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Distribution of M-channel subunits KCNQ2 and KCNQ3 in rat hippocampus

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Abstract

Neuronal M-channels are low threshold, slowly activating and non-inactivating, voltage dependent K^+ channels that play a crucial role in controlling neuronal excitability. The native M-channel is composed of heteromeric or homomeric assemblies of subunits belonging to the Kv7/KCNQ family, with KCNQ2/3 heteromers being the most abundant form. KCNQ2 and KCNQ3 subunits have been found to be expressed in various neurons in the central and peripheral nervous system of rodents and humans. Previous evidence shows preferential localization of both subunits to axon initial segments, somata and nodes of Ranvier. In this work, we show the distribution and co-localization of KCNQ2 and KCNQ3 subunits throughout the hippocampal formation, via immunostaining experiments on unfixed rat brain slices and confocal microscopy. We find intense localization and colocalization to the axonal initial segment in several regions of the hippocampus, as well as staining for non-neuronal cells in the area of the lateral ventricle. We did not observe colocalization of KCNQ2 or KCNQ3 with the presynaptic protein, synaptophysin.

Keywords

M channel; hippocampus; confocal microscopy; immunostaining; neurophysiology

1. Introduction

M-type K^+ channels were first described in sympathetic ganglion neurons (Brown and Adams, 1980; Constanti and Brown, 1981) and its name relates to their inhibition by stimulation of muscarinic acetylcholine receptors (Delmas and Brown, 2005). The functional channel is composed of heteromeric or homomeric assemblies of subunits belonging to the Kv7/KCNQ family. In total, five members of this family are known (KCNQ1–5). KCNQ1-containing channels are mostly found in the heart and epithelia and KCNQ4 homomers predominate in the inner ear and auditory cortex, whereas KCNQ2-, KCNQ3- and KCNQ5-containing channels are expressed throughout the nervous system, with KCNQ2/3 heteromers being the most abundant form (Brown and Yu, 2000; Robbins,

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2001; Roche et al., 2002; Schroeder et al., 2000; Shah et al., 2002; Wang et al., 1998; Wickenden et al., 2001). These findings are supported by experiments showing that KCNQ2 and KCNQ3 subunits can be co-immunoprecipitated from brain lysates (Cooper et al., 2000), as well as electrophysiological measurements showing that current density is reduced when either KCNQ2 or KCNQ3 subunits are mutated in the pore, thus acting as a dominant negative for the tetrameric channel (Schroeder et al., 1998). Moreover, numerous studies have showed that mutations in KCNQ2 or KCNQ3 genes underlie the epileptic syndrome called benign neonatal familial convulsions (Biervert et al., 1998; Jentsch, 2000; Singh et al., 1998), underscoring the importance of these subunits to neuronal discharge in the brain.

Functionally, M-current is a slowly activating and non-inactivating voltage dependent K^+ current with a threshold voltage for activation near −60 mV. Hence, it plays a crucial role in controlling neuronal excitability, by influencing the resting membrane potential, spike frequency adaptation and burst suppression (Gu et al., 2005; Lawrence et al., 2006; Yue and Yaari, 2004; Zaika et al., 2006). M channel control of neuronal discharge is thought to localize to the axon initial segment (AIS) (Pan et al., 2006) and is lost when the targeting of KCNQ channels to this compartment is disturbed (Shah et al., 2008). Apart from this functional role, a plethora of studies have investigated the pathways modulating KCNQ/M-channels (for reviews, see Delmas and Brown, 2005; Hernandez et al., 2008).

KCNQ2 and KCNQ3 subunits have been found to be expressed in various neurons in the central and peripheral nervous system. Cooper and colleagues found extensive KCNQ2 immunoreactivity throughout the mouse brain, with many regions presenting the most intense labeling at neuronal somata (Cooper et al., 2001). In the hippocampal formation, the most intense staining was found in the mossy fibers of the dentate gyrus and *stratum lucidum* of CA3. Moderate labeling was observed at cell somata throughout the principal cell layers (pyramidal cell layer of CA1 and CA3 and granule cell layer of dentate gyrus), and these were revealed to be mostly parvalbumin-positive interneurons. However, in sections of human brain, somatodendritic staining for both KCNQ2 and KCNQ3 was reported to be present on principal cells in different regions of the hippocampal formation (hilus, CA3 and subiculus), with a subpopulation expressing KCNQ2, but not KCNQ3, also in the neuropil (Cooper et al., 2000).

Strong evidence for localization of KCNQ2 subunits to the AIS and nodes of Ranvier has been found in a variety of central and peripheral neurons, with a subset co-expressing KCNQ3 (Devaux et al., 2004). However, Pan et al., using several antibodies against KCNQ3, showed that both KCNQ2 and KCNQ3 subunits concentrated at the AIS and nodes of Ranvier in various neurons in the mouse, including the CA1 region of the hippocampus (Pan et al., 2006). Localization to the AIS has been shown to require the presence of ankyrin-G, the same protein responsible for the localization of voltage-gated Na^+ channels to this region. Moreover, selective disruption of KCNQ targeting to the AIS in CA1 pyramidal neurons significantly depolarized the resting membrane potential and increased evoked, as well as spontaneous, action-potential firing (Shah et al., 2008).

In this work, we show the distribution and co-localization of KCNQ2 and KCNQ3 subunits throughout the rat hippocampal formation via immunostaining experiments on unfixed rat brain slices and confocal microscopy. Furthermore, we used synaptophysin as a presynaptic marker, in order to investigate a possible localization of the channel at pre-synaptic terminals. We find intense localization of the M-channel subunits to the AIS in several regions of the hippocampal formation, as well as staining for other structures in this region of the brain. However, we did not see co-localization of KCNQ2 or KCNQ3 with the pre-synaptic protein, synaptophysin.

2. Materials and Methods

Antibodies

Rabbit anti-KCNQ2 antibodies were raised against the an amino-terminal epitope of human KCNQ2 as previously described (Roche et al., 2002) and purified by affinity chromatography. Guinea-pig anti-KCNQ3 antibodies raised against the amino-terminal of human KCNQ3 were given to us by Ed Cooper (Baylor College of Medicine, Houston, TX) (Devaux et al., 2004; Pan et al., 2006). The mouse anti-synaptophysin antibody was purchased from Sigma-Aldrich (cat #S5768), and FITC-conjugated anti-mouse or anti-rabbit antibodies and rhodamine red-conjugated anti-rabbit or anti-guinea pig antibodies were obtained from Jackson Immunoresearch. The nucleic-acid staining dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Invitrogen.

Immunohistochemistry

Unfixed brain slices were prepared from two to three week-old Sprague-Dawley rats that were euthanized in accordance with the rules of IACUC at UTHSCSA, and decapitated. A total of six rats were used of either gender. Brains were briefly rinsed in 0.9% saline solution and subsequently placed on dry ice for 10–15 minutes until completely frozen. All further steps were carried out at −20°C. Whole brains were mounted on a cryostat holder using OCT mounting media and ten to twenty slices containing the dorsal part of the hippocampus region were collected. For orientation, “The Rat Brain in Stereotaxic Coordinates” (*Paxino G., Watson C. 1998, 4th ed. Academic Press ISBN-13: 978-0125476171*) atlas was used. Starting with the frontal lobe, the brain was sectioned until the hippocampal region corresponding to plate 30 was reached and the cut tissue was discarded. At this point, 16-μm thick cryostat sections were cut and thaw-mounted onto pre-cooled Superfrost Plus slides (Fischer Scientific). The margins of the slides were previously labeled with the “Pap Pen” hydrophobic slide marker to allow solution to remain on the slide during antibody incubation and washing steps.

Slices were incubated for 30 min in blocking buffer containing 5% donkey serum and 0.1% saponin in PBS and incubated overnight in primary antibody at dilutions of 1:500 for anti-KCNQ2 and anti-synaptophysin and 1:200 for anti-KCNQ3. The next day, slices were carefully washed 3x with phosphate-buffered saline (PBS), incubated with the secondary antibody (1:300) (plus DAPI, when indicated, at 1:200) for one hour and finally washed again 3x with PBS. Coverslips were mounted onto slides using Vectashield media (Vector Labs) and sealed with nail polish at room temperature. Imaging was performed either immediately after complete drying or the next day (in this case, the slides were kept at 4°C until use).

Imaging

Imaging was performed on a Nikon Swept-Field Confocal (SFC) located in the confocal microscopy core facility in the Department of Physiology. This rig consists of a TE2000E2 inverted motorized microscope system and the Prairie Technologies Livescan SFC capable of slit-scan or pinhole-mode, coupled to a CoolSnap HQ2 camera. For excitation of FTIC and rhodamine red, we used the 488 nm line of an argon laser or a 563 nm solid-state laser, respectively. All images show snap-shots of 3D reconstructions of Z-stacks taken in pinhole mode using a 40X objective (400X total magnification) with 500 ms exposure time and 50% laser power. The range of the Z-dimension was set manually.

3. Results

3.1 Introduction to Results

The hippocampal formation is part of the limbic system and is mainly responsible for long term memory and spatial navigation. Its structure consists of three distinct formations: the dentate gyrus (DG), hippocampus proper (or *cornu ammonis*, CA, subdivided in CA1, CA2 and CA3) and the subiculum. In brief, the hippocampus consists of a principally uni-directional network of glutamatergic excitatory neurons, with inputs from the entorhinal cortex (EC) along the perforant pathway to granule cells located in the DG. These send "mossy" fibers to neurons in CA3 and from there, monosynaptic innervations are made to CA1 neurons via Schaffer collaterals, forming the so called tri-synaptic circuit of glutamatergic principal cells (Graumann and Sasse, 2005). CA1 pyramidal cells send projections to neurons located in subiculum and from there, in turn, back to the EC, completing the circuit. In addition to the principal glutamatergic neurons, numerous interneurons influence network activity and output of the pyramidal cells (Somogyi and Klausberger, 2005). To determine the expression profile of the M-channel subunits KCNQ2 and KCNQ3 in different regions of the hippocampus, we performed immunostaining experiments on unfixed rat brain slices. Since KCNQ/M-channels have been shown to be poorly expressed in neonatal rat hippocampus, with markedly increased expression in the subsequent three weeks (Safiulina et al., 2008), we used two to three week-old rats for our studies. Brains slices were co-stained for KCNQ2 or KCNQ3 and synaptophysin as a synaptic marker. Evidence of their distribution in different regions of the hippocampus was collected via confocal microscopy. Fig. 1 shows a 40X image of a typical hippocampal slice that was fixed and stained with hematoxylin and eosin (H&E) to clearly show the pertinent cell layers. Indicated on this image are the approximate positions of each 400X image shown below. All of these images represent 3D reconstructions of Z-stacks. Occasionally, the nucleic-acid staining dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was used in order to label cell nuclei.

3.2 Expression of KCNQ2 and KCNQ3 in the CA region of the hippocampus

The hippocampus proper is subdivided in CA1, CA2 and CA3 regions, with CA2 controversial whether it can be classified as an identifiable region in the rat (Amaral and Witter, 1995). Thus, we restricted our investigations to CA1 and CA3. These regions contain principal excitatory neurons (pyramidal neurons) with their cell bodies densely packed within the pyramidal cell layer (*stratum pyramidale*, Sp). Pyramidal cells have their basal and apical dendrites extending in *stratum oriens* (So) and *stratum radiatum* (Sr), respectively. The axons of the pyramidal neurons pass *stratum oriens* and travel further towards the subiculum, through the fiber-containing alveus (white matter of the hippocampus). Fig. 2 shows Z-stack reconstruction images of the CA1 region stained for KCNQ2 (Fig. 2A) or KCNQ3 (Fig. 2B), and synaptophysin. Synaptophysin is an integral membrane glycoprotein present in presynaptic vesicles of all central neurons, thus visualizing synaptic formations. Via synaptophysin staining, we could easily identify *stratum pyramidale* with its cell bodies. In addition, DAPI staining (blue) in Fig. 2B confirms the localization of pyramidal cell bodies in this region. Both KCNQ2 and KCNQ3 staining exhibited a tubular shape with localization to the proximal portion of the presumed axon initial segments (AIS) of cells with somata located in the pyramidal cell layer. These patterns indicate expression of the channel subunits to the AIS of the neurons. Localization of KCNQ2 and KCNQ3 to the AIS in CA1 hippocampal neurons has previously been described (Pan et al., 2006), and is confirmed here. The AIS is the area of the axon near the soma that contains a high density of voltage-gated Na^+ channels, which are responsible for the initial depolarization that leads to the initiation of the action potential (Rasband, 2010). Thus, it is not surprising to find expression of M-channel subunits in this area, given their

widely-accepted role in controlling neuronal excitability. Moreover, we did not see any co-localization of either KCNQ2 or KCNQ3 with synaptophysin in this region, consistent with the lack of synapses at the AIS. Therefore, we presumed that this structure represents the AIS, without further control experiments. In the CA3 region, similar organizational patterns of pyramidal neurons with their dendrites and axons are present. In addition, specific for CA3 is a narrow, non-cellular layer located just above the *stratum pyramidale*. This layer is called *stratum lucidum* (S_l) and contains mossy fibers originating from the DG which synapse here on the dendrites of the pyramidal neurons (Amaral and Witter, 1995).

Fig. 3 shows the expression patterns of KCNQ2 and KCNQ3 subunits in CA3. Both KCNQ2 (Fig. 3A) and KCNQ3 (Fig. 3B) staining is shown in red, whereas green again indicates synaptophysin expression. We notice that DAPI staining (blue) in Fig. 3B shows less cell nuclei staining than in CA1 (Fig. 2B), which corresponds to the pyramidal cells in this region being less densely arranged then in CA1 (Graumann and Sasse, 2005). Consistent with this also, less KCNQ staining is present. Both anti-KCNQ2 and anti-KCNQ3 antibodies label structures that can be identified as AIS of the pyramidal cells (arrows), extending from *stratum pyramidale* (Sp) to *stratus oriens* (So). However, in contrast to the abundant co-localization of KCNQ2 and KCNQ3 in CA1, the expression of KCNQ3 in CA3 is relatively modest (see also Fig. 5 below). In addition, KCNQ2, but not KCNQ3, staining shows diffuse expression within the pyramidal cell layer (indicated by the asterisk in Fig. 3A), suggesting the presence of this subunit at cell somata. Finally, in both CA1 and CA3 regions, colocalization of the M-channel subunits with synaptophysin was not observed.

3.3 Expression of KCNQ2 and KCNQ3 in dentate gyrus of the hippocampus

The dentate gyrus region of the hippocampus comprises three cell layers (starting from outside): molecular, granule and polymorphic cell layers. The principal cell types of the dentate gyrus are the granule cells that are densely packed into the thusly-named granule cell layer (*stratum granulare*, Sg). From here, granule neurons send their dendritic extensions to *stratum moleculare* (Smo), whereas their unmyelinated axons, “mossy fibers,” cross through the polymorphic cell layer (*stratum multiforme*, Sm), where they give rise to multiple thinner collaterals, before entering the CA3 region (Amaral and Witter, 1995). Fig. 4 shows Z-stack reconstruction images of dentate gyrus in brain slices stained for KCNQ2 (Fig. 4A) or KCNQ3 (Fig. 4B) and synaptophysin. In this region of the hippocampus, different expression patterns of KCNQ2 and KCNQ3 were observed. There is an intense labeling of KCNQ2 at the level of the AIS, extending from *stratum granulare* (Sg) to *stratum multiforme* (Sm). This layer is intensely labeled with anti-synaptophysin antibody (Fig. 4A, green), but no colocalization with the M-channel subunit could be observed. KCNQ3 was also found to be expressed at the level of the AIS of granule cells, as indicated by the arrows in Fig. 4B. However, in addition, we observed diffuse expression of KCNQ3 subunits at the cell somata of a few cells, as indicated by the asterisks in Fig. 4B. These could be either principal excitatory neurons from the granule cell layer, or other types of cells, such as basket cells or GABAergic interneurons, as they are known to be present within, or just slightly below, the granule cell layer (Amaral and Witter, 1995). Also, it has been previously shown that parvalbumin-positive interneurons are labeled by anti-KCNQ2 antibodies (Cooper et al., 2001).

3.4 Colocalization of KCNQ2 and KCNQ3 in all regions of the hippocampus

After investigating the expression of KCNQ2 and KCNQ3 individually throughout the hippocampus, we then wanted to know if these two subunits of the classic M-channel colocalize within the same cells or sub-cellular domains. Therefore, we performed simultaneous immunostaining using antibodies against both KCNQ2 and KCNQ3. Again, evidence was collected via confocal microscopy and is again presented as image

reconstructions of 3D Z-stacks. Fig. 5 shows colocalization of both M-channel subunits in the different regions of the rat hippocampus. In the CA1 region (Fig. 5A), both subunits were seen to be present at the AIS of the pyramidal cells and colocalization is shown in the merged image. Similarly, in the CA3 region (Fig. 5B), many AIS structures were labeled for both KCNQ2 and KCNQ3. Consistent with Fig. 2, less cell nuclei (stained by DAPI) and by implication, less KCNQ3-stained AIS, were present. However, when images are merged, notable yellow regions reveal colocalization of both subunits, indicating the presence of KCNQ2/3 heteromers, although there was significant immunolabeling of KCNQ2 only. The dentate gyrus with *stratum granulare* (Sg), *stratum moleculare* (Smo) and *stratum multiforme* (Sm) is shown in Fig. 5C. Again, DAPI staining was used in order to visualize cell nuclei in *stratum granulare*. Considerable staining for KCNQ2 was obvious in the AIS (green) but much less for KCNQ3 (red), with modest areas of co-localization of the two subunits indicated in yellow when the two images are merged. In summary, abundant KCNQ2/3 heteromers in the AIS of the CA1 region is indicated; and a mixture of KCNQ2/3 heteromers and KCNQ2 homomers is likely in CA3 and dentate gyrus.

3.5 Expression of KCNQ3, but not KCNQ2, in cells in the lateral ventricle region

Interestingly, we found very intense staining for KCNQ3, but not KCNQ2, subunits in the region of the lateral ventricle (Fig. 6). Figs. 6A and 6B show staining for KCNQ2 or KCNQ3, respectively, together with synaptophysin. The lack of synaptophysin staining reveals other than neuronal tissue and DAPI staining confirms the presence of multiple cells within the ventricle. These cells are most probably either ependymal cells, or cells that are integral to the blood vessels that build the choroid plexus. Intense staining for KCNQ3 (Fig. 6B), but no staining for KCNQ2 (Fig. 6A) could be detected in this area. The shape and arrangement of the labeling indicate localization of the subunit at the cell membrane. However, since no neurons are found in the ventricle region, the cells expressing KCNQ3 subunits here must be non-neuronal (see *Discussion*). Similar results were observed when co-staining for both KCNQ2 (green) and KCNQ3 (red) was conducted (Fig. 6C). Again, no green signal was detected, confirming that KCNQ2 subunits are not expressed in this region. However, intense red staining indicates the clear presence of KCNQ3 subunits. The cellular attribution of these findings remains uncertain, and needs further investigation for a clear statement. Unfortunately, due to complete consumption of the specific anti-KCNQ3 antibody used in this study, we were not able to explore this aspect in more detail.

3.6. Summary of results

Table 1 summarizes the results of this study regarding the localization of KCNQ2 and KCNQ3 in the four regions of the rat hippocampus studied here. We found robust expression of KCNQ2 in the dentate gyrus, CA1 and CA3, but not the lateral ventricle. For KCNQ3, we found robust expression in CA1, with noticeable but reduced expression in the dentate gyrus and CA3. We found strong expression of KCNQ3, but not KCNQ2, in cells of the lateral ventricle. For dentate gyrus, CA1 and CA3, the expression pattern was generally consistent with previous results in the mouse and human, including the dominant perisomatic/AIS expression pattern. For the lateral ventricle, the finding of M-type channels is novel, with interesting implications, as discussed below.

4. Discussion

4.1 Overview of Discussion

A number of studies have shown expression of KCNQ2 and KCNQ3 subunits at the AIS and nodes of Ranvier in the peripheral and central nervous systems (Devaux et al., 2004; Pan et al., 2006; Rasmussen et al., 2007). This is supported by our data showing the presence of these M-channel subunits at the AIS of pyramidal and granule cells throughout the

hippocampus. Such localization is in accordance with their function in controlling neuronal excitability, since AIS and the nodes of Ranvier are the sites where synaptic inputs are integrated and action potentials are generated and propagated, respectively. In the dentate gyrus, KCNQ3, but not KCNQ2, staining was diffusely present at the cell somata of a few neurons in the granule cell layer, in addition to the AIS. In order to conclude whether these are principal excitatory cells, or rather inhibitory interneurons, further co-labeling experiments (*e.g*. using parvalbumin antibodies) would be necessary. Due to the limited availability of the effective and specific KCNQ2 and KCNQ3 antibodies used in this study, we were not able to conduct further experiments along these lines. In the CA3 region, in contrast, the anti-KCNQ2, but not anti-KCNQ3, antibodies resulted in diffuse staining for channels at the somata of the pyramidal neurons. Thus, these findings indicate that other compositions besides KCNQ2/3 heteromers are present and should be functional in hippocampal neurons. Although many labs report KCNQ3 homomers to generate only very small currents in heterologous systems, they may function better in native tissue, and such homomeric channels may thus play an important role in the brain. Alternatively, KCNQ3 subunits in neurons without KCNQ2 subunits may express in heteromeric combination with KCNQ5 subunits, since KCNQ3/5 heteromers have been reported to express well in different heterologous systems (Lerche et al., 2000; Wickenden et al., 2001). Unfortunately, no selective antibody against KCNQ5 was available for our experiments to test this hypothesis.

4.2 Technical Considerations

A number of labs have reported the identification of endogenous KCNQ channels in native neurons to be challenging. We initially attempted to immunostain hippocampal slices fixed with 4% paraformaldehyde, but did not obtain staining that clearly differentiated from non-specific controls. Likewise, we performed immunostaining experiments, using these same antibodies, on fixed, cultured sympathetic or hippocampal neurons, but again did not observe significant specific labeling. These results are in accord with those of the Cooper lab, who most recently performed their immunostaining on unfixed nerve fibers or cryostat-sectioned brain slices (Devaux et al., 2004; Pan et al., 2006). Like that group, we also find KCNQ2 and KCNQ3 to strongly localize to the AIS, a location found by a recent paper to be fully consistent with current-clamp recordings and nerve conductance modeling (Shah et al., 2008). As for the Cooper lab (E. Cooper, *personal communication*), we find that fixing brain slices to preclude specific immunostaining by a variety of antibodies tested (data not shown), although we did not find any need for “antigen retrieval,” *e.g*., by incubation of slices in a microwave oven. These technical challenges are in stark contrast with our experience with cloned KCNQ1–4 channels heterologously-expressed in tissue-culture cells, which we find to be easily and reproducibly labeled by a variety of antibodies, with fixation presenting no problem. We speculate that the endogenous channels may be closely associated with cytoskeletal or other proteins, such an ankryn-G (Pan et al., 2006), that may block access of antibodies to intracellular antigens when the cells are fixed.

4.3 Implications for neurotransmitter release

With respect to the presynaptic localization of M-channels and its consequential role in tuning neurotransmitter release, the literature is contradictory. This issue has been repeatedly investigated in functional studies using different specific activators and blockers of M-channels. Thus, activation of M-channels by flupirtine or retigabine resulted in an inhibition of depolarisation-induced release of various transmitters, including noradrenaline, aspartate and GABA, from rat hippocampal synaptosomes (Martire et al., 2004), and of dopamine from rat striatal synaptosomes (Martire et al., 2007). The M-channel blocker, linopirdine, was reported to increase the frequency of miniature EPSCs in hippocampal neurons, whereas the novel opener, NH6, decreased it, consistent with a pre-synaptic site of action

(Peretz et al., 2007). In contrast, retigabine was found to enhance inhibitory postsynaptic currents (IPSCs), but not excitatory postsynaptic currents (EPSCs) in the cortex, with the former action appearing to arise post-synaptically (Otto et al., 2002).

Similar conflicting results are reported in the peripheral nervous system. Thus, in sympathetic neurons, linopirdine indeed triggered noradrenaline release, but this effect was abolished by tetrodotoxin and was thus suggested as not mediated by a presynaptic mechanism (Kristufek et al., 1999; Lechner et al., 2003). Likewise, inhibition of M currents via M_1 receptors triggered noradrenaline release in a similar tetrodotoxin-sensitive manner, whereas retigabine was reported not to affect spontaneous or electrically evoked release of tritiated noradrenaline (Lechner et al., 2003). On the other hand, retigabine and the higher-potency linopirdine derivative, XE991, were found to enhance, or reduce, the release of noradrenaline from sympathetic neurons, respectively, as determined by microamperometry (Hernandez et al., 2008). Lastly, M current has recently been shown to regulate the functional release of NE from sympathetic neurons onto cardiomyocytes, observed as changes in the beating frequency of the latter in sympathetic neuron/cardiomyocyte co-cultures, although it is not known if this action is solely due to an increase in action-potential firing (Zaika et al., 2011).

Finally, KCNQ channels have been shown as modulated by the pre-synaptic protein, syntaxin (Regev et al., 2009), suggesting a novel role for a pre-synaptic localization for these channels. In this work, we did not observe obvious co-localization of KCNQ2 or KCNQ3 subunits with synaptophysin. Based on these negative results, we therefore cannot provide more evidence supporting a role for M channels in the tuning of neurotransmitter release by regulation of the pre-synaptic resting potential or input resistance in the hippocampus. Interestingly, such a role seems clear for KCNQ5 channels in the calyx of Held (Huang and Trussell, 2011). Thus, although we cannot exclude pre-synaptic localization of M-type channels in the hippocampal formation, we do conclude that localization of KCNQ2 and KCNQ3 to the AIS, where action potentials are widely thought to be generated, is at least the predominant role for these channels in this part of the brain.

4.4. KCNQ channels in the lateral ventricle

Intriguingly, we found strong expression of KCNQ3, but not KCNQ2, in cells contained in the choroid plexus of the lateral ventricle. The shape of the staining indicates localization at the cell membrane. However, since no neuronal cells are present in the choroid plexus of the lateral ventricles, these data indicate that KCNQ3 subunits are expressed also in non-neuronal cells in the brain. The imaging is consistent with these cells either being of the ependymal layer, which contains ciliated epithelial cells whose role is to secrete and circulate cerebral spinal fluid and the subventricular zone which has been suggested to be source of multi-potent adult neural stem cells (Gage, 2000), or cells underlying blood vessels in this area. The first possibility is particularly interesting, given the phylogenetic-lineage relationship between the ependymal layer in the forebrain and the sensory epithelia of the cochlea (Wei et al., 2008). Both tissues have embryonic origin from the neural ectoderm layer, and share certain proteins expressed in the organ of Corti and spiral ganglia neurons (Stankovic et al., 2004; Zecevic, 2004). Among M-type channels, the inner ear is most noted for expression of KCNQ4, mutations in which cause inherited syndromes of hearing loss due to the death of hair cells and of spiral ganglia neurons (Coucke et al., 1999; Kubisch et al., 1999; Lv et al., 2010). KCNQ4 expression has also been shown to extend to central auditory tracts (Kharkovets et al., 2000). Interestingly, expression of KCNQ2 and KCNQ3 has recently been shown in the mammalian cochlea, with KCNQ3 particularly in satellite cells of the spiral ganglia (Jin et al., 2009), whose role is likely similar to the ependymal cells of the forebrain. Thus, the KCNQ3 expression seen here in the lateral

ventricle could have common origin to cochlear nuclei, with common KCNQ3 expression arising from this phylogenetic linkage.

Since expression of the KCNQ gene family has been identified in vascular and non-vascular smooth muscle cells (VSMCs) (Greenwood and Ohya, 2009), our staining could show KCNQ3-containing channels in the VSMCs in the wall of the vessels of the choroid plexus. However, whereas there is clear evidence for KCNQ1, KCNQ4 and KCNQ5 expression at the mRNA and protein level, as well as function in murine vascular contractility (Mackie et al., 2008; Yeung et al., 2007), no evidence has been found so far for expression there of KCNQ2 or KCNQ3. Thus, these data are consistent with a role for M channels in cerebral vasospasm, in which persistent constriction of cerebral arteries results in ischemic neurological deficits and consequential high morbidity and mortality (Rahimi et al., 2006; Suarez, 2006). Such a finding also underscores the emerging role of M-type currents as a neuroprotective mechanism during ischemic/hypoxic stroke (Boscia et al., 2006; Gamper et al., 2006), presenting a possible novel mode of therapeutic intervention against cerebrovascular events.

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Research Highlights

- The localization of M-type (KCNQ) K^+ channels in the hippocampal formation was systematically studied using immunostaining and confocal microscopy.
- The immunolocalization revealed strong expression in axon initial segments and somata, but relatively little in dendrites or synaptic sites.
- Co-localization of KCNQ2 and KCNQ3 subunits show most M channels in the hippocampal formation to be KCNQ2/3 heteromers, but clear evidence for KCNQ2 or KCNQ3 homomers was found.
- Immunolocalization of KCNQ3, but not KCNQ2, was found in the lateral ventricles, of as yet unknown physiological function.

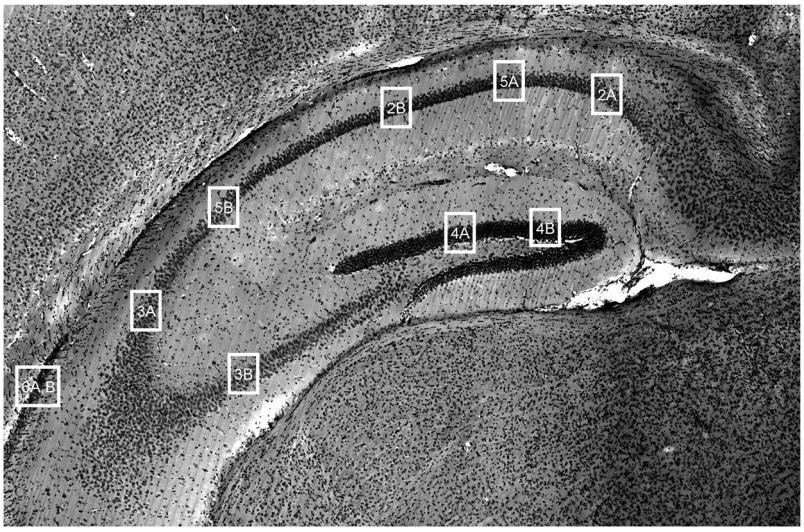

Fig. 1.

Shown is a 40X magnification image of a fixed whole-brain slice stained with hematoxylin and eosin (H&E), showing the hippocampal region studied in this paper. The approximate locations of each Z-stack set of images taken in the figures shown hereafter in this paper are indicated by the white boxes, with the figure numbers indicated.

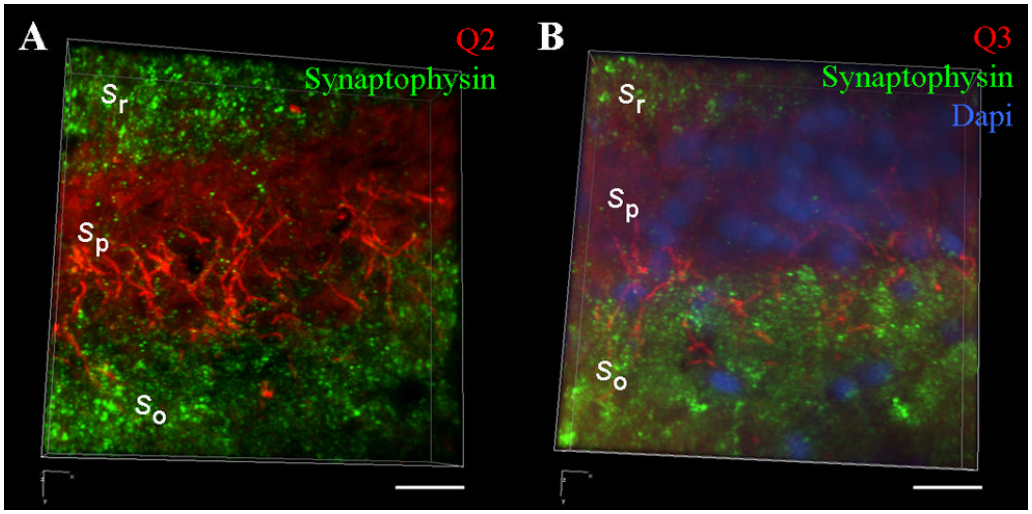

Fig. 2.

3-D reconstructions showing expression of KCNQ2 and KCNQ3 in the CA1 region of the hippocampus. Unfixed rat brain slices were stained for either KCNQ2 (Q2, A) or KCNQ3 (Q3, B) (red) and synaptophysin (green). Both KCNQ2 and KCNQ3 subunits are present in the CA1 region of the hippocampus. The tubular shape of the staining and the localization in the proximity of the pyramidal cell layer (*stratus pyramidale*, Sp) indicate the localization of the channel subunits to the axonal initial segment (AIS) of the neurons. No colocalization of either KCNQ2 or KCNQ3 with synaptophysin could be observed. *Stratum oriens* (So) and *stratum radiatum* (Sr) are indicated. Together with KCNQ3, DAPI staining (blue) was used in order to visualize the cell nuclei. Scale bar 20 μm.

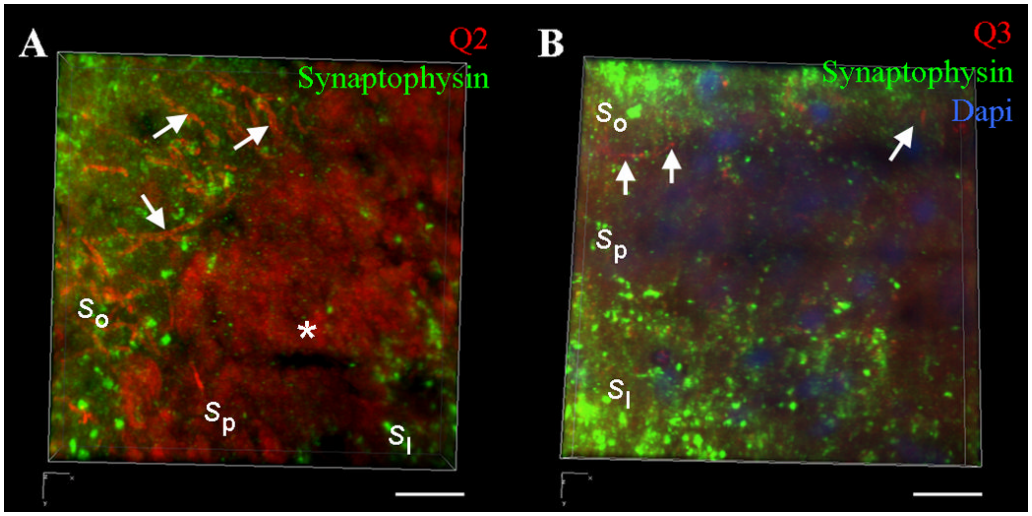

Fig 3.

3-D reconstructions showing expression of KCNQ2 and KCNQ3 in the CA3 region of the hippocampus. Unfixed rat brain slices were stained for either KCNQ2 (A) or KCNQ3 (B) (red) and synaptophysin (green). Both subunits are expressed at the AIS of pyramidal neurons (arrows). Asterisks indicate diffuse expression of KCNQ2 at the neuronal somata within the stratum pyramidale (Sp). No colocalization of either KCNQ2 or KCNQ3 with synaptophysin could be observed. Stratum lucidum (Sl) and stratum oriens (So) are indicated. In (B), cell nuclei were visualized by staining with DAPI (blue). Scale bar 20 μm.

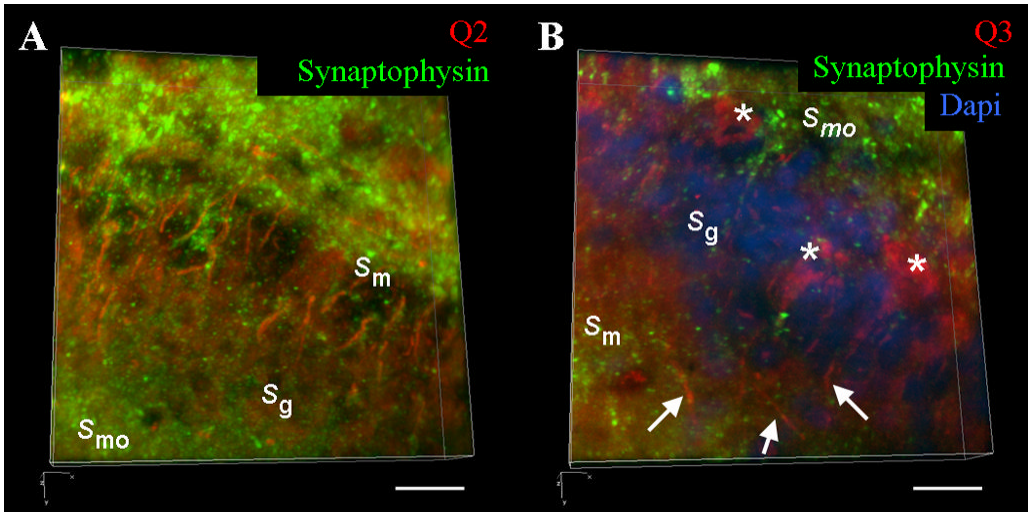

Fig 4.

3-D reconstructions showing expression of KCNQ2 and KCNQ3 in dentate gyrus of the hippocampus. Unfixed rat brain slices were stained for either KCNQ2 (A) or KCNQ3 (B) (red) and synaptophysin (green). Intense staining for synaptophysin is present in *stratum multiforme* (Sm) and *stratum moleculare* (Smo), whereas neuronal cell bodies are concentrated within granule cell layer (*stratum granulare*, Sg). Both anti-KCNQ2 (A) and anti-KCNQ3 (B) antibodies label the neuronal AIS. In addition, KCNQ3 expression is observed in the cell somata of a few neurons, indicated by the asterisks in (B). Blue shows nuclear staining by DAPI. No presynaptic localization of either KCNQ2 or KCNQ3 could be observed. Scale bar 20 μm.

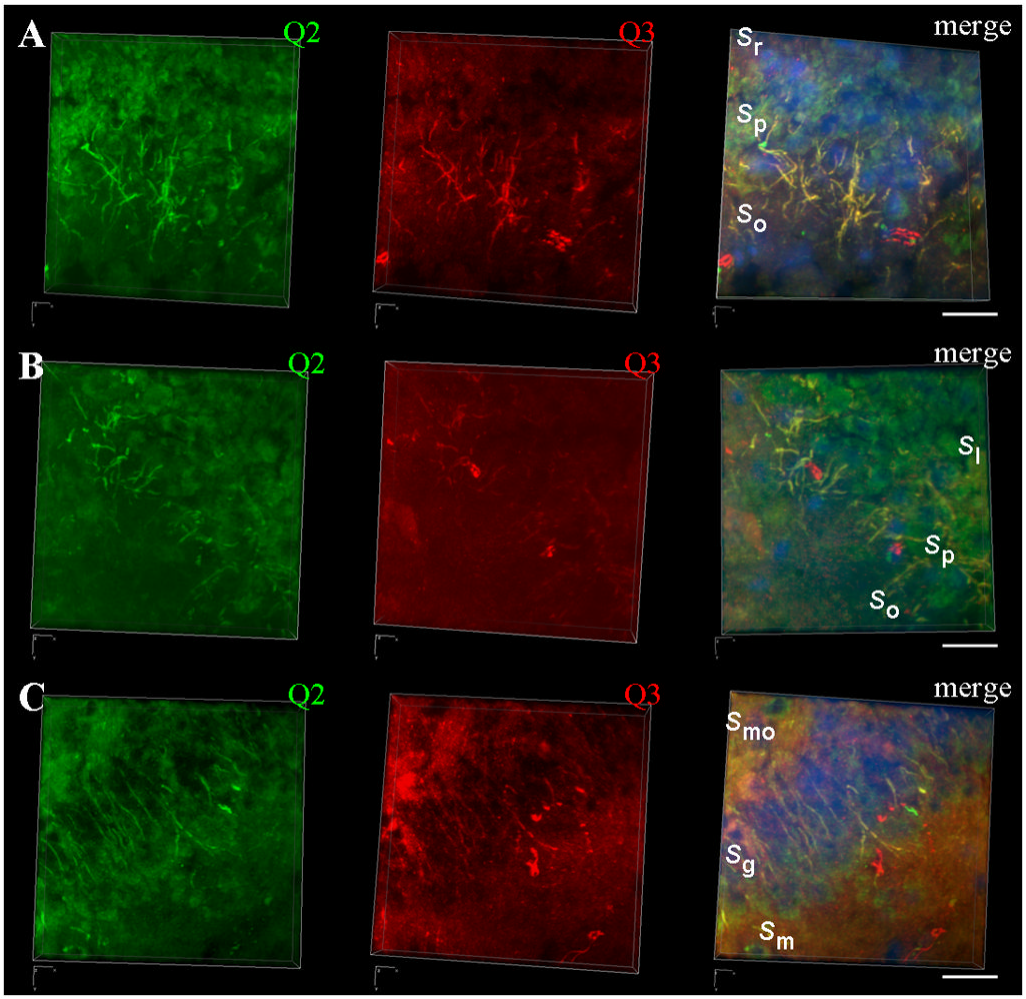

Fig 5.

3-D reconstructions showing colocalization of KCNQ2 and KCNQ3 throughout the hippocampus. Unfixed rat brain slices were stained for KCNQ2 (green) and KCNQ3 (red). (A) The CA1 region in hippocampus shows the presence of both KCNQ2 and KCNQ3 at the level of the AIS of pyramidal neurons as well as their colocalization (merged image). (B) Similarly, in the CA3 region, although less densely expressed, KCNQ2 (green) and KCNQ3 (red) subunits colocalize to the AIS (yellow in merged image). (C) Shown is the dentate gyrus, with expression of M-channel subunits KCNQ2 (green) and KCNQ3 (red) at the AIS of neurons in the granule cell layer. Merged images reveal colocalization of both subunits. In all merged images, blue indicates DAPI staining of the cell nuclei. *Stratum radiatum* (Sr), *stratum pyramidale* (Sp), *stratum oriens* (So), *stratum lucidum* (Sl), *stratum granulare* (Sg) *stratum multiforme* (Sm) and *stratum moleculare* (Smo) are indicated. Scale bar 20 μm.

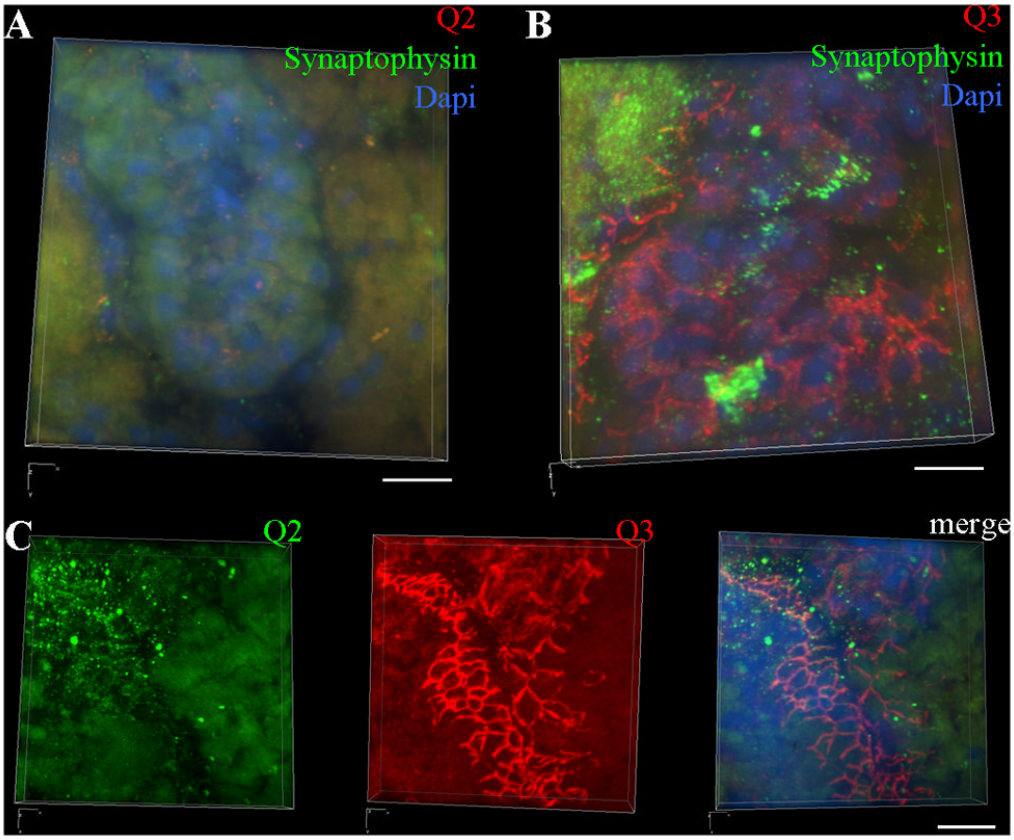

Fig 6.

3-D reconstructions showing the lateral ventricle stained for KCNQ2, KCNQ3 and synaptophysin. (A) Double-staining reveals the lack of both KCNQ2 (red) and synaptophysin (green), in this region. DAPI staining (blue) indicate the presence of cell somata within the ventricle. (B) Anti-KCNQ3 antibody shows intense staining at the membrane of the cells contained in the lateral ventricle, most probably cells of the choroid plexus. No specific staining for synaptophysin was observed, whereas cell nuclei were visualized by DAPI staining (blue). (C) A different slide was co-stained for both M-channel subunits. Again, KCNQ3 (red) shows intense membrane localization, whereas no expression of KCNQ2 could be observed. Scale bar 20 μm.

Table 1

Overview of the previous and current data regarding expression of KCNQ2 and KCNQ3 subunits in different regions of the hippocampus. Q2, KCNQ2; Q3, KCNQ3; LV, lateral ventricle; ND, not done.

	Dentate Gyrus			CA1			CA3			LV
	mouse	**rat**	**human**	**mouse**	**rat**	**human**	**mouse**	**rat**	**human**	**rat**
Q2	(Cooper et al., 2001)	Robust, *this study*	(Cooper et al., 2000)	(Cooper et al., 2001) (Devaux et al., 2004) (Pan et al., 2006)	Robust, *this study*	ND	(Cooper et al., 2001) (Devaux et al., 2004)	Robust, *this study*	(Cooper et al., 2000)	Absent, *this study*
Q3	ND	Modest, *this study*	(Cooper et al., 2000)	(Devaux et al., 2004) (Pan et al., 2006)	Robust, *this study*	ND	(Devaux et al., 2004)	Modest, *this study*	(Cooper et al., 2000)	Robust, *this study*