vGLUT2 and therefore most likely thalamic input, was impossible at the light microscopic level, but this question could be addressed in the future by immunogold labelling using electron microscopy.

5-HT2c – a serotonin receptor subunit

Another antibody against 5-HT2c – the 2c subunit of a serotonin receptor – was tested with various dilutions ranging from 1:20 to 1:500. However, only a very weak nuclear labelling in all cells was observed, although a receptor-like labelling of the cell membrane was expected. Interestingly, when this antibody was also tested in a much higher dilution (1:10000 and 1:100000), a specific labelling of the plasma membranes of neurons was observed in layer VI of the prefrontal cortex, but no labelling in superficial layers or the hippocampus.

N200

The co-localisation of PV and N200 – a neurofilament - was tested as well, but of 28 counted cells in layer II and III of the mPFC only one PV-expressing cell was negative for N200. Further investigations may verify whether this molecular marker could be used to distinguish between subpopulations of PV-expressing cells.

Kv3.1

An antibody against the potassium channel Kv3.1 was tested for co-localisation with PV and of 164 observed cells only 3 PV positive cells did not express this potassium channel.

Kv4.3

A Kv4.3 antibody raised in rabbit was tested as well, but instead of an expression pattern on the cell surface a dotted nuclear labelling was observed in all concentration ranging from 1:500 to 1:100000. A co-localisation study with PV indicated that the labelling was not on the cell surface, but in the nucleus (**Figure 18**). Additionally, an antibody raised in goat was also tested, but showed similar non-specific results as the rabbit antibody.
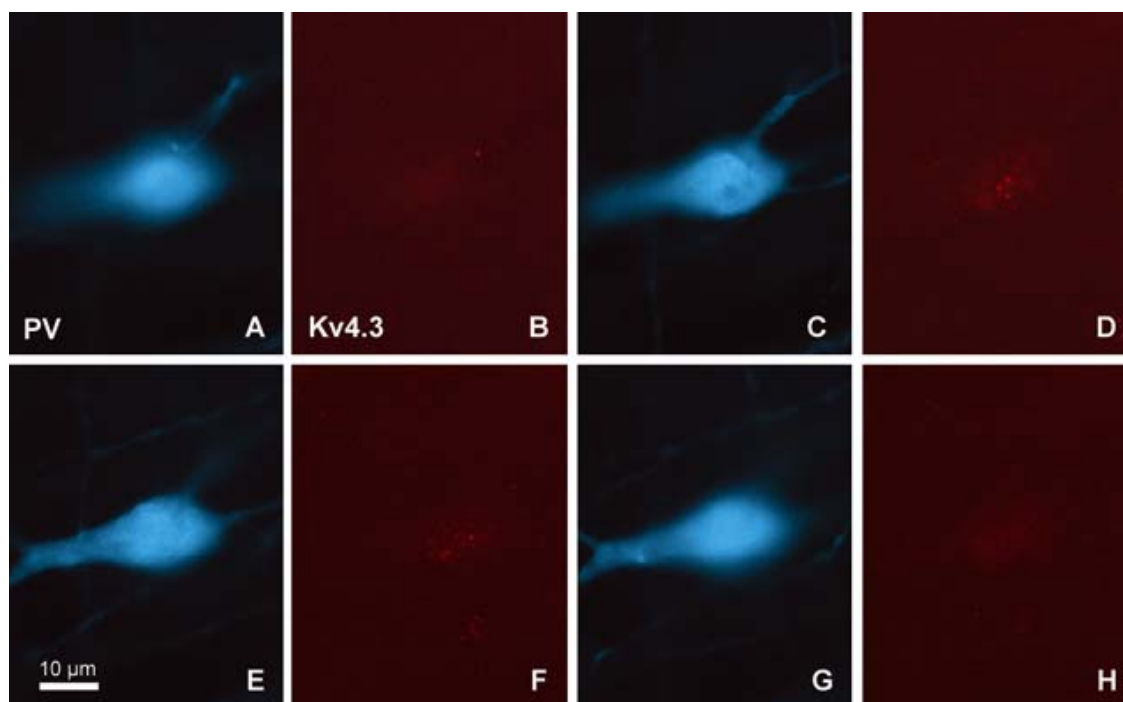
Figure 18: Fluorescence micrographs showing a PV (A,C,E,G) positive cell in different focuses (from top to bottom) acquired with a conventional fluorescence microscope and the corresponding images showing Kv4.3 labelling (B,D,F,H). The Kv4.3 labelling cannot be encountered on the cell surface, but in the nucleus.

D1 dopamine receptor

A subtype specific antibody against the D1 dopamine receptor was tested in concentrations ranging from 1:20 to 1:100000 (optimal concentration between 1:1000 and 1:10000) and showed strong labelling in the substantia nigra and the striatum, but only a few dots (presumably boutons) in the prefrontal cortex.

GluR2

A GluR2 antibody was also tested and showed dotted labelling throughout the section (like boutons) and also some dotted nuclear labelling of interneurons (somata in stratum radiatum and stratum oriens of the hippocampal CA1 region were labelled). Also somata in the prefrontal cortex had this unspecific nuclear labelling and therefore, this antibody cannot be used in our material.

NO sensitive guanylyl cyclase α1 subunit

Another promising candidate was a nitric oxide (NO) sensitive guanylyl cyclase α1 subunit (GCα1) specific antibody, which was used at an optimal dilution of 1:10000 and showed receptor-like labelling in both the hippocampus and the prefrontal cortex. A co-localisation study with PV and Somatostatin (SM) was performed, but

unfortunately, the immunoreaction of the GCα1 only seemed to have worked in the hippocampus (**Figure 19**), but not in the prefrontal cortex, although both other antibodies worked in the prefrontal cortex as well. Therefore, the prefrontal cortex of the same brain as the hippocampal sections was cut and the immunoreaction repeated on those sections. This time the reaction worked in both the hippocampal and the prefrontal section, although it was somewhat weaker in the mPFC. Many cells expressed this protein at relatively low level especially in layer II/III, whereas in layer V fewer cells were strongly immunopositve for this molecular marker.

In conclusion, this molecular marker might allow to distinguish subpopulations of PV-expressing cells in the hippocampus (possibly axo-axonic and basket cells), but whether it can be used in the mPFC is a matter of further investigations.
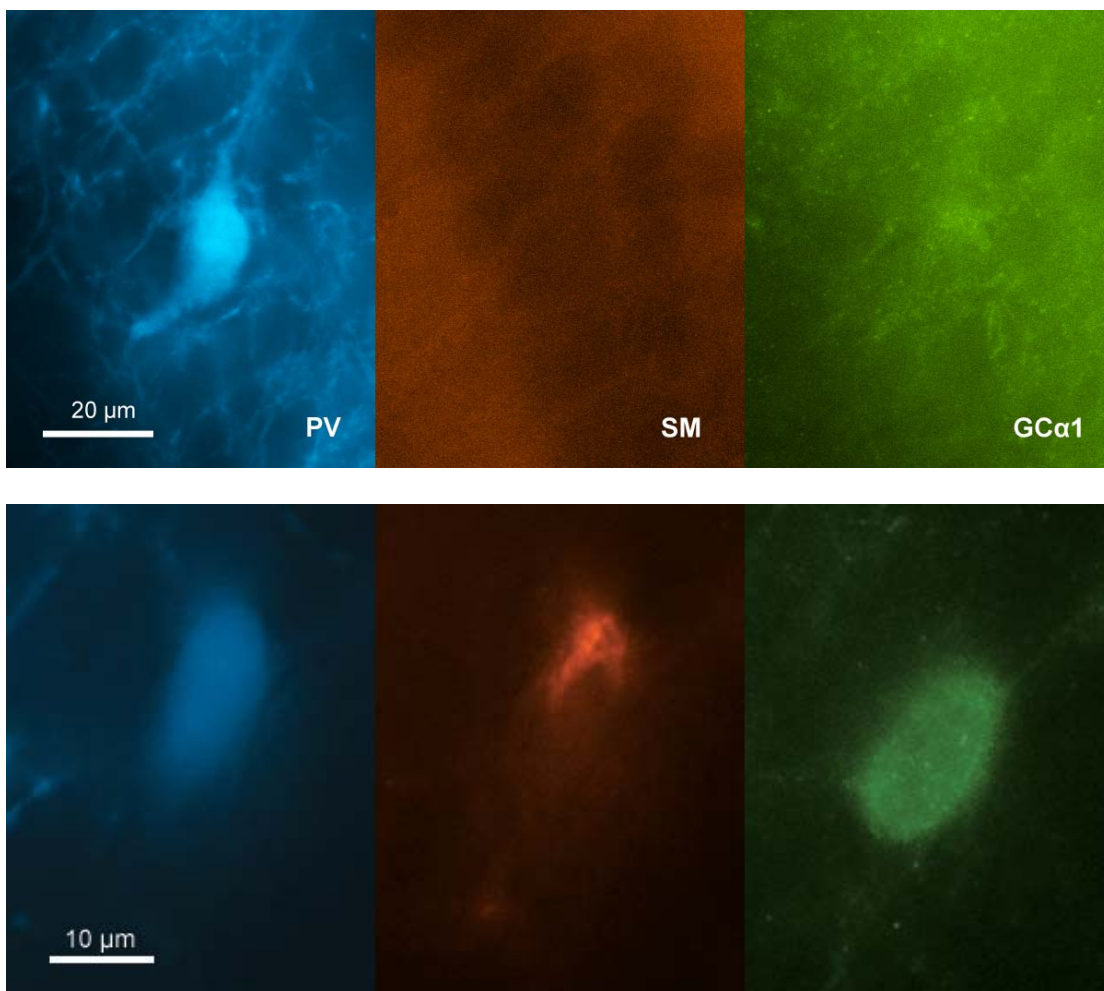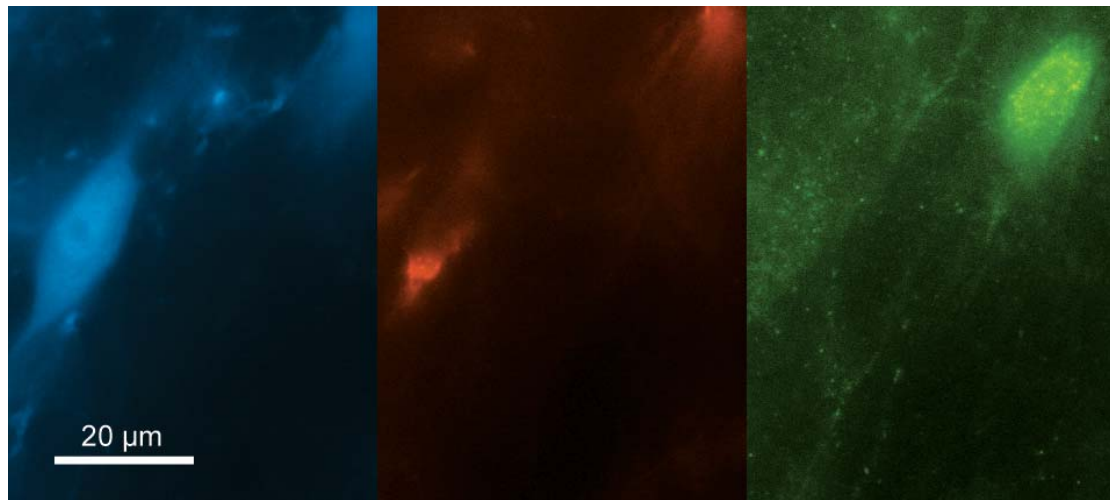
Figure 19: Fluorescence micrographs showing co-immunolabelling for PV in blue, SM in red and GCα1 in green. The first row depicts a cells in stratum pyramidale of the hippocampal CA1 area immunopositive for PV, but immunonegative for SM and GCα1. In the second row a cell in stratum oriens is shown that is positive for all three molecules tested in this co-localisation study. In the third row a cell is shown, which is positive for PV and SM, but negative for GCα1 and another cell positive for GCα1 and PV (weak), but negative for SM captured from stratum oriens.

CB-SM-NPY

Furthermore, to further investigate CB expressing cells, a co-localisation of CB, SM and Neuropeptide tyrosin (NPY) was carried out. Of 41 CB expressing cells 30 % also expressed SM, 10 % also NPY, 10 % both markers, whereas 50 % expressed CB only. Out of the four in vivo labelled CB expressing cells, one expresses SM and NPY, whereas the other three only expressed CB; this reflects the ratio of the co-localisation study.

VIP

In order to verify whether distinct subsets of PV expressing cells receive differential amounts of input from vasoactive intestinal peptide (VIP) expressing interneurons as suggested previously (David et al., 2007), a co-localisation of PV and VIP was performed. In Figure 20 an example of a bouton expressing VIP forming a putative synapse onto a dendrite expressing PV is shown, but only very few similar examples were observed.

In conclusion, mGluR1α, N200 and GC α1 could be used to further characterize labelled cells in the mPFC, and vGLUT2- and VIP-input could be investigated using confocal or electron microscopy.

Thomas Pollak   20/01/2008
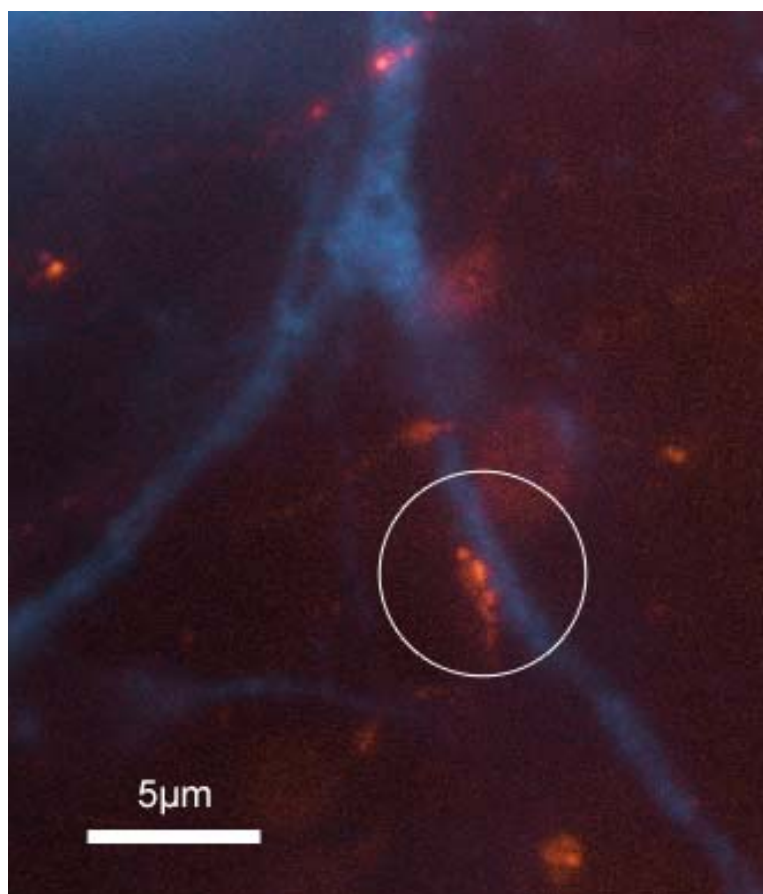

Figure 20: Fluorescence micrograph showing VIP positive boutons (orange, in circle) in close proximity to and putatively forming synapses onto a PV positive dendrite (blue).

Discussion

In the present thesis I have analysed the molecular expression profile and postsynaptic targets of GABAergic interneurons in the medial prefrontal cortex. Of twelve labelled cells the postsynaptic targets were identified and these cells can be divided into three categories: axo-axonic cells, which target exclusively axon initial segments, CB-expressing dendrite targeting cells and basket cells, which either express PV or co-express PV and CB and target apical dendrites and somata additionally to small dendrites and spines of pyramidal cells. Approximately 25% of synapses of interneurons target dendritic spines in the cat visual cortex (Beaulieu et al., 1992) and these spines usually also receive excitatory input (Jones and Powell, 1969). Innervation of spines has been found for basket and dendrite targeting cells, but not the axo-axonic cell. Because spines are electrically compact, GABAergic synapses are in a unique position to shape the excitatory input onto the spine (Dehay et al., 1991) and it has been shown that interneurons target predominantly spines that receive thalamo-cortical input (Kubota et al., 2007). These spines are larger and express AMPA receptors in contrast to spines that receive cortico-cortical input (Kubota et al., 2007) and co-innervation of interneurons of spines receiving thalamo-cortical input might be a mechanism to compensate for reduced synaptic plasticity of these terminals. Interestingly, it seems that from the interneurons investigated in this study all of them innervate dendritic spines apart from axo-axonic cells.

Basket cells

About 50% of all inhibitory interneurons are considered to be basket cells (Markram et al., 2004) and the percentage of the different target cell domains in this study for basket cells is consistent with previous findings in the neocortex (Kisvarday et al., 1985; Kubota et al., 2007; Somogyi et al., 1983a). In the CA1 area of the hippocampus basket cells express either PV, or CCK together with either VIP or vGLUT3 (Klausberger et al., 2005), whereas in the neocortex basket cells can express both PV and CB and many neuropeptides including CCK, NPY and occassionally SM or VIP (Markram et al., 2004). At least some cells co-express PV and CB in the mPFC and could be identified as basket cells in this study. There is one recorded cell that expresses CCK, which might also be a basket cell, but its postsynaptic targets have not been investigated yet.

Basket cells in the hippocampus are considered to provide temporal coordination for pyramidal cells (Klausberger et al., 2003) and considering that basket cells in the mPFC are also correlated to network oscillations, they might have the same function.

Axo-axonic cell

In this study one axo-axonic cell has been investigated. In the hippocampus axo-axonic cells are considered to express PV (Katsumaru et al., 1988) and in the neocortex to express either PV or CB (Markram et al., 2004), but this particular cell was tested for both molecular markers and turned out to be negative for both of them. Another calcium-binding protein expressed in certain GABAergic interneurons is Calretinin (Acsady et al., 1996), but this molecular marker was not tested yet.

Axo-axonic cells target exclusively axon initial segments (Somogyi et al., 1983b) and are therefore in the unique position to control the output of postsynaptic pyramidal cells (Buhl et al., 1994). The electrophysiological data of this recorded axo-axonic cell is not evaluated yet, but in the hippocampus it has been shown that axo-axonic cells fire just after the peak during theta oscillation and at the beginning of sharp wave bursts, but are silent subsequently, whereas basket cells fire at the descending phase of theta and are modulated to sharp wave oscillations (Klausberger et al., 2003). This suggests a differential contribution of basket cells and axo-axonic cells to the temporal coordination of pyramidal cells with the latter considered as controlling the output of pyramidal cells through inhibition. In contrast, it was reported that axo-axonic cells can depolarize pyramidal cells dependent on their membrane potential and chloride concentration in the axon-initial segment (Szabadics et al., 2006). Therefore, the function of axo-axonic cells and their contribution to network oscillations is not only dependent on their input, but also on the electrophysiological state of the postsynaptic target.

CB-expressing dendrite targeting cells

On the other hand, CB only expressing cells exclusively target small dendrites and dendritic spines and they are interestingly not correlated to local spindle and CA1 theta oscillations. Two of these cells have remarkably spiny dendrites and considering that most interneurons have fewer spines than pyramidal cells (Fairen et al., 1984), this might suggest that these cells mature slower than other cells (Wang et al., 2004). Martinotti cells in the neocortex also have spiny dendrites (Wang et al., 2004), but they

always express SM and for oriens-lacunosum moleculare (O-LM) cells – the pendant to Martinotti cells in the hippocampus – it has been shown that they are correlated to the trough of hippocampal theta oscillations (Klausberger et al., 2003). Since these cells have not been reconstructed yet, it is difficult to compare them with classes of cells already described in the literature or to suggest possible functions of these cells. But considering that most pyramidal cells are correlated to local spindle oscillations (Steriade and Amzica, 1998), there might be a subpopulation that is not correlated to these network oscillation. These CB-expressing cells might provide feedback inhibition for such pyramidal cells.

In conclusion, interneurons innervating distinct domains of pyramidal cells – axon initial segments; somata and apical dendrites; distal dendrites – shape the firing of pyramidal cells in different ways, with axo-axonic cells having the strongest influence on the output of their target cells (Buhl et al., 1994).

Heterogeneity of mPFC interneurons

The mPFC integrates input from several different brain regions including the limbic system (hippocampus and amygdala), the midline thalamus, various cortical areas and monoaminergic nuclei of the brain stem (Hoover and Vertes, 2007), whereas the CA1 area of the hippocampus has fewer extrinsic inputs and therefore less variation amongst its cell population (Somogyi and Klausberger, 2005).

An example to illustrate the heterogeneity of interneurons in the mPFC is a co-localisation study of PV, SM, mGluR1α and CB, which showed that PV and mGluR1α are much more frequently co-localized in the mPFC than in the CA1 of the hippocampus (Ferraguti et al., 2004). Furthermore, in the mPFC PV, CB and mGluR1α can be expressed either by themselves or in all sorts of combinations, except for PV and SM, which are not co-expressed. In contrast, bistratified and O-LM cells in the CA1 region of the hippocampus co-express PV and SM (Klausberger et al., 2003; Klausberger et al., 2004).

Examples for different expression in different layers of the mPFC are the serotonin receptor subunit 2c, which is predominantly expressed by cells in deeper layers (Liu et al., 2007a), vGLUT2, which is localized in boutons mainly in layer I/III/V (unpublished observation) and is mainly expressed in thalamic terminals, and GCα1 (NO sensitive guanylyl cyclase subunit α1), which shows high expression levels in relatively few

layer V neurons (similar to hippocampal CA1 (Szabadits et al., 2007)), whereas in layer II/III many cells seem to express this molecular marker at relatively low levels.

The above mentioned examples illustrate which complexity of interneuron types can be expected in the mPFC and that it is not always straightforward to classify these cells. Therefore, not only molecular markers, but also postsynaptic targets, *in vivo*-firing patterns, dendritic arborisation and presynaptic input (expression profile of boutons innervating dendrites and/or soma of a labelled cell, but also postsynaptic receptor expression) are considered to reliably define cell types (Markram et al., 2004; Somogyi and Klausberger, 2005).

Future directions

Because the recording and labelling of single cells in freely-moving animals has not yet been possible for technical reasons, all recordings were performed in anaesthetized rats. How does that relate to decision making? During this drug induced brain state of rats, network oscillations occur that were also observed during sleep, like spindle oscillations (7-14 Hz) during slow wave sleep (0.5-4 Hz) in the neocortex (Steriade and Amzica, 1998), or theta oscillations (4-12 Hz) in the hippocampus (Buzsaki, 2002), which also occur during exploratory behaviour. Therefore, single cell recordings simultaneous with the LFP of the mPFC and hippocampus - to obtain information about oscillatory brain activity - allow investigating the *in vivo*-firing patterns of single cells, but also the anatomy including postsynaptic targets and molecular expression profiles of these recorded cells. In order to relate these results to behaving rats there are two possible ways. First of all, these results can be used to identify certain types of interneurons according to their *in vivo*-firing patterns alone obtained by other recording methods, which in fact is currently being done by Timothy Senior, MRC Anatomical Neuropharmacology Unit, Oxford, UK. Secondly, after technical difficulties are circumvented single cell recording methods could be applied to behaving animals to verify the results from anaesthetized animals.

Furthermore, molecular expression profiles can be used to classify neurons in different brain areas. In addition, genetic manipulation of subpopulations of neurons expressing a certain marker could be used to measure the contribution of these subpopulations to network oscillation. After a promoter that controls the expression of a certain protein in a certain subpopulations of interneurons is identified, this promoter could be used in a construct to control the expression of genes that allow the manipulation of the cell's

activity with, for example, light (channelrhodopsin-2 (Deisseroth et al., 2006)) or reversible manipulation with a pharmacological drug such as zolpidem (Wulff et al., 2007). This would be a way to investigate causality – which subpopulations contribute to which kind of oscillations and to which degree – which is not possible with the experimental procedure used in this study.

mPFC in health and disease

Understanding the circuitry of the mPFC and the role of interneurons in these circuits is not only important to comprehend working memory and decision making in the healthy brain, but also has implications for brain dysfunction. It was suggested that alteration in GABAergic inhibition originating from distinct interneuron subclasses, in particular axo-axonic cells, is associated with schizophrenia (Lewis et al., 1999) and that abnormal synchronization of hippocampus and mPFC might contribute to attention deficit/hyperactivity disorder (Willis and Weiler, 2005) as well as to schizophrenia (Friston and Frith, 1995).

In conclusion, PV- and PV/CB-basket cells in the mPFC contribute to the temporal coordination of local pyramidal cells, whereas CB-expressing dendrite targeting cells might provide feedback inhibition of certain pyramidal cells that are not correlated to rhythmic brain activity. In order to further characterize these cells, in particular PV/CB co-expressing cells and cells that were not positive for any markers tested so far, they could be tested for some of the novel molecular markers. In order to understand the contribution of certain subpopulations of interneurons to local and distributed network oscillations during behaviour, these results can be used to support and help the understanding of results from experiments in behaving animals. A combination of behavioural experiments and anatomical analysis will eventually lead to an understanding which cells or cell types contribute to which kind of brain activity and resulting behaviour.

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In order to learn ultra microtome cutting and electron microscopy (EM) I have quite a long list of supervisors. Richard Hewer showed me for the first time how to use an ultra microtome and he did a really great job, although he also just learned this technique a couple of months ago. But I also learned a lot from David Roberts, who for example showed me how to use some features of the EM and explained to me in the EM, how to recognize axon initial segments. From Jojanneke Huck I learned re-embedding and from Yannis Dalezios, a visiting Professor from the University of Crete, I got a lot of very helpful technical advice.

A lab without technicians would not work at all, but it works well, if you have great technicians as in Peter Somogyi's lab. From Wai-Yee Suen I learned a lot about immunohistochemistry, Freeze/thaw and DAB reaction. Kristina Detzner as a German tries to bring some organisation into the chaos and is doing a great job. She was always very supportive and when you ask her for a favour, for example, to wash your sections, she would even mount them. It was a pleasure to work with both of them.

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Curiculum Vitae

Personal Information

- Nationality: Austria
- Date and place of Birth: 15th January 1981, Zwettl

Education

Since October 2002   University of Vienna

Molecular Biology

1. diploma passed (equivalent to BSc)

diploma project at Oxford University, 2. diploma exam will be at the end of January 2008 (equivalent to MSc)

From October 2001 to January 2005   University of Vienna

Nutritional Science

1. diploma passed

From 1995 to 2000   Vienna

Secondary College for Chemical Technology

passed with distinction

From 1991 to 1995   Stift Zwettl

Secondary Modern School with Emphasis on Music

passed with distinction

From 1987 to 1991   Jahrings

Elementary School

passed with distinction

Abilities

- Experience in working in laboratories:

  Chemistry: photometry, HPLC, titrimetry,…

  Molecular Biology: genotyping (mouse & plant tissue), northern & western blotting, antibody purification, tissue culture,…

Neuroscience: dissecting of anatomical structures,

Immunohistochemistry,

Electron Microscopy

of serial, ultrathin sections,…

- Computer know-how (Word, Excel, Power Point, Adobe Photoshop and Illustrator, Endnotes,…)
- Language skills: *English*: 7.5 points on the IELTS-Test, lived in Australia and England for the last 20 months

Hobbies

- **Playing clarinet in:** Oxford University Wind Orchestra,

Oxford Millennium Orchestra,

Also playing piano, saxophone, accordeon, keyboards, bassoon,

- **Sports:** jogging, gym, scuba diving, horse riding, skiing,...
- reading, attending the opera and the theatre

Experiences

Visiting Student at the MRC Anatomical Neuropharmacology Unit, Oxford University, in Dr. Thomas Klausberger's lab: from December 2006 until November 2007 working on a project with the aim to characterize GABAergic Interneurons in the prefrontal cortex

Exchange Student at the University of Queensland: in semester 1 in 2006 and additionally in July and August 2006

Work experience student in Prof. Linda Richards' lab involved in a project with the aim to characterize a point mutation in the human leader sequence of the DCC gene

Work experience student in Dr. Peter Schlögelhofer's lab: in September and October 2005 working on project to characterize a mutation in the FANCD2 gene in *Arabidopsis thaliana*

Work experience student in Prof. Gerhard Wiche's lab: in July 2005 doing antibody purification, cell transfection and

immunolabelling, and protein expression in *Escherichia coli*

Study trip to Costa Rica: organised by the University of Vienna (12.07.04 – 16.08.04): 2 weeks guided tours through the different kinds of rainforests and a 3 week project concerning *Bromeliaceae* at the tropical research station in the "Rainforest of the Austrians"

***ofi*-Austrian Research Institute for Chemistry and Technology**: occupied from August 2002 until February 2006 in the department for analytical chemistry (one day per week), where I did different standard analysis on all sorts of plastic materials, and in November 2006 in the QLAB (the department for food quality control)

Military Service: from July 2000 until August 2001 at the Gardemusik Wien where I played bass clarinet and clarinet in B flat

Wilhelmsburger Lederfabrik: occupied in August 1999

Ankerbrot AG: occupied in August 1998 doing food quality control

Herberts (now Dupont): occupied in August 1997 in the laboratory

Scholarships

FWF-scholarship: for diploma thesis (£3,849.00 for one year)

MRC living allowance: for diploma thesis (£3,900.00 for one year)

Joint Study Scholarship: for study abroad at the University of Queensland (£1,312.00 for one term)

TOP-Scholarship: for study abroad at the University of Queensland (£583.00 for one term)