Cell-type specific functions of calcium- and voltage-dependent potassium channels in the entorhinal-hippocampal memory system

Doctoral thesis by

Pedro Mateos-Aparicio

Division for Physiology, Department of Molecular Medicine, Institute of Medical Basic Sciences, PB 1103 Blindern, N-0317 Oslo, Norway

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Preface

The work presented in this thesis was performed at the Department of Physiology (currently named Section for Physiology, Department of Molecular Medicine) at the Institute of Basic Medical Sciences, located in the Faculty of Medicine, University of Oslo, from February 2009 to August 2015. My work has been supported by EMBIO stipend and the Norwegian Research Council.

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I want to write sincere words in memory of professors and staff in our department that sadly passed away during this period; my thoughts are with their families and friends.

Finally, I would like to express my deepest gratitude to my family and friends in Spain, Norway and other countries. Their constant and unconditional support, patience, and lovely words helped me a lot during this process.

List of papers

This thesis is composed by five papers, named by Roman numerals throughout the text. Three of them (papers I, II and V) are published, whereas papers III and IV are manuscripts in preparation.

Paper I* **Mateos-Aparicio P**, Murphy R, and Storm JF (2014), Complementary functions of SK and Kv7/M potassium channels in excitability control and synaptic integration in rat hippocampal dentate granule cells. JPhysiol, 592: 669–693.doi:10.1113/jphysiol.2013.267872.

Paper II Nigro MJ, **Mateos-Aparicio P**, Storm JF (2014) Expression and functional roles of Kv7/KCNQ/M-channels in rat medial entorhinal cortex layer II stellate cells. *JNeurosci*, 34:6807–6812. doi: 10.1523/JNEUROSCI.4153-13.2014.

Paper III **Mateos-Aparicio P**, Storm JF (2016) Functions and muscarinic modulation of Kv7/M channels in mossy fiber boutons of rat dentate gyrus granule cells. *Manuscript*.

Paper IV **Mateos-Aparicio P**, Hönigsperger C, Storm JF (2016) Dorsoventral differences in the sAHP and excitability of dentate gyrus granule cells of rats and mice. *Manuscript*.

Paper V Wang K**, **Mateos-Aparicio P****, Hönigsperger C, Raghuram V, Wu WW, Ridder MC, Sah P, Maylie J, Storm JF, Adelman, JP (2016). IK1 channels do not contribute to the slow afterhyperpolarization in pyramidal neurons. *eLife*, *5*, e11206. http://doi.org/10.7554/eLife.11206. doi:10.7554/eLife.11206.

* The granule cell model included in this paper is freely available online at:
http://senselab.med.yale.edu/modeldb/
The ModelDB accession number is 169240.

** Shared first author.

1. Introduction

The human nervous system contains about 10^{12} neurons, nearly 10000 morphological types and probably a higher number of functional classes (Johnston and Wu, 1995). To achieve such an extraordinary functional specialization, neurons express a vast diversity of ion channels that tightly control the electrical activity within their different subcellular compartments (Trimmer, 2015). Potassium (K^+) channels is the most diverse group of ion channels in nature. Probably all cells have K^+ channels but their specific roles are unknown in many neuronal types (Hille, 2001). The diversity of K^+ channels was initially noticed in the 1970s, when a series of studies reported differences in tetraethylammonium (TEA) sensitivity of delayed-rectifier K^+ channels of frog heart and skeletal muscle (Hille, 1967, Stanfield, 1970a, b, 1983). Since then, advances in molecular biology techniques, development and refinement of patch-clamp techniques have massively increased our knowledge about the molecular diversity, biophysical properties, and functions of these channels in different neuron types (Rudy, 1988, Storm, 1990, Baxter and Byrne, 1991, Rudy et al., 1991, Coetzee et al., 1999, Trimmer, 2015).

In general, K^+ channel opening leads to efflux of K^+ ions, hyperpolarizing the membrane potential of the cell and reducing the excitability. However, depending on their specific subunit composition and distribution, they control a wide range of neuronal features such as action potential repolarization, afterpotentials, spike frequency adaptation, synaptic integration, neurotransmitter release, resting membrane potential or temporal integration of signals, among others. K^+ channels of the same type located in morphologically different neurons may control different aspects of neuronal activity. Conversely, different channel types in different neurons may functionally converge by controlling similar features. Even within a single neuron, the roles of a specific K^+ channel type may vary depending on the subcellular compartment under consideration.

CA1 pyramidal neurons have been a model of study for ion channel functions during the last 40 years (Storm, 1990, Johnston and Wu, 1995, Andersen, 2007), in part due to easier identification, recording, and viability in slice preparations. In this thesis, I studied several functions of Ca^{+2}-activated K^+ channels and Kv7/M-channels related to afterhyperpolarizations in three different types of principal neurons in the entorhinal-hippocampal system, with a particular focus on granule cells of the dentate gyrus (Papers I, III,

and IV). In addition, we provided experimental evidence that help to clarify controversial issues in current literature, such as Kv7/M-channel function in stellate neurons of the entorhinal cortex (Paper II) or whether IK1 channels contribute to the slow afterhyperpolarization of CA1 pyramidal neurons (Paper V).

1.1. The entorhinal-hippocampal system: focus on the dentate gyrus

1.1.1. The entorhinal-hippocampal system

The parahippocampal formation (PHF) and the hippocampal formation (HF or hippocampus) constitute a functional brain system located in the medial temporal lobe of the mammalian brain involved in episodic memory and spatial navigation. The PHF consists of a group of several related brain regions adjacent to the HF: the presubiculum, the parasubiculum, the entorhinal cortex, the perirhinal cortex and the postrhinal cortex (van Strien et al., 2009). The HF is divided in three subregions: the dentate gyrus (DG), the hippocampus proper (subdivided in CA3, CA2 and CA1 areas) and the subiculum. Both PHF and HF contain a variety of cell types that are linked to different aspects of spatial information processing.

The circuitry consisting of the entorhinal cortex and the HF (so-called "the entorhinal-hippocampal system") is of key importance in memory formation and spatial navigation. The entorhinal cortex is a six-layered cortical structure that provides the major cortical input to the HF. Superficial layers (mostly II and III) of the entorhinal cortex project to the hippocampus through the perforant path (contacting DG and CA3) and temporoammonic path (contacting CA1), whereas reciprocal projections from CA1 and subiculum contact deep layers (V/VI) of the entorhinal cortex (Andersen, 2007, van Strien et al., 2009). Traditionally, the entorhinal cortex is divided in two areas showing cytoarchitectonic differences (Canto and Witter, 2012), the medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC). The MEC contains two main types of excitatory neurons, stellate and pyramidal cells, which can be distinguished by differences in their morphology and functional properties (Klink and Alonso, 1993, 1997, Yoshida and Alonso, 2007, Yoshida et al., 2011). Furthermore, stellate cells are thought to constitute a major part of the "grid cells", since several lines of evidence indicate that most of the stellate cells show hexagonal grid-like spatial firing fields (Fyhn et al., 2004, Hafting et al., 2005), although recent work suggested that pyramidal cells may show grid firing pattern as well (Tang et al., 2014). Grid field spacing and size vary along the dorsoventral axis of the MEC, being more dispersed and wider towards ventral MEC (Hafting et al., 2005, Brun et al., 2008). This finding motivated an extensive search for dorsoventral

differences in the intrinsic properties of stellate neurons, which include high expression of HCN channels (Garden et al., 2008, Pastoll et al., 2012). A direct link between dorsoventral gradients of HCN1 expression and the effect on intrinsic properties and spatial grid firing was shown in HCN1 knock-out mice (Nolan et al., 2007, Giocomo and Hasselmo, 2008, 2009, Giocomo et al., 2011). However, the study of other ion channel types is more limited in these neurons. For example, the expression and functions of Kv7/M-channels has been debated (Paper II), as different studies suggested the presence or absence of these channels in stellate neurons (Yoshida and Alonso, 2007, Heys et al., 2010, Heys and Hasselmo, 2012, Boehlen et al., 2013).

Hippocampal neurons fire in spatially restricted fields ("place fields") that also vary along the septotemporal axis of the hippocampus (Jung et al., 1994, Leutgeb et al., 2007). CA3, CA1 and DG neurons at least, show larger place fields towards ventral hippocampus, which may indicate different coding schemes along the septotemporal axis (Royer et al., 2010, Yartsev, 2010). Although several forms of functional differentiation within the hippocampus are well known (Moser and Moser, 1998, Fanselow and Dong, 2010, Strange et al., 2014), many studies of the intrinsic properties of hippocampal neurons have traditionally assumed homogeneous properties within each of the main cell populations. However, recent studies showed that CA1 neurons are heterogeneous and their properties differ along the septotemporal axis (Kim et al., 2012a, Marcelin et al., 2012a, Marcelin et al., 2012b, Honigsperger et al., 2015, Kim and Johnston, 2015). A direct link between septotemporal differences in ion channel functions and place field scaling is still missing, however, but the increasing attention on this line of research may lead to future progress in this respect.

1.1.2. The dentate gyrus

1.1.2.1 Functional anatomy of the dentate gyrus

The dentate gyrus is part of the hippocampal formation in the medial temporal lobe of the mammalian brain. It consists of three layers, which is typical for the allocortex. The molecular layer is the outermost layer and contains the dendrites of granule cells, a few sparsely distributed interneurons and axons from different sources. The second layer, the granule cell layer, is composed by densely packed granule cell bodies. The dorsal part of the layer is termed the supra-pyramidal blade and its opposite, the infra-pyramidal blade. The granule cell body layer also contains somata of different types of interneurons (Hosp et al., 2014). The third layer of dentate gyrus is called the polymorphic layer or hilus. This layer is enclosed by

the granule cell body layer which contains several different cell types and the axons from the granule cell body layer to the CA3 area, termed as the mossy fiber projection. Although there is a clear distinction between these three layers, there are contacts between different cell types across layers that constitute several, well defined local circuits (Larimer and Strowbridge, 2008).

The dentate gyrus receives inputs from a variety of sources. The major input comes from the entorhinal cortex through the so-called perforant pathway. The perforant pathway is divided in two parts based, among other factors, on the region of origin. In rodents, these divisions originate in the MEC and LEC and are therefore called the medial and lateral perforant paths. The medial perforant path contacts the middle third of granule cell dendrites and the lateral perforant path form synaptic contacts with the distal one third of granule cell dendrites (Witter, 2007).

The main output projection of the dentate gyrus is the mossy fiber projection. The unmyelinated axons of the granule cells were named "mossy fibers" by Ramón y Cajal (1909), due to the presence of varicosities along the length of these axons (Andersen, 2007). The mossy fibers traverse the hilus and terminate in *stratum lucidum* of CA3, but do not cross the border between CA3 and CA2. A distinctive feature of these axons is the presence of large *en passant* synaptic contacts or "boutons". Mossy fiber boutons (MFBs) are typically 3-5 μm size but can be as large as 8 μm. Each granule cell stablishes an average of 15-20 synaptic contacts with different CA3 pyramidal cells whereas each CA3 neuron receives synaptic contacts from 72 different granule cells (Amaral et al., 2007). This remarkable pattern of connectivity has important implications on the computations performed by the dentate gyrus. When traversing the hilus, mossy fibers contact the proximal dendrites of mossy cells, basal dendrites of basket cells and other hilar interneurons. Mossy fiber axons give rise to a plexus of axon collaterals in the hilus, typically more or less perpendicular to the direction of the parent axon. The majority of mossy fiber collaterals from a single granule cell terminate on hilar interneurons, establishing up to 160-200 synaptic contacts (Amaral et al., 2007).

1.1.2.2. Morphology and electrophysiology of granule cells

The granule cells (DGCs) are the largest population of hippocampal cells and the principal cells of the dentate gyrus. The estimated number of granule cells per hemisphere is ~1 million

neurons in rats, 5 millions in the monkey and ~15 million in humans (Seress, 2007). Granule cells have a remarkably different morphology compared to other cell types in the hippocampus. Their name comes from their relatively small, spherical-shaped soma (~10 μm diameter). One of the most characteristic morphological features of these neurons is their cone-shaped apical dendritic tree that crosses the molecular layer. Mature granule cells located in the supra- or infra-pyramidal blade extend their dendrites throughout the molecular layer and reach the hippocampal fissure or the ventricular surface, respectively (Amaral et al., 2007). Different studies showed different average total dendritic lengths of granule cells but in general, total dendritic length values fall within a 2500-4500 μm range (Schneider et al., 2012). Several studies reported a shorter mean total dendritic length of granule cells in the infrapyramidal blade compared to the suprapyramidal blade (Rahimi and Claiborne, 2007). The maximum lateral spread of mature granule cell dendrites is typically around 300 μm (Claiborne et al., 1990, Williams et al., 2007), but dendritic morphology vary depending on the position of the granule cell within the layer (Shapiro and Ribak, 2005). Granule cell dendrites are densely covered with spines in the whole molecular layer, it is estimated a spine density of 1.6 and 1.3 spines/μm in the suprapyramidal and infrapyramidal blades, respectively (Rahimi and Claiborne, 2007). The mossy fiber axons of granule cells have a diameter of 0.2-0.5 μm and extend a collateral axonal plexus (up to 2300 μm) in the hilus before reaching the *stratum lucidum* of the CA3 area (Amaral et al., 2007). The mossy fiber bundle carries the output information of the dentate gyrus to the CA3 area in a unidirectional way. In the next paragraphs I will describe the electrophysiological properties of DGCs.

Mature granule cells have a highly negative resting membrane potential and high input resistance compared to pyramidal cells of the hippocampus (Spruston and Johnston, 1992). The input resistance of immature granule cells can be as high as 4 GΩ and decreases to values between 100-300 MΩ at mature stages (Liu et al., 1996, Schmidt-Hieber et al., 2004). The membrane time constant values of granule cells from rats, monkeys or humans fall within the range 20-50 ms (Spruston and Johnston, 1992, Staley et al., 1992, Williamson et al., 1993, St John et al., 1997, Schmidt-Hieber et al., 2004). Therefore, the resting properties of granule cells seem to be similar across species and markedly different from hippocampal pyramidal cells.

The active properties of these cells are shaped by a number of voltage-dependent conductances present in the somato-dendritic compartment as well as in the axon. The small

caliber of granule cell dendrites has limited the knowledge about their integrative properties and ion channel composition. However, an elegant study by Krueppel et al. (2011) revealed that granule cell dendrites, contrary to CA1 pyramidal neurons, lack regenerative dendritic events (dendritic spikes), and display linear integrative properties and strong attenuation of incoming signals. Furthermore, a recent study (Brunner et al., 2013) provided evidence for an mGlu2-activated K^+ conductance in proximal dendrites. This implies that release of glutamate by different sources targeting the proximal one third of granule cell dendrites may represent a precise and selective inhibition mechanism, through dendritic-branch-specific modulation. Much of the knowledge about active properties of granule cells originates from somatic recordings. DGCs express a transient inactivating A-type potassium current (I_A), which controls excitability, spike repolarization, interspike interval and the latency to the first action potential induced by current injection (Beck et al., 1992, Beck et al., 1997a, Riazanski et al., 2001, Ruschenschmidt et al., 2006). Somatic voltage clamp recordings together with real-time polymerase chain reaction (RT-PCR) and immunohistochemistry pointed towards Kv3.3 and Kv3.4 subunits as the main contributors for this current (Beck et al., 1992, Riazanski et al., 2001). Furthermore, a small DTX-sensitive delay current (I_D) mediated by Kv1 channels, has been reported to control the action potential delay response of mice granule cells (Kirchheim et al., 2013). In response to hyperpolarizing current pulses, granule cells show a small or absent sag, indicative of low expression of HCN channels, in contrast to CA1 pyramidal neurons (Stabel et al., 1992). Moreover, strong expression of Kv7/M channel subunits is restricted to the mossy fibers (Cooper et al., 2001). The role of Kv7/M channels in DGCs is part of this thesis and will be discussed further detail (Papers I and III).

As many other neurons in the brain, DGCs generate afterhyperpolarizations following action potentials. Different types of calcium channels (N-, T- or L-type) contribute to shape afterpotentials in granule cells (Zhang et al., 1993, Beck et al., 1997b, Valiante et al., 1997, Aradi and Holmes, 1999). Ca^{+2} –activated K^+ currents of BK and SK types have been described in somatic (Beck et al., 1997a, Sailer et al., 2002) and MFB recordings (Alle et al., 2011), but their functional roles are not entirely clear. In paper I, we studied the roles of SK channels controlling afterhyperpolarizations and somatic excitability.

The action potential threshold of granule cells is significantly more depolarized than that of pyramidal neurons (Kress et al., 2008, Kress et al., 2010). Voltage-gated Na^+ channels in DGCs represent a good example of compartmentalized ion channel function. In absence of a

morphologically defined axon initial segment, the proximal region of the mossy fibers (~15-30 um) contains ~2 to 9 times higher density of Na^+ channels with gating kinetics 2 times faster than somatic Na^+ channels (Schmidt-Hieber and Bischofberger, 2010). Furthermore, Na^+ channels in mossy fiber boutons show ~50% faster inactivation kinetics than proximal axon channels (Engel and Jonas, 2005, Schmidt-Hieber and Bischofberger, 2010). Specialized Na^+ channels in mossy fiber boutons can amplify the presynaptic action potential and enhance Ca^{+2} inflow (Engel and Jonas, 2005). Therefore, Na^+ channels in granule cells have different gating kinetics in three different cellular compartments (soma, proximal and distal axon).

In MFBs, presynaptic action potential repolarization is mainly mediated by Kv1 and Kv3 channels (Geiger and Jonas, 2000, Alle et al., 2011). Mossy fiber boutons are equipped with P/Q-, N- and R-type Ca^{+2} channels that are differentially recruited by presynaptic voltage waveforms (Li et al., 2007). Interestingly, no evidence of T-type channels was found in direct presynaptic recordings, suggestive of proximal-distal axon variations. Two distinct populations of BK channels seem to coexist in these terminals, but they do not contribute to action potential repolarization under basal conditions. Therefore, BK channels in mossy fiber boutons may represent an emergency brake and become activated under conditions of reduced Kv3 channel availability (Hu et al., 2001, Alle et al., 2011).

Compared to *in vitro* studies, fewer have investigated intracellular granule cell activity *in vivo*. Although granule cells mean firing rate *in vivo* is low , it was demonstrated that physiological patterns of DGC firing contain periods of high frequency firing that effectively drive CA3 neuron discharges (Henze et al., 2002). The development of *in vivo* recording techniques allowed the first whole-cell patch-clamp recordings of DGCs in awake rodents (Anderson and Strowbridge, 2014, Pernia-Andrade and Jonas, 2014). Granule cells in awake animals fired at low frequencies in spite of high frequency of subthreshold events. Interestingly, the neurons fired preferentially bursts of action potentials, which have been reported to increase the probability of CA3 discharge (Henze et al., 2002). Furthermore, EPSCs and IPSCs were coherent with the extracellular theta and gamma rhythm, respectively (Pernia-Andrade and Jonas, 2014). Finally, whole-cell patch-clamp recordings in awake mice during locomotion revealed transient alpha-oscillations of the membrane potential associated with movement onset (Anderson and Strowbridge, 2014). Further studies will be required to dissect the specific inputs regulated by alpha oscillations in DGCs.

1.2. SK channels

Small conductance calcium-activated potassium (SK) channels are tetrameric channels gated directly by intracellular calcium, with high sensitivity in the nanomolar range. They are voltage independent with low single channel conductance (10-20 pS) (Faber, 2009, Adelman et al., 2012). SK channels were identified by Blatz and Magleby (1986), some years after apamin, a bee venom toxin that shows remarkable selectivity for SK channels, was shown to block a voltage-insensitive Ca^{+2}-activated K^+ current (Hugues et al., 1982). Molecular cloning of SK channels identified three types of subunits, SK1, SK2 and SK3 (Kohler et al., 1996), which are members of the same gene family $K_{Ca}2.1$, $K_{Ca}2.2$ and $K_{Ca}2.3$, respectively. Each of these genes can generate multiple mRNA splice variants (Shmukler et al., 2001, Wittekindt et al., 2004, Faber and Sah, 2007, Faber, 2009, Adelman et al., 2012). Furthermore, SK2 isoforms with structural differences in their intracellular N-terminal domain have also been described (Strassmaier et al., 2005). The functional significance of the molecular diversity of SK channels and the physiological relevance of each splice variant or isoform are not completely understood.

Each SK channel subunit consists of six transmembrane domains (S1-S6) with a pore-forming loop located between S5 and S6 and both C- and N-terminals on the cytoplasmic side. The calcium sensitivity of SK channels is conferred by calmodulin which is bound to calmodulin-binding domain within the C-terminal (Schumacher et al., 2001, Li et al., 2009, Adelman, 2015). It is estimated that one calmodulin binds to each subunit of the tetramer, and the binding of calcium to calmodulin induces opening of the channel (Xia et al., 1998, Keen et al., 1999, Schumacher et al., 2001, Adelman et al., 2012, Adelman, 2015).

In-situ hybridization and immunohistochemical studies of SK channels have shown a close match between mRNA transcript and protein expression distributions throughout the rat and mice brain (Stocker and Pedarzani, 2000, Sailer et al., 2002, Sailer et al., 2004). SK1 and SK2 subunits are highly expressed in the neocortex, hippocampus, amygdala, cerebellum and brainstem whereas SK3 subunits are highly expressed in the midbrain, thalamus, cerebellum and hypothalamus (Sailer et al., 2002, Sailer et al., 2004). In the hippocampus, CA1 and CA3 pyramidal cells showed high levels of mRNA transcripts for SK1 and SK2 subunits, while low or moderate levels of all subunits were observed in dentate gyrus (Stocker and Pedarzani, 2000). Immunohistochemistry of SK proteins revealed the highest SK1 protein expression levels associated to the molecular layer and mossy fiber system in the *stratum lucidum* of the

CA3 area, whereas low or virtually absent expression was found in the granule cell layer (Sailer et al., 2002). SK2 proteins were associated mostly to *stratum oriens* and *radiatum* in CA1 region, most likely associated with dendritic terminals of pyramidal cells, whereas the dentate gyrus showed low levels of SK2 protein. Finally, SK3 subunits showed moderate expression in the hippocampus, particularly within the hilus and the end of mossy fibers (Sailer et al., 2002).

The roles of SK channels in the regulation of neuronal excitability have been extensively reviewed (Vogalis et al., 2003, Stocker, 2004, Bond et al., 2005, Faber and Sah, 2007, Pedarzani and Stocker, 2008, Faber, 2009, Adelman et al., 2012). The opening of SK channels causes K^+ efflux resulting in membrane hyperpolarization and shunting, thus reducing the excitability of neurons. Their specific role within a neuron is determined by different factors such as their subcellular location, kinetics and type of Ca^{+2} sources, distance to Ca^{+2} sources, and subunit composition, among others. In general, SK channels contribute to afterhyperpolarizations, spike frequency adaptation, regulation of synaptic transmission, synaptic plasticity, as well as learning and memory processes (Faber, 2009, Adelman et al., 2012). Calcium influx evoked by action potentials can activate SK channels, which show cumulative activation during and after a train of action potentials. SK channels contribute to the mAHP in many different neuronal types (Faber, 2009, Adelman et al., 2012). In the hippocampus, SK channels underlie an apamin-sensitive component of the mAHP current (I_{mAHP}) of CA1 pyramidal neurons (Stocker et al., 1999, Stackman et al., 2002, Gu et al., 2005). However, current clamp recordings showed that the mAHP in CA1 neurons is generated by Kv7/M channels with no significant contribution of SK channels (Storm, 1989, Gu et al., 2005). This suggests that the voltage pulse used in voltage clamp experiments to elicit tail I_{AHP} currents triggers a higher Ca^{+2} influx than sodium action potentials. In fact, when Na^+ spikes were blocked, SK channels were readily activated by Ca^{2+} spikes and the related mAHP was blocked by apamin (Gu et al., 2008). Finally, SK channels located in CA1 dendrites can modulate synaptic transmission through interplay with NMDA receptors (Ngo-Anh et al., 2005, Gu et al., 2008, Lin et al., 2008). Recently, it was shown that SK channels can control the excitability of CA1 neurons under conditions in which Kv7/M channel function is compromised such as hyposmolarity, representing a “second line of defense” that prevents neuronal hyperexcitability (Chen et al., 2014).

In paper I, we addressed the functions of SK and Kv7/M channels in granule cells of the dentate gyrus and found that both channel types underlie different but complementary aspects of excitability control in DGCs.

1.3. Kv7/M channels

Kv7/M channels are the molecular correlates of the low threshold, slow (both activation and deactivation), and non-inactivating M-current (I_M) (Wang et al., 1998). This current can be modulated by several transmitters and hormones through activation of G-protein coupled receptors (Delmas and Brown, 2005), as first observed for muscarinic acetylcholine receptors, hence its name (Brown and Adams, 1980, Halliwell and Adams, 1982, Brown and Passmore, 2009). Although Kv7/M subunits can form homotetrameric channels, they are typically expressed in heteromeric form (Hadley et al., 2003). Kv7/M channel subunits (Kv7.1-7.5) are encoded by the KCNQ gene family (KCNQ1-5). Among the five Kv7 subunits, Kv7.1 channel subunits are expressed in the heart and peripheral epithelial and smooth muscle cells, and Kv7.2-7.5 expression is restricted to the nervous system. Whereas neuronal Kv7/M channels are mainly composed by Kv7.2, Kv7.3 and Kv7.5 subunits, Kv7.4 subunit expression is found in the auditory system (Kubisch et al., 1999, Cooper et al., 2001, Spitzmaul et al., 2013). Kv7.2 and Kv7.3 subunits co-localize with Na^+ channels in the axon initial segment (AIS) and nodes of Ranvier of neurons in the hippocampus, cerebral and cerebellar cortex, the ventral horn, and the sciatic nerve (Cooper et al., 2001, Devaux et al., 2004, Pan et al., 2006). Clustering of Kv7/M channels in axons is due to the presence of an ankyrin-G binding domain located in the intracellular C3 region of Kv7.2 and Kv7.3 subunits (Chung et al., 2006, Pan et al., 2006). Interestingly, this sequence shows a high homology with the Na^+ channel domain that binds to ankyrin-G (Pan et al., 2006, Cooper, 2011). Finally, the importance of Kv7/M channels in brain function is highlighted by mutations in KCNQ genes that lead to several hereditary disorders (Jentsch, 2000) like dominant deafness (Kubisch et al., 1999) or some forms of epilepsy (Biervert et al., 1998, Peters et al., 2005).

The modulation of the M-current by a variety of neurotransmitters and intracellular second messenger is of major importance in neuronal physiology and has been extensively studied since I_M was discovered (Delmas and Brown, 2005). The inhibition of I_M by acetylcholine acting through muscarinic receptors was the first modulatory mechanism described for this current and received considerable attention over the years (Brown and Adams, 1980). For example, stimulation of G(q) protein-coupled receptors (GPCRs) such as the M1 muscarinic

acetylcholine receptor, activates phospholipase-C (PLC) which in turn hydrolyzes membrane phosphatidylinositol-4,5-biphosphate (PIP2), generating the second messengers dyacilglycerol (DAG) and inositol triphosphate that activate protein kinase C (PKC) and increase intracellular calcium levels, respectively (Suh and Hille, 2002, Suh et al., 2006, Brown et al., 2007, Kosenko et al., 2012). Each one of these steps can modulate the M-current through different mechanisms (Kosenko et al., 2012). Depletion of membrane PIP2 and rise of cytosolic calcium can both inhibit I_M (Suh and Hille, 2002, Brown et al., 2007, Bal et al., 2010, Zaydman and Cui, 2014). However, other products of this pathway, such as arachidonic acid, can also increase the M-current (Schweitzer et al., 1990, Villarroel, 1994).

In the hippocampus, CA1 pyramidal neurons express perisomatic Kv7/M channels that control excitability, action potential threshold, EPSP summation, mAHPs, and subthreshold resonance (Hu et al., 2002, Vervaeke et al., 2006, Hu et al., 2007, Hu et al., 2009). In previous studies, pharmacological disruption of the Kv7/M channel-ankyrin-G binding showed that Kv7/M channels in the AIS of CA1 neurons regulated the action potential threshold and resting membrane potential (Shah et al., 2008). The role of AIS Kv7/M channels in the control of EPSP-spike coupling is mainly due to their influence in the action potential threshold, as axonal Kv7/M channels did not influence EPSP summation (Shah et al., 2011). In addition, some studies reported that presynaptic Kv7/M channels affect transmitter release onto CA1 pyramidal neurons (Vervaeke et al., 2006, Peretz et al., 2007). However, whether presynaptic Kv7/M channels are present in other axons and regulate their synaptic transmission remains to be directly tested. The existence of dendritic Kv7/M channels in CA1 pyramidal cells is more controversial. Whereas some studies suggested that Kv7/M channels are expressed in dendrites of CA1 pyramids and interneurons (Chen and Johnston, 2004, Lawrence et al., 2006, Yue and Yaari, 2006), direct recordings from apical CA1 dendrites combined with focal application of Kv7/M channel blockers or enhancers showed no effect on excitability (Hu et al., 2007).

In CA3 pyramidal neurons, Kv7/M channels have contrasting roles depending on their subcellular localization (Vervaeke et al., 2006). On the one hand, they control the somatic mAHP, ADP, and bursting; on the other hand, they can regulate excitability and transmitter release in the Schaffer collaterals when these axons are sufficiently depolarized, for example by activity-induced increase of extracellular K^+ (Vervaeke et al., 2006). Furthermore, another study showed that Kv7/M channels in the CA3 area can modulate kainate-induced gamma

oscillations *in vitro* by decreasing phase coupling of CA3 pyramidal neuron spikes (Leao et al., 2009).

Less is known about the functions of Kv7/M channels in the largest population of hippocampal neurons, the granule cells of the dentate gyrus. Immunohistochemical studies showed that Kv7.2 and Kv7.3 subunits are strongly expressed in the mossy fiber tract in the hilus and the *stratum lucidum* in the CA3 area (where mossy fibers contact CA3 neurons), while only scattered staining was found in the granule cell layer, mostly co-localized with parvalbumin-positive interneurons (Cooper et al., 2001, Klinger et al., 2011), suggesting that Kv7/M channel expression is restricted to the axons of DGCs. In this thesis, we tested the roles of Kv7/M channels in DGCs both at the somatic compartment (Paper I) and mossy fiber boutons (paper III). In addition, the presence and functions of Kv7/M channels in MEC layer II stellate neurons, which provide the main input to DG, remain under debate (Hetka et al., 1999, Yoshida and Alonso, 2007, Heys et al., 2010, Heys and Hasselmo, 2012, Boehlen et al., 2013). We addressed this issue in paper II and our results showed that Kv7/M channels are present and control excitability of these neurons. In addition, we provided explanations that help to clarify the current debate in literature.

1.4. Afterhyperpolarizations

Action potentials are often followed by periods of membrane potential hyperpolarization called afterhyperpolarizations (AHPs). AHPs are important, but not the sole, controllers of neuronal excitability by contributing to spike frequency adaptation at different time scales. A wide variety of AHP sequences and kinetics are observed across neuronal types. In many mammalian central neurons, 3 types of AHPs can be distinguished according to their duration and underlying ionic mechanisms. For example, principal neurons in the mammalian hippocampus, exhibit a typical sequence of 3 AHPs that have been thoroughly studied over the last 30 years.

The fast AHP (fAHP) lasting 1-5 ms, follows single action potentials and is generated by fast-activating K^+ channels involved in action potential repolarization (Storm, 1987a, b). Kv3 channels underlie action potential repolarization and fAHP in a variety of principal neurons (Rudy et al., 1999, Rudy and McBain, 2001) and interneurons (Lien and Jonas, 2003). In addition, BK channels underlie spike repolarization and fAHPs in many central neurons, such as hippocampal CA1 and CA3 pyramidal neurons (Storm, 1987a, Hu et al., 2001), and

hippocampal interneurons (Zhang and McBain, 1995). In DGCs, both BK and Kv3 channels are expressed (Knaus et al., 1996, Riazanski et al., 2001), but their contributions to the fAHP seem different than in pyramidal neurons. Somatic recordings from DGCs of mice lacking β4 BK channel subunits showed increased BK activity that shortened action potentials and increased high-frequency firing causing temporal lobe seizures (Brenner et al., 2005). Strikingly, BK channel expression is stronger in MFBs than in DGC somata. Nevertheless, Kv3 channels dominate the AP repolarization and fAHP in mossy fiber boutons (Alle et al., 2011), leaving the role of BK channels in MFBs unclear. A thorough analysis of the role of BK channels mediating the fAHP in CA1 pyramidal neurons concluded that the activation of these channels supported high frequency firing by sharpening action potentials and producing fAHPs that limit both activation of other K^+ channels and Na^+ channel inactivation (Gu et al., 2007). In addition, the rapid inactivation of BK channels during high frequency firing causes spike broadening, spike frequency adaptation, and reduction of the fAHP (Shao et al., 1999, Gu et al., 2007). Therefore, the mechanisms underlying this type of afterhyperpolarization have counterintuitive effects on membrane excitability, supporting high frequency firing when activated and causing spike frequency adaptation when inactivated, in contrast to other AHPs with slower kinetics (Gu et al., 2007). Finally, some studies revealed that the fAHP amplitude in CA1 pyramidal neurons was reduced after learning a hippocampus-dependent trace eyeblink task (Matthews et al., 2008, Matthews et al., 2009), although the precise chain of events leading to this reduction is not clear.

An afterhyperpolarization of medium duration (so-called medium afterhyperpolarization or mAHP) typically follows the fAHP after single spike, but its amplitude can increase with the number of action potentials (Storm, 1989). The mAHP is responsible for early spike frequency adaptation during sustained firing, excitability control and prevention of bursting activity. Initial studies on the mAHP mechanisms of CA1 pyramidal neurons suggested that Kv7/M channels at depolarized potentials and HCN channels at hyperpolarized potentials were responsible for mAHP and related excitability control (Storm, 1989, Williamson and Alger, 1990). Some years later, other studies suggested that SK channels contributed to the mAHP and excitability control in these neurons (Stocker et al., 1999, Stackman et al., 2002, Bond et al., 2004, Gerlach et al., 2004). Because SK channels underlie the mAHP and related excitability control in many regions of the brain (Adelman et al., 2012), this idea was commonly generalized to all neurons. However, a series of studies systematically investigated this issue in CA1 pyramidal neurons and confirmed the initial findings that Kv7/M channels

and HCN channels generated the mAHP at different membrane potentials (Gu et al., 2005, Gu et al., 2008). In addition, they also found that whereas apamin had little or no effect under current clamp recordings, voltage clamp recordings revealed a prominent apamin-sensitive mAHP current (Gu et al., 2005). Furthermore, genetic deletion of Kv7/M channels effectively suppressed the mAHP and reduced the I_{mAHP} in CA1 neurons (Peters et al., 2005, Tzingounis and Nicoll, 2008). Recently, another study concluded that SK channels can control the excitability of CA1 neurons when Kv7/M channel activity is compromised, such as hyposmolarity (Chen et al., 2014). In parallel, examples of Kv7/M-mediated mAHPs were found in neurons from other brain regions, such as cholinergic and GABAergic neurons in the pedunculopontine nucleus (Bordas et al., 2015). The example of CA1 pyramidal neurons illustrates that the mechanisms controlling neuronal excitability are defined by the specific expression and subcellular distribution of different ion channels in a cell-type dependent manner. Other neurons within the entorhinal-hippocampal system express a non SK-mediated mAHP as well, such as stellate neurons in layer II MEC (Khawaja et al., 2007, Pastoll et al., 2012).

Following the mAHP, activation of a slow AHP (sAHP) underlie a classical form of spike frequency adaptation that limits the cell excitability after a train of action potentials (Madison and Nicoll, 1982). Also, learning hippocampal-dependent eyeblink conditioning task resulted in reduced sAHP, enhanced excitability of CA1 pyramidal neurons (Matthews et al., 2009, Oh et al., 2009, Oh et al., 2010), and interneurons (McKay et al., 2013). Aging, however, induces opposite intrinsic plasticity in CA1 neurons, which showed enhanced sAHP amplitudes and reduced excitability (Matthews et al., 2009, Oh et al., 2010). The sAHP was first described more than 30 years ago (Hotson and Prince, 1980, Madison and Nicoll, 1982, Lancaster and Adams, 1986) and since then, a great deal of research focused in elucidating the properties of the underlying current and its molecular correlates. The sAHP current (I_{sAHP}) in most neurons is a voltage-insensitive, Ca^{+2}-dependent K^+ current with slow activation and decay kinetics that is strongly regulated by a variety of neuromodulators (Andrade et al., 2012). However, variability across brain regions and cell types has been reported. For example, a longer-lasting (up to 20 s) sodium-ATPase-mediated sAHPs after intensive firing were observed in some invertebrate neurons (Pulver and Griffith, 2010) as well as non-mammalian (Parker et al., 1996, Zhang and Sillar, 2012) and mammalian neurons (Kim and von Gersdorff, 2012), including CA1 and layer V pyramidal neurons (Gulledge et al., 2013).

Recently, it was suggested that the intermediate-conductance calcium-activated K^+ channels (IK1, SK4) are the main determinants of the sAHP in CA1 pyramidal neurons (King et al., 2015). In collaboration with 2 independent groups, we tested this hypothesis under a variety of experimental conditions (Paper V) but failed to replicate the findings reported in King et al. (2015). Some studies have suggested Kv7/M channels as partial mediators of the sAHP in DGCs (Tzingounis and Nicoll, 2008) and CA3 pyramidal neurons (Tzingounis et al., 2010). Furthermore, another study showed that the sAHP amplitude of DGCs is reduced by a K_{ATP} channel inhibitor (Tanner et al., 2011). The results mentioned above have led to the recent hypothesis that the sAHP may not be the result of a single channel type but rather mediated by several ion channels depending on the brain region, cell type and neuronal background (Andrade et al., 2012).

2. Aims of the study

The principal objective of this thesis project was to address the functions of K^+ channels in different cell types within the hippocampal formation, with a special focus on granule cells of the dentate gyrus. Much work on K^+ channel functions and AHPs has been done in CA1 pyramidal neurons in the last 40 years. The functions of Ca^{+2}-activated K^+ channels and Kv7/M channels in AHPs and excitability control have been thoroughly studied as well. However, DGCs have received far less attention than pyramidal neurons in spite of being the largest cell population in the hippocampus. We aimed to test whether the functions attributed to SK and Kv7/M channels in CA1 pyramidal neurons apply also to other neurons in the entorhinal-hippocampal system, in particular in DGCs and MEC layer II stellate cells. Also, we wanted to test a recent proposal that IK1 channels may underlie the sAHP in CA1 pyramidal neurons, whose underlying mechanism remains unknown after 40 years of research. In particular, our work shed light on the following groups of questions:

1. What are the mechanisms underlying the medium afterhyperpolarization (mAHP) and excitability control in DGCs? Are SK or Kv7/M channels the main contributors to these phenomena? How do these channel types control the excitability of DGCs? (Paper I).
2. Do stellate neurons in layer II of the MEC express functional Kv7/M channels? How do they affect the electrical properties of these neurons? Why did other studies obtain seemingly negative results? (Paper II).
3. What are the functions of Kv7/M channels in mossy fiber boutons? Can muscarinic modulation affect these Kv7/M channels and thus modulate the electrical properties of distal mossy fibers? (Paper III).
4. In Paper I, we noticed variability in the sAHP amplitude of DGCs, and asked: does this variability follow any pattern? Are there any dorsoventral differences in intrinsic properties of DGCs, as reported for CA1 pyramidal neurons? (Paper IV).
5. A recent paper suggested that IK1 channels are the main contributors to the sAHP in CA1 pyramidal neurons (King et al., 2015). This was surprising since previous studies reported lack of IK1channels in the brain. Therefore, we directly tested this hypothesis under a variety of experimental conditions. Are IK1 channels the major determinant of the sAHP in CA1 pyramidal neurons or in other pyramidal neurons such as those of the basolateral amygdala? (Paper V).

3. Methods

The specific information of all experiments is detailed in the Methods section of each paper included in this thesis. In general, rats or mice were deeply anesthetized by isoflurane inhalation and decapitated. The brain was removed and quickly placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF). Horizontal, coronal, or parasagittal slices (300-400 μm thick) were prepared using a vibratome. Slices were subsequently incubated for 30 minutes (30-33 ºC) in a holding chamber filled with sucrose ACSF saturated with 95% O_2 5% CO_2. After heat-bath incubation, slices were stored at room temperature during each experimental session. For recording purposes, slices were transferred to a recording chamber perfused with ACSF saturated with 95% CO_2 / 5% O_2 and heated to 30-33 ºC or 34-35 ºC. The perfusion rate was 2-3 ml/min. Recordings were performed under visual guidance with an infrared differential interference contrast (IR-DIC) video microscope.

3.1. Whole-cell patch-clamp recordings

The main technique used in this thesis was patch-clamp recordings in whole-cell configuration (Sakmann and Neher, 2009). In this technique, application of constant positive pressure to the patch pipette allows for its movement through the slice tissue while keeping a clean tip. Under visual guidance, the pipette is carefully approached towards the neuron of interest. When contact is stablished, a dimple on the cell membrane is observed and quick release of positive pressure produces the sealing of the membrane around pipette tip. Coordinated application of gentle negative pressure by mouth and hyperpolarization of the electrode by 60-70 mV (close to the resting potential of the cell) should achieve a high resistance GΩ seal formation. Seal resistance is monitored throughout the process by measuring the current response to a -10 mV pulse. Next, application of brief suction pulses by mouth helps to break the patch and stablish whole-cell configuration. If the pipette capacitive transients were cancelled during cell-attached configuration (Giga-seal formation), transition to whole-cell mode appears as a sudden increase in the amplitude and duration of the capacitive currents (Sakmann and Neher, 2009).

Somatic whole-cell recordings were obtained from DGCs (papers I and IV), MEC layer II stellate cells (paper II) and CA1 pyramidal neurons (paper V). For somatic whole cell recordings, patch pipettes were pulled from borosilicate glass tubes (outer diameter 1.5 mm / inner diameter 0.86 mm) in a vertical puller and filled with potassium-based intracellular

solution. Recordings in whole-cell configuration have a number of advantages such as 1) visual control of neuronal structures, 2) relatively easy to do, 3) low resistance, 4) access to the electrical activity of the whole neuron, or 5) possibility of intracellular and extracellular pharmacology. The main disadvantage associated with this technique is the dialysis of the cytoplasmic components, as the pipette solution and cytoplasm equilibrate over time after whole-cell establishment. Therefore, the choice of intracellular solution influences the outcome of the recordings (Kaczorowski et al., 2007). In addition, voltage clamp measurements are prone to error and especially problematic in neurons with complex dendritic processes due to space clamp errors (Williams and Mitchell, 2008).

3.2. Whole-bouton patch-clamp recordings

Subcellular patch-clamp techniques allow for direct recordings of the electrical activity in neuronal processes such as dendrites or axons with unprecedented resolution. In paper III, presynaptic MFB recordings in the CA3 region were obtained following protocols described previously (Bischofberger et al., 2006). Recording pipettes were made from thick-walled borosilicate glass tubing (outer diameter 2 mm / inner diameter 0.6 mm) pulled in a horizontal puller. Somatic whole-cell recordings from DGCs give little information about the electrical properties of their big presynaptic terminals. Whole-bouton recordings overcome this limitation and previous research showed that the active properties of mossy fiber boutons differ markedly from the somatic compartment, providing invaluable information (Engel and Jonas, 2005, Alle et al., 2011). However, patching small structures have a number of disadvantages such as increased difficulty, reduced success rate, or increased access resistance. In addition, structures with high input resistance (typically >1 GΩ) require a high seal resistance (> 5 GΩ), in order to avoid artefactual leak currents between the pipette and the membrane that distort voltage measurements (i.e. accurate membrane potential), among other parameters (Wilson et al., 2011).

3.3. Morphological reconstructions

In papers III and IV, 0.2-0.3% biocytin was included in the recording pipette, which allowed for post-*hoc* staining and morphological reconstruction of recorded neurons. Detailed protocol information is given in the Methods section of each paper. Neuronal structures were reconstructed and analyzed with Neurolucida software package.

4. Results and Discussion

4.1. Paper I

In the hippocampus, the roles of SK and Kv7/M channels controlling the mAHP and excitability control in CA1 pyramidal neurons have been extensively debated in the past 35 years (Storm, 1989, Stocker et al., 1999, Stackman et al., 2002, Vogalis et al., 2003, Bond et al., 2004, Kelly and Church, 2004, Gu et al., 2005, Gu et al., 2008, Chen et al., 2014). However, this issue remained less explored in other hippocampal neurons. In paper I, we addressed this problem in DGCs by combining whole-cell patch clamp recordings in DGCs with pharmacology and computational modelling.

We found that specific SK and Kv7/M channel blockers reduced the mAHP of DGCs. SK channel blockade largely abolished the mAHP after a train and single action potentials, whereas Kv7/M channel blockade had quite weak effect on the mAHP after train of spikes and no effect after single action potential. Intriguingly, blocking (XE991) or enhancing (retigabine) Kv7/M channel activity reduced the sAHP amplitude after a train of spikes. SK and Kv7/M channel blockade had several different but complementary effects in controlling excitability, spike frequency adaptation, and postsynaptic integration. Our results showed that SK channels reduced the cell´s excitability and increased early spike frequency adaptation, but had no influence on subthreshold input resistance or spike threshold. Consequently, SK channels seem to play strictly suprathreshold roles. In contrast, Kv7/M channels reduced neuronal excitability and increased spike frequency adaptation by reducing subthreshold input resistance and controlling action potential threshold. These effects contributed to a Kv7/M channel-mediated weakening of EPSP-spike coupling.

These results suggest that the roles of SK and Kv7/M channels in the hippocampus and other brain areas are cell-type dependent. In CA1 pyramidal neurons, a clear apamin-sensitive AHP current (I_{aAHP}) was reported in several studies (Stocker et al., 1999, Stackman et al., 2002, Bond et al., 2004, Gu et al., 2005). However, under current clamp conditions, Kv7/M channel blockers abolished the mAHP and increased related excitability of the cell whereas apamin failed to affect the mAHP and excitability (Storm, 1989, Williamson and Alger, 1990, Gu et al., 2005, Gu et al., 2008, Chen et al., 2014). Paradoxically, our results and those of others suggest that the opposite applies to DGCs. Although these studies showed a small apamin-sensitive current (Beck et al., 1997a, Sailer et al., 2002) under voltage clamp, SK channel

blockers consistently reduced the mAHP and early spike frequency adaptation. Therefore, our results suggest that the roles of K^+ channels in the hippocampus are cell-type dependent.

We reported intriguing effects of both XE991 and retigabine on the sAHP amplitude of DGCs. Previously, some studies suggested that Kv7/M channels may underlie a component of the I_{sAHP} in mouse DGCs (Tzingounis and Nicoll, 2008) and the sAHP and its underlying current in CA3 pyramidal neurons (Tzingounis et al., 2010, Kim et al., 2012b). A recent hypothesis suggested that the sAHP might be the result of several types of ion channels acting in concert (Andrade et al., 2012). A direct contribution of Kv7/M channels to the sAHP implies that application of XE991 would reduce the sAHP amplitude and retigabine would increase it. However, we found that both XE991 and retigabine reduced the sAHP. A similar sAHP reduction by retigabine was observed in CA1 pyramidal neurons (Gu et al., 2005), which is consistent with the idea that any Kv7/M channel contribution to the sAHP might be indirect. Several hypotheses are possible: 1) retigabine opened Kv7/M channels already before the spike train, which partly occluded further opening after the spikes. Computational modelling in our paper is consistent with this idea and suggested that the size of the retigabine effect on the mAHP depended on the relative increase in M-conductance (g_M) during the spike train. 2) It is possible that Kv7/M channels enhancement produced local shunting and membrane polarization in other compartments, limiting the opening of voltage-gated Ca^{+2} channels (Ca_v). 3) Our model failed to reproduce the experimentally observed sAHP reduction by Kv7/M channel blockade, suggesting that such effect might be indirect. For example, Kv7/M channels may interact with intracellular calcium sensors that control the sAHP (Kim et al., 2012b). Future research will be needed to address the relation between Kv7/M channels and the sAHP in detail.

Recently, a study tested the effect of cholinergic stimulation on Kv7/M channel function in DGCs (Martinello et al., 2015). Cholinergic suppression of Kv7/M channels lowered the axon potential threshold, enhanced EPSP-spike coupling and increased the excitability of DGCs, confirming the results obtained in our paper. Interestingly, Martinello et al. (2015) showed that the cholinergic inhibition of Kv7/M channels was mediated by axonal T-type Ca^{+2} channels. Immunogold staining showed muscarinic M1 receptor and $Ca_v3.2$ subunit expression along the mossy fibers. An analysis of Ca^{+2} channel types in mossy fiber boutons found no evidences of T-type channels (Li et al., 2007), suggesting that muscarinic M1 receptors in mossy fibers might not suppress the M-current in MFBs or the mechanism of

inhibition is different from that in the proximal mossy fiber. In Paper III, we tested the effects of muscarinic receptor activation on Kv7/M channels by direct patch-clamp recordings in MFBs.

Paper I demonstrates that in DGCs, SK and Kv7/M channels control complementary aspects of excitability in the soma. The physiological relevance of these findings lay in the marked frequency-dependence of information transfer from DG to CA3 (Henze et al., 2002). Granule cell firing can recruit local inhibitory circuits of the DG in a frequency-dependent manner, for example, bursting by a granule cell evokes discharge of CA3 pyramidal neurons during a short period of time and triggers feed-forward short-term plasticity in local interneurons, resulting in enhanced inhibition onto pyramidal cells (Mori et al., 2004, Mori et al., 2007). In addition, it has been showed that DGCs *in vivo* fire predominantly (65%) in short bursts of 3-6 spikes (Pernia-Andrade and Jonas, 2014). Therefore, it seems that bursting in DGCs is more relevant than single action potentials and the mechanisms underlying control of early spike frequency adaptation, action potential threshold or EPSP-spike coupling are relevant for DG function. Future research is needed to address the relevance of SK and Kv7/M channels during DG network function *in vivo*.

4.2. Paper II

The medial entorhinal cortex contains spatially modulated neurons (Fyhn et al., 2004, Witter and Moser, 2006, Schmidt-Hieber and Hausser, 2013). The stellate neurons in the layer II of MEC are thought to be the neural correlates of the so-called "grid cells" (Hafting et al., 2005), although a fraction of pyramidal neuron population in layer II may exhibit grid-like firing pattern (Tang et al., 2014). Stellate neurons in MEC also provide the main input to the dentate gyrus (Witter, 2007, van Strien et al., 2009).

In paper II, we describe the whole-cell voltage and current clamp recordings that we performed to determine the existence and impact an M-current in layer II MEC stellate neurons. Our voltage clamp recordings provided for the first time direct evidence for a voltage dependent outward current with slow kinetics and sensitivity to the Kv7/M channel blocker XE991, typical of M-current. In addition, we found that bath application of 20 mM TEA blocked a large fraction (65%) of the I_{tail} amplitude, consistent with previous reports in other cell types (Hadley et al., 2000, Hadley et al., 2003, Battefeld et al., 2014). These results provided an explanation for why Heys and Hasselmo (2012) failed to show a significant M-current in stellate neurons, since they included 20 mM TEA in the bath during their recordings.

Current clamp experiments combined with pharmacology demonstrated that blockade (XE991) or enhancement (retigabine) of Kv7/M channels affected the excitability of the stellate neurons. In particular, retigabine significantly reduced input resistance and firing frequency while XE991 prevented the effect of retigabine. In addition, blockade of Kv7/M channels increased the firing frequency in response to 1 s long depolarizing pulses and reduced late spike frequency adaptation.

The relevance of Kv7/M channels in layer II MEC stellate neurons has been debated in the last 10 years, since several reports presented conflicting results on this matter. On one side, contradictory results from the same lab first showed that blockade of Kv7/M channels reduced both resonance frequency and strength at subthreshold membrane potentials (Heys et al., 2010), similar to previously reported in CA1 pyramidal cells (Hu et al., 2002). Later, a different study from the same group concluded that the M-current is not expressed in stellate neurons (Heys and Hasselmo, 2012). On the other side, other studies suggested that Kv7/M channels influence the excitability, membrane potential oscillations and resonance in stellate

neurons, although the effect of Kv7/M channel blockers or enhancers seemed weak (Yoshida and Alonso, 2007, Boehlen et al., 2013). In our paper, we showed several lines of convergent evidence for the presence of M-current in stellate neurons and provided explanations for previous controversial findings, which can be fully explained by methodological factors.

Our results also suggested that the remaining TEA- and XE991- resistant tail current might be due to Kv7.5 channels, which are expressed in MEC and are more resistant to TEA and XE991 (Hadley et al., 2000, Schroeder et al., 2000, Huang and Trussell, 2011, Battefeld et al., 2014). Future research will be required to address several important points regarding: 1) the subcellular distribution, 2) subunit composition and 3) functions within specific subcellular compartments of Kv7/M channels in stellate neurons and principal neurons in other layers of entorhinal cortex.

4.3. Paper III

Past research revealed that hippocampal mossy fiber boutons (MFBs) have specialized electrical properties due to specific subcellular expression of different types of ion channels (Geiger and Jonas, 2000, Ruiz et al., 2003, Engel and Jonas, 2005, Alle et al., 2009b, Alle et al., 2011, Ruiz and Kullmann, 2012). We investigated the roles of Kv7/M channels in DGCs by somatic whole-cell patch-clamp recordings (Paper I) and others reported that cholinergic activation of DGCs inhibited Kv7/M channels via increased T-type Ca^{+2} channel activity (Martinello et al., 2015). In both studies, the observed Kv7/M channel-dependent functions are likely to be mediated by channels located in the proximal axon of DGCs. However, previous results from collaboration between German and Norwegian labs showed that MFB express significant M-currents that mediate M-resonance (Alle et al., 2009a, Murphy et al., 2010, Storm et al., 2010, Alle et al., 2012), which seems to be absent in somatic recordings of DGCs (Krueppel et al., 2011). The latter difference between somatic and MFB recordings may be suggestive of proximal-distal axonal differences in DGCs. Meanwhile cholinergic M1 receptors were detected along mossy fibers (Martinello et al., 2015) as well as Kv7/M channels (Cooper et al., 2001). In this study we used presynaptic whole-bouton recordings to investigate whether Kv7/M channels in MFBs are also modulated by cholinergic receptors.

In unequivocally identified MFBs, we injected trains of mock EPSC waveforms at a frequency that produced no summation of these subthreshold signals. However, when Kv7/M channels were blocked by bath application of XE991, summation of the EPSP waveforms clearly increased, suggesting that activation of Kv7/M channels in MFBs attenuates subthreshold depolarizing signals. In addition, after each mock EPSP train we observed an mAHP-like hyperpolarization that was abolished by XE991, similar to the Kv7/M channel-dependent mAHP seen in CA1 pyramidal neurons (Gu et al., 2005). Surprisingly, bath application of nonselective acetylcholine muscarinic receptor agonists muscarine (10 μM) or oxo-M (10 μM) failed to increase the summation ratio or block the Kv7/M-dependent mAHP, indicating that cholinergic receptors may not inhibit Kv7/M channels in MFBs.

Our results showed that Kv7/M channels in MFBs control summation of subthreshold signals at more negative potentials than showed in CA1 pyramidal neurons. Similar results were reported in the calyx of Held (Huang and Trussell, 2011), where Kv7.5 channels have more negative voltage dependence (V_{half} ~ -54 mV) than reported in other glutamatergic axons (Vhalf ~ -30 mV) expressing Kv7.2-7.3 channels (Battefeld et al., 2014). Previous voltage

clamp experiments showed a V_{half} ~ -50 mV for I_M in MFB (Alle et al., 2012), which is consistent with results from the calyx of Held. Thus, Kv7/M channels in MFBs control subthreshold depolarizations at membrane potential values consistent with the presence of Kv7.5 channels.

A key point for discussion is whether Kv7/M channels control the resting membrane potential of MFBs as they do in the calyx of Held. Previous slice work showed that the resting membrane potential of mossy fibers is close to -90 mV, too negative for any Kv7/M channel activity (Alle and Geiger, 2006). However, recent *in vivo* patch-clamp recordings indicated that the resting membrane potential of DGCs may be around -70 mV (Anderson and Strowbridge, 2014, Pernia-Andrade and Jonas, 2014) which is in the range of activation for Kv7.5 channels (Huang and Trussell, 2011). Nevertheless, assuming a resting membrane potential of about -80 mV for DGCs, barrages of *in vivo* synaptic potentials (Pernia-Andrade and Jonas, 2014) or oscillations (Munoz et al., 1990, Anderson and Strowbridge, 2014) are likely to propagate along the mossy fibers (Alle and Geiger, 2006), potentially activating Kv7/M channels at membrane potential values consistent with our experimental results.

In our study, we failed to inhibit Kv7/M-dependent features with acetylcholine muscarinic receptor agonists. Neither muscarine nor oxo-M had any effect on mock EPSP summation and mAHP. This is a surprising finding, since a hallmark of Kv7/M channels is their modulation by muscarinic receptors (hence the name M-channels or M-current). In addition, Martinello et al. (2015) showed that oxo-M irreversibly inhibited Kv7/M channels via basal increase of T-type Ca^{+2} channel activity. An interesting possibility is that Kv7/M channels and their regulation in MFBs may differ from those at proximal locations along the mossy fibers, as reported for Na^{+} channels (Engel and Jonas, 2005). For example, Li et al. (2007) found that T-type Ca^{+2} currents are absent in MFBs, which may support the above mentioned hypothesis. If so, the lack of Kv7/M inhibition reported in Paper III may be due to the absence of T-type Ca^{+2} channels in MFB, which represents subcellular differences within DGCs. In any case, more research is needed to further test our conclusions and test other possibilities such as wash-out or buffering of intracellular signals needed for muscarinic inhibition of Kv7/M channels, and to explore possible Kv7/M channel modulation by membrane lipids at mossy fiber synapses (Delmas and Brown, 2005, Carta et al., 2014).

4.4. Paper IV

It is widely accepted that the hippocampus is not a homogeneous structure. Multiple discrete domains of gene expression overlap with gradients in functional connectivity and neuronal activity (Moser and Moser, 1998, Fanselow and Dong, 2010, Strange et al., 2014, Kesner and Rolls, 2015) along the longitudinal axis of the hippocampus (dorsoventral or septotemporal axis). In the past few years, an increasing body of evidence from CA1 pyramidal neurons suggested that the intrinsic properties of hippocampal neurons may also vary along its septotemporal axis (Dougherty et al., 2012, Marcelin et al., 2012a, Marcelin et al., 2012b, Dougherty et al., 2013, Honigsperger et al., 2015, Kim and Johnston, 2015).

In paper IV, we tested this idea in the granule cells of the dentate gyrus. We analyzed the sAHP and *f*/*I* responses of DGCs at different locations along the hippocampal axis *in vitro*. Our current clamp recordings suggested that the sAHP and related spike frequency adaptation increase towards dorsal DG both in rats (young and adult) and mice. These results were confirmed in different slice preparations and using different intracellular recording solutions. In addition, voltage clamp recordings showed larger amplitude and slower kinetics of I_{sAHP} in dorsal DGCs. Morphological reconstructions of biocytin-filled DGCs revealed that total dendritic or axonal lengths cannot explain the observed dorsoventral differences.

Our study represents the first direct, *in vitro* evidence for dorsoventral differences in the excitability of DGCs. In the dentate gyrus, dorsoventral differences in DG function, maturation and neurogenesis were reported previously (Piatti et al., 2006, Leutgeb et al., 2007, Rahimi and Claiborne, 2007, Jinno, 2011, Piatti et al., 2011, Kesner, 2013, Kheirbek et al., 2013, Lyttle et al., 2013, Tannenholz et al., 2014, Wu and Hen, 2014), suggesting that the hippocampus in general and the dentate gyrus in particular may perform different computations along the septotemporal axis (Yartsev, 2010). In order to conclusively validate the functional relevance of the results presented in paper IV, it is necessary to perform intracellular recordings of DGCs along the septotemporal axis *in vivo*. Successful intracellular recordings of DGC activity *in vivo* are restricted to the dorsal DG (Munoz et al., 1990, Henze et al., 2002, Anderson and Strowbridge, 2014, Pernia-Andrade and Jonas, 2014), as the dorsal DG is more accessible for pipette penetration. However, *in vivo* whole-cell patch clamp recordings are now regularly done in many labs, so it is expected that this question will be addressed in the coming years.

The dentate gyrus is a key structure that limits limits downstream spread of activity from the hippocampus during epileptic seizures (Heinemann et al., 1992, Lothman et al., 1992, Hsu, 2007, Krook-Magnuson et al., 2015). In addition, DGCs can undergo intrinsic plasticity, i.e. changes in their intrinsic biophysical properties, after epileptic seizures in animal models as well as in humans (Young et al., 2009, Wolfart and Laker, 2015). Mossy fiber sprouting is commonly observed in epileptic dentate gyrus, resulting in the formation of aberrant recurrent synapses between DGCs with enriched postsynaptic kainate receptors which affect the cellular excitability (Epsztein et al., 2010, Artinian et al., 2011, Artinian et al., 2015, Crepel and Mulle, 2015). In pyramidal neurons from both hippocampal CA1 and CA3 areas, kainate receptors can inhibit the sAHP via metabotropic actions (Melyan et al., 2002, Fisahn et al., 2005, Chamberlain et al., 2013). Therefore, an interesting possibility to be tested in separate studies is the interaction between the sAHP and kainate receptors of DGCs under normal and epileptic conditions along the septotemporal axis of the hippocampus.

4.5. Paper V

The identity of the channels underlying the sAHP is a major question in current neuroscience (Andrade et al., 2012). Recently, a study suggested that the intermediate-conductance Ca^{+2}-gated K^+ channels (SK4, IK1, KCa3.1) are a critical determinant of the sAHP in CA1 pyramidal neurons (King et al., 2015). In this study, we collaborated with two other laboratories (John P. Adelman´s group in Portland and Pankaj Sah´s group in Brisbane) to test the validity of the above mentioned hypothesis under a variety of experimental conditions.

Results from Adelman´s group showed that either extracellular or intracellular application of TRAM-34, a specific blocker of IK1 channels, failed to block or reduce the I_{sAHP} recorded in CA1 pyramidal neurons at room temperature. In contrast, TRAM-34 abolished the current generated by IK1 channels expressed in HEK293 cells at room temperature. Furthermore, voltage clamp recordings in wild-type and IK1 null mice revealed a similar and prominent I_{sAHP} that was largely eliminated by carbachol application. Pankaj Sah´s group found no effect of bath-applied TRAM-34 on voltage and current clamp recordings of the I_{sAHP} or the sAHP in basolateral amygdala (BLA) pyramidal neurons at 32 ºC. Similarly, the excitability of these neurons was not affected by the IK1 channel blocker. Finally, our lab performed current clamp recordings of both the sAHP and *f-I* responses in CA1 pyramidal neurons from slices and organotypic cultures, at 34 ºC. Whereas the other two laboratories used $KMeSO_4$-based intracellular recording solution, we used a different K^+-gluconate-based solution. Despite our different recording conditions, the results showed a similar lack of effect of bath-applied TRAM-34 on the sAHP amplitude and related excitability in CA1 pyramidal neurons. Importantly, all the three laboratories showed that the TRAM-34 insensitive sAHP or I_{sAHP} was blocked after carbachol or noradrenaline application at the end of the experiments. In summary, results from three independent laboratories under a variety of experimental conditions failed to replicate the effect of TRAM-34 on the sAHP or its underlying current (I_{sAHP}), converging to the same conclusion: IK1 channels do not underlie the sAHP in pyramidal neurons. Therefore, the mechanisms underlying the sAHP remain unknown (Andrade et al., 2012).

The results presented in paper V are in clear discrepancy with those reported in King et al. (2015). IK1 expression is mainly found in peripheral tissue such as blood and epithelial cells, or myenteric neurons (Ishii et al., 1997, Joiner et al., 1997, Begenisich et al., 2004, Nguyen et al., 2007, Wulff et al., 2007, Pedarzani and Stocker, 2008). However, it is under debate

whether IK1 channels are expressed in central neurons and more specifically, in hippocampal neurons. Recent studies from the same group found suggested that IK1 protein expression is widely distributed in the brain (Turner et al., 2015), suggesting potential physiological roles of these channels in central neurons (Engbers et al., 2012, King et al., 2015). In our study, three independent laboratories were unable to detect any effect of TRAM-34 in both hippocampal CA1 and BLA pyramidal neurons under a variety of conditions, in contrast to the clear effect reported by King et al. (2015). Intriguingly, King et al. (2015) only showed time-plots of control experiments without drug application and did not report any time-plot of the drug effect. In absence of time-plots of drug effects, run-down of the sAHP cannot be excluded, especially when the intracellular pipette solution is KMeSO4-based (Kaczorowski et al., 2007).

Methodological aspects may explain the differences between these studies, since King et al. (2015) used synaptic stimulation in the *stratum radiatum* and electrode solution exchange, which we did not use in our study. As shown in our Fig. 3, we bath-applied TRAM-34 for at least 30 minutes prior recordings. If TRAM-34 blocks the sAHP, recordings after 30 minutes of drug application should show reduced or absent sAHP. However, this was not the case.

In conclusion, we could not replicate the results from King et al. (2015), suggesting that IK1 channels do not underlie the sAHP in CA1 or BLA pyramidal neurons. Our data showed that time-plots of the experimental results are of major importance in this issue.

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