The role of KCNQ channels in the thalamus

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1. **Summary**

The present study aims to supply evidence for a functional role of KCNQ channels in the thalamus. KCNQ channels are low-threshold activated $K^+$ channels (Jentsch, 2000) mediating a slow, non-inactivating and hyperpolarizing $K^+$ current that has been termed M-current for its sensitivity to muscarinic modulation (Adams and Brown, 1982). In the CNS, the activation of the M-current plays a crucial role in dampening neuronal excitability and in regulating spike frequency adaptation (Cooper et al., 2001). A channel dysfunction in early stages of life has been suggested to be the cause of several diseases related to altered excitability, such as epilepsy and neuropathic pain. While its role has been well investigated in hippocampal, striatal and cortical neurons (Tatulian et al., 2001 Yue and Yaari; 2004; Gunthorpe et al., 2012), investigation of a functional role of these channels in thalamic neurons was neglected for decades, due to the lack of spike frequency adaptation in thalamocortical relay (TC) neurons. In the work presented here, it is suggested that the relay and processing of sensory information in TC neurons is influenced by KCNQ channels. Using a combined *in vitro* and *in vivo* approach, it is shown that in the somato-sensory VB nucleus:

1. KCNQ2 and KCNQ3 channels are present on the membrane of TC neurons;
2. KCNQ channels in TC neurons play a crucial role in dampening neuronal excitability, hyperpolarizing the cell and, thus, in favouring burst-like activity over tonic activity;
3. KCNQ channels are modulated by endogenous and exogenous analgesic compounds, such as $\beta$-endorphin and diclofenac;
4. Activation of thalamic KCNQ channels attenuates thermal nociception, as evidenced by an increase of the latency to pain-related behaviour in freely behaving animals;
5. The subunit composition of KCNQ channels changes during development.

For the first time, a systematic study of the functional role of KCNQ channels in TC neurons of the somato-sensory VB nucleus is provided. The activation of KCNQ channels seems to
mediate an analgesic effect by favouring burst-like activity, rendering these channels a potential target in pain treatment. Moreover, the developmental changes of channel subunit composition may be important for the organization of the somato-sensory system, suggesting a possible pathophysiological mechanism in pain sensation.
2. Introduction

Since decades the assumption that KCNQ channels are not relevant for thalamic cell functioning, was generally accepted among researchers. More recently, some evidences of their expression and functional role in this brain region have been reported (Cooper et al., 2001; Saganich et al., 2001; Kasten et al., 2007). The present study is the first systematic analysis of the expression of KCNQ channels as well as their corresponding M-current in thalamic ventrobasal (VB) neurons. Moreover, we demonstrate that the modulation of KCNQ channels in thalamic VB cells is important for the regulation of some aspects of somato-sensory perception.

2.1. The thalamo-cortical system

The thalamus is considered the major gateway to the cortex because most of the information conveyed to the cortex passes through this brain region. This relation between thalamus and cortex is referred in the term of “thalamo-cortical system” (Sherman and Guillery, 2006). In rodents, the largest part of the diencephalon is occupied by the thalamus. It consists of many nuclei that can be functionally and structurally differentiated based on the type of information they are processing (Groenewegen and Witter, 2004). Following these categorization five main groups can be identified: sensory/motor relay, nonspecific, associative, limbic, and ‘gatekeeper’ nuclei. The relay nuclei receive and convey sensory, motor, visceral and associative information from the ascending pathways to other brain areas and to different layers of the cortex (Steriade, 1991). Nuclei belonging to the relay category are: ventral posterior lateral (VPL; somatic sensation) and medial (VPM; somatic sensation and taste), lateral (LGN; vision) and medial geniculate (MGN; auditory), ventral anterior (VA; motoric) and ventral lateral (VL; motoric) nucleus. The nonspecific group is formed by the midline and intralaminar nuclei that are involved in the perception and relay of information coming from visceral and arousal regions of the brainstem and forebrain.
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(emotional pain; Houghton et al., 2001; Ren et al., 2009). The associative group of thalamic nuclei is comprised of dorsomedial (DM), pulvinar and lateral posterior nuclei which play a role in the integration of different sensory stimuli, their interpretation and formation of a response. The limbic group consists of anterior (AD) and lateral dorsal (LD) nuclei, and is involved in processes of emotion and memory. The only nucleus of the “gatekeeper” group is the reticular thalamic nucleus (RTN or NRT) which regulates the communication between thalamus and cortex. Unlike other thalamic nuclei it is composed exclusively of interneurons (IN; Houser et al, 1980) and projects only to other thalamic targets but not to the cortex (Carman et al, 1964; Steriade et al, 1984).

Most thalamic nuclei consist mainly exclusively of thalamo-cortical relay (TC) neurons which function in two basic modes: transfer mode (tonic) and oscillatory mode (burst). The transfer mode occurs during wakefulness and is characterized by a firing rate depending on the incoming excitation strength, a modality which is considered fundamental for the relay function of TC neurons, namely, the ability to transfer information to the cortex. On the other hand, during the oscillatory mode the tonic firing rate decreases and the neurons fire bursts of action potentials separated by quiescent periods, a modality which is characteristic during drowsiness and slow wave sleep (Steriade et al., 1988; Weyand et al., 2001). The regulation of the shift between the two firing modes and maintenance of a certain firing pattern requires a change in the membrane potential (Guillery and Sherman, 2002) and it is governed largely by the modulation of ion channels (intrinsic properties of TC neurons) and membrane receptors via release of several endogenous compounds (Llinàs and Steriade, 2006).

Briefly, the ion channels involved in the regulation of the two firing patterns are:

- $\text{Na}^+$ channels (inactivating $\text{Na}^+$ channels, sensitive to TTX and persistent $I_{\text{Na}}$ (Jahnsen and Llinas, 1984a, b));

- $\text{K}^+$ channels (leak-channels, $\text{Ca}^{2+}$-activated $\text{K}^+$ channels, voltage-dependent $\text{K}^+$ channels which mediate the transient $\text{K}^+$ current ($I_{\Lambda}$; McCormick and Pape, 1990) and
the hyperpolarization-activated cation current ($I_h$, Pape, 1996; Luthi and McCormick, 1998));

- various Ca$^{2+}$ channels, chiefly responsible for the change in the firing pattern is the T-type Ca$^{2+}$ channel which is activated at potentials negative to $-60$ mV and gives rise to a Ca$^{2+}$ conductance ($I_T$) and consequently to a depolarization which may become regenerative and activates Ca$^{2+}$-dependent spikes that cause the high frequency burst of Na$^+$-dependent action potentials which crown the crest of the Ca$^{2+}$ spike. This mechanism arises at hyperpolarized values of the membrane potential and mainly regulates the burst activity (Llinas and Steriade, 2006).

Likewise, the release of transmitters from different afferent inputs to the thalamus can regulate the firing patterns via modulation of ionotropic and metabotropic receptors (McCormick, 1992):

- the release of glutamate (Glu) from the synaptic terminals of neurons from different layers of the cortex and from sensory afferents, activate TC neurons, causing then to favour the tonic mode via activation of ionotropic and metabotropic receptors;

- the release of acetylcholine (ACh) from the parabrachial region of the brainstem and noradrenaline (NA) from the locus coeruleus inhibits the K$^+$ conductance via activation of metabotropic receptors in the thalamus such that TC neurons are more likely to fire in tonic mode (Steriade, 2006; Sherman and Guillery, 2006);

- the release of serotonin (5-HT) from the raphe nucleus and histamine from the hypothalamus promotes tonic firing via activation of metabotropic receptors.

- the release of GABA from interneurons within the thalamus activates both ionotropic and metabotropic receptors, thus promoting burst firing in TC neurons.

For many years, the dichotomy of tonic firing/wakefulness and bursting/sleep was considered a dogma and the tonic firing was thought to be the only mechanism to relay information to the cortex (Weyand et al., 2001). But, evidence shows that the burst firing can occur during
wakefulness when a sudden stimulus is conveyed to the thalamus, such as visual stimuli (Guido et al., 1992; Weyand et al., 2001) or noxious stimuli conveyed via the spino-thalamic tract (Jahnsen et al., 1984). Thus, new questions arise concerning the functional meaning of bursts occurring during the relay mode of TC neurons. Many authors claim that such an event is required to create a situation in which TC neurons are more prone to receive stimuli, a condition which has been defined “readiness status” (Steriade, 1988) or “wake up call” (Sherman and Guillery, 2006). However, its role is still not well understood.

2.2. The pain system

Some thalamic nuclei, such as the VB and intralaminar nuclei, have been shown to be involved in pain sensation and discrimination. In order to further clarify their role in this aspect of the somato-sensory perception a brief overview on the pain system is necessary. The expression “pain system” refers to a broad and various set of pathways responsible for perception and discrimination of pain. Sensation of noxious stimuli requires the incoming signals to be converted into a form that can be interpreted by networks of the peripheral and central nervous system (PNS and CNS, respectively; Riley and Boulis, 2006). Different types of stimuli are processed in different ways, depending on the nature and quality of the stimulus per se. For example, some sensory-discriminative aspects of pain, like duration, localization and intensity are associated with the body surface and, in this case, the perception involves the visual, auditory and somato-sensory systems (Auvray et al., 2010). The emotional and affective aspects of pain are processed from different brain regions, such as the limbic system (Bourbia et al, 2010, Pedersen et al, 2007). Since these systems are often structurally and functionally overlapping, it is very difficult to schematically classify pain stimuli and their processing. Briefly, the sensory processing involves a bidirectional system which consists of ascending and descending components. In the first case, the pain information is transmitted by peripheral nociceptors to the spinal neurons that project through the ascending fibres (spino-
thalamic, spino-mesencephalic, spino-reticular and lemnisci), to the sensory relay nuclei in
the thalamus. Depending on the vigilance state, pain information partially processed by the
thalamus might be conveyed further to the cerebral cortex. Additionally, the information
containing the emotional component is directed to the brainstem and deep brain structures,
which results in the negative reinforcement mediated by the limbic system, usually induced
by pain. After discrimination the processed information is carried backwards from central
regions to the periphery via descending routes (Willis and Westlund, 1997, 2008).

Pain stimuli, resulting e.g. from tissue damage, lead to nociceptive pain, which is a
physiological response that helps to protect the organism. It serves as an alarm signal
indicating injuries and/or danger and leads to the initiation of appropriate reactions.
Physiologically, nociceptive pain is a normal transient process: its symptoms disappear as
soon as the painful stimulus is removed or the damage is healed and importantly, perception
of it can be modulated with analgesic compounds (Butera, 2007). Many different types of
pain can be characterized by their cause and elicited symptoms: acute pain usually refers to a
short (mechanical or thermal) stimulus; while visceral pain is typically longer lasting induced
by the release (or injection) of inflammatory substances inside the body. In some cases acute,
normally transient pain, might convert into a long-lasting chronic and neuropathic pain
(Bennet et al., 2012; Attal et al., 2012). Neuropathic pain, as a particular case of chronic pain,
involves an abnormal processing of sensory input usually occurring after direct injury, viral
infection, damage to nerve or non-nervous tissue. It is characterized by a spontaneous
hypersensitive pain response that typically persists long after the original nerve cause has
healed (Campbell and Meyer, 2006). This unusually heightened pain response could be
observed as hyperalgesia (an increased sensitivity to a noxious pain stimulus) or allodynia (an
abnormal pain response to non-noxious stimulus, e.g. cold, warmth, touch). This kind of pain
is chronic and debilitating because pain episodes are often resistant to currently available
pharmacotherapies (Butera, 2007).
2.3. VB complex and pain perception

The ventrobasal complex (VB) in rodents, occupies a large part of the ventrolateral thalamic area (Groenewegen and Witter, 2004; Fig. 1). It extends rostromedial to the MGN and is bordered by the medial lemniscus and NRT. It is the main relay station for sensory information from the periphery to the cortex and consists of two nuclei: the ventral posterolateral nucleus (VPL) and the ventral posteromedial nucleus (VPM). Both nuclei consist mostly of projecting neurons and only very few GABA-ergic neurons (Price, 1995). The main sensory inputs are directly coming from somato-sensory afferents of the spino-thalamic tract, originating in the dorsal column nuclei and the trigeminal complex of the spinal cord (Lenz, 1992). In addition, the VPM and VPL receive afferents from different layers of the somato-sensory cortex, many subcortical areas including other thalamic nuclei (such as the NRT) and brainstem serotonergic, cholinergic and adrenergic nuclei (Steriade et al., 1988). These afferents are conveying nociceptive, tactile and kinaesthetic information from the head and the rest of the body (Groenewegen and Witter, 2004).

The first evidence that pointed to an involvement of the VB complex in nociception was reported in the late 1980s. It was shown that, in addition to previously described mechano-receptive cells, the VPL contains many nociceptive and thermo-receptive neurons (Peschanski et al, 1980b; Hellon and Misra, 1973). Moreover, it was shown that the response of nociceptive VPL neurons to a noxious stimulus is enhanced during the formalin test, an acute inflammation test, (Guilbaud et al, 1986, 1987a, 1987b, 1990) and that the response is more
prominent during the latest phase of the test, when the inflammation reaches its peak (Huang et al., 2006). Additionally, the intra-thalamic injection of the local anaesthetic lidocaine induces a profound impairment in the ability to discriminate thermal noxious stimuli in monkeys (Bushnell et al., 1991). Electrophysiological recordings performed in anesthetised animals suggest also a somatotopic organization of VB because the response of neurons, localized in different parts of VPL, can be evoked by mechanic and thermal stimulation of different body parts (Koyama et al. 1998; Houghton et al., 2001; Wang et al., 2009). In addition, recently, neuronal thalamic response to noxious and innocuous stimuli was assessed in mice using the f-MRI technique (Bosshard et al., 2010).

The crucial role of the VB complex in pain and somato-sensory discrimination has since been further established. Nevertheless, many aspects of pain sensation, such as the onset of different aversive behaviours at different time points following the stimulus presentation, are still not well understood (Price et al, 1995). For example, the pain response to the formalin test consists of two different phases: an early and late phase; characterized by different pain-related behaviours elicited by different mechanisms which both involve VB activation. Interestingly, both phase-related pain perceptions can be relieved by intrathalamic injections of opioids and other antinociceptive substances (Potes et al, 2006; Pozza et al, 2010; Porro et al, 2003).

Several reports revealed changes of the electrical behaviour of thalamic neurons, especially in the VB complex, after injuries to the peripheral nerves (Guilbaud et al, 1990; Rinaldi et al 1991; Rasmusson et al., 1996), ascending pain pathways (Hirayama et al., 1989; Radhakrishnan et al., 1999; Miki et al., 2000; Gerke et al., 2003; Gwak et al., 2010) and during chronic inflammation, such as monoarthritis (Neto et al., 2008). These findings suggest that the thalamus is involved in the onset and maintenance of neuropathic pain and allodynia, a condition that can be reverted or ameliorated by pharmacological modulation of the thalamic somatosensory nuclei (Saadè et al., 2006; Ueda et al., 2010).
2.4. KCNQ channels

KCNQ channels (Kv7 channels according to the new nomenclature) are low-threshold voltage-activated K\(^+\) channels mediating a hyperpolarizing K\(^+\) current. KCNQ genes encode 5 members of the KCNQ channel family: KCNQ1-5 (Brown and Passmore, 2009). Of these, four channels (KCNQ2-KCNQ5) are expressed in different cell types of the nervous system, while the expression of KCNQ1 is restricted to the heart, peripheral epithelial and smooth muscle cells (Jentsch, 2000; Robbins, 2001).

Each KCNQ subtype exhibits a high homology degree with other proteins belonging to the broad K\(^+\) channels family. KCNQ channels are tetramers consisting of four subunits (Fig. 2). Every subunit is built by six trans-membrane domains (S1-6) and a single pore P-loop that forms the selectivity filter. An intracellular part of the S4 domain plays a role as a voltage sensor. The amino- and carboxy-termini of the protein are located on the intracellular side of the membrane and are rich in phosphorylation sites for the intra-cellular modulation of the channel function (Schwake et al., 2006; Howard et al., 2007; Hernandez et al., 2008).

In vitro experiments demonstrated that all five known KCNQ proteins can form homomeric channels, but only a certain combination leads to formation of heteromeric channels, such as KCNQ3 and 5, KCNQ3 and 4 as well as KCNQ2 and 3 (Schroeder et al., 1998, 2000; Jentsch, 2000; Lerche et al., 2000).

The heteromeric channel resulting from the association of KCNQ2 and KCNQ3 is the most abundant in the nervous system and it seems to be the molecular substrate of the M-current (Wang et al., 1998). This current was first identified and described more than 30 years ago by Brown and Adams (1980) in sympathetic neurons of the frog. Because it is modulated by muscarinic agonists it was called M-current and the channels underlying it, were thus termed M-channels (Brown and Adams, 1980; Marrion et al., 1989).

M-current shows a characteristic time and voltage dependency that result in a hyperpolarization of the membrane potential when the cell is exposed to an excitatory
stimulus (Delmas and Brown, 2005). Therefore, KCNQ channels are not fully open at RMP and they play a crucial role in regulating the excitability (e.g. spike frequency adaptation) of various peripheral and central neurons, such as hippocampal pyramidal cells and striatal neurons (Yue et al., 2004; Peters et al., 2005; Gu et al., 2005; Gunthorpe et al., 2012). KCNQ channels are expressed at different cellular locations, such as somato-dendritic and axonal and terminal sites. This broad distribution enables them to participate in both pre- and post-synaptic modulation of basal and stimulated neurotransmission, both inhibitory and excitatory (Vervake et al., 2006; Peretz et al., 2007; Hansen et al., 2008).

Fig. 2. a KCNQ channel subunits have a conventional shaker-like $K^+$ channel structure, with 6 transmembrane domains (S1–S6), a single pore (P)-loop that forms the selectivity filter of the pore, a positively-charged fourth transmembrane domain (S4) that acts as a voltage sensor and a long intracellular carboxy-terminal tail. Four such subunits make up a functional KCNQ channel. The carboxy terminus contains a conserved domain (A domain) that determines the subunit specificity of the KCNQ channel assembly. b The carboxy-terminal tail of KCNQ2 subunits has binding sites for several potential regulatory molecules, as depicted. CaMI and CaMII represent the two binding sites for calmodulin (CaM). Modified from Delmas and Brown (2005).

2.4.1. KCNQ channel expression in the CNS

The KCNQ subunits 2 and 3 are expressed in the CNS and are localized at key sites for neuronal network oscillations (Cooper et al., 2001). Strong KCNQ2 immunoreactivity has been detected in the thalamus (NRT, VPL, VPM), a subpopulation of cholinergic neurons of the medial habenula, cerebral cortex, hypothalamus, midbrain, all neuronal populations of the
hippocampus, basal ganglia, substantia nigra, striatum and ventral-tegmental area (Cooper et al., 2001).

KCNQ3 is mostly expressed in the same regions as KCNQ2. An in situ hybridization study showed that centrally localized KCNQ3 channels are highly expressed in the forebrain: cortex (all layers), hippocampus (CA2 and CA3), septum, basal ganglia and thalamus (VB complex, MGN and LGN; Saganich et al., 2001).

KCNQ4 is expressed in all cell types of the auditory system, especially in the cochlea (Liang et al., 2006). There is also evidence for KCNQ4 expression in murine arteries in co-localization with KCNQ5 (Ng et al., 2011). KCNQ5 is broadly expressed in the cortex, amygdala, caudate-putamen, hippocampus and in the skeletal muscles (Lerche et al., 2000).

2.4.2. Developmental profile of KCNQ channels

The expression of the four KCNQ channels in the brain differs among various regions. Moreover, the stoichiometry of heteromeric channels varies also. For example, KCNQ3 can form heteromeric channels by co-assembling with KCNQ2, 4 and 5 (Jentsch, 2000). When it is co-assembled with KCNQ2 but also KCNQ5, it mediates M-current with an amplitude current that is much bigger than the current mediated by a homomeric KCNQ3 channel (Brown and Adams, 1980; Marrion, 1997; Schroeder et al., 2000). Various explanations for this phenomenon can be found in the literature. Some authors claim that the difference in current amplitudes is due to the existence of several splice variants of KCNQ2 (Pan et al., 2001; Smith et al., 2001; Hadley et al, 2003). Other authors find the cause for this difference in the stoichiometry of the heteromeric KCNQ2/KCNQ3 channels (Cooper et al., 2001; Hadley et al., 2003), although the impact of the exact stoichiometry or subunit combination on the channel function are not fully understood.

Regarding the phenomenon of varying current amplitudes dependent on the subunit composition, the developmental profile of KCNQ channel expression should be taken into
consideration. In fact, many recent studies show differences in the protein expression of KCNQ2 and KCNQ3 during ontogenesis. Hadley and colleagues (2003) have shown a developmental delay in the expression of KCNQ3 in native ganglionic neurons from rats. They found an increased expression starting at post-natal day (P) 7. This remained elevated until P45, whereas there was no change in the expression of KCNQ2 and KCNQ5. Previous studies pointed out that in the mouse brain, the KCNQ2 levels are high already at P7, whereas KCNQ3 levels are very low at P3 and do not increase considerably before P30 (Tinel et al., 1998). Interestingly, the sensitivity to linopirdine (a KCNQ blocker) increases with the age of the animals, reflecting developmental changes in KCNQ channel expression (Safiulina et al., 2008). In line with above findings are results obtained in humans: the expression of KCNQ2 in hippocampus is high during foetal life and decreases after birth, whereas expression of KCNQ3 increases in late foetal life and stays high during infancy. From a pathophysiological point of view, an imbalance in the expression patterns of KCNQ channels could be the cause of neonatal epilepsy syndromes (Pena et al., 2006; Kanaumi et al., 2008).

2.4.3. Muscarinic signal transduction

In the early 80s Brown and colleagues proved that activation of the G-protein cascade, via activation of muscarinic receptors M1 and M3, is able to reduce M-current (Brown et al., 1980). All subsequent experiments trying to understand the signal transduction mechanism that modulates M-channel activity were based on this finding. In particular, *in vitro* experiments conducted in Gqα deficient mice, identified subclasses of G-protein: Gq and G11, as candidates triggering the signal transduction pathway that modulate M-channel activity (Pfallinger et al., 1988; Haley et al., 1998, 2000).

The subunit Gqα first triggers the activation of phospholipase-C (PLC; Fig. 3), an enzyme that is responsible for the hydrolysis of phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P2 or PIP2) and formation of two compounds: inositol-1,4,5-triphosphate (Ins(1,4,5)P3) and...
diacylglycerol (DAG). Both are second messengers and can trigger activation or deactivation of many intra-cellular mechanisms, mainly phosphorylations and changes in the intra-cellular Ca\(^{2+}\) concentrations (by inducing Ca\(^{2+}\) release from the internal stores; Marrion et al., 1997; Suh et al., 2002, 2004; Ford et al., 2003; Zhang et al., 2003, 2010). It is believed that depletion of PIP\(_2\) and increase of intra-cellular Ca\(^{2+}\) are the main mechanisms responsible for the closing of M-channels. Summarizing, any signal that triggers synthesis of PIP\(_2\), decreases the intracellular Ca\(^{2+}\) concentration (like cellular hyposmotic conditions; Caspi et al., 2009) or de-phosphorylates channel proteins could lead to an enhancement of KCNQ channel activation (Li et al., 2005; Hernandez et al., 2008).

**Fig. 3.** KCNQ2–5 channels bind phosphatidylinositol-4,5-bisphosphate (PIP2). PIP2 is required for KCNQ channel opening. M1 muscarinic and bradykinin (BK) receptors (B2R) couple to Gq/11 G-proteins and activate phospholipase-C (PLC). This leads to hydrolysis of PIP2 to produce inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) (IP3) and diacylglycerol (DAG). The local increase in IP3 produces a vigorous release of Ca\(^{2+}\) that is sufficient to bind to KCNQ-attached calmodulin and close the channels. Modified from Delmas and Brown, 2005.
2.4.4. Modulation of M-current

The activation of several receptors due to agonist binding can lead to a modulation of M-channels. For example, ACh released from the cholinergic fibres binds to the muscarinic receptors M1/M3 (MACHr) and inhibits KCNQ channels, which in turn induces a slow depolarization of the postsynaptic neuron (Gahwiler et al., 1985; Cruzblanca, 2006). Moreover, the release of serotonin in the human cortex (McCormick and Williamson, 1989), activation of endocannabinoid CB1 receptors (Schweitzer, 2000) and release of glutamate and purinergic agonists in the superior cervical ganglia (SCG) via activation of mGluR1 and P2Y, respectively, can suppress M-current (Charpak et al., 1990; Delmas and Brown, 2005; Hernandez et al., 2008; Schicker et al., 2010). Besides neurotransmitters, several other endogenous messengers can negatively modulate KCNQ channels: bradykinin (via activation of B2 receptors; Delmas and Brown, 2005; Zaika et al., 2007), angiotensin II (via activation of AT1 receptors; Zaika et al., 2006), the nerve growth factor (NGF, Jia et al., 2008) and the luteinizing hormone (LHRH; Bley et al., 1990).

Unfortunately, much less is known about the endogenous activation of KCNQ channels. There is controversial evidence regarding substance P (SP) which was shown to either augment the M-current in DRG neurons (Linley et al., 2012; Lin et al., 2012), or inhibit KCNQ channels in sympathetic bullfrog neurons (Adams et al., 1983; Bley et al., 1990). In the hippocampal CA1 region, somatostatin, corticostatin and dynorphine A have been reported to activate M-current, thereby hyperpolarizing the $V_m$ and reducing neuronal excitability (Moore et al., 1988, 1994; de Lecea et al., 1996; Madamba et al., 1999a, b). In CA1, an enhanced M-current is due to the activation of somatostatin subtype 4 receptors (SSR4; Qiu et al., 2008) and a subsequent activation of arachidonic acid metabolites pathway (Schweitzer et al., 1990). Moreover, in neurons of the solitary tract somatostatin produces hyperpolarization and depresses excitability via an increased M-current (Jaquin et al., 1988).
The pharmacological modulation of KCNQ channels using different exogenous compounds is a specific and selective approach for channel characterization studies. Over the last years, a variety of K⁺ blockers and enhancers that strongly and specifically modulate M-current through direct binding to KCNQ channels have been identified (Wulff et al., 2009). One of those specific channel blockers is linopirdine. It induces closure of KCNQ channels (Aiken et al., 1995), and shows low affinity for other K⁺ channels (Lamas et al., 1997). A similar mechanism of action is typical for other specific blockers, XE991 which shows an even higher affinity and selectivity for native M-currents than linopirdine (Wang et al., 1998, 2000; Robbins et al., 2001; Schroeder et al., 2001; Peretz et al., 2007). The application of KCNQ blockers was useful for the identification of M-currents in neurons of the dorsal root ganglion and the spinal cord, in which XE991 induces depolarization and an increase of the spike frequency (Otto et al., 2006; Rivera-Arconada et al., 2005).

Among the channel openers are flupirtine, retigabine (Linley et al., 2012; Dalby-Brown et al., 2006), BMS-204352 (maxipost; Schroder et al., 2001; Korsgaard et al., 2005), diclofenac, meclofenamic acid, NH6 (Peretz et al., 2005, 2007), ICA-27243 (Wickenden et al, 2008), several acrylamides (Bentzen et al., 2006; Blom et al., 2009) and zinc-pyrithione (Xiong et al., 2008). The most frequently used KCNQ channel opener and enhancer is retigabine which was shown to activate M-current mediated by heteromeric KCNQ2/KCNQ3 channels among the others (Main et al., 2000; Rundfeldt and Netzer, 2000b; Wickenden et al., 2000). Retigabine modifies the voltage-dependence of these channels, increases the activation rate, favours the open configuration of the channels and slows down the deactivation phase (Tatulian and Brown, 2003). It was also found to induce spike frequency adaptation in hippocampal (Yue and Yaari, 2005) and spinal cord neurons (Rivera-Arconada and Lopez-Garcia, 2005). However, at concentrations higher than the one required to modulate KCNQ channels, retigabine can interact with the GABA-ergic system indirectly by reducing GABA release (Kapetanovic et al., 1995; Rundfeldt and Netzer, 2000a; van Rijn and Willems-van
Bree, 2003) and directly by allosteric modulation of the major GABA<sub>A</sub> receptor isoforms in the brain (Wafford, 2005; Gunthorpe et al., 2012). Further investigations showed that retigabine does not interact with the ionotropic glutamatergic receptors and that it only weakly inhibits Na<sup>+</sup> and Ca<sup>2+</sup> channels (Gunthorpe et al., 2012).

### 2.5. KCNQ channels in health and disease

The peculiarity of the hypothesized mechanism of action of KCNQ channels and their role in dampening the cell excitability emphasize their importance in regulation of many physiological processes. Dysfunction of KCNQ channels causes severe functional consequence that leads to the development of pathophysiological states, and several examples of KCNQ-associated diseases are described in literature (Jentsch, 2000; Brown, 2008). A mutation of the cardiac KCNQ1 protein most probably induces an alteration in Ca<sup>2+</sup> signalling and has been linked to the onset of the Hereditary Long-QT Syndrome (LQTS; Park et al., 2005). Mutations in genes encoding for KCNQ4 and KCNQ5 lead to many other severe diseases like cardiac arrhythmia (Wang et al., 1996), impaired transepithelial transport (Neyroud et al., 1997), and progressive hearing loss (Kubisch et al., 1999). Mutations in KCNQ2 and KCNQ3 heteromeric channels are responsible of benign familial neonatal convulsions (BFNC) and other forms of epilepsy (Bievert et al., 1998; Singh et al., 1998; Charlier et al., 1998; Jentsch, 2000). Moreover, mutations in either KCNQ2 (Watanabe et al., 2000) or KCNQ3 as homomeric channels can also induce several epileptic syndromes of particular severity (Jentsch, 2000). Compared to other KCNQ channels, KCNQ2/3 heteromeric channels produce larger currents (Brown and Adams, 1980; Wang et al., 1998; Schroder et al., 1998), with slightly different kinetics and sensitivity to openers and inhibitors (Hadley et al., 2000) they are very promising targets for novel anticonvulsive drugs. It was already shown that retigabine attenuates epileptoform activity in ex vivo and in vivo models of epilepsy (Armand et al. 1999; 2000; Dost and Rundfeldt, 2000; Wuttke and Lerche, 2006).
Additionally, it attenuates the symptoms in pharmacoresistant cases of epilepsy in human preparations, and it successfully completed phase I and II clinical trials as an anticonvulsive drug, currently being in phase III trial (Porter et al., 2007a, b).

2.6. KCNQ channels and pain

Pain perception in the nervous system can be regulated by many endogenous compounds, among which are substance P and bradykinin. In fact, intra-peritoneal injection of substance P and bradykinin induces acute visceral and inflammatory pain in mice (Potter et al., 1962; Mayer et al., 1999; Laird et al., 2000; Kamp et al., 2001). Both substances are also negative modulators of KCNQ channels, suggesting involvement of these channels in pain processing. On the other hand, KCNQ channels are positively modulated by dynorphine, an opioid involved in analgesia and anti-nociception (Tung et al., 1982; Tiseo et al., 1988; Kapitzke et al., 2005). Further evidence shows that KCNQ channels expressed in peripheral nerve endings are involved in the machinery of receiving and transducing pain signals (Belmonte and Viana, 2008). In peripheral myelinated axons they have been functionally characterized (Devaux et al., 2004) and were found to co-localize with Na$^+$ channels in nodes of Ranvier (Devaux et al., 2004; Pan et al., 2006). In isolated segments of human sural nerves, retigabine reduced the excitability of unmyelinated C-type fibres which mediate pain sensation (Lang et al., 2008). Moreover, KCNQ channels are broadly expressed on the cell bodies of primary afferents in dorsal root ganglia (DRG), where they are involved in inducing hyperexcitability during inflammation (Passmore, 2003; Linley et al., 2008). Also, in a large proportion of dorsal horn neurons M-like currents can be recorded after application of retigabine (Rivera-Arconada et al., 2004, 2009; Passmore et al., 2012).
2.6.1. KCNQ channels and acute pain

The role of KCNQ channels in modulating acute pain is a controversial issue. Upon activation of KCNQ channels via i.p injection of retigabine in rats, Dost and colleagues (2004) showed a small increase in latency in the tail-flick test, while in the same test Blackburn-Munro and colleagues (2003) observed no changes in the perception of acute pain after injection either of retigabine or XE991. Moreover, retigabine and flupirtine attenuate nociception during the second phase of the formalin test, a model of acute pain inflammation (Blackburn-Munro et al., 2003; Hirano et al., 2007; Capuano et al., 2011).

2.6.2. KCNQ channels and neuropathic pain

The localization (among other K\(^+\) channels) and mechanism of action of KCNQ channels also suggest their involvement in appearance and maintenance of the neuropathic pain syndrome. This holds true for both, peripheral and central components of the nociceptive system (Rivera-Arconada et al., 2009) and many findings support this theory. Utilization of neuropathic pain models in rodents is a reliable method to reproduce human neuropathic conditions and to evaluate the role played by KCNQ channels. Data derived from these studies showed that at the peripheral level there is a change in expression of Na\(^+\) and K\(^+\) channels, with consequent appearance of ectopic activity associated with neuropathic symptoms (Rasband et al., 2001; Bostock et al., 2005; Hains et al., 2005; Devor, 2006; Roza et al., 2008). Likewise, in trigeminal inflammatory neuropathic model (TRG), a transient outward K\(^+\) current (I\(_{\text{A}}\)) was shown to be strongly suppressed when compared with I\(_{\text{A}}\) of naïve animals (Takeda et al., 2011). Moreover, the partial nerve ligation (PSLN) in rats induces a neuropathic pain syndrome which is characterized by a down-regulation of the KCNQ2 gene in the latest stage. This suggests a role of KCNQ2 channels in the maintenance of the neuropathic state (Rose et al., 2011).
In the past, the therapy for neuropathic pain included the use of anticonvulsive drugs (gabapentin and pregabalin; Sindrup and Jensen, 1999), however, it was accompanied by many side effects. Recently Blackburn-Munro and colleagues (2005) showed that the negative side effects are due to the higher concentrations needed for the analgesic effect while the dosage required to attenuate anxious behaviour causes less side effect. In the same study, they showed that retigabine, like many other KCNQ channel openers, has a positive effect on the treatment of neuropathic pain at relatively low concentrations. In other neuropathic models with chronic constriction and nerve injury, the injection of retigabine and flupirtine attenuates allodynia in the pin prick stimulation to the injured paw (Blackburn-Munro and Jensen, 2003). Moreover, in rats with nerve ligation the same compounds induce an analgesic effect to thermal and tactile stimulation. Such effects can be reverted by application of the specific blocker linopirdine (Dost et al., 2003). The efficacy of analgesic effect induced by KCNQ openers is comparable to the effect induced by derivates of opioids such as tramadol, in models of mechanic neuropathic pain (Dost et al., 2003), morphine in a model of inflammation-induced neuropathic pain (Xu et al., 2010), and ketamine in an acid-induced model of allodynia (Nielsen et al., 2004). The evidence of KCNQ channel modulation in the periphery and at the central level of the nervous system highlights that these channels are good candidates for specific pain treatments (Bi et al., 2011)
3. **Aim of the study**

Epilepsy, cardiac syndromes and neuropathic pain are serious and severe diseases caused by cell excitability dysfunctions and not well understood (Bi et al., 2011). The involvement of KCNQ channels in regulating cell excitability and therefore, the appearance and/or maintenance of such diseases is relatively new concept introduced only over the last decade. These channels are widely expressed in the CNS and evidence of their presence in the thalamus was presented in the literature (Cooper et al., 2001; Saganich et al., 2001). Based on the non-adapting firing properties of thalamic neurons, a potential functional role played by KCNQ channels in the thalamus was neglected for decades. Recently, evidence of functional expression has been shown in TC neurons where the inhibition of KCNQ channels enhances the firing rate thereby modulating the TC neurons excitability (Kasten et al., 2007). This finding opened a new scenario for the study of the role of KCNQ channels in the thalamus. In fact, we suggest that the modulation of the KCNQ channels expressed in the thalamus can alter the regulation of excitability of TC neurons and subsequent regulation of the relay function, i.e. the processing of the sensory information eventually conveyed to the cortex. Therefore, this study aims to:

- understand the functional role of KCNQ channels in the thalamic ventro-basal nuclei (VB complex);
- identify possible endogenous compounds which can modulate KCNQ channels in the thalamus;
- assess the subunit compositions by analysing the current amplitude before and after pharmacological modulation in TC neurons, in heterologous expression systems and in genetically modified mice (KCNQ3+/−);
- determine the role of the VB complex and KCNQ channels in the acute pain sensation.

By combining different *in vitro* and *in vivo* techniques, we show a consistent expression of KCNQ channels in VB were these channels are functionally involved in regulating the cell
excitability. This, in TC neurons, influences the two different firing patterns characteristic of these neurons. The evidence herein presented, clearly shows the activation of the slow hyperpolarizing M-current and the strong effect on the firing rate: reduction of action potential number and frequency after retigabine application and a respective increase after XE991 application. The application of synthetic compounds, with different specificity for the different KCNQ subunits, helped to understand the nature of the M-current in the thalamus and its functional role in the somato-sensory system information processing.

In freely behaving animals, a correlation between KCNQ activity and pain sensation has been found: the intra-thalamic injection of retigabine increased the latency to pain behaviour. This effect was region-specific since injection in neighbouring nuclei failed to produce an analgesic effect.
4. **Material and Methods**

4.4. **Ex vivo experiments**

4.4.1. Tissue preparation

All animal work has been approved by local authorities (review board institution: Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; approval ID numbers: 8.87-51.05.2010.117).

C57Bl/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred in the animal facility of the Institute of Physiology I and their offspring served as subjects at post-natal day 15-30. Animals were group-housed in standard dark-light conditions (12 h in dark/light cycle) with food and water *ad libitum*. The animals used for the electrophysiological recordings were anesthetised with isoflurane and decapitated; a block of brain tissue was rapidly removed from the skull and placed in chilled (2-4 °C) oxygenated slicing solution containing (in mM): sucrose, 200; glucose, 10; PIPES, 20; KCl, 2.5; MgSO₄, 10; CaCl₂, 0.5; pH 7.35 (adjusted with NaOH). Thalamic slices were prepared as coronal sections of 300 µm thickness on a vibratome (Leica VT 1200, Leica, Wetzlar, Germany). Prior to recording, slices were kept in an incubation chamber in standard artificial cerebrospinal fluid (ACSF) which contained (in mM): NaCl, 125; KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 24; MgSO₄, 2; CaCl₂, 2; glucose, 10; pH 7.35 (adjusted with carbogen; a mixture of 95% O₂ and 5% CO₂).

4.4.2. Patch-clamp recordings in acute brain slices

Whole-cell recordings were performed on visually identified TC neurons of the ventro-basal complex (VB) at room temperature. Slices were transferred to a recording chamber where they were kept in a solution containing (in mM): NaCl, 120; KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 22; C₆H₁₂O₆, 20; CaCl₂, 2; MgSO₄, 2; pH 7.35 (adjusted with carbogen).
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Electrical activity was measured with electrodes made out of borosilicate glass capillaries (GC150TF-10, Clark Electromedical Instruments, Pangbourne, UK) pulled on a vertical puller and connected to an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Pipettes were filled with one of two solutions which contained (in mM):

1) standard solution: NaCl, 10; K-Gluconate, 88; K_3-citrate, 20; HEPES, 10; BAPTA, 3; Phosphocreatin, 15; MgCl_2, 1; CaCl_2, 0.5; Mg-ATP, 3; Na-GTP, 0.5.

2) NaCl, 10; CsMeSO_4, 82; K-BAPTA, 11; HEPES, 10; KCl, 1; TEA-Cl, 15; 4-AP, 5; Qx-314-Cl, 3.35; Phosphocreatin, 15; MgCl, 1; CaCl, 1; Mg-ATP, 3; Na-GTP, 0.5. This solution was used for experiments in which blocking of potassium channels was required.

The electrode solutions were set to pH 7.25 (with KOH) and an osmolality of 295 mOsmol/l. Typical electrode resistance was 2-3 MΩ and the series resistance throughout recordings was in the range of 5-15 MΩ and regularly monitored during recordings. Electrophysiological experiments were governed by Pulse software (HEKA Elektronik, Lambrecht/Pfalz, Germany) operating on an IBM-compatible PC. Recorded current and voltage traces were corrected for liquid junction potential (V_m = V_p -10 mV; where V_m = membrane potential and V_p = potential of the pipette). Series resistance compensation of 25% was routinely used.

The resting membrane potential (RMP) was assessed immediately after breaking the membrane patch, thus achieving the whole cell configuration, to avoid dilution of the intracellular environment. The passive and active membrane properties were recorded under current clamp conditions by holding the cell at -65 mV and injecting hyperpolarizing and depolarizing currents (duration of 1s, amplitude from -100 pA till +240 pA, in 20 pA increments). The input resistance (R_{in}) was calculated from the voltage shift during a current injection of -60 pA. The cell capacitance (C) was calculated as C = τ/R and the τ (membrane time constant) was obtained by a monoexponential fit of the hyperpolarizing voltage-deflection elicited by a -60 pA current–injection during the current clamp recordings.
4.4.3. Voltage-clamp analysis

The changes in current amplitude were measured in the voltage clamp mode maintaining the same recording conditions and adding routinely: mibefradil (2 µM), ZD7228 (30µM) and TTX (0.5 µM) to the bath solution. The membrane potential was held at -65 mV and thereafter depolarized to -45 mV for 4 s, followed by a repolarization to -60 mV for 3 s. This protocol was chosen with respect to the slow activation and deactivation with the latter resulting in the typical M-channel tail current. This voltage step was preceded by a short depolarizing step (from -65 mV to -40 mV, 80 ms), a pre-pulse, which aims to inactivate fast transient membrane currents. The step protocol was repeated over time: during control conditions and during drug application. The amplitude of the current was analysed at the end (the last 500 ms) of the depolarizing voltage step, when the activation of M-current was maximal. Current amplitudes were compared under control conditions, in the presence of drugs and after washing out. The difference between the mean of the recordings at control conditions and in the presence of the drugs is termed drug-evoked current (also named sensitive current, in the following). The values are expressed in percent of control values (control = 100%). The visual representation of the sensitive current is obtained by subtraction of the current during drug application from the current under control conditions.

The recordings to analyse the family of currents have been performed under voltage clamp conditions holding the membrane potential at -85 mV and applying hyperpolarizing and depolarizing steps lasting for 4 s starting from -65 mV to -25 mV with an increment of 15 mV. The protocol is shown in Fig. 4 and it was preceded by the pre-pulse, it was repeated undero control conditions and in the presence of retigabine.

A ramp protocol was used to establish the current-voltage relationship (I/V). The neuron was held at –35 mV to open the M-channels and ramped to –135 mV (100 ms) and re-polarised to holding potential. The change in the current is proportional to the change in the holding voltage (1 ms = 10 mV). Then, the value of the reversal potential of the sensitive current was
obtained empirically and compared to the value calculated by application of Nernst’s equation, considering a temperature of 22-23 °C and the composition of the electrode solutions (K⁺ concentration of 108 mM).

4.4.4. Current-clamp analysis

The current clamp experiments were performed using the bath solution without blockers. Tonic firing was induced by current injection of four different amplitudes (50, 100, 150 and 200 pA) lasting for 2.5 s when the cell was held at ~ -60 mV by the injection of a DC current. The bursting mode (bursts of action potentials crowning a low-threshold Ca²⁺-peak) was elicited by injecting 50 pA depolarizing steps from hyperpolarized potentials of ~ -65 mV. The number of action potential during tonic firing and burst was calculated using a custom-made Matlab script (courtesy of Dr. Patrick Meuth). Neurons with a Vₘ more positive than – 55 mV and non-overshooting action potentials were discarded.

4.4.5. Drugs

For pharmacological tests drugs were added to the external solution and all the concentrations herein reported are final concentrations. Linopirdine (10 µM), oxotremorine-M (10 µM), pirenzepine (10 µM), 4-DAMP (10 µM), carbachol (25 µM), DAMGO (1 µM), leu-enkephalin (500 nM), nociceptin (1 µM), endomorphin-1 (1 µM) and dynorphin A (1 µM) were purchased from Tocris Bioscience, (Bristol, UK). ZD7288 (30 µM), mibebradil (2 µM), XE991 (20 µM), diclofenac (20 µM), meclofenamic acid (20 µM) were purchased from Abcam Biochemicals (Cambridge, UK). Nifedipine (1 µM) was purchased from Sigma (Taufkirchen, Germany); TTX (0.5 µM) was purchased from Biotrend (Cologne, Germany); retigabine (30 µM) was purchased by LGM Pharma (Boca Raton, Florida). ICA27243 (30 µM) and maxipost (20 µM) were kindly provided by Dr. Dalby-Brown from Neurosearch (Ballerup, Denmark).
In case substances were dissolved in dimethylsulfoxide (DMSO), solvent concentration did not exceed 0.1%. The solvent alone had no effect on the investigated parameters.

4.4.6. Cell culture transfection

The experiments described in this paragraph have been conducted by the candidate in collaboration with Prof. Oliver’s group at the University of Marburg. Chinese hamster ovary (CHO) cells were maintained in MEM Alpha Medium (Invitrogen GmbH, Darmstadt, Germany) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (both from Invitrogen). For experiments, cells were plated on glass cover slips (Carl Roth, Karlsruhe, Germany) and transfected with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). The transfection vectors were pBK-CMV containing human KCNQ2 (NM_172106.1) and human KCNQ3 (NM_004519.2), and were expressed under the control of CMV promoter. The cells were co-transfected with pEGFP-C1 (Clontech, Saint-Germain-en-Laye, France) to identify transfected cell. Plasmids coding for KCNQ2 and KCNQ3 subunits were transfected at various concentration ratios while the total amount of the DNA was kept constant: KCNQ2/3 1:1, KCNQ2/3 9:1 and KCNQ2/3 1:9. Experiments on transfected cells were performed between 24 h and 48 h post-transfection.

4.4.7. Patch-clamp recordings in CHO culture cells

Patch clamp recordings were performed in the whole cell configuration with an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Data were sampled with an ITC-18 interface (HEKA Elektronik, Lambrecht, Germany) governed by PatchMaster software (HEKA) via a Macintosh PowerPC (Apple Inc, Cupertino, CA, USA). Currents were low-pass filtered at 2 kHz and sampled at 5 kHz, and whole-cell capacitance was compensated in the whole-cell configuration. Patch electrodes were pulled from borosilicate glass (Sutter Instruments Company, Novato, CA, USA) to an open pipette electrode resistance of 1.5-3
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MΩ when back-filled with intracellular solution containing (in mM): 135 KCl, 2.41 CaCl₂ (0.1 µM free Ca²⁺), 3.5 MgCl₂, 5 HEPES, 5 EGTA, 2.5 Na₂ATP, pH 7.3 (with KOH), 290-295 mOsm/kg. Series resistance (Rₛ) typically was below 8 MΩ and Rₛ compensation (80-90%) was applied throughout the recordings. Access to the basolateral membrane of OHCs was achieved by gently removing surrounding tissue with a suction pipette. In transfected CHO cells the robust expression of KCNQ2 and KCNQ3 was verified by GFP fluorescence. All experiments were performed at room temperature (23°C). Membrane potentials shown were not corrected for liquid junction potentials (\(V_m = V_p - 4 \text{ mV}\); Leitner et al., 2012).

4.4.8. Voltage-clamp analysis

The protocol used during the experiments on CHO transfected cells is a series of hyperpolarizations and depolarization from -100 mV to +40 mV with an increment of 10 mV (\(V_h = -60 \text{ mV}\); see Fig. 20). By calculating the difference between the current at the beginning of each step with the current at the end (steady state) on the same step the current-voltage relationship has been obtained.

4.4.9. Reverse transcription RT-PCR assays

Total RNA was prepared from freshly dissected tissue by extraction with Trizol reagent according to the manufacturer’s instructions (RNeasy Lipid Tissue, Qiagen, Hilden, Germany). First-strand cDNA was primed with random hexamer primers (Invitrogen Life Technologies) from 0.5-1 µg of RNA and synthesized using the SuperScript II enzyme (Invitrogen) at 42°C for 50 min. Normalization was carried out against an endogenous housekeeping gene transcript for β-actin. PCR was performed in a 20-µl reaction the mixture using 0.5 U Taq polymerase (Qiagen); mixture in all cases contained 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 20 pmol of each primer using the following cycling protocol: 3 min at
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94°C; 35 cycles (25 cycles in case of β-actin): 30 s at 94°C, 1 min at 58°C, 1 min at 72°C; with a final elongation for 7 min at 72°C.

The following primers were used:

*KCNQ1 (nucleotides 556-984), accession No. NM_008434*

for: GCCACCGGGACCCTCTTCTG

rev: GATGC GGCCGGACTCGTTCA

*KCNQ2 (nucleotides 979-1500), accession No. NM_001003824*

for: CAAGTACCCTCAGACCTGGAAC

rev: CAGCTCTTTGGGCACCTTGCT

*KCNQ3 (nucleotides 1798-2265), accession No. NM_152923*

for: CCAAGGAATGAACCATATGTAGCC

rev: CAGAAGAGTCAAGATGGGCAGGAC

*KCNQ4 (nucleotides 732-1085), accession No. NM_001081142*

for: CATCGGGTTCTCTGGTGCTCATCTT

rev: TAGGCCCCGGCTTGCTCCGTG

*KCNQ5 (nucleotides 984-1183), accession No. NM_001160139*

for: CCATTGTTCTCATCGCTTCA

rev: TCCAATGTACCAGGCTGTA
-actinβ (nucleotides 333-1160), accession No. NM_007393 for: ATCTGGCACCACACCTTCTACAAT

rev: CTGCTTGCTGATCCACATCTGC

4.4.10. Immunofluorescence

Mice (23-27 days old) were deeply anaesthetized using pentobarbital (50 mg/kg body weight) and transcardially perfused with PBS, followed by an ice-cold 4% PFA/PBS for 35–40 minutes. Brains were removed, post-fixed overnight in 4% PFA/PBS and cryoprotected with 25% sucrose. Coronal sections (40 µm) were cut at the level of the VB and collected in Tris-buffered saline (TBS). After several washings with TBS, sections were blocked with 10% normal horse serum (NHS), 2% BSA, 0.3% Triton X-100 in TBS for 2 h to minimize nonspecific binding before incubation of slices with primary antibodies in 2% NHS, 2% BSA, 0.3% Triton X-100 in TBS at 4 °C for 16-18 h. The following antibodies were used: rabbit anti-KCNQ2 or rabbit anti-KCNQ3 (1:500 for both, Alomone Labs, Jerusalem, Israel) and mouse anti-NeuN (1:200, Millipore). After washing (3 x 10 min with TBS), sections were exposed to Cy2-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit IgG (1:300 for all, Dianova, Kerala, India) for 1.5 h, washed again and cover slipped with Immumount. For negative controls occlusion of the primary antibody from the staining procedure was routinely performed with no positive immunological signal detected. For additional negative controls pre-adsorption of the primary antibody using a control antigen was performed according to the manufacture’s instruction (Alomone Labs, Jerusalem, Israel).

In brief, KCNQ2 (or KCNQ3) antibody was pre-incubated at its working dilution (see above) with corresponding control peptide antigen for 1 h. The antigen-antibody complexes were spun down by centrifugation, and the resulting supernatant was used in place of the primary
antibody. No staining was obtained by incubation with the pre-adsorbed serum. Pre-adsorption procedure was specific and did not influence other primary antibody stainings.

All images were obtained using confocal laser scanning microscope (Nikon eC1 plus, Duesseldorf, Germany). Densitometric analysis of immunofluorescence was performed by using the fluorescence measuring function of ImageJ software (public domain, National Institutes of Health). One hundred square areas (30 µm × 30 µm) were placed on different positions of VB images and the mean fluorescence intensity was determined after background subtraction. This analysis was repeated for three slices taken from different animals.

4.5. In vivo experiments

4.5.1. Surgery

All animal work has been approved by local authorities (review board institution: Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; approval ID numbers: 84-02.04.2011.A177).

C57Bl6J male mice (P30) were purchased from Charles River (Germany) and group-housed for one week in the animal facility in order to allow recovery from shipping stress. After recovery period, animals were individually housed for an additional week and later underwent the surgery. The animals were anaesthetized with i.p. (intra-perithoneal) injection of pentobarbital (50mg/kg) supplemented with a subcutaneous injection of analgesic agent Carprofen (25ml/kg; Rimadyl, Pfitzer GmbH, Berlin, Germany). Additionally, 10 minutes before the start of the surgery all tissues to be incised were injected with an analgesic agent (2% xylasine, Astra Zeneca GmbH, Wedel, Germany), all pressure points were covered with 2% xylasine gel and corneas were protected with Bepanthen eye gel (Bayer, Leverkusen, Germany). Animals were mounted in the stereotaxic apparatus (ASI Instruments, Ins., Waren, MI, USA) via ear and incisor bars. The skin on the top the skull was incised, the soft tissue
covering the skull was removed and the levels of bregma and lambda were equalized in the horizontal plane. Holes in the skull were drilled bilaterally above VB complex according to stereotaxic coordinates obtained from Brain Atlas (Paxinos & Franklin, 2001) that were: 1.7 mm posterior from bregma, 1.85 mm lateral from bregma and 3.3 mm ventral from the cortical surface. The left brain hemisphere was implanted with a custom made guide canula (12 mm long, ID = 250 µm, OD = 360 µm, Polymicro Technologies, Phoenix, AZ, USA), the right hemisphere was implanted with self made microelectrode array (eight recording electrodes) coupled with a second guide canula. Implants were secured in place with dental cement and animal was allowed to recover for at least four days before a hot plate tests was performed.

4.5.2. Hot plate test

The hot plate test was performed as previously described (Kuraishi et al., 1983). The hot plate apparatus herein used differs from the ones available on the market because of an additional sliding Plexiglas platform parallel to the plate. The platform creates the floor of the testing cylinder and was designed such that placing and removal of the animal from the plate was possible without delay and without causing damage to the injection system mounted on the animal’s head. In fact, this was necessary since animals were connected, via injection canula (1m long, ID = 40 µm, OD = 150 µm, Polymicro Technologies, USA) to the Hamilton syringe for the intra-thalamic injection and via cable to the amplifier for electrophysiological recordings. Both syringe and amplifier cable were connected to a swivel in order to avoid any restriction movements. The temperature of the plate was set at 55 ± 1 °C. The latency was defined as the time elapsed from the placements of the animal on the plate until the time when the first pain behaviour was noticed. The following behaviours were considered as signs of pain: jumping, paws licking or shaking (Crockett et al., 1977). To prevent any skin damage of the animal’s paws, after a maximum of 20 s the animal was removed (cut off time). Every
animal was subjected the test twice, before and after drug injection (volume = 300 nl/site, speed 100 nl/min). The interval between the injection and the second trial depended on the administered drug and was: 60 min for retigabine (3 mM, 300 nl) and DMSO (0.1% in 0.9% NaCl); 40 min for XE991 (2 mM, 300 nl); 10 min for muscimol (0.3 nM, 300 nl) and DAMGO (1 µM, 300 nl); 30 min for β-endorphin (0.2 nM, 30 nl). The interval time corresponded to the time taken to achieve maximum analgesic effects, as has been assessed experimentally.

4.5.3. Histological verification of injection sites

All injected solutions contained the fluorescent dye Alexa-488 in order to mark the injection site. At the end of the experiment, the animals were given an overdose of isoflurane and were decapitated. The brain was quickly removed, placed in buffered 4% paraformaldehyde (PFA) for eight hours and then transferred to cryoprotective solution containing 30% sucrose for 12 h. Subsequently, 50 µm thick coronal slices containing the VB were cut, mounted on glass slides and inspected for fluorescence with 10× objective of a confocal microscope (Fluoview 300, Olympus, Hamburg, Germany). For documentation digital photographs were taken. Only animals in which the injection sites were verified to be correct in both hemispheres were considered for further statistical analysis.

4.5.4. Physiological release of β-endorphins

To assess the possible role of β-endorphin as an endogenous KCNQ channel modulator, a protocol to induce the physiological release of β-endorphin was developed. In our protocol we combined physical exercise and the ingestion of sweet palatable food because both conditions have been shown to induce β-endorphin release in mice and rats (Werme et al., 2000; Dum et al., 1983; Takeda et al., 2000; Mizushige et al., 2006). The animals underwent 7 training sessions (one/day) consisting of 20 min running in a running wheel (TSE Systems GmbH,
Material and Methods

Berlin, Germany) followed by receiving highly palatable food like chocolate milk (low fat milk with 6% chocolate powder, Nesquick, Nestlé, France) and pieces of chocolate-covered waffles (Kit-Kat, minimal chocolate content 27%, Rowntree Macintosh GmbH, Hamburg, Germany). Training was conducted in the evenings, when rodents are naturally active. For every animal, the total wheel rotations were counted and the total distance covered in 20 minutes was calculated (wheel diameter = 12 cm). At the end of the last training session (i.e. at the end of the 7th day) the animals were subjected to hot plate test and sacrificed directly after. The brains and blood of three animals were used for the quantification of β-endorphin content with the ELISA test and the brains of the other two remaining animals were used for the *ex vivo* electrophysiological recordings.

4.5.5. Enzyme Linked Immuno-Absorbent Assay (ELISA)

For the quantification of β-endorphin levels the 96 wells ELISA-EIA kit for mouse was used (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany). Immediately after the hot plate test, the animals were sacrificed, blood was collected in plastic tubes containing anti-coagulant (heparin or EDTA) and the brain was quickly dissected, collecting separately thalamus, hippocampus and cerebellum. To separate the plasma, blood was immediately centrifuged at 1600 rpm for 15 minutes at 4°C and subsequently stored at -80 °C till the day of extraction. The brain tissue was homogenised in 5ml/g lysis buffer (10 mM Tris, pH 7.0), centrifuged at 1600 rpm for 15 minutes at 4°C, the supernatant was collected and kept at -80°C till the day of extraction. The ELISA kit has a high sensitivity (Minimum Detectable Concentration = 0.15 ng/ml), a necessary criterion for our purposes, because of the small amount of tissue. The extraction of endorphins was performed as described before (Hofbauer et al., 2000) and the Absorbance (A) value for every sample was obtained using a standard reader. To convert the absorbance values in ng (nano grams), these values were plotted in a semi logarithmic sigmoid curve obtained from the concentrations of a known protein (control curve, included
in the ELISA Kit). Thus, the values empirically calculated were referred to the total amount of tissue collected before the extraction and expressed in ng/mg for the tissue and in ng/ml for the plasma.

4.5.6. Statistics

All results were presented as mean ± SEM. Statistical significance of the data with Gauss distribution was evaluated by Student’s t-test. Multiple comparisons were done by ANOVA or Repeated Mesures ANOVA. For current-clamp data, custom written MatLab routines were used to analyze AP characteristics during burst and tonic firing. OriginPro software (version 8G, OriginLab, Friedrichsdorf, Germany), Statistica (Statsoft, Hamburg, Germany) and Prism (version 5, GraphPad software, San Diego, USA) were used for data analysis and figure plotting.
5. Results

Electrophysiological characteristics of KCNQ channels in thalamocortical (TC) neurons of the ventrobasal complex (VB) were recorded using the patch clamp method in acute brain slices. Based on the recordings from 50 cells passive properties of the membrane were estimated and were: the resting membrane potential (RMP) was $-65.8 \pm 1$ mV, input resistance ($R_{in}$) = $221.9 \pm 26.6$ MΩ and capacitance ($C_s$) value was $81.7 \pm 4.5$ pF and it remained stable during the recordings. The application of retigabine and XE991 hyperpolarize and depolarize the cell, respectively, but in general, drug application did not significantly change the RMP and the $R_{in}$ values.

5.1. M-current in TC neurons

KCNQ channels are known to be activated by slow depolarizing stimuli (Brown et al., 1982). In order to assess in which conditions an outward current resembling M-current can be voked, the first step of our experiment it was the analysis of a family of outward currents. The experiment was performed in VB TC neurons, in voltage clamp mode using the protocol shown in Fig. 4. It consists of a series of slow depolarizing steps from $-65$ mV till $-25$ mV ($V_h = -85$ mV). For every voltage step the corresponding evoked current with a certain amplitude and kinetics can be observed. The recordings were performed under control conditions, in the presence of retigabine and/or XE991 to assess which was their effect on the evoked outward currents (Fig. 4A, B and C). The calculated sensitive currents shown in Fig. 4D and E revealed typical kinetics for an M-current-like at positive more positive than $-55$ mV.
In the following experiments, changes in the current amplitude were measured from VB TC neurons in voltage clamp mode using the protocol shown in Fig. 5. The membrane potential was held at -65 mV (a value resembling the RMP of a VB TC neuron), thereafter depolarized to -45 mV for 4 s and followed with hyperpolarization to -60 mV for 3 s. To verify that the M-current in VB TC neurons is mediated by KCNQ2 and KCNQ3 channels, during electrophysiological recordings a specific channel opener retigabine (30µM) as well as specific blockers XE991 (20µM) and linopirdine (10µM) were applied.

The application of retigabine facilitated the opening of the KCNQ-channels, inducing an M-current (Fig. 5A). The calculated retigabine-sensitive current (Fig. 5B, obtained by subtraction of the current recorded during retigabine application from the current recorded under control conditions) revealed well known M-current characteristics: slow activation and slow deactivation, the latter visible in the tail currents. The effect of retigabine on the current amplitude was almost completely prevented by co-administration of XE991 or linopirdine (control, 100 ± 12.8 %; retigabine, 171.2 ± 23.6 %; retigabine + XE991, 102.9 ± 16.4 %; retigabine + linopirdine, 107.3 ± 8 %, Fig. 5C). The application of XE991 alone reduced the

**Fig. 4. M-current is activated by positive voltage steps in the presence of retigabine.** The upper panel shows the evoked currents, in VC mode, in response to increasing depolarizing voltage steps from -65 mV to -25 mV, under control conditions, after application of retigabine and in retigabine + XE991 (A, B and C, respectively). The calculated sensitive currents in E and D show the M-current like kinetics at positive potential, in the presence of retigabine.
Results

Current amplitude, indicating open M-channels at RMP conditions. A competitive effect between retigabine and XE991 was observed during co-administration of both compounds while the pre-incubation with XE991 prevent channels activation (73.4 ± 18.9 %, Fig.1C; Repeated Measures ANOVA, F (4, 31) = 4.57; p = 0.0051; Newman-Keuls post-hoc test: retigabine vs. control: #p < 0.05; retigabine + XE991 and + linopirdine vs. retigabine: *p < 0.05; XE991 vs. retigabine: **p < 0.01; n = 7; Fig. 5C).

Fig. 5. Retigabine activates M-current in TC neurons. The application of retigabine (30 µM) induces M-current as shown in the example traces in A while the sensitive current reveals the slow activation and deactivation kinetics (B). The bar graph in C shows a significant increase in the current amplitude due to the application of retigabine alone compared to control conditions (retigabine vs. control: #p < 0.05). Moreover, retigabine application in combination with different M-channel blockers (XE991, 20 µM; linopirdine, 10 µM) and the application of XE991 alone induce significantly smaller effects (retigabine + XE991 and + Lino vs. retigabine: *p < 0.05; XE991 vs. retigabine **p < 0.01; n = 7).
5.2. M-channels regulate cell excitability in TC neurons

Next, electrophysiological experiments were performed in the current-clamp mode to assess that M-channels are involved in regulation of cell excitability and shape tonic and burst firing modes which are characteristic for TC neurons (Steriade et al., 1988). Tonic firing was evoked by injection of direct current steps of four different amplitudes (50, 100, 150 and 200 pA) while the cell was held at ~-60 mV. The application of retigabine during evoked tonic firing, slightly hyperpolarized the cell and significantly reduced the number of action potentials when compared to control conditions (Fig. 6C) and shifting the firing pattern to burst-like activity (Figs. 6A and B; Repeated Measures ANOVA, F (7, 71) = 2.3; *p = 0.0322; Newman-Keuls post-hoc test: *p < 0.05 vs. respective controls; n = 11/4; Fig. 6C). On the other hand, XE991 slightly depolarized the cell and augmented the number of action potentials (Figs. 7A, B) with a significant effect due to the treatment (Repeated Measures ANOVA; F (5, 41) = 9.5; p < 0.001.; Newman-Keuls post-hoc test: ns; n = 7/3, Fig. 7C).
Fig. 6. **M-channel enhancement reduces tonic firing.** In A and B example traces recorded from VB TC neurons show a firing pattern that was induced by a depolarizing pulse (200 pA of 2.5 of duration). ACSF without (A) and with retigabine (B) was used. The bar graph shows the effects of drug application on the number of action potentials during control conditions (white bars) and in the presence of retigabine (filled bars, C) during the respective current injections (*p < 0.05 vs. respective control; n = 11/4).
Results

5.3. Effect of M-channel modulation on burst firing

From more hyperpolarized potentials (> -60 mV), injection of 50 pA depolarizing steps elicited bursts of action potentials crowning a low-threshold Ca$^{2+}$ spike. The application of both retigabine and XE991 influenced the number of action potentials in a burst (Fig. 8A-D). Application of retigabine induced a decrease in the number of burst action potentials (control, 4.1 ± 0.49; retigabine, 2.2 ± 0.42; paired t-test, t = 3.6; *** p= 0.0048; n=13/3) while XE991 significantly increased that number (control, 3.3 ± 0.66; XE991, 5.2 ± 0.55; paired t-test, t = 4.8; *** p = 0.0004; n = 11/3).
Results

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5.4. Muscarinic modulation of M-channels

Since M-channels were first identified as K$^+$ channels that are sensitive to muscarinic compounds (Marrion, 1989), a set of experiments using muscarinic agonists and antagonists has been performed.
**Oxotremorine M (Oxo-M).** The muscarinic agonist oxotremorine-M (Oxo-M, 10 µM) was tested during recordings in VC mode using the protocol shown in the inset of Fig 5. Activation of muscarinic receptors (MACHR) by Oxo-M inhibited M-channels and reduced the amplitude of the retigabine-evoked current (Fig. 9; control, 100 ± 8.6 %; retigabine 147.4 ± 19.5 %; retigabine + Oxo-M, 123.7 ± 16.9 %). The application of XE991 in combination with Oxo-M completely reversed the effect of retigabine, reducing the current to control values (100.7 ± 9.9%; Repeated Measures ANOVA, F (3, 15) = 9.24; p<0.0001; Newman-Keuls post-hoc test: retigabine vs. control # p < 0.05; retigabine + Oxo-M vs. retigabine * p < 0.05; retigabine + Oxo-M + XE991 vs. retigabine ** p < 0.01; n = 6/2).

**Carbachol.** Carbachol (CChol) is a cholino-mimetic compound able to activate MACHR with less specificity than Oxo-M. The experiments in VC mode were performed as described above (see Fig. 9). The M-current evoked by application of retigabine was inhibited by the application of CChol (25µM; control, 100 ± 9 %; retigabine 154 ± 13.1 %; retigabine + CChol, 122 ± 15.9 %). The co-application of CChol and XE991 completely reverted the effect.
Results

induced by retigabine (88 ± 16.8 %; Repeated Measures ANOVA, F (3, 31) = 15.54; p < 0.0001; Newman-Keuls post-hoc test: control vs. retigabine: ###p < 0.001; retigabine + CChol vs. retigabine: **p < 0.01; retigabine + CChol + XE991 vs. retigabine: *p < 0.05; n = 8/4; Fig. 10).

Muscarinic antagonists. Next, two subtype-specific muscarinic antagonists (pirenzepine for M1 and 4-DAMP for M3, 10 µM) were tested to assess if AChR inhibition affected the M-current amplitude. Both compounds showed a weak effect when applied in the presence of retigabine (control, 100 ± 13.7 %; retigabine, 189.4 ± 21.8 %; retigabine + pirenzepine + 4-DAMP, 198 ± 23.6 %, Fig. 11A). The co-application of Oxo-M and the muscarinic antagonists slightly reduced the M-current but, the effect was not significant (186 ± 21.5 %, Fig. 11A). The application of a drug cocktail containing additionally XE991 had stronger effect (data not shown; Repeated Measures ANOVA, F (3, 30) = 36.37; p < 0.001; Newman-Keuls post-hoc test: retigabine and retigabine + pir. + 4-DAMP vs. control: ###p < 0.001; n = 11/4). No effect of the two muscarinic antagonists was observed when the compounds were applied before retigabine (control, 100 ± 17.2 %; Pir + 4-DAMP, 100.2 ± 16.2 %; retigabine +

**Fig. 10. M-current is inhibited by carbachol.** The bar graph shows the changes in current amplitude as consequence of respective drug application. The application of retigabine significantly increases the current amplitude, while co-applications with the muscarinic agonist CChol and the specific M-channel blocker XE991 significantly decrease the evoked current (regabine vs. control: ###p < 0.001; retigabine + CChol vs. retigabine: **p < 0.01; retigabine + Oxo-M + XE991 vs. retigabine: *p < 0.05; n = 6/2).
pir + 4-DAMP, 180.2 ± 28 %; Repeated Measures ANOVA, F (2, 23) = 13.36, p < 0.0001; Newman post-hoc test: Pir + 4-DAMP + retigabine vs. control: ##p < 0.001; Pir. + 4-DAMP vs. retigabine + Pir. + 4-DAMP: **p < 0.01; n = 8/2; Fig. 11B).

5.5. The reversal potential of the current induced by retigabine and mediated by KCNQ channels

In order to confirm that the current evoked by retigabine is carried by K\(^+\) ions, the current-voltage relationship (I/V) was analysed and the reversal potential of the M-current was calculated. The following ramp protocol was used: the cell was held at -35 mV, and then it was hyperpolarized rapidly, in a ramp-like manner, (10 ms) to -135 mV and subsequently repolarized to −35 mV. Only open and non de-inactivating channels can follow this protocol: holding the cell at -35 mV keeps the KCNQ channels open and the changes in the voltage induce changes in the conductance of the channels and consequently, in the mediated current. The retigabine-sensitive current was obtained by graphical substraction and the resulting I/V

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 11. Block of M1/M3 AChR prevents the effect of Oxo-M but not retigabine. The application of the two M1/M3 AChR antagonists slightly increased the retigabine-activated current (retigabine + Pir + 4-DAMP vs. control: ###p < 0.001; n = 11/4, A). The application of both muscarinic antagonists alone fails to induce an M-current, but this is successfully achieved by co-application of retigabine (Pir + 4-DAMP + retigabine vs. control: ##p < 0.001; Pir + 4-DAMP vs. retigabine + Pir + 4-DAMP: *p < 0.01; n = 8/2; B).
curve reversed at a potential of -98 ± 2 mV (~ 22 °C, n = 5; Fig. 12) which is close to $E_K$ (-103 mV under the present recording conditions).

**Fig. 12. The reversal potential of the current mediated by KCNQ channels.** A shows examples of the fast ramp protocols under control conditions (black trace), during retigabine and XE991 application (light and dark grey, respectively). The retigabine-sensitive current obtained as described before (see Material and Methods), shows a reversal potential very close to the $E_K$ (-98.2 mV, B).

### 5.6. M-channel modulation in VB TC neurons is not influenced by Ca$^{2+}$ currents

It has been shown that the activation of KCNQ channels can be modulated by changes of the intra- and extra-cellular Ca$^{2+}$ concentrations (Hernandez et al., 2008) and that retigabine has a weak effect on activation of Ca$^{2+}$ channels (Rundfeldt et al., 2000). Since the activation protocol for M-current can activate also fast-transient Ca$^{2+}$ currents (T-type Ca$^{2+}$ current), in all VC experiments, a preceding short depolarizing pulse was applied, that completely inactivated T-type Ca$^{2+}$ current (Budde et al. 1992; Fig. 13A, inset). Moreover, retigabine had no effect on the pre-pulse sensitive current (Fig. 13A). To further determine the specificity of the retigabine effect we isolated the Ca$^{2+}$ current by blocking all K$^+$ and Na$^+$ currents using an intra-cellular solution containing CsMeSO$_4$ (substitute of K$^+$ inside the cell) and with several K$^+$ and Na$^+$ channel blockers. Under these conditions, retigabine had no effect on the recorded currents indicating that retigabine modulates K$^+$ channels but not Ca$^{2+}$ currents (Fig. 13B).
Modulation of M-channels by endogenous compounds

To identify possible endogenous agonists or modulators of M-channels in the VB, a series of experiments was performed using bath application of endogenously released substances or their exogenous derivatives.

The opioid dynorphin A (Dyn A) has been shown to enhance M-current in the hippocampus (Tatulian et al., 2003). Experiments were performed in VC mode and three different concentrations of Dyn A (250 nM, 500 nM and 1 µM) were used. Only Dyn A at 1 µM concentration was able to evoke an XE991-sensitive response in 3 out of 25 neurons recorded (control, 100 ± 47.8 %; Dyn A, 118 ± 43.9 %; Dyn A + XE991, 107 ± 40.8 %, values were calculated for responding cells; Fig. 14A). However, effects were not significant.

Fig. 13. Retigabine exerts no effect on Ca$^{2+}$ currents. In the recording protocols used in this study, the VC step for the activation of M-channels is preceded by a pre-pulse which is a short depolarizing step (-45 mV for 80 ms) that inactivates transient currents. In A a magnification of the pre-pulse evoked current is shown. Current traces in the presence and absence of retigabine are shown. B shows example traces of VC recordings performed with CsMeSO$_3$ in the presence (black) and absence of retigabine (red).

5.7. Modulation of M-channels by endogenous compounds

To identify possible endogenous agonists or modulators of M-channels in the VB, a series of experiments was performed using bath application of endogenously released substances or their exogenous derivatives.
The sensitive current did not reveal the typical slow kinetics of M-current. Example traces recorded from a responding cell are shown in Fig. 14 Ba and Bb respectively. In current clamp experiments, the application of Dyn A (1 µM) increased the number of action potentials during tonic firing when a current step of 100 pA was injected into the cell (control, 9 ± 1.9; Dyn A, 21 ± 8.5) although the RMP remained stable through all the experiment (-67 ± 1 mV before and -66 ± 0.2 mV after Dyn A application; data not shown).

**Fig. 14. Dynorphin A does not modulate M-channels.** The application of dynorphin A fails to activate M-current in 22 out of 25 recorded cells. The bar graph summarizes the effect of the three responding cells during control conditions, after application of Dyn A and during co-application with XE991 (A) as shown in the example traces and the dynorphin sensitive current in Ba and Bb, respectively.

Since the κ-opioid receptor agonist Dyn A did not modulate the M-current, opioid agonists with different receptor specificity were probed in the following:

**DAMGO.** We tested an analogue of morphine DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin), a synthetic peptide with high affinity for µ- and δ–opioid receptors. In VC experiments DAMGO did not affect the outward current (n = 3/1), while in CC experiments it remarkably reduced the number of action potentials (upon injection of 100 pA current steps) compared to control conditions (n = 3/1; 9.0 ± 1.9 and 5.0 ± 3.0, respectively). The RMP was
slightly hyperpolarized compared to baseline (-67 ± 1 mV and -69 ± 1 mV, respectively; data not shown).

**Endomorphin-1.** The application of endomorphin-1 (1 µM), which specifically binds to µ-opioid receptors and is involved in arousal, induced no effect on the outward current in VC as well as in CC experiments (n=5/2; data not shown).

**Leu-Enkephalin.** The enkephalins (Leu- and Met-Enkephalin) constitute an opioid family with a high affinity for µ- and δ–opioid receptors, but not for κ–receptors. In VC recordings, the application of Leu-Enkephalin (Leu-Enk, 500 nM) augmented an M-like current in 5 out of 22 TC neurons, and the co-application of XE991 reversed the evoked effect (control, 100 ± 15.4 %; Leu-Enk, 111 ± 13.4; Leu-Enk + XE991, 96 ± 13.7; Repeated Measures ANOVA, F (2, 14) = 11.04, p = 0.005; Newman-Keuls post-hoc test: Leu-Enk vs. Control, #p < 0.05; Leu-Enk + XE991, *p < 0.01; n = 5/5; Fig. 15A). The example traces depicted in Fig. 14Ba reveals slow kinetics of the Leu-Enk sensitive current.

In CC experiments Leu-Enk increased the number of action potentials in comparison to the control, upon injection of 50, 100 and 150 pA current steps (Fig. 15C; respectively for different amplitudes of the current steps: control, 1 ± 1; 26 ± 10.6; 15 ± 9; Leu-Enk, 8 ± 4.2; 57 ± 19.6; 23 ± 14; Repeated Measures ANOVA, F (5, 27) = 3.145; p = ns; n = 6/2).
Results

β-endorphin. The endogenous β-endorphin belongs to the opioid family although it does not bind to any of the opioid receptors. It is synthesized and released in the brain as well as in the blood stream after physical activity, strong trauma, stress and pain (Dum et al., 1983; Werme et al., 2000). Because of the role it plays in pain sensation (Stanojevic et al., 2007), β-endorphin was here tested as a plausible M-channel modulator. In VB TC neurons, the application of β-endorphin (1 nM) during VC recordings augmented the outward current and the effect was reversed by co-application of XE991 (control, 100 ± 16.1 %; β-endorphin, 114 ± 16.8 %; β-endorphin + XE991, 74 ± 15 %; Repeated Measures ANOVA, F (2, 11) = 3.3; p
The positive effect of β-endorphin was recorded in 5 out of 12 neurons. In the example traces and the calculated sensitive current shown in Fig. 16Ba and Bb an M-like current kinetics can be seen.

![Graph showing the effect of β-endorphin on current amplitude](image)

**Fig. 16.** β-endorphin increases M-like current in TC neurons. β-endorphin in VB TC neurons nominally increases M-like current (A) inducing an effect that can be reversed by co-application of XE991 (p = 0.107; n = 5/3). In B the example traces (a) and the sensitive current (b) show the typical slow activation and de-activation of M-current.

**Nociceptin.** Several studies linked the peptide nociceptin to pain sensation and to the modulation of M-channels (for review see Meis et al., 2003). To assess a similar link, in VB TC neurons, nociceptin (1 µM) was applied in VC experiments. Application of nociceptin augmented the depolarization-induced current. The effect was reversed by co-application of XE991 (control, 100 ± 15.9 %; nociceptin, 119 ± 13.3 %; nociceptin + XE991, 93 ± 14 %; Repeated Measures ANOVA, F (2, 17) = 16.13, p = 0.0007; Newman-Keuls post-hoc test: nociceptin vs. control ##p < 0.01; nociceptin + XE991 vs. nociceptin **p < 0.01; n = 6/1; Fig. 17A). However, the nociceptin-sensitive component of the current (Fig. 17Bb) did not show the typical M-current kinetics (slow activation and de-activation) suggesting the contribution of other K⁺ currents. To clarify this issue, we first blocked M-channels with the specific blocker XE991 (20 µM) and then applied nociceptin, under this conditions, nociceptin still
evoked a K⁺ current increase (control, 100 ± 8.2 %; XE991, 86.1 ± 21 %; nociceptin + XE991, 101 ± 22 %; Repeated Measures ANOVA, F (2, 11) = 2.74, p = 0.142; n = 4/1; Fig. 17C). In an additional set of experiments, M-channels were activated with retigabine and subsequently nociceptin was applied. In this case, we observed the typical augmentation of the current due to retigabine and a further increase by nociceptin (control, 100 ± 10.9 %, retigabine, 138 ± 25.4 %; nociceptin + retigabine, 166 ± 36.6 %; Repeated Measures ANOVA, F (2, 11) = 2.48, p = 0.163; n = 4/1; Fig. 17D). Taken together, nociceptin activated an outward current with only little XE991-sensitivity.

**Fig. 17. Effect of nociceptin on the outward current.** Nociceptin increases the outward current (**p < 0.01), and the effect was reversed by co-application of XE991 (20 µM; ***p < 0.01; A). The same experiment has been conducted applying nociceptin in the presence of XE991 (C) and in the presence of retigabine (D), in both cases nociceptin elicits an effect on the outward current.
5.8. Novel synthetic compounds can modulate M-channels

Further experiments to prove the nature of the M-channels in TC neurons of the VB were performed using the synthetic compounds ICA27243 and maxipost (kindly provided by Dr. Dalby-Drown, Neurosearch, Copenhagen, Denmark).

ICA27243. ICA27243 (30µM) is a specific opener for heteromeric M-channels composed of KCNQ2 and KCNQ3 (Wickenden et al., 2008). Its application increased an M-like current in VC experiments and the effect could be reversed by co-application of XE991 (control, 100 ± 21.3 %; ICA25243, 115.2 ± 22.7 %; ICA25243 + XE991, 99.1 ± 20.4%; Repeated Measures ANOVA, F(2,12) = 9.39, p=0.0035; Newman-Keuls post-hoc test: ICA25243 vs. control: ##p< 0.01; ICA25243 + XE991 vs. ICA27243: **p< 0.01; n = 7/2; Fig. 18A). A further proof of the specificity of the effect induced by this substance is given by applying ICA27243 after blocking M-channels with XE991. Under these conditions, ICA27243 failed to evoke an increase of the current (Fig. 18C). Example traces and the calculated ICA27243-sensitive current showed the slow activation typical for M-current (Fig. 18Ba and Bb, respectively).
Maxipost. The molecule BMS204352, also named maxipost, is an opener preferring KCNQ4 and KCNQ5 subunits (Schroder et al., 2001; Dupuis et al., 2002). This substance therefore allowed evaluating the contribution of KCNQ4 and KCNQ5 subunits to the assembled M-channels in VB. Application of maxipost (20 µM) induced an increase of M-current and the extent of this increase was similar to the one induced by ICA27243 application. The effect was reversed by application of XE991 (control, 100 ± 10.5 %; maxipost, 117.3 ± 11.6 %; maxipost + XE991, 97.9 ± 9.6 %; Repeated Measures ANOVA, F (2, 6) = 18.09; p = 0.0029; Newman-Keuls post-hoc test: maxipost vs. control: **p<0.0; maxipost + XE991 vs. maxipost:
** Results

The example traces (Fig. 19Ba) and the sensitive current evoked by maxipost (Fig. 19Bb) show the typical M-current kinetics.

**p < 0.01; n = 4/1; Fig. 19A).** The example traces (Fig. 19Ba) and the sensitive current evoked by maxipost (Fig. 19Bb) show the typical M-current kinetics.

**Fig. 19. Maxipost positively modulates M-channels.** The application of the specific M-channel modulator maxipost increases M-current in TC VB neurons (A). The effect is reversed by co-application of XE991 (maxipost vs. control: **p<0.01 M; maxipost + XE991 vs. maxipost: **p < 0.01; n = 4/1, A). B) An example trace is shown in a and the maxipost-sensitive component of the outward current is shown in b.

5.9. Anti-inflammatory drugs modulate M-channels

Diclofenac and meclofenamic acid are non-steroid anti-inflammatory drugs used in the treatment of pain. The mechanism of action is a specific inhibition of cyclooxigenase II (Cox II) and prostaglandin synthesis (PG), involved in the inflammatory response triggered by noxious stimuli. Thus, diclofenac and meclofenamic acid exhibit analgesic and anti-inflammatory effect (Menassè et al., 1978). Additionally, they have been found to modulate KCNQ channels in primary cell cultures (Peretz et al., 2005).

**Diclofenac.** The application of diclofenac (20 µM) induced significant enhancement of M-current and this effect was sensitive to XE991 (control, 100 ± 14.6 %; diclofenac, 132.6 ± 17.5 %; diclofenac + XE991, 93.9 ± 11.9 %; Repeated Measures ANOVA, F (2, 16) = 14.8, p = 0.0002; Newman-Keuls post-hoc test: diclofenac vs. control: **p < 0.01; diclofenac + XE991 vs. diclofenac: **p < 0.001; n = 9/3; Fig. 20A). The example traces and the calculated
Results

diclofenac-sensitive current show slow activation and deactivation, typical M-current characteristics (Fig. 20Ba and Bb, respectively). Further evidence supporting the specificity of diclofenac activation of M-current is shown in Fig. 20C, when M-current is blocked by XE991, diclofenac fails to induce any increase in the evoked current amplitude.

**Fig. 20. Diclofenac augments M-current in VB TC neurons.** The bar graph in A shows the current amplitude under control conditions and in the presence of the anti-inflammatory drug diclofenac. The diclofenac effect can be reversed by prior co-application of XE991 (diclofenac vs. control: **p < 0.01; diclofenac + XE991 vs. diclofenac: ***p<0.001; n = 9/3). In C the prior application of XE991 almost prevent the diclofenac effect. The example traces (Ba) and the calculated diclofenac-sensitive current (Bb) show the slow activation and deactivation kinetics typical for M-current.

**Meclofenamic acid.** Meclofenamic acid (20µM) increased M-current to a similar extent as diclofenac. The co-application with XE991 reverted this effect further confirming specific action on M-current (control, 100 ± 13.2 %; meclofenamic acid, 125.6 ± 10.2 %;
Results

meclofenamic acid ± 7.95 %; Repeated Measures ANOVA, F (2, 6) = 11.32, p= 0.0092; Newman-Keuls post-hoc test: meclofenamic acid vs. control: ##p < 0.01; meclofenamic acid + XE991 vs. meclofenamic acid: *p < 0.05; n = 4/3; Fig. 21). However, the kinetics of activation and deactivation did not exhibit the slow time course of the M-current (Fig. 21Ba and Bb).

Fig. 21. Meclofenamic acid modulates M-channels in TC neurons. The application of the anti-inflammatory compound meclofenamic acid significantly increases the current amplitude (A), and the co-application of XE991 reversed this effect (##p < 0.01 meclofenamic acid vs. control, *p < 0.05 meclofenamic acid + XE991 vs. meclofenamic acid; n= 4/3). Example traces and the calculated meclofenamic acid-sensitive current are shown in Ba and b, respectively.)
5.10. Genetic suppression of KCNQ3 influences M-channels currents

In order to evaluate the extent to which KCNQ3 contributes to the M-current, VC experiments were performed in the VB TC neurons of mice that were deficient for one allele of KCNQ3 (KCNQ3<sup>+/−</sup>). In these heterozygous mice, retigabine- and XE991-evoked effects were much smaller compared to the results obtained from the wild type (WT) animals (control, 100 ± 8.5 %; retigabine, 123.8 ± 14.5 %; retigabine + XE991, 116 ± 13.7 %; Repeated Measures ANOVA, F (2, 16) = 5.1, p= 0.0193; Newman-Keuls post-hoc test: retigabine vs. control: *p< 0.05; n = 9/2; Fig. 22A). Amplitudes and kinetics of the evoked current are depicted in Fig. 22Ba and Bb. Moreover, the amplitudes of the retigabine-sensitive currents measured in KCNQ3<sup>+/−</sup> and WT mice were significantly different (retigabine WT + 62.8 ± 9.2 %; retigabine KCNQ3<sup>+/−</sup>, 23.8 ± 14.5 %; unpaired t-test; t = 2.4, *p = 0.0271; n = 13/4 and n = 9/2 respectively; Fig. 22C).

Table 1. Summary of the characteristics of the M-current in VB TC neurons, including results of the pharmacological modulation.

<table>
<thead>
<tr>
<th>property</th>
<th>specification</th>
</tr>
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<tbody>
<tr>
<td>voltage dependency</td>
<td>subthreshold activation no inactivation</td>
</tr>
<tr>
<td>kinetics</td>
<td>slow activation slow deactivation</td>
</tr>
<tr>
<td>exogenous activator</td>
<td>retigabine diclofenac ICA27243 maxipost</td>
</tr>
<tr>
<td>endogenous activator</td>
<td>β-endorphin ?</td>
</tr>
<tr>
<td>exogenous blocker</td>
<td>XE991 linopirdine muscarinic agonists</td>
</tr>
</tbody>
</table>
5.11. Different roles of M-channel subunits in modulation of the M-current

The findings from KCNQ3+/− mice and evidence from previous studies show that differences in the current amplitude may be caused by different subunit composition of the channel. This warranted further experiments which were performed in collaboration with the Institute of Physiology of the Philipps University Marburg (Head of the department: Prof. Dr. Oliver).

We used CHO heterologous expression system to co-express KCNQ2 and KCNQ3 in three different concentration ratios of the transfection vector: KCNQ2/3 1:1, 9:1 and 1:9. Expressed channels were challenged by a series of hyperpolarizing and depolarizing voltage steps (from...
Results

-100 mV to +40 mV in 20 mV increment) while the cell was held at -60 mV (see inset of Fig. 23). Figs. 23A, B and C show a clear gradation in current amplitudes: KCNQ2/3 1:1 > KCNQ2/3 9:1 > KCNQ2/3 1:9. Furthermore, the current voltage relationship (I/V plot) obtained from cells with different ratios of KCNQ2 and KCNQ3 is shown in Fig. 23D, show typical voltage activation.

To further assess the properties of monomeric channels, the CHO cells were transfected with the same concentration of either KCNQ2 or KCNQ3. By this, we were able to estimate the individual contribution of KCNQ2 and KCNQ3 to the current amplitude, at high and at low concentrations (data not shown).

Fig. 23. M-channels expression in CHO cells. The expression of different ratios of KCNQ2 to KCNQ3 in CHO cells leads to differences in current amplitude in the following gradation: KCNQ2/3 1:1 > KCNQ2/3 9:1 > KCNQ2/3 1:9, as shown in example traces A, B and C. The I/V plot of KCNQ2/3 1:1 shows the typical voltage activation (D).
5.12. The expression of KCNQ2 and KCNQ3 in VB is age-dependent

PCR analysis was conducted in collaboration with Dr. Kanyshkova from Physiology I, University of Muenster, to check if KCNQ2 and KCNQ3 mRNAs are present in thalamic VB. Surprisingly, the analysis revealed also the mRNA expression of KCNQ4 (Fig. 24A). Then, additional immunohistochemical (IHC) stainings were performed to analyse the protein expression using specific antibodies against KCNQ2, KCNQ3 and KCNQ4 (Fig. 24B), and, while strong signal was observed for KCNQ2 and KCNQ3, no protein expression was found for KCNQ4. Moreover, further investigations, performed with two different secondary antibodies for KCNQ2 and KCNQ3, showed that they are co-expressed and co-localize in the membrane of VB TC neurons (Fig. 24C). The IHC staining has been performed at four postnatal ages (p10; p20; p30 and p90). Our staining revealed that KCNQ2 is already expressed at p10. However, the highest level of fluorescence is reached at p20 and this level of expression is maintained throughout the adulthood (Fig. 25). An opposite trend could be observed for KCNQ3, which is highly expressed in juvenile mice (p20 and p30), but not in adulthood (Fig. 26).
Fig. 24. Expression of KCNQ channels in the VB nucleus of thalamus. A shows the mRNA expression of KCNQ channel isoforms in thalamic VB nucleus. The bands corresponding to KCNQ2, KCNQ3 and KCNQ4 are very strong, whereas KCNQ1 and KCNQ5 are not detected. B shows the protein expression of KCNQ2 and KCNQ3 (but not KCNQ4) in mouse VB complex. NeuN is a specific marker for neuronal cells (green) and KCNQ2/3 channels are labelled in red. In the bottom row KCNQ4 is labelled in green and DAPI (blue) is used as cell marker (scale bar = 20 µm). C shows the co-expression of KCNQ2 and KCNQ3 in VB. All stainings were performed in mice at P20.
Fig. 25. Age profile of KCNQ2 channel expression in the VB nucleus of the thalamus. A, B, C and D show the expression of KCNQ2 channels in VB nucleus of the thalamus at different ages. The expression of this channel is strong at early post-natal stages. A developmental down-regulation of the channel can be observed with increasing age. In the panel at the bottom, a double staining with the dendritic marker is shown when the expression of KCNQ2 is maximal, at p20.
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5.13. In vivo pharmacological modulation of KCNQ channels

The characterization in vitro of the KCNQ channels, performed in the VB TC neurons, provided information concerning KCNQ channel expression, their subunit composition and

Fig. 26. Age profile of KCNQ3 channel expression in the VB nucleus of thalamus. A, B, C and D show the expression of KCNQ3 channel in thalamus VB at different ages. The expression of this channel is strong at the beginning of the adulthood. A developmental up-regulation of the channel can be observed with increasing age. In the panel at the bottom, a double staining with the dendritic marker is shown at p20.
their sensitivity to given substances. However, the physiological role of KCNQ channels in the thalamus remains unclear. Several lines of evidence showed involvement of both VB complex and KCNQ channels in pain sensation modulation (Pozza et al. 2010; Linley et al., 2008). The following in vivo study was designed in order to explore the contribution of KCNQ channels to thalamic sensory functions, in particular their involvement in discrimination and processing of thermal noxious stimuli. In collaboration with Dr. Hanna Szkudlarek (Institut of Physiology I, University of Muenster), we performed hot plate test in freely behaving animals, which were implanted with intra-thalamic guide canulas, for local injections of substances (see Material and Methods). The hot plate test is an acute pain test using thermal pain stimuli evoked by heating a base plate to 55 ± 1°C. The latency to the appearance of pain related behaviours is then measured (jumping, licking, shaking of the fore-or back-paws are considered as pain-related behaviours; Crockett et al., 1976; Kuraishi et al., 1983). The graph in Fig. 27 shows that the intra-thalamic injection of retigabine significantly increases the latency in the hot plate test compared to the latency observed during the control trials (300 nl, 3 mM in 0.1 % DMSO in 0.9 % NaCl; control, 6.7 ± 0.3 s; retigabine, 11.5 ± 0.3 s). The intra-thalamic injection of the specific KCNQ channel blocker XE991 significantly decreases the latency (300 nl, 2 mM in 0.9 % NaCl; control, 6.4 ± 0.4 s; XE991, 4.0 ± 0.3 s). Moreover, the co-injection of XE991 and retigabine (300 nl, 2 mM and 3 mM, respectively) induced a slight change in the latency, which was not statistically significant (control, 5.5 ± 0.4 s; retigabine + XE991, 7.1 ± 0.7 s). Similarly, the injection of the vehicle (0.1% DMSO in 0.9 % NaCl) causes no significant change in the observed latency (control, 7.2 ± 0.4 s; DMSO, 6.2 ± 0.4 s; Repeated Measures ANOVA; F (7, 48) = 26.38; p<0.001; Newman-Keuls post-hoc test: retigabine and XE991 vs. respective control: *** p < 0.001; retigabine vs. DMSO, XE991 and retigabine + XE991: p < 0.001; XE991 vs. DMSO, retigabine + XE991: p < 0.001; n = 7; Fig. 27).
The animals were subjected to the hot plate test twice (control and post-injection trial). To exclude the possibility that learning or conditioning mechanisms influenced the second performance, we performed the test in naïve, retigabine-injected animals which had not experienced the control trial. Importantly, the observed latencies to pain in these naïve, retigabine-injected animals were not different from the latencies observed in retigabine-injected animals that had first been subjected to a control trial (naïve + ret, 10 ± 0.6 s; retigabine preceded by control, 11.5 ± 0.3 s).

All the compounds were co-injected with the fluorescent dye Alexa 488 to track and verify the injections sites using a fluorescent microscope. Only animals in which both injections site were within the VB complex were taken for the statistical analysis, Fig. 28 shows two example of the histological verification: a correct and a non correct one (A and B, respectively). The histological evaluation of the animals used for the experiments presented in Fig. 27 is summarize in the scheme in Fig. 28C and D where different color codes correspond to the different injected compounds.

**Fig. 27. M-channel modulation influences the pain response in mice.** The latency to pain-related behaviour in the hot plate test increased after intra-thalamic injection of retigabine. The injection of XE991 alone decreases this latency and co-injection of the two compounds does not significantly change latency, nor does injection of the vehicle DMSO (***p < 0.001 retigabine and XE991 vs. respective control; n = 7).
Results

5.14. Retigabine is not metabolized within 24 h in the mouse brain

Previous reports suggest that retigabine is not fully metabolized within 24 h post-injection (Rundfeldt et al., 2000). To assess this possibility, we tested the animals 24 h after the first trial. Latency values (10.5 ± 0.3 s) were still increased, suggesting that retigabine is active after 24 h following the injection. Additionally, the effect of the co-injection was the same, either when XE991 was injected 24h after retigabine or co-injected simultaneously, the same day (retigabine day 1, 11.9 ± 2.8 s; XE991 injected one day afterwards, 7.6 ± 1.2 s; n = 5).

Fig. 28. Histological verification of the injection site. To track and verify the injection site, the fluorescent indicator Alexa 488 was co-injected with all substances. Fluorescence microscopy also allowed to estimate the spatial distribution of the injected volume. A) example photomicrograph of injection site after preparation of coronal slices. Green fluorescence shows the spatial spread of the injected fluid (dashed line). The injection site was within the borders of VB. B) The injection site is not within the VB. In C and D is shown a schematic representation of all the verified injection sites within VB, for the difference injected compounds. Note the orientation legend: d (dorsal), v (ventral), m (medial), l (lateral).
5.15. Effect of a combined intra-thalamic and intra-peritoneal modulation on pain behaviour

Next, we tested if the analgesic effects observed in animals after intra-thalamic injections of the different substances were comparable to the effects induced by a systemic administration of the same substances. In addition, we aimed to understand the role of thalamic KCNQ channels in pain processing performing simultaneously intra-thalamic and intra-peritoneal (i.p.) injection of substance evoking the opposite effect (opener and blocker).

In a first series of experiments, the animals were tested under control conditions and then, simultaneously injected with retigabine applied i.p (0.5 mg/ml) and with XE991 applied intra-thalamically (2 mM, 300 nl). This manipulation, did not cause changes in the latency (control, 7.2 ± 0.7 s; after injections, 7.6 ± 1.8 s; paired t-test, t = 0.23; p = 0.83, n = 4). In one animal of this group, the XE991 injection site was not located in the VB region and a big increase in the latency was observed, probably reflecting only the retigabine effect (latency = 20.3 s).

In a second series of animals, XE991 (2 mg/kg) was injected i.p. and retigabine (3 mM, 300 nl) was injected intra-thalamically. Under these conditions, no significant change in the latency occurred (control, 8.9 ± 0.1 s; after injections, 9.6 ± 4 s; n = 2). The sole i.p. injection of XE991 was able to decrease the latency compared to the control trial (control, 5.3 s; XE991, 3.3 s).

Control experiments showed that the intra-thalamic injection of DMSO (0.1 % in 0.9 % NaCl, 300 nl) does not affect the action of an i.p. injection of retigabine on hot plate pain behaviour latencies (control, 6 ± 0.6 s; retigabine, 15.3 ± 2 s; paired t-test, t = 6.4, p = 0.02; n = 3).

5.16. The analgesic effect of retigabine in comparison to known analgesic drugs

In the following, two well known analgesic compounds were used: the µ-opioid receptor agonist DAMGO (1 µM, 300 nl, in 0.9 % NaCl) and the GABAergic agonist muscimol (0.3 nM, 300 nl, in 0.9 % NaCl). Both compounds affectively increased the latency in the hot plate
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test (retigabine, 11.5 ± 0.3 s; DAMGO, 15.8 ± 1.3 s; muscimol, 13.3 ± 1.61 s; one way
ANOVA, F(5, 29) = 22.13, p < 0.0001; Tukey post hoc test: DAMGO, muscimol and
retigabine vs. respective controls: p < 0.001; retigabine vs. DAMGO: p < 0.01; n = 4, 6, 7;
Fig. 29).

![Graph showing latency in s for retigabine, DAMGO, and muscimol](image)

**Fig. 29. Comparison of the effect of retigabine on pain threshold to the effect of other analgesic compounds.** The intra-thalamic injection of the analgesic compounds DAMGO and muscimol (an opioid-like compound and GABAergic agonist, respectively) significantly increase the latency on the hot plate test compared to respective controls (DAMGO, muscimol and retigabine vs. respective controls: ***p < 0.001). The effects of retigabine and muscimol are similar whereas the effect of DAMGO is significantly larger compared to the retigabine-induced effect (p < 0.01).

### 5.17. β-endorphin can modulate pain response

Our *in vitro* patch-clamp experiments suggested β-endorphin as a possible endogenous modulator of KCNQ channels. Therefore we tested this opioid-like compound in freely behaving animals. The intra-thalamic injection of β-endorphin increased the latency in the hot plate test (control, 7.4 ± 0.3 s; β-endorphin, 10.2 ± 0.7 s; n = 10) and that effect was partially reversed by co-application with XE991 (control, 9.1 ± 1.3 s; β-endorphin + XE991, 6.5 ± 1.6 s; n = 2; One way ANOVA, F (3, 23) = 4.67, p = 0.0124; Tukey post hoc test: β-endorphin vs. control: *p < 0.05; Fig. 30). Nevertheless, this finding does not necessarily imply an
involvement of KCNQ channels since, β-endorphin could induce the effect via activation of other channels.

\[
\text{Fig. 30. β-endorphin modulates pain response in the hot plate test. The intra-thalamic injection of β-endorphin induces an increase in the latency compared to the control response, while the co-injection of β-endorphin and XE991 decreases the latency.}
\]

**5.18. Physiological release of β-endorphin does not affect KCNQ channel behaviour**

β-endorphin plays a role in the modulation of pain sensation (present study, Bach, 1997; Tseng, 2001). In the following experiments we aimed to assess the effect of endogenous β-endorphin production on pain sensation in vivo and M-current amplitudes ex vivo. Therefore, we developed a protocol for triggering a physiological release of β-endorphin. The protocol included a 7-day training consisting of 20 minutes daily running in a running wheel, followed by enriched dietary supplement composed of milk and chocolate. Both procedures are known to induce the release of β-endorphin in mice (Dum et al., 1983). At the end of the training week, the animals were tested in the hot plate test. Analysis of the obtained results revealed no change in the latency (7.5 ± 1.4 s; n = 6). In line with this result, no changes in M-current properties were found during ex vivo recordings performed in VC mode (data not shown). Despite the lack of effect on the latency and on the M-current amplitude, we could show in the Enzyme Linked-Immunooabsorbent Assay (ELISA) that physical exercise combined with
especially palatable food induced an increase of endogenous β-endorphin levels in the plasma but not in the thalamic tissue (Table 2).

<table>
<thead>
<tr>
<th>tissue</th>
<th>ng/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalamus control</td>
<td>0.85 ± 0.41</td>
</tr>
<tr>
<td>Thalamus runners</td>
<td>0.30 ± 0.14</td>
</tr>
<tr>
<td>Plasma control</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>Plasma runners</td>
<td>0.67 ± 0.31</td>
</tr>
</tbody>
</table>

Table 2. The concentration of β-endorphin in thalamus and in the blood plasma in control animals compared to trained animals (“runners”). A nominal increase in the β-endorphin concentration could be observed in the “runners” group compared to control in the plasma but not in thalamus (control; n = 2, runners: n = 4).
6. Discussion

For decades, the assumption that KCNQ channels are not functionally relevant for thalamic cell functioning was generally accepted among researchers. The main reason for this consideration was related to the lack of spike frequency adaptation in TC neurons, a hallmark of neurons with functional M-current (Adams and Brown, 1982; Jentsch, 2000). However, the work presented here disproves this generally accepted assumption. For the first time, consistent evidence for the mRNA and protein expression of KCNQ channels as well as functional M-current in thalamic VB neurons is presented. We show that these channels play an important role in regulating cell excitability. Moreover, they are able to regulate changes in the thalamic firing pattern during tonic and burst firing mode. In in vivo experiments, we demonstrate that the modulation of KCNQ channels in VB neurons is important for the regulation of pain sensation. The findings presented herein provide a new perspective on pain information processing in the thalamic VB, and we suggest KCNQ channels in the thalamus as new possible candidate in pain therapy.

6.1. KCNQ channels are expressed and functionally active in thalamus VB

KCNQ channels have been identified and characterized in PNS as well as in many CNS regions. Evidence for KCNQ2 (immunoreactivity) and for KCNQ3 (in situ hybridization) expression has been detected in hippocampus, cortex and thalamus (in the latter, mainly in the NRT and in the somato-sensory thalamus; Cooper et al., 2001; Geiger et al., 2006; Saganich et al., 2001). Our results corroborate these findings. We observed clear signals for KCNQ2 and KCNQ3 mRNA and protein expression in PCR studies and IHC experiments, respectively. Despite previous expression studies, revealing clear signals of KCNQ channel expression in the thalamus, rather little evidence for a possible functional role of these channels in this brain region has been presented yet. In this respect, the expression of KCNQ2 channels in the NRT, a region controlling thalamic oscillation, was described and the authors
speculated about the possibility of depolarizing NRT cells by inhibition of KCNQ channels (Cooper et al., 2001; Kasten et al., 2007).

The result obtained in the course of this study verified protein expression of KCNQ channels. In order to assess their functional role in the thalamus, we performed an electrophysiological characterization of these channels by application of the KCNQ channel opener retigabine, which is considered one of the most effective and specific, among the openers, for the subunits 2-5 (the lack of effect on Ca\(^{2+}\) currents corroborates the specificity of retigabine, see Fig. 13; see also Tatulian and Brown, 2003; Gunthorpe et al., 2012), and by application of the specific blocker XE991 (Rivera-Arconada et al., 2005). In the present study, the application of the specific blocker was a constant procedure. In fact, the application of the opener supplies information about the presence of the channels in the region of interest, while the application of the specific blocker, in combination with the opener or alone, prior the opener application, allows assessing the specificity of the drug and, therefore, the specificity of our experimental approach.

In VC experiments, the application of retigabine evokes a non-inactivating low-threshold K\(^{+}\) current (see Figs. 4B and 5A). The retigabine-sensitive component of the current show typical kinetics of an M-current (see Fig. 5B): slow activation and de-activation and no-discernible inactivation as reviewed by Brown and colleagues (2009). On the other hand, the application of the blocker XE991 during control conditions decreased a current with very similar properties, although to a smaller extent in comparison to the retigabine-activated current (see Fig. 5C). These voltage-clamp experiments demonstrated that M-current is present in the VB nucleus. Nevertheless, at RMP, its presence is less obvious, because only a small number of the channels is active at this potential, as shown by the small effect induced by the blocker alone, during control conditions (see Fig. 5). This finding is fully supported by the literature which reports a similar small effect of M-current blockers at RMP values in cell
cultures and hippocampal neurons (Tatulian et al., 2001; Otto et al., 2002; Yue and Yaari, 2004). In the same studies, the authors showed that M-current activation influenced neuronal firing properties by reducing the neuronal subthreshold excitability, thus, facilitating the rise of one or more action potentials, triggered by a small depolarization. Our results, obtained from current clamp experiments, suggest a similar effect on the firing properties of TC neurons. The application of the specific activator retigabine, causes a reduced number of stimulus-evoked action potentials. On the other hand, the application of the blocker XE991 causes an increased number of stimulus-evoked action potentials thereby affecting the tonic firing mode of TC neurons (see Figs 6 and 7).

The modulation of KCNQ channels in TC neurons also affects the burst firing mode in a significant way when it is elicited by injection of a short depolarizing pulse from hyperpolarized membrane potentials (see Fig. 8). The application of retigabine reduces the number of Na$^+$ dependent action potentials crowning the Ca$^{2+}$ spike, while the application of XE991 increases this number. This is the first evidence of M-current induced effects on tonic and burst firing in VB TC neurons. Previous studies in LD TC neurons (Kasten et al., 2007) and in hippocampal neurons (Yue and Yaari, 2004) already showed that retigabine attenuates high frequency or burst firing evoked by prolonged depolarizing stimuli. It was assumed that this effect was related to a modulation of KCNQ channels by two mechanisms involved in of the action potential rise: a facilitation of the afterdepolarization phase after XE991 application (when the channels are closed) and a prolongation of the middle after hyperpolarization (mAHP) phase, observed after retigabine application, with the latter reducing the maximal firing rate (Storm, 1989; Gunthorpe et al., 2012).

Taken together, we can conclude that the expression of KCNQ channels in the thalamus has an important functional impact on the excitability of TC neurons. As with cortical and hippocampal neurons, M-current hyperpolarizes TC neurons in response to a depolarizing stimulus and tunes the neurons’s response (Hetka et al., 1999; Kasten et al., 2007). In fact,
this physiological role was described as “neuronal brake” from Brown (1982) and its removal “tunes up” the neuron leading to an alteration in the firing properties (Brown et al., 1982). Such a functionally important control mechanism serves a different role in the thalamus, where the intrinsic properties and peculiar anatomical connections of TC neurons contribute to the occurrence and maintenance of the two firing patterns. In fact, the change in their firing rate code is responsible of the typical TC neuron relay function that consists in discriminating information (somato-sensory and motoric informations), eventually conveyed to the cortex or to other high brain regions.

M-current was originally described as muscarine-sensitive current (Brown, 1982; Adams and Brown, 1982), and in the present study, muscarinic modulation of KCNQ channels has been demonstrated using the muscarinic agonists oxotremorine-M and carbachol, which inhibit M-current following previous current activation by retigabine (see Figs. 9 and 10). Muscarinic agonists modulate KCNQ channels via activation of second messenger cascades (Selyanko et al., 1992). In line with this, the application of the specific blocker XE991 exerts a stronger effect, most likely via a direct blockade, compared to Oxo-M or carbachol, that do not directly bind to the KCNQ channels. The release of acetylcholine in the thalamus from the brainstem ascending fibres directly regulates the shift between the two firing modes, promoting the tonic firing when the M1 and M3 muscarinic receptors are activated (McCormick., 1993; Steriade et al., 1993; Bista et al., 2012) and bursting when M2 receptors are activated (McCormick and Prince, 1986). This supports our hypothesis of KCNQ channels being important modulators in the somato-sensory system. In this respect, it is important to point out that KCNQ channels are involved in regulation of hippocampal oscillation during sleep and thus, they are inhibited during wakefulness, this evidence supports the role of these channels in regulating TC excitability and their firing modes (Hu et al., 2009).
6.2. Endogenous modulation of M-channels

The present work has demonstrated that KCNQ channels are expressed and conduct the M-current in thalamic neurons of the VB. Given the rather low basal activity in VB neurons and the inhibition of KCNQ channels by transmitters of the ascending brainstem system, the question arising is under which conditions these channels exert a strong functional impact. These questions may be answered by the identification of endogenous compounds that are able to activate the otherwise masked M-current in VB neurons. Previous studies identified a multitude of endogenous compounds active in pain sensation, which modulate M-channels in different brain regions. Thalamic VB neurons were already shown to be sensitive to opioid and opioid-like substances (Benoist et al., 1983; Okuyama et al., 1985), therefore, we here concentrated on those substances that have been associated with central pain procession.

Nociceptin is an opioid-like compound binding to a specific opioid-like receptor (OLR; Meis et al., 2003) and Leu-enkephalin is an opioid that binds to μ- and δ-opioid receptors (Moore et al., 1994). Both compounds were shown to enhance a K$^+$ current (M-current was among them) in the hippocampus in vitro (Madamba et al. 1999; Meis et al., 2003; Inyushkin, 2005). In voltage-clamp recordings from VB neurons shown in this study, leu-enkephalin and nociceptin activated an outward current sensitive to XE991 that cannot be considered an M-current because of the different kinetics showed by the sensitive components of the current (see Figs. 15 and 17). Similar results were obtained from the application of dynorphin A and β-endorphin (see Figs. 14 and 16), only some of the TC neurons responded to substance application in a way that resembled M-current. This set of experiments revealed a kind of unspecific effect of XE991 application, which is decreasing evoked outward currents which either do not show M-current kinetics or only resemble them. Such effect could be explained by taking into consideration that the activation of the opioid receptors may influence M-current kinetics or that we observed due different effects: the increment in the outward current due to additional K$^+$ currents, probably conducted by inward-rectifier potassium channels.
(Meis et al., 2003; Amano et al., 2000) and the XE991 effect on the basal M-current. Many factors could contribute to this result, and further investigation will be necessary.

Although an endogenous compound for KCNQ channel activation could not be identified, an endogenous compound that is known to inhibit the M-current is acetylcholine (ACh). Cholinergic projections from the brainstem massively innervate thalamic cells. Thereby, they influence the regulation of sleep and wakefulness, memory and sensory cognition (Steriade et al., 1988). More recently, a number of studies have shown a strong participation of muscarinic and nicotinic ACh receptors in pain modulation at the peripheral level (Ueda et al., 2011; Motta et al., 2011). However, their role in the central nervous system is still unclear. The presence of KNCQ channels in the thalamus and their sensitivity to the muscarinic receptor activation could play a role in somato-sensory discrimination at the central level.

6.3. **Exogenous modulation of M-channels in the mouse VB**

6.3.1. Specific KCNQ modulators regulate M-channels

Based on the inconclusive findings discussed in the literature above and the ambiguous results observed after the application of β-endorphin and dynorphin A (discussed in the previous paragraph), the next step was to investigate to which extent this lack of clarity may depend on differences in the channel subunit composition in different brain areas. Therefore, we tried to assess the channel subunit expression by applying selective agonists of different KCNQ subunits. A large number of different and specific synthetic modulators of M-current are known (Wulff et al., 2009). None of these compounds have yet been tested on VB neurons. The effects observed for ICA27243, a selective enhancer of KCNQ2 and KCNQ3 heteromeric channels, and maxipost, specific activator of KCNQ4 and KCNQ5 subunits, in VB, are similar to previous data obtained in the presence of retigabine (Dalby-Brown et al., 2005). Although the magnitude of the effect is smaller for ICA27243 and maxipost, the sensitive components of the current clearly reveal slow activation and deactivation (see Figs. 18 and
These features are typical M-current kinetics, which suggests that M-current of thalamic VB neurons is mediated by both hetero- and/or homomeric channels composed of KCNQ2/3/4. Similar results were already published for striatum-neurons where the effects of ICA27243 and maxipost were weaker compared to retigabine, but both compounds have previously been considered good tools to distinguish between the different channel subtypes (Blom et al., 2010). Indeed, in this study they were very useful to assess from the electrophysiological point of view, that the heteromeric channel formed by KCNQ2 and KCNQ3, is abundant and functioning in VB, supporting our IHC findings on the protein expression level. Additionally, we can conclude that KCNQ4 and maybe KCNQ5 give some contribution to the M-current, as suggested by the effect of maxipost.

6.3.2. Specific analgesic and anti-inflammatory compounds regulate M-channels

The hypothesised role of M-channels in regulating the pain sensation still remains unclear. So far, there are only a few hints pointing to a participation of KCNQ channels in acute, chronic and inflammatory pain sensation (Blackburn-Munro et al., 2003, 2005; Dost et al., 2003; Takeda et al., 2011). Many of the novel synthesised KCNQ channel modulators (ICA27243, maxipost; Leventhal et al., 2007; Mark et al., 2007), as well as some already known compounds (diclofenac, meclofenamic acid) showed analgesic effects in different pain and inflammation animal models (Wulff et al. 2009). The ability of diclofenac and meclofenamic acid to enhance M-channels has been demonstrated before (Peretz et al., 2005). Here, both compounds are tested for the first time in the VB. Indeed, diclofenac and meclofenamic acid specifically modulate KCNQ2 and KCNQ3 channels. Their effect, in particular the one induced by diclofenac, is very strong and specific (see Figs. 20, 21). This was confirmed by blocking KCNQ channels before diclofenac application thereby preventing all M-channel activation. The possibility that other K⁺ currents, such as Iₐ and Iₖ, could contribute to this
effect, was also excluded by experiments in which both currents were blocked by 4-AP and TEA in concentrations not influencing M-current (see Fig. 20C).

Considering all the findings in vitro, it is concluded that KCNQ channels in the thalamus are expressed both as homo- and heteromeric channels and can be activated by analgesic drugs. This supports the presumed role of thalamic M-channels in central pain sensation. The tested compounds can overcome the physiologic inhibition of M-channels (Linley et al., 2012). Consequently, these channels might act as targets for a future therapeutic strategy in pain and inflammatory pain treatment.

6.4. KCNQ channels modulate acute pain sensation in freely behaving mice

To further confirm our hypothesis and to understand if channel activation or inhibition is required for pain sensation in VB, the in vitro findings were translated into an in vivo model. The experiments were designed according to previous findings showing that the injection of opioid-like compounds into the VB induce relief and decrease in pain related behaviour (Porro et al., 2003; Potes et al., 2006; Pozza et al., 2010). To investigate the effect of KCNQ channel modulation in freely behaving animals, the hot plate test was used (55 °C) as cute pain model assay. Using a small Hamilton syringe, compounds were injected into the VB via a guide canula. The animals were then tested twice, i.e. before and after intra-thalamic injection of the substance, and the latency was defined as the time elapsed from the placement of the animal onto the plate until the time when the first pain behaviour was noticed (see Material and Methods). The experiment showed a clear effect of retigabine and XE991 on the latency to pain behaviour during the hot plate test: an increase in the case of retigabine and a decrease in the case of XE991 (see Fig. 27). XE991 partially or fully antagonised the analgesic effect of retigabine in animals injected with both XE991 and retigabine (see Fig. 27, see also Dost et al. 2004). It is concluded that KCNQ channels play an important role in modulating thermal acute pain discrimination. This role of KCNQ channels is neither well
known nor consistently documented in the literature; most of the studies focus on their role in neuropathic pain (Rivera-Arconada et al., 2009; Rose et al., 2011). Indeed, the effect of a pharmacological modulation of KCNQ channels on acute pain seems to be comparable to the effects of well known analgesic compounds, such as DAMGO and muscimol, after intrathalamic injection (see Fig. 29), supporting the findings in vitro and the role of these channels as possible therapeutic targets in pain therapy.

6.5. The activation of the relay VB complex is required for pain sensation mediated by M-channels

The VB complex is considered as the relay nucleus for somato-sensory information, such as nociceptive, tactile and kinaesthetic input, and couples the periphery to the cortex (Groenewegen and Witter, 2004). Because of that, KCNQ modulators were injected in other brain regions than the VB complex as negative control experiments (see Fig. 28). In those cases, there was no effect on the latency to pain behaviour, showing that the modulation of pain sensation via KCNQ channels is a VB-specific effect. As an additional control, simultaneous systemic and thalamic injections of retigabine and XE991 were performed in order to compare the effects induced by the two compounds in the periphery and at a central level, respectively. During the hot plate test, the injection of retigabine into the thalamus was antagonized by a systemic injection of XE991, and vice versa. However, no antagonism is observed when the injection site in the brain was not the VB. This suggests that there is a specific involvement of the VB complex in the modulation of pain perception, and that such a modulation requires the activation of KCNQ channels. Moreover, it shows that the drugs, when directly injected into the brain region of interest, remain largely confined into the injection site.

Although these experiments depict a very clear scenario including VB neurons, pain sensation and KCNQ channels, many aspects of this regulation remain unknown. The experimental
setup applied in this study does not enable to discriminate between changes in pain threshold or in sensation per se. Both will evoke an increased latency. Additional experiments will be necessary to test compound effects in vivo in KCNQ2 and/or KCNQ3 deficient mice. Alternatively, the same hot plate test can be performed before and after retigabine injection, starting at a temperature that does not cause pain. By gradually increasing the temperature the threshold can be assessed and any shift will be registered.

Based on the data presented herein, these future experiments would further corroborate the hypothesis that KCNQ channels are viable targets for acute pain treatment.

Taken together, the findings from the experiments in vitro and in vivo shed light on the functional role of M-channels in the thalamic VB complex. We showed that KCNQ channel activity influences tonic and burst firing of thalamic VB neurons. The increased burst activity after the activation of KCNQ channels and the consequent appearance of the anti-nociceptive effect upon thermal stimulation suggest that M-current affects pain sensation as well as the information relay of the VB complex to the cortex, normally associated to the tonic activity.

### 6.6. A possible functional meaning for a difference in the channel subunit composition

The IHC staining (see Figs. 25 and 26) shows a clear trend for the expression of the subunits KCNQ2 and KCNQ3, respectively up- and down-regulated during the pre-weaning period and expressed at more stable levels during adulthood (after-weaning period). This subtype-specific profile of development has been considered physiologic both in humans and rodents (Tinel et al., 1998; Kanaumi et al., 2008), and any alterations at the expression level was associated with the onset of more or less severe forms of epilepsy (Pena et al., 2006). The expression of KCNQ2 and KCNQ3 in the CHO heterologous expression system clearly showed that a change in subunit composition leads to alterations in the current amplitude (see Fig. 23). These findings are in line with previous studies that associated the differences in
current amplitudes to differences in subunit composition which, in VB, appear to be age-
dependent (Cooper et al., 2001; Hadley et al., 2003). Differences in the current amplitude
could depend on differences in the expression of KCNQ genes. In fact, this can produce
different splice variants and consequently, different KCNQ2 subunits can combine with
KCNQ3 to form the channel, as suggested by Pan and colleagues (2001).

The role of different subunit composition potentially mediating different current amplitudes
gives rise to speculations. Future experiments could clarify their role in somato-sensory
perception processing such as learning and motor perception (Markus et al., 1987), circadian
rhythms (Tamasy et al., 1980) and pain. Indeed, developmental differences were observable
in the acute thermal pain sensation of young and adult rats. Markus and Petit (1987)
performed the tail flick test, in which the tail of the animal is exposed to a strong red light
with a constant intensity, and the latency to move the tail away from the light source was
taken as readout. They found that the response to painful stimuli is shorter in young animals
when compared to adults (Markus and Petit, 1987).

Since KCNQ channels are involved in pain sensation (Rundfeldt et al., 2000, Delmas and
Brown, 2005) and since the data presented herein show their particular expression pattern
before and after weaning, a direct correlation may exist. Possibly, the difference in the onset
of pain-related behaviour is influenced by KCNQ subunit compositions. It has been found
previously that the age differences in pain responses were associated to incomplete maturation
of the neuroanatomical and neurochemical systems (Ba and Seri, 1993; Falcon et al., 1996).

In our case, the developmental profile of the channels has been shown in in vitro experiments,
whereas the involvement of the channels in acute thermal pain sensation was shown in in vivo
experiment. Altogether, the findings support the suggested hypothesis of a KCNQ channel-
dependent modulation of pain sensation in VB neurons.

For future investigations, the experiments on the hot plate could be performed with animals of
different ages in order to test possible developmental differences. A pharmacological
modulation on the same animals could be tested simultaneously. Nevertheless, brain surgery on young animals is difficult and rarely put into practice. In general, the study presented herein raises many questions concerning the pain sensation in rodents, but also in human neonates and infants. In order to improve pain treatment in children and adolescents, human ability to perceive and handle pain stimuli should be addressed in future studies.

6.7. Concluding remarks

KCNQ channels are involved in the modulation of pain sensation and in regulating cellular excitability, but such an effect can only occur when the channels are activated. Experimentally, this was achieved by the application of retigabine and typical analgesics. The effect of the specific blocker XE991 suggests that these channels were partially activated at RMP. This particular aspect of KCNQ channel activation may depend on the modulating muscarinic pathway, which most likely inhibits the current during wakefulness, favouring tonic firing and the relay function of TC neurons. In this scenario, extrinsic stimuli, such as acute thermal noxae, are required in order to open the channels. This would then cause a burst-like firing, which may underlie the analgesic effect observed in the hot plate test. It is likely that KCNQ channels are involved in a physiological response, like pain behaviour, which, under pathophysiological conditions, may alter the channels’ function. Unfortunately, the physiological conditions activating KCNQ channels in the thalamus are still unknown. Moreover, the knowledge about the pathophysiology of these channels, which is commonly a starting point to understand the physiological mechanisms, is still insufficient. Further and more detailed experiments, both in vitro and in vivo, will be necessary to clarify this.
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8. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxy thyrosine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>AChR</td>
<td>Acetylcholine receptor</td>
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<tr>
<td>ACSF</td>
<td>Artificial Cerebro-Spinal Fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Antero-dorsal</td>
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<tr>
<td>AT1</td>
<td>Angiotensin receptor type 1</td>
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<tr>
<td>B2</td>
<td>Bradykinin receptor type 2</td>
</tr>
<tr>
<td>BFNC</td>
<td>Benignal Familial Neonatal Convulsion</td>
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<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
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<tr>
<td>CamK</td>
<td>Calcium Calmodulin Kinase</td>
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<tr>
<td>CChol</td>
<td>Carbachol</td>
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<tr>
<td>CHO</td>
<td>Chinese Hamster Ovarium</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>Cs</td>
<td>Membrane Capacitance</td>
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<td>CsMetSO_{4}</td>
<td>Cesium Metal Sulphate</td>
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<tr>
<td>DAMGO</td>
<td>[D-Ala{\textsuperscript{2}}, N-MePhe{\textsuperscript{4}}, Gly-ol]-enkephalin</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
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<tr>
<td>E_{K}</td>
<td>Potassium Equilibrium Potential</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno-Adsorbent Assay</td>
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<tr>
<td>GABA</td>
<td>Gamma amino-butyric acid</td>
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<td>GABAA</td>
<td>GABA receptor type A</td>
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<tr>
<td>Glu</td>
<td>Glutamate</td>
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<tr>
<td>I{\textsubscript{A}}</td>
<td>A-type potassium current</td>
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<td>IN</td>
<td>Interneurons</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>LGN</td>
<td>Lateral Geniculate Nucleus</td>
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<tr>
<td>LQTS</td>
<td>Long QT-Syndrome</td>
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<tr>
<td>M1</td>
<td>Muscarinic receptor type 1</td>
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<tr>
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<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
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<tr>
<td>MGN</td>
<td>Medial Geniculate Nucleus</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>Na⁺</td>
<td>Sodium</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NMDA</td>
<td>N-Methyl, D-Aspartate</td>
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<td>P2Y</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PSLN</td>
<td>Partial Sciatic Ligation of the Nerve</td>
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<tr>
<td>Rₘᵢₙ</td>
<td>Input Resistance</td>
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<tr>
<td>RMP</td>
<td>Resting Membrane Potential</td>
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<tr>
<td>SCG</td>
<td>Superior Cervical Ganglia</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SSR4</td>
<td>Somatostatin receptor type 4</td>
</tr>
<tr>
<td>TC</td>
<td>Thalamo-cortical</td>
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<tr>
<td>TRG</td>
<td>Trigeminal</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TTX</td>
<td>Tetradotoxin</td>
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<td>VA</td>
<td>Ventro-anterior</td>
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<tr>
<td>VB</td>
<td>Ventro-basal</td>
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<tr>
<td>V_h</td>
<td>Holding Potential</td>
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<tr>
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<td>Ventro-lateral</td>
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<td>Ventro-postero lateral</td>
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<tr>
<td>VPM</td>
<td>Ventro-postero medial</td>
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Contribution of collaborators

Dr. Hanna Szkudlarek performed together with the candidate the behavioural studies. In details, Dr. Szkudlarek performed the surgery implanting the canula and electrodes. We performed together the behavioural studies and the micro thalamic injections. The candidate performed the analysis of the behavioural data while Dr. Szkudlarek performed the analysis of the *in vivo* electrophysiological data.

Dr. Tatyana Kanishkova performed all the immunohistological experiments and kindly provided the data.

The candidate spent two weeks in the laboratory of Prof. Dr. Dominik Oliver, Marburg University, where she performed all the experiments in the CHO cells. Prof. Oliver contributed with helpful suggestions for the experimental approach and provided everything needed for the experiments (including chemicals, drugs and the set up).
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