ON THE MECHANISM OF ACTION OF THE NON-OPIOID ANALGESIC FLUPIRTINE

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1. LIST OF ABBREVIATIONS

5-HT .......................................................... 5-hydroxytryptamine (serotonin)
aEPSC .......................................................... autaptic excitatory postsynaptic currents
aIPSC .......................................................... autaptic inhibitory postsynaptic currents
AIS ........................................................... axonal initial segment
AMPA ...................................................... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS .......................................................... ammonium persulfate
ATP .......................................................... adenosine triphosphate
BCA .......................................................... bicinchoninic acid
Bis-tris .................................................... Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan
BMI ........................................................... bicuculline methionide
cAMP ........................................................ cyclic adenosine monophosphate
CaV channels .............................................. voltage-gated Ca\(^{2+}\) channels
CNQX ...................................................... cyano-2,3-dihydroxy-7-nitroquinoxaline
CNS ........................................................ central nervous system
COX ........................................................ cyclooxygenase
dAD .......................................................... diacyl glycerol
DH ........................................................... dorsal horn
DMEM ...................................................... Dulbecco’s modified Eagle’s medium
DRG ........................................................ dorsal root ganglia
EDTA ....................................................... ethylenediaminetetraacetic acid
EPSC ........................................................ excitatory postsynaptic current
FCS .......................................................... fetal calf serum
GABA ....................................................... gamma amino butric acid
GIRK channels ........................................ G-protein coupled inwardly rectifying K\(^+\) channels
GPCR ........................................................ G protein coupled receptor
HB ........................................................... homogenizing buffer
HEK cells ............................................... human embryonic kidney cells
HEPES .................................................... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hiFCS ....................................................... heat inactivated fetal calf serum
IPSP ........................................................ inhibitory postsynaptic potential
I\(_{\text{M}}\) ......................................................... M-current (current through M-channel)
IP$_3$ .............................................................. inositol 1,4,5-triphosphate
mIPSC ............................................................... miniature inhibitory postsynaptic currents
mEPSC ............................................................... miniature excitatory postsynaptic currents
MOPS ................................................................. 3-(N-morpholino)propanesulfonic acid
mRNA ............................................................... messenger ribonucleic acid
Na$_V$ channels ...................................................... voltage-gated Na$^+$ channels
NBQX .................. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NGF ...................................................................................... neuronal growth factor
NKA ................................................................. neurokinin A
NMDA ................................................................. N-methyl-D-aspartate
NO ................................................................. nitric oxide
NSAIDs ................................................................. non-steroidal anti-inflammatory drugs
PBS ................................................................. phosphate buffered saline
PCR ................................................................. polymerase chain reaction
PLC ................................................................. phospholipase C
PGE ................................................................. prostaglandin E
PIP$_2$ ................................................................. phosphatidylinositol 4,5-bisphosphate
PKC ................................................................. protein kinase C
PNS ................................................................. peripheral nervous system
PVDF ................................................................. polyvinylidene fluoride
rpm ................................................................. rotations per minute
SCG ................................................................. superior cervical ganglia
SDS ................................................................. sodium dodecyl sulphate
SDS–PAGE .................. sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNEPCO .......................................................... Selective Neuronal Potassium Channel Openers
TEMED ........................................................... tetramethylethyldiamin
TRP channels ................................................ transient receptor potential-generating channels
TTX ................................................................. tetrodotoxin
VR ................................................................. valinoid receptor
2. ABSTRACT

Pain is “an unpleasant sensory and emotional experience” and it represents the response of the central nervous system to tissue damage or to changes in the secondary structural and/or functional nervous system adaptations that take place either centrally or peripherally. Many factors contribute to the pain perception, sensitisation, transmission and modulation. Consequently, there are multiple possibilities for the pharmacological pain control. Depending on the type of analgesic drug, the compound exerts its action by inhibiting the formation of chemical mediators that lead to pain sensation and inflammatory response, or it acts directly on the peripheral or central nervous system. The classical analgesics in clinical use belong to one of the following classes: opioids, COX inhibitors, antiepileptic drugs, local anaesthetics, and cannabinoids. A more recently discovered drug is flupirtine, which is a non-opioid analgesic agent characterized as Selective NEuronal Potassium Channel Opener (SNEPCO). However, the mechanism by which flupirtine exerts its analgesic action, is not yet fully understood and is the purpose of this study.

Using electrophysiological experiments on cultured neurons isolated from hippocampal, spinal dorsal horn and dorsal root ganglia, as well as transfected tsA cells, we investigated the effects of flupirtine on different voltage- and ligand- gated ion channels.

Therapeutic flupirtine concentrations (<10 µM) did not affect glycine or ionotropic glutamate receptors. Flupirtine shifted the gating of Kv7 K⁺ channels to more negative potentials and the gating of GABA_A receptors to lower GABA concentrations. This latter effect was more pronounced in dorsal root ganglion and dorsal horn, than in hippocampal neurons; this difference was paralleled by differences in the expression pattern of GABA_A receptor subunits in these tissues as determined by immunoblots. Also, the potentiating effect of flupirtine on the GABA- mediated current appeared to be different on phasic and tonically active GABA_A receptors.

This work reveals GABA_A receptors as novel sites of action for the non-opioid analgesic flupirtine. Thus, flupirtine is not a SNEPCO as inferred up to now, but rather exerts its analgesic actions by combining two therapeutic principles, the potentiation of both, GABA_A receptors and Kv7 channels.
3. ZUSAMMENFASSUNG


Therapeutisch wirksame Konzentrationen von Flupirtin zeigten keinen Effekt auf Glycin- oder ionotrope Glutamatrezeptoren, begünstigten jedoch die Öffnung von K\text{v7} K⁺ Kanäle und die Aktivierung von GAB\text{A} Rezeptoren bei niedrigeren Konzentrationen von GABA. Letzteres war in Neuronen vom Hinterhorn und Hinterwurzelganglien starker ausgeprägt, als in Neuronen von Hippocampus. Dieser Unterschied wurde in Western Blot Experimenten mit dem Expressionsmuster verschiedener Untereinheiten der GAB\text{A} Rezeptoren verglichen. Zudem zeigte sich die verstärkende Wirkung von Flupirtin unterschiedlich auf GABA-Ströme in Bezug auf phasische und tonische GAB\text{A} Rezeptoren.
Diese Arbeit beschreibt GABA$_A$ Rezeptoren als neues Angriffsziel für das nicht-"opioide Analgetikum Flupirtin. Somit ist Flupirtin kein SNEPCO, wie bisher behauptet, sondern es entfaltet seine analgetische Wirkung, indem es die Aktivierung sowohl der GABA$_A$ Rezeptoren als auch der K$_V$7 Kanäle verstärkt.
4. INTRODUCTION

4.1 Pain and nociception

Pain is officially defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, as described in terms of such damage” (Merskey and Bogduk, 1994).

Pain can be classified as nociceptive or neuropathic pain. In nociceptive pain, or nociception, information about tissue trauma is registered by peripherally located nerve terminals and transmitted to the central nervous system (Julius and Basbaum, 2001). In the case of neuropathic pain, in contrast, no direct tissue damage can be registered, but secondary structural and/or functional nervous system adaptations take place either centrally or peripherally (Baron, 2000). In both cases, pain is the response of the central nervous system to these changes. Thus, the pain pathway comprises peripheral as well as central components.

The primary sensors for painful stimuli are the terminals of primary afferent fibers, called nociceptors. Mechanical, chemical or thermal stimuli will trigger action potential generation when applied to the nociceptive terminals. While acute pain is the physiological response to an excessive noxious stimulus and has a protective role, neuropathic pain is associated with pathological increase in sensitivity conditions, leading to hyperalgesia (increased response to noxious stimuli), allodynia (nociceptive response to non-painful stimuli) or spontaneous pain (in absence of any precipitating stimulus). Chronic pain states can result from different pathophysiological conditions, such as tissue damage (inflammatory pain) or nerve injury (neuropathic pain).

4.2 Nociceptive system

The perception of pain includes three stages: 1. activation of peripheral nociceptors; 2. generation of a neuronal signal and 3. transmission of this signal to the higher brain regions. As result, the nociceptive pathway consists of at least three neurons: 1. a peripheral sensory neuron located in the dorsal root ganglia (DRGs); 2. a second order neuron in the dorsal horn of the spinal cord and 3. a third order neuron, which is generally located in the thalamus.
Under normal conditions, pain is associated with tissue trauma that can be caused by various types of noxious or potentially tissue-damaging stimuli, including mechanical, thermal or chemical stimuli (Cesare and McNaughton, 1997). These noxious stimuli activate primary afferent fibers of peripheral nerves. Based on their anatomical and functional criteria, the primary afferent fibers can be divided into three groups: Aβ-, Aδ- and C-fibers. They all present a different conduction velocity and thus, transmit different types of sensory information (Julius and Basbaum, 2001). Aβ-fibers are large in diameter and highly myelinated, allowing them to quickly conduct action potentials from their peripheral to central terminals. They have low activation thresholds and respond to light touch, therefore, mediating low intensity mechanical, tactile and proprioceptive inputs. Aδ-fibers, with smaller diameter and less myelin, are slower conducting, possess higher activation thresholds and respond to thermal and mechanical stimuli. C-fibers are the smallest type of primary afferents and are completely unmyelinated, thus having very slow conductance. They have the highest threshold for activation and therefore detect selectively “painful” stimuli. Both Aδ- and C-fibers are termed as nociceptors and respond to noxious stimuli. Experiments on human subjects have shown that activity in the Aδ-fibers causes a sensation of sharp, well localized pain, whereas C-fibers activity causes a dull burning pain (Beissner et al., 2010).

Fig. 4.1. A schematic overview of the main circuits mediating physiological pain, modified from (Kuner, 2010). Noxious stimuli activate peripheral primary afferent fibres with the cell body located in the dorsal root ganglion and the axons terminating in the dorsal horn of the spinal cord. From here, pain signal is transmitted to the higher brain centre.
Fig 4.1 shows a schematic overview of the main pathways mediating pain. Cell bodies of primary afferent neurons that innervate the periphery are located in the dorsal root ganglia (DRG). From here, all primary sensory nociceptors send extensions via the dorsal roots to the dorsal horn of the spinal cord where they form the first synapse in the pain pathways, the so called “pain synapse”. Most of the nociceptors (C-fibers and some of the Aδ-fibers) end in the superficial layers of the dorsal horn (lamina I and II), while inputs from non-nociceptive fibers form synapses in deeper laminae. From the dorsal horn, projected axons transmit pain messages via the spinothalamic tract to higher brain centers including the reticular formation, thalamus and somatosensory cortex (Basbaum and Jessell, 2000). Beside the spinal projection neurons with their extensions leaving towards higher brain centres, plenty of interneurons are located in the dorsal horn, with their axon and dendrites remaining in the spinal cord. They can be divided in two classes: excitatory and inhibitory interneurons. Excitatory interneurons use glutamate, while most of the inhibitory interneurons use gamma amino butyric acid (GABA) and/or glycine as their main transmitter.

4.3 Pain perception

There is a wide range of initial stimuli, including those of thermal and mechanical origin, and the peculiar ability of the nociceptors is to transform all of them in electrical signaling. This is possible due to the diversity of the specialized receptors present in the membrane of the nerve terminals. Various ionotrophic and metabotropic receptors are involved in this process (Caterina and Julius, 1999; Cesare and McNaughton, 1997; McCleskey and Gold, 1999).

Transient receptor potential-generating (TRP) channels are ion channels involved in thermo sensation. Six subgroups of this family are known and each of them show different thermal activation threshold (Tominaga and Caterina, 2004). In humans, thermo sensation can be accompanied by the sensation of pain, when the temperatures are below 15°C or above 43°C (LaMotte and Campbell, 1978; Tillman et al., 1995). One member of the TRP channels family is the TRPV1 channel, also known as the vaniloid receptor (VR1) or the capsaicin receptor, since it gets activated by heat (> 43°C) and capsaicin (Caterina and Julius, 2001; Caterina et al., 1997; Szallasi and Blumberg, 1999; Tominaga et al., 1998). TRPV1 is a nonselective cation channel with a thermal activation threshold of about 43°C,
expressed predominantly in sensory neurons where it generates heat-evoked currents (Tominaga and Caterina, 2004). Mice lacking VR1 showed the inability to detect painful heat, while their responses to noxious mechanical stimuli were not affected (Caterina et al., 2000; Davis et al., 2000). Electrophysiological analysis of heat-evoked currents in native DRG neurons as well as transfected cells, suggests that heat directly activate VR1 (Kirschstein et al., 1997; Tominaga et al., 1998). However, the temperature threshold for VR1 activation is decreased in acidic conditions (pH ≤ 5.9), when the receptor can be activated even at room temperature (Tominaga et al., 1998). In DRG neurons as well as in heterologous system, TRPV1 response was reported to be potentiated by adenosine triphosphate (ATP) and bradykinin (Sugiura et al., 2002; Tominaga et al., 2001). This effect is mediated by second messenger signaling initiated upon activation of the metabotropic purinergic and bradikynin receptors, respectively (Bhave et al., 2002; Numazaki et al., 2002; Rathee et al., 2002; Yousuf et al.).

**P2X<sub>3</sub> receptor** is another well investigated ion channel important for the pain perception. It belongs to the ionotropic purinergic receptors (Khakh et al., 2001) and is activated by extracellular ATP. As essential metabolite, ATP is widely distributed in various tissues and released into the extracellular compartment upon tissue damage. Holton P. was the first to propose that ATP might acts as a neurotransmitter (Holton, 1959). Meanwhile, enough evidence has been collected for the role of ATP as a neurotransmitter and neuromodulator at the primary afferent nerve terminals (Krishtal et al., 1988; MacKenzie et al., 1999; North and Barnard, 1997; Sawynok and Sweeney, 1989). In cell culture experiments using electrophysiological or imaging measurements, DRG sensory neurons as well as dorsal horn neurons have been found to respond to exogenously applied ATP by depolarization or increase in intracellular free Ca<sup>2+</sup>-concentrations (Bouvier et al., 1991; Grubb and Evans, 1999; Jahr and Jessell, 1983, 1985; Rae et al., 1998). Moreover, ATP has been shown to increase glutamate release by acting on presynaptic terminals of primary afferent neurons in the spinal cord (Gu and MacDermott, 1997). Of the seven P2X receptor sub-units, P2X<sub>3</sub> is expressed in sensory neurons, primarily in a subset of C-fibers (Burnstock, 2000; Vulchanova et al., 1998). Thus, P2X<sub>3</sub> containing receptor was focus of numerous pain studies. Upon tissue damage, released ATP activates P2X<sub>3</sub> receptors and initiate the nociceptive signal (Burnstock, 2001). In animal models of neuropathic pain, P2X<sub>3</sub> receptor was reported to be upregulated in DRG and dorsal horn neurons (Novakovic et al., 1999). Further evidence deliver experiment with P2X<sub>3</sub>-null mice, these showing reduced pain-
related behavior in response to injection of ATP and formalin (Cockayne et al., 2000). In electrophysiological recordings, dorsal root ganglion neurons from these mice showed a loss of the rapidly desensitizing ATP-induced currents (Cockayne et al., 2000).

4.4 Pain sensitisation

Any type of tissue injury is followed by an inflammatory response, which in turn amplifies the pain perception by increasing the sensitivity of nociceptive terminals to thermal and mechanical stimuli. This mechanism is mediated by a wide range of chemical factors, which are released by the direct tissue damage, the sensory fibres or the recruited immune cells. These chemical mediators are often referred to as ‘inflammatory soup’ (Julius and Basbaum, 2001) (Fig. 4.2) and include substance P, bradykinin, ATP, cytokines, chemokines, prostaglandins, growth factors, proteases, protons, nitric oxide (NO) and many others (Linley et al., 2010). Many of these mediators (e.g. bradykinin, histamine, serotonin) have been known to have endogenous pain-inducing effects by either sensitising or directly exciting the peripheral terminals of nociceptive neurons (Keele, 1967). Their effect on sensory neurons is mediated by specific receptors, which can be grouped in three categories: (1) G protein coupled receptors (GPCRs), e.g. for bradykinin, proteases and histamine; (2) tyrosine kinase receptors, e.g. growth factor receptors, TrkA and TrkB, and (3) ionotropic receptors/ion channels, e.g. the purinergic P2X receptors. The latter group has been already described in the previous section; here, I will concentrate on the chemical transducers that participate in pain pathways by targeting specific second-messenger signalling cascades.

**Fig 4.2. Inflammatory mediators released after tissue injury induce complex molecular response modified from (Julius and Basbaum, 2001).** Different inflammatory mediators such as lipids (prostaglandins), peptides (bradykinin), neurotrophins (neuronal growth factor) and neurotransmitters (serotonin and ATP) are released upon tissue injury. Here, they sensitize or excite primary afferent fibres terminals via their cell-surface receptors. Indicated in the box are some of these factors and the corresponding molecular targets.
Bradykinin

Bradykinin is generated at sites of tissue injury and it is known to be a major inflammatory mediator (Dray and Perkins, 1993). Bradykinin activates specific receptors, B1 and B2, with B2 being the major receptor type involved in acute inflammatory processes (Couture et al., 2001). They are G protein–coupled receptors acting via $G_{q/11}$ signalling cascades. This includes the activation of phospholipase C (PLC), which induces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) in diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). DAG remains bound to the membrane, while IP$_3$ diffuses into the cytosol, where it leads to release of Ca$^{2+}$ from intracellular stores and activation of protein kinase C (PKC). Application of bradykinin to primary or cultured sensory neurons induced immediate membrane depolarisation (Burgess et al., 1989) or augmentation of the heat-evoked currents (Cesare and McNaughton, 1996). In animal as well as human studies, application of bradykinin resulted in spontaneous pain and increased sensitivity to painful and non-painful stimuli (Couture et al., 2001; Dray and Perkins, 1993). It has been shown recently that the major effects of bradykinin on the excitability of nociceptors are mediated by PLC- and Ca$^{2+}$-dependent inhibition of M-type K$^+$ channels and by simultaneous opening of Ca$^{2+}$-activated Cl$^-$ channels. Both these effects contribute to membrane depolarization in primary sensory afferents, resulting in the generation of ascending nociceptive signals (Liu et al., 2010).

Serotonin

Serotonin (5-hydroxtryptamine [5-HT]) has long been shown to have a role in the regulation and modulation of nociception (Eide and Hole, 1993). 5-HT is released in damaged tissue from immune cells (Andén and Olsson, 1967; Dray, 1995; Lehtosalo et al., 1984) and the increase in concentration shows a maximum level about 10 to 30 minutes after mechanical injury (Maeno et al., 1991) and after 3 h after carrageenan-induced inflammation (Zhang et al., 2000). Intradermally applied serotonin was found to produce a dose-dependent hyperalgesia in rat studies employing the mechanical paw withdrawal nociceptive threshold and the extremely short onset time suggest the hyperalgesic effects of serotonin to be exerted by direct action on primary afferent neurons (Taiwo and Levine, 1992). Using polymerase chain reaction (PCR) analysis the presence of messenger ribonucleic acid (mRNA) for different serotonin receptor subtypes was found to be expressed in rat peripheral sensory and sympathetic ganglia (Pierce et al., 1996). In human studies, patients with fibromyalgia showed increased intramuscular serotonin concentration (Ernberg
et al., 1999) and serotonin injection into the masseter muscle of healthy volunteers, induced pain and allodynia (Ernberg et al., 2000b) that could be antagonised by granisetron, specific 5-HT3 receptor blocker (Ernberg et al., 2000a). Topical application of ondansetron, another 5-HT3 receptor antagonist, induced dose-dependent attenuation of inflammatory and nociceptive effects of intradermally injected capsaicin in humans (Giordano et al., 1998).

Neuronal growth factor (NGF)

NGF is a signalling molecule first described for its role in differentiation and survival of peripheral sensory and sympathetic neurons. Now it is known that NGF is also associated with different functional activities of cells of the immune and endocrine system and it is released at sites of injury and inflammation (Aloe et al., 1992; Weskamp and Otten, 1987). Both subcutaneous and systemic application of NGF induced hyperalgesia (Andreev et al., 1995; Bennett et al., 1998; Lewin et al., 1993) and these hyperalgesic responses were diminished when anti-NGF antibodies (Lewin et al., 1994; Woolf et al., 1994) or NGF-specific immunoadhesin molecules (McMahon et al., 1995) were used in experimental inflammatory assays. NGF applied to cultured DRG neurons produced sensitisation of capsaicin-evoked currents and this effect may involve the activation of VR1 (Shu and Mendell, 1999). NGF acts through binding to the TrkA tyrosine kinase receptor that activates mitogen-activated protein (MAP) kinase and PLC-γ signalling pathways (Ganju et al., 1998).

Prostaglandin E2

Prostaglandins are potent proinflammatory agents, synthesized from arachidonic acid by the cyclooxygenase (COX) enzymes. Prostaglandin E2 (PGE2) is able to modulate peripheral as well as central sensitizing effect of nociceptive information, as shown in both, human and experimental studies. Thus, PGE2 applied subcutaneously in human volunteers produced hyperalgesia to mechanical and chemical stimuli, but did not induce spontaneous pain (Ferreira, 1972). Moreover, monoclonal antibodies against PGE2 inhibited the hyperalgesic effect of prostaglandin in carrageenan-induced rat paw edema model and substantially reduced the dorsoflexion response of mice to phenylbenzoquinone injection (Mnich et al., 1995). The central sensitising properties have been demonstrated in studies where intrathecal administration of prostaglandin dose-dependently induced hyperalgesia and allodynia in conscious mice (Minami et al., 1994; Uda et al., 1990). Regarding
the mechanism by which PGE$_2$ modulates pain perception, an involvement of tetrodotoxin (TTX)-resistant Na$^+$-channels has been suggested. Electrophysiological recordings in DRG neurons treated with PGE$_2$ revealed an increase in the neuronal excitability, due to a shift towards hyperpolarisation in the voltage dependence of the channel activation. This effect was suggested to involve phosphorylation of the channel by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (England et al., 1996; Fitzgerald et al., 1999; Gold et al., 1998).

**Substance P**

Substance P is a neuropeptide widely distributed in the nervous system, but especially in nociceptive primary afferent neurons and the dorsal horn. Several studies support the concept that substance P and neurokinin A (NKA), another neuropeptide, are involved in the nociceptive signalling from primary afferent fibres to the dorsal horn of the spinal cord. Co-expression in primary afferent fibres and co-release upon noxious stimulation of these two neuropeptides has been demonstrated in numerous studies (Boehmer et al., 1989; Dalsgaard et al., 1984; Duggan et al., 1990; Hokfelt et al., 1975; Hope et al., 1990; Levine et al., 1993). Iontophoretic application of substance P followed by extracellular single-unit recordings in the lumbar spinal cord of anaesthetized cats demonstrated its role in transmission of nociceptive inputs at the first afferent synapse (Radhakrishnan and Henry, 1991; Salter and Henry, 1991). Substance P activates Neurokinin 1 (NK1)-, while NKA activates NK2-receptors. Changes in the density of NK1-receptors may contribute to sensitization. Thus, immunocytochemical experiments demonstrated up-regulation of NK1-receptors in the superficial laminae of the dorsal horn in two distinct rat models of persistent pain (Abbadie et al., 1996).

### 4.5 Pain transmission

Voltage-gated Na$^+$-channels enable the propagation of the pain signal, while the transmission of the pain signal at the synapse is mediated by voltage-gated Ca$^{2+}$-channels. At the spinal cord level, glutamate is the main neurotransmitter released from the presynaptic neurons and thus, postsynaptic glutamate receptors are responsible for transmission of the pain signal to the higher brain regions (Basbaum et al., 2009).
Voltage-gated Na\textsuperscript{+}-channels

Voltage-gated Na\textsuperscript{+} (Na\textsubscript{V}) channels are crucial in generating and propagating electrical signal in neurons. They are primarily found at the axonal initial segment (AIS) and the Nodes of Ranvier in myelinated neurons (Leterrier et al., 2010). Nine members of the Na\textsubscript{V} channel family are known: Na\textsubscript{V}1.1-Na\textsubscript{V}1.9 (Catterall et al., 2005), with different members showing different kinetics, voltage-dependencies and pharmacological properties. According to their sensitivity to tetrodotoxin (TTX), they can be classified in two groups: TTX-sensitive Na\textsubscript{V} channels, including Na\textsubscript{V}1.1-Na\textsubscript{V}1.6 which show sensitivity to nanomolar concentrations of TTX and TTX-resistant Na\textsubscript{V} channels containing Na\textsubscript{V}1.7-Na\textsubscript{V}1.9 (Catterall et al., 2005; Rush et al., 1998). DRG neurons express mRNA for up to five sodium channels: Na\textsubscript{V}1.1, Na\textsubscript{V}1.6, Na\textsubscript{V}1.7, Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 (as well as Na\textsubscript{V}1.5 at low levels), with Na\textsubscript{V}1.7, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 being specific for peripheral nervous system (PNS) neurons, while Na\textsubscript{V}1.1 and Na\textsubscript{V}1.6 expression is common to both peripheral and central nervous system (CNS) (Black et al., 1996; Dib-Hajj et al., 2010).

The impact of Na\textsubscript{V} channels in the development and maintenance of many types of chronic pain syndromes has been documented in various studies (Dib-Hajj et al., 2009; Lai et al., 2004; Waxman and Hains, 2006). Studies in animal models have shown that transcriptional regulation of sodium channel genes in DRG neurons can result in neuronal hyperexcitability (Waxman, 2001). Thus, Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 were downregulated in small rat DRG neurons after axotomy (Decosterd et al., 2002; Dib-Hajj et al., 1998; Sleeper et al., 2000) and reduced in human DRG neurons after injury (Coward et al., 2001; Coward et al., 2000). Other studies have shown in immunocytochemical settings that Na\textsubscript{V}1.7 and Na\textsubscript{V}1.8 were upregulated in painful human neuromas (Black et al., 2008; Kretschmer et al., 2002). In behavioral studies, Na\textsubscript{V}1.8 knock-out animals showed an increase in the pain threshold to mechanical pressure and deficits in inflammatory pain (Akopian et al., 1999; Kerr et al., 2001). In electrophysiological recordings using the same mice, reduced responses to suprathreshold mechanical stimuli were observed (Matthews et al., 2006).

Voltage-gated Ca\textsuperscript{2+}-channels

Voltage-gated Ca\textsuperscript{2+} (Ca\textsubscript{V}) channels are membrane proteins that respond to changes in membrane potential by opening a Ca\textsuperscript{2+}-selective pore. They can be grouped in three families: Ca\textsubscript{V}1 channels with Ca\textsubscript{V}1.1-1.4 corresponding to the L-type Ca\textsubscript{V} channels; Ca\textsubscript{V}2 channels with Ca\textsubscript{V}2.1 (P/Q-type), Ca\textsubscript{V}2.2 (N-type) and Ca\textsubscript{V}2.3 (R-type) and the Ca\textsubscript{V}3 family with Ca\textsubscript{V}3.1-3.3, corresponding to the T-type Ca\textsubscript{V} channels (Catterall et al., 2003).
P/Q- and N-type CaV channels are localized at the presynaptic nerve terminals where they mediate calcium entry and trigger neurotransmitter release (Catterall and Few, 2008). In ascending pain pathways, T-type and N-type CaV channels play the major role (Zamponi et al., 2009). Located in cell bodies and nerve endings of primary afferent fibres, T-type CaV channels contribute to the regulation of the neuronal excitability by influencing the initiation of action potential series (Todorovic and Jevtovic-Todorovic, 2006). A combination of behavioural, immunohistological, molecular and electrophysiological studies demonstrate an upregulation of the T-type CaV channel expression in different neuropathic rat pain models (Jagodic et al., 2008; Jagodic et al., 2007). Moreover, decreased sensitivity to pain was observed in rats with gene knockout or antisense knockdown of the CaV3.2 isoform, or after intrathecal administration of specific T-type CaV channel inhibitors (Bourinet et al., 2005; Choi et al., 2007; Dogrul et al., 2003; Matthews and Dickenson, 2001; Todorovic et al., 2002). N-type CaV channels are highly expressed at the presynaptic end of the primary afferent neuron where they initiate Ca2+-mediated neurotransmitter release as response to incoming action potentials (Westenbroek et al., 1998). Mice lacking N-type CaV channels show increased threshold to a painful stimulus and reduced symptoms of neuropathic pain (Saegusa et al., 2001).

Spinal glutamate receptors
At the spinal cord level, independent of the type of nociceptor, the major excitatory neurotransmitter used for further signal transmission is glutamate. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) type of glutamate receptors have distinct contribution to the nociceptive transmission (D’Mello and Dickenson, 2008). Activation of postsynaptic AMPA receptors by glutamate release from sensory afferent neurons generates excitatory postsynaptic currents (EPSCs) at this level. Summation of subthreshold EPSCs will then result in transmission of action potential to higher order neurons. Thus, AMPA-type of glutamate receptors are responsible for the baseline level of nociception and for the intensity and duration of the pain stimulus. Under normal physiological conditions, NMDA type of glutamate receptors are voltage-dependently blocked by Mg2+ ions. Upon repetitive and high frequency stimulation of C-fibers, different peptides (such as substance P (Battaglia and Rustioni, 1988)), are coreleased with glutamate, resulting in a prolonged slow depolarization of the postsynaptic membrane. This depolarization will allow removal of the Mg2+-blockage and activation of NMDA receptors. The activation of NMDA receptors occurs superimposed on the baseline
activity and leads to an amplification and prolongation of the response of the spinal dorsal horn neurons. This mechanism is called “wind-up” (Dickenson and Sullivan, 1987) and it plays an important role in hyperalgesia and neuropathic pain (Price et al., 1994; Seltzer et al., 1991; Woolf and Thompson, 1991).

4.6 Pain modulation

Opioids

Opioid peptides are defined as peptides with opiate-like pharmacological effects and are widely distributed in the nervous system. In the dorsal horn, they are found in interneurons and in the descending pathways from the midbrain to the dorsal horn. They are also produced by many non-neuronal cells, including endocrine and exocrine glands and cells of the immune system and thus, they play a regulatory role in many different physiological systems. I will concentrate here on their role in pain modulation at the spinal cord level.

Three main types of opioid receptors have been described from binding studies and bioassays, and they are termed μ, δ and κ receptors and they all are GPCRs, signalling mainly through Gi/o-proteins. Upon activation, they can inhibit cAMP production, and/or directly interact with ion channels in the cell membrane, like K⁺ and Ca²⁺ channels. Presynaptic localisation of opioid receptors on C-fibre terminals was suggested in different imaging studies showing expression on small DRG neurons as well as in the superficial layers of the dorsal horn (Abbadie et al., 2001; Cheng et al., 1996; Fields et al., 1980). In congruency with this suggestion, other studies showed a reduction in the number of opioid receptors after C-fibre destruction (Besse et al., 1990; Gamse et al., 1979). However, opiate binding was not completely eliminated, implicating also a postsynaptic localisation of the opioid receptors. Indeed, opioid receptor mRNA was found in the dorsal horn of the spinal cord (Kemp et al., 1996; Mansour et al., 1995; Todd and Spike, 1993). Thus, opioid peptides modulate pain perception by acting on both, pre- and postsynaptic opioid receptors. Presynaptically, they inactivate voltage gated Ca²⁺ channels, thus, inhibiting the neurotransmitter release (Schroeder et al., 1991; Soldo and Moises, 1998) and postsynaptically, they open membrane K⁺ channels, thus, decreasing the neuronal excitability (Grudt and Williams, 1994; Yoshimura and North, 1983). Spinal administration of opiates resulted in potent analgesic effect in both rodents and humans, but failed to affect other sensory functions (Yaksh, 1981). In animal studies, application of opioids inhibited electrophysio-
logical responses of dorsal horn neurons to C-fibre (but not A-fibre) stimulation (Grudt and Williams, 1994; Jurna and Heinz, 1979; Le Bars et al., 1976), while opioid receptor agonists were shown to inhibit substance P release evoked by stimulation of primary sensory neurons (Aimone and Yaksh, 1989; Chang et al., 1989; Jessell and Iversen, 1977; Kondo et al., 2005; Yaksh et al., 1980).

**Spinal GABA<sub>A</sub> receptors**

Most of the studies concentrate on the physiology and pharmacology of GABA<sub>A</sub> receptors in the higher brain regions and the role of GABA in the spinal cord has been underestimated for a long time. However, GABA<sub>A</sub> receptors are also widely expressed in the spinal cord (Malcangio and Bowery, 1996), where GABA and glycine act as co-transmitters at some central connections (Jonas et al., 1998; Todd et al., 1996; Triller et al., 1987). Under physiological conditions, GABAergic interneurons control the transmission of nociceptive stimuli from the periphery through the spinal cord to higher CNS regions and prevent the excitation of pain-specific neurons by non-noxious stimuli (Fig. 4.3). In consistence with this knowledge, it has been shown that loss of inhibitory GABAergic transmission in the superficial dorsal horn of the spinal cord underlies several forms of chronic pain (Moore et al., 2002). Moreover, increased spinal GABA concentration due to application of GABA uptake inhibitors (tiagabine) or GABA transaminase inhibitors (vigabatrin) reduced pain-related behaviours in rodent models (Buckett, 1980; Laughlin et al., 2002). Intrathecal application of the benzodiazepine midazolam seems to improve perioperative analgesia in humans, as shown in a recent meta-analysis (Ho and Ismail, 2008).

A reduction of inhibitory transmission, called disinhibition, may contribute to spontaneous pain, hyperalgesia and allodynia (Sandkuhler, 2009). Disinhibition may result from downregulation and/or cell death in the dorsal horn following nerve injury (Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002; Scholz et al., 2005; Whiteside and Munglani, 2001). Also, application of antagonists for GABA<sub>A</sub> and glycine receptors leads to increased transmission from afferent primary neurons in inflammatory and neuropathic pain states (Schoffnegger et al., 2008; Torsney and MacDermott, 2006).
Figure 4.3. Schematic representation of pain control by spinal inhibitory neurotransmission modified from (Zeilhofer et al., 2009). Nociceptive (Aδ- and C-fibers, magenta) and non-nociceptive (Aβ-fibers, blue) fibers synapse in the dorsal horn directly to the spinal projection neurons (large red circles) or to interneurons. Excitatory interneurons (small red circles) connect Aβ fibers with pain-specific projection neurons. Inhibitory interneurons (green circles) control spinal nociception through axo-axonic synapses with primary afferent nociceptors (presynaptic inhibition) and axo-dendritic and axo-somatic synapses with the intrinsic dorsal horn neurons (postsynaptic inhibition). GABA mediates both, pre- and postsynaptic inhibition, while glycine contributes to postsynaptic inhibition only. Inhibitory dorsal horn neurons can also be activated through descending antinociceptive pathways (yellow).

4.7 Analgesics

Analgesics are drugs capable of relieving pain and providing analgesia. As described above, perception, transmission and modulation of pain is a very complex system. Consequently, there are multiple possibilities for the pharmacological pain control. Depending on the type of analgesic drug, the compound acts directly on the nervous system (pain propagation), peripheral or centrally, or it exerts its action by inhibiting the formation of chemical mediators that lead to pain sensation and inflammatory responses. The classical analgesics in clinical use belong to one of the following classes: opioids, COX inhibitors, antiepileptic drugs, local anaesthetics, and cannabinoids (Guindon et al, 2007).

Although capsaicin induces pain by selectively activating unmyelinated nociceptive neurons, repetitive administration was shown to induce a reversible antinociceptive and anti-inflammatory action after an initial undesirable effect (Dray, 1992). This is due to the
desensitization and an inactivation of sensory neurons (Winter et al., 1995) and includes different mechanism, like receptor inactivation, blockage of voltage-gated Ca\(^{2+}\) channels and proteolytic enzyme activation (Dray, 1992). In both rats and mice, neonatal treatment with capsaicin induced analgesic responses to noxious mechanical and chemical stimuli (Faulkner and Growcott, 1980; Gamse, 1982; Hayes et al., 1981). When administrated systematically, capsaicin was shown to induce loss of C-fibre terminals (Chung et al., 1990; Chung et al., 1985). In humans, systemic or local administration of capsaicin reduced the inflammatory heat and noxious chemical hyperalgesia and reduced post-surgical, osteoarthritis and fibromyalgia pain (Fraenkel et al., 2004; Remadevi and Szallisi, 2008). To date, low-concentration capsaicin creams are used as topical treatment of peripheral neuropathic pain (McCormack, 2010).

Classical non-steroidal anti-inflammatory drugs (NSAIDs) exert their analgesic and anti-inflammatory effects by inhibiting the activity of COX, the enzyme responsible for the synthesis of prostaglandins (Vane, 1971; Vane et al., 1998). The prototypic agent is acetylsalicylic acid (aspirin), but NSAIDs represent a heterogeneous group of agents, including salicylates, acetic and propionic acids, and pyrazolones and anthranilic acids and they all present similar efficacy in the analgesic properties (Eisenberg et al., 1994; Kostamovaara et al., 1998; Riedemann et al., 1993; Tramer et al., 1998). However, different agents present different adverse effects. Gastrointestinal ulceration, renal dysfunction, impaired haemostasis and asthmatic reactions are the principal adverse effects associated with NSAIDs (Power and Barratt, 1999; Stalnikowicz and Rachmilewitz, 1993) and these result from their action as non-selective inhibitors of both isoforms of COX (COX-1 and COX-2). While COX-1 is found in different tissues and its activation is a response to physiological stimuli, COX-2 is an inducible isoenzyme found in inflamed tissue where it is produced as a response to different inflammatory stimuli and tissue trauma.

Opioids, such as morphine and heroin, exert their effects by mimicking effects of endogenous opioid peptides called endorphins. They belong to the most potent analgesics and are widely used in the treatment of severe pain. However, their use is hampered by tolerance development during prolonged administration and severe adverse effects, such as respiratory depression, sedation, nausea and constipation (MacPherson, 2000; Stein et al., 2009). With the intention to reduce total opioid use as well as the incidence of side effects, there is an increase in recommendations for combination of opioids with other adjuvant medications (Brodner et al., 1998; Kehlet and Dahl, 1993).
Ketamine, as the classical NMDA receptor antagonist (Anis et al., 1983; Thomson et al., 1985) has a well-documented analgesic effect (Rabben et al., 1999; Schmid et al., 1999). However, the blockage of NMDA receptor may not be (alone) responsible for the analgesic effect, since ketamine is known as a multimodal drug (Hirota and Lambert, 1996) being able to bind at various receptors. Thus, beside the NMDA receptor, ketamine has been shown to bind with different affinities at other receptors, such as opioid, monoaminergic and muscarinic receptors (Crisp et al., 1991; Durieux, 1995; Hustveit et al., 1995; Smith et al., 1987). Moreover, ketamine also showed a dose-dependent blockage of Na\(^+\) channels, revealing local anaesthetic properties (Frenkel and Urban, 1992).

Local anaesthetic drugs, with lidocaide being the prototypic agent, prevent pain transmission by directly blocking voltage-gated Na\(^+\) channels and thus, inhibiting the generation and propagation of excitative inputs (Butterworth and Strichartz, 1990). Local anaesthetics are not specific for the Na\(^+\) channels in the pain pathway, but they can bind to any type of nerve fibre, reversibly blocking the neuronal activity. Thus, they can cause both sensory and motor paralysis in the area innervated by the respective nerve trunk (Catterall and Mackie, 2005). Although there is a great individual variation, for most patients treatment with local anaesthetics causes the sensation of pain to disappear first, followed by loss of the sensation of temperature, touch, deep pressure and finally motor function. In congruency with these observations, experiments showed that nociceptive A\(\delta\)- and C-fibres are blocked more rapidly and at lower concentrations than the large myelinated non-nociceptive fibres (Ford et al., 1984; Franz and Perry, 1974).

More recently discovered, ziconotide, a specific blocker of presynaptic N-type Ca\(\text{V}\) channels (Hirata et al., 1997; Nielsen et al., 1996), has been shown to possess a potent analgesic activity (MacPherson, 2000). The analgesic effect of ziconotide results from the inhibition of Ca\(^{2+}\)-dependent neurotransmission from the primary afferent neurons to the 2\(^{\text{nd}}\) order neurons in the spinal cord (Klotz, 2006). Its efficacy has been demonstrated in both, animal (Sluka, 1998; White and Cousins, 1998) and human (Brose et al., 1997) studies. With a long-term efficacy and a reduced spectrum of adverse effects (Penn and Paice, 2000), ziconotide was proposed as new alternative analgesic for the treatment of severe acute and chronic pain (Klotz, 2006; Miljanich, 2004).

The anticonvulsant carbamazepine was first introduced for pain relief about half of a century ago (Blom, 1962) and numerous double-blind controlled studies showed carbamazepine to be significantly more effective than placebo in patients suffering from trigeminal neuralgia (Swerdlow, 1984). Subsequent studies revealed oxcarbazepine, a deri-
vate of carbamazepine, to have comparable analgesic effect but substantially fewer adverse
events than carbamazepine in the treatment of trigeminal neuralgia (Carrazana and Mikoshi,
2003). Both, carbamazepine and oxcarbazepine, reduce the hyperexcitability of pe-
ripheral nerves by blocking voltage-sensitive Na\(^+\) channels (Dickenson et al., 2002; White,
1999). When tested on cat cutaneous afferent fibres, oxcarbazepine was able to inhibit the
generation of high-frequency firing, without impairing normal impulse conduction
(Ichikawa et al., 2001). Peripherally applied carbamazepine in the hindpaws of adult rats
induced dose-dependent analgesia in the injected paw (Todorovic et al., 2003).

Gabapentin, a GABA analogue, is another anticonvulsant agent, with incompletely
understood mechanisms of action. Different theories have been suggested, including en-
hancement of non-vesicular GABA release, modulation of certain types of Ca\(^{2+}\) currents
and inhibition of voltage-activated Na\(^+\) channels (Taylor et al., 1998). Numerous animal
(Gillin and Sorkin, 1998; Hwang and Yaksh, 1997; Jun and Yaksh, 1998) and human
(Backonja et al., 1998; McGraw and Kosek, 1997; Rosenberg et al., 1997; Rosner et al.,
1996) studies have revealed gabapentin as an efficient analgesic agent. Although analogue
of GABA, binding studies showed little affinity of gabapentin at GABA\(_A\) or GABA\(_B\) re-
ceptors (Suman-Chauhan et al., 1993). Moreover, in different rat models of induced neuro-
pathic pain, the antinociceptive effect of intrathecally applied gabapentin could not be re-
versed by the GABA\(_A\) and/or GABA\(_B\) receptor antagonists, bicuculline and CGP 35348,
respectively (Hwang and Yaksh, 1997; Yoon et al., 2003). Thus, it is generally believed
that GABA receptors are not involved in the antinociceptive mechanisms of gabapentin.
Gabapentin was found to have a specific binding site at the \(\alpha_2\delta\) subunit of voltage-gated
Ca\(^{2+}\) channels (Gee et al., 1996), by which it inhibits Ca\(^{2+}\)-currents and consecutively neu-
rotransmitter release and neuronal excitability (Fink et al., 2002; Quintero et al., 2011; Ste-
fani et al., 1998). In the postoperative pain model, intrathecal injection of different modula-
tors of the binding at the \(\alpha_2\delta\) subunit of the Ca\(^{2+}\) channels attenuated the analgesic effect of
gabapentin (Cheng et al., 2003). Moreover, \(\alpha_2\delta\)-1 subunit was found to be upregulated in
DRGs and spinal cord of rats in gabapentin-sensitive, but not in gabapentin-insensitive
pain models (Li et al., 2004; Luo et al., 2002; Luo et al., 2001). These findings suggest that
\(\alpha_2\delta\) subunits of voltage-gated Ca\(^{2+}\) channels are involved in the analgesic effect of gabapen-
tin. Pregabalin, another GABA analogue has shown even greater potency in animal pain
models (Field et al., 1997; Jun and Yaksh, 1998). However, side effects such as dizziness,
somnolence, ataxia and headache have been reported in up to 27% of cases (Backonja et
al., 1998; Rowbotham et al., 1998).
Agonists at GABA<sub>A</sub> receptors as well as substances that increase the GABA concentration, like the GABA uptake inhibitor tiagabine and GABA transaminase inhibitor vigabatrin, show significant antinociceptive effects in several animal models (Jasmin et al., 2003; Kendall et al., 1982; Laughlin et al., 2002; Malan et al., 2002). Due to the wide distribution of GABA<sub>A</sub> receptors in the CNS, as well as the diversity in the receptor subtypes, however, application of GABA<sub>A</sub> receptor modulators in the treatment of pain will correlate with various unwanted side-effects. Thus, development of drugs selective for specific GABA<sub>A</sub> receptor subtypes might be a key point in the clinical treatment of pain. GABA<sub>A</sub> receptors containing δ together with α<sub>4</sub> subunits have been found to mediate the analgesic action of gaboxadol, an agonist at the GABA-binding site (Krogsgaard-Larsen et al., 2004). Indeed, α<sub>4</sub> subunit knockout mice did not respond to the analgesic effects of gaboxadol (Chandra et al., 2006). However, when used in patients with chronic pain of malignant origin, gaboxadol showed strong sedation as side effect, in addition to the analgesic effect (Kjaer and Nielsen, 1983). Spinal GABA<sub>A</sub> receptors containing the α<sub>2</sub> and/or α<sub>3</sub> subunit have been shown recently to mediate pronounced analgesia (Knabl et al., 2008) and their activation by the specific ligand L-838,417 induced analgesia in both, inflammatory and neuropathic, pain models in rats and did not show any unwanted side effects. These new findings may achieve improvement of the analgesic therapy by modulating specific subtypes of GABA<sub>A</sub> receptors.

4.8 Flupirtine

Flupirtine (Katadolon<sup>®</sup>) is a non opioid analgesic drug used in the clinic since the early 1980s in the treatment of a number of pain states (Heusinger, 1987), such as cancer pain (Luben et al., 1994), post-surgery (Moore et al., 1983), arthritis (Herrmann et al., 1987) and muscular pain (Mueller-Schwefe, 2003). Various test procedures predictive of analgesic activity were carried out in animal models, demonstrating the analgesic effect of flupirtine (Carlsson and Jurna, 1987; Goodchild et al., 2008; Nickel, 1987; Nielsen et al., 2004). Its efficacy was repeatedly compared with different established analgesics. Flupirtine has been shown to have pain-relieving properties comparable with those of dihydrocodeine after abdominal hysterectomy (Moore et al., 1983) and of pentazocine (Galasko et al., 1985) or diclofenac (Mastronardi et al., 1988) after orthopaedic surgeries. In patients suffering from cancer pain, flupirtine was significantly more effective in pain reduction than pentazocine (Scheef, 1987) or tramadol (Luben et al., 1994) and caused in both stud-
ies less (intense) side effects. Under therapeutic dosages, plasma concentrations of flupirtine in humans reach up to 5-6.5 µM (Hlavica and Niebch, 1985; Hummel et al., 1991).

Despite its routine usage in the clinic, the mechanism of action that leads to the analgesic effect of flupirtine is not fully understood. However, the analgesic action of flupirtine is supposed to be independent of the opioid pathway. This assumption is supported by studies showing that the pain-relieving property of flupirtine is not reduced by the general opioid receptor antagonist naloxone (Nickel, 1987; Nickel et al., 1985) or naltrexone (Swedberg et al., 1988; Vaupel et al., 1989) and that flupirtine shows no affinity to any of the known opiate receptors in binding studies (Nickel, 1987). It has been shown that flupirtine needs an intact noradrenergic system in the central nervous system to elicit its antinociceptive activity, without having any relevant affinities to α₁- or α₂-adrenoreceptors (Szelenyi et al., 1989).

In addition to the analgesic effect, flupirtine shows neuroprotective (Boscia et al., 2006) and muscle relaxant effects. Muscle relaxation has been demonstrated to depend on the GABAergic system, since it could be abolished by the GABA<sub>A</sub> receptor antagonist bicuculline. However, the benzodiazepine binding site is not involved, since the specific antagonist flumazenil did not alter the myorelaxant action of flupirtine (Nickel et al., 1990). Schwarz et al, however, suggested that NMDA receptors might be involved in the mediation of the muscle relaxant effect of flupirtine, since intraperitoneal or intrathecal administration of flupirtine depressed the spinal polysynaptic flexor reflex (NMDA receptor mediated), but did not influence the monosynaptic Hoffmann reflex (non-NMDA receptor mediated) (Schwarz et al., 1994; Schwarz et al., 1995).

Neuroprotection by flupirtine has been demonstrated in both, in vivo (Block et al., 1997; Rupalla et al., 1995) and in vitro systems (Muller et al., 1997; Perovic et al., 1994; Rupalla et al., 1995). As a possible mechanism of action to explain the neuroprotective effect of flupirtine, an NMDA receptor antagonistic activity was suggested. However, in binding studies it could be shown that flupirtine has no affinity for any of the characterized binding sites at NMDA receptors (Osborne et al., 1998). Moreover, flupirtine was found to indirectly inhibit NMDA receptors by activating the G-protein coupled inwardly rectifying K<sup>+</sup> (GIRK) channels, as shown in electrophysiological recordings of cultured rat hippocampal (Jakob and Krieglstein, 1997) and superior colliculus (Kornhuber et al., 1999a) neurons. The involvement of a G-protein was confirmed by blockage with pertussis toxin (Jakob and Krieglstein, 1997). Thus, it seems that flupirtine accounts for its neuroprotective properties by stabilizing the membrane resting potential through activation of GIRK.
channels and following indirect inhibition of NMDA receptors. Since no cardiac side effects are notable, flupirtine might selectively influence neuronal $K^+$ channels and therefore was proposed to be the prototype of a new class of drugs with analgesic, muscle-relaxant and neuroprotective properties, the so called Selective Neuronal Potassium Channel Openers (SNEPCO) (Kornhuber et al., 1999b).

Flupirtine is a derivative of triaminopyridine (Fig. 4.4) and very similar in structure to retigabine. Like retigabine (Rundfeldt, 1997), flupirtine acts as a neuronal KCNQ/Kv7 $K^+$ channel opener (Gribkoff, 2003). Thus, it facilitates the gating of KCNQ/Kv7 channels by shifting the current-voltage relation to more negative potentials, as demonstrated for recombinant (Martire et al, 2004), as well as native, KCNQ/Kv7 channels (Wladyka and Kunze, 2006).

![Fig. 4.4. Chemical structure of flupirtine](image)

_(ethyl-N-[2-amino-6-(4-fluoro-phenyl-methylamino) pyridine-3-y)] carbamate)
4.9 Aims of the study

With this study, we intended to investigate the possible mechanisms by which flupirtine exerts its analgesic effect. For this, we conducted electrophysiological experiments on primary neuronal cells in culture and semiquantitative analysis using immunoblots of membranes derived from tissue from different regions of the central and peripheral nervous system. More precisely, the following questions were addressed:

- Does flupirtine activate Kv7/M-channels heterologously expressed in tsA cells? And is this effect comparable with the effect in native neurons?
- Does flupirtine affect neurotransmitter release from presynaptic nerve terminals?
- Does flupirtine directly interact with postsynaptic ligand gated ion channels, like glutamate-, glycine- and GABA\textsubscript{A} receptors?
- Does flupirtine differentiate between phasic and tonically-active GABA\textsubscript{A} receptors?
- Is the effect of flupirtine on GABA\textsubscript{A} receptors depending on their localisation in different regions of the central and peripheral nervous system?
- Does the different effect of flupirtine in distinct nerve tissues correlate to the different expression patterns of GABA\textsubscript{A} receptor subunits?
- Is the effect of flupirtine on neuronal M-currents different in pain-related and non pain-related neurons?
- Are both, Kv7/M-channels and GABA\textsubscript{A} receptors, activated/modulated by concentrations of flupirtine corresponding to the therapeutic plasma concentrations?
5. MATERIALS AND METHODS

5.1 Tissue preparation and cell culture

All tissues were collected from Sprague-Dawley rats which have been killed by decapitation in full accordance with all rules of the Austrian animal protection law (see http://ris1.bka.gv.at/AppI/findbgbl.aspx?name=entwurf&format=pdf&docid=COO_2026_100_2_72288 and the Austrian animal experiment by-laws (see http://www.ris2.bka.gv.at/Dokumente/BgbLPdf/2000_198_2/2000_198_2.pdf).

5.1.1 Hippocampal and dorsal horn cell cultures

Hippocampi and dorsal horns of the spinal cord were dissected from neonatal Sprague-Dawley rats. The tissue was collected in ice-cold PUCK/Kyn solution [PUCK solution containing (in mM): NaCl (137), KCl (5.4), Na2HPO4x2H2O (1.1), KH2PO4 (1.1) and glucose (6.1) adjusted to pH 7.3 with NaOH and supplemented with 1mM kynurenic acid]. Subsequently, the tissue was incubated in papain (25 U/mL in L-15 Leibovitz medium, supplemented with 1 mM kynurenic acid) for 15-30 min at 37°C. Enzyme activity was stopped by transferring the tissue for 2-3 minutes in PUCK/Kyn solution containing 25% heat inactivated fetal calf serum (hiFCS). Thereafter, a single cell suspension was obtained by triturating the tissue in high glucose (4.5 g/l) Dulbecco’s modified Eagle’s medium (DMEM) containing 10% hiFCS and 5 µg/mL insulin, 5 µg/mL transferring, 5 ng/mL Na-selenite, 18 µg/mL putrescine, 10 nM progesterone, 2 mM MgCl2, 25000 IU/L penicillin and 25 mg/L streptomycin. For trituration, three fire-polished Pasteur pipettes with decreasing tip diameter were used.

~50 000 cells were seeded into micro-chambers created by glass rings (inner diameter 10 mm) placed in the center of 35 mm culture dishes (Nunc, Roskilde, Denmark) coated with poly D-lysine 0.1 mg/mL and containing 2 ml culture medium. After 2-3 hours, the rings were carefully removed and the next day, medium was changed to 3 ml penicillin- and streptomycin-free culture medium. After 3-5 days, 1 µM cytosine arabinoside was added to the culture medium to inhibit further proliferation of non-neuronal cells.

To obtain hippocampal microisland cultures, ~120 000 cells were seeded into uncoated 35 mm culture dishes containing DMEM supplemented with 10% hiFCS. Medium
was changed after 1-2 hours, and cytosine arabinoside (1 µM) was added after 2 days. This treatment resulted in growth of islands consisting of one or few glial cells. One week later, the island cultures were treated for 60 min with 1 mM glutamate and 3 mM CaCl₂ to remove surviving neurons and subsequently washed 1x with phosphate buffered saline (PBS, containing in mM: NaCl 137, KCl 2.7, Na₂HPO₄·2H₂O 8.1 and KH₂PO₄ 1.8, adjusted to pH 7.4 with NaOH). Subsequently, hippocampal culture medium, containing freshly dissociated neurons (~15 000 neurons per dish) was added and 2 days later, the further proliferation of the glial cells was inhibited by addition of 1 µM cytosine arabinoside.

All cultures were kept in a water-saturated, 5% CO₂ atmosphere at 37°C and used for measurements after 10-15 days.

5.1.2 Superior cervical ganglia and dorsal root ganglia cell cultures

Superior cervical ganglia (SCG) were collected from 4 to 6 days old rats and dorsal root ganglia (DRG) were dissected from 10-14 days old rats. In both cases, ganglia were collected in ice-cold tyrode solution containing (in mM): NaCl (150), KCl (4), MgCl₂·6H₂O (2), glucose (10) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10) adjusted to pH 7.4 with NaOH. The tissue was incubated in collagenase (775 U/ml) and dispase (1.5 U/ml) for 30 minutes at 37°C and subsequently in trypsin solution (745 U/ml) for 15 minutes at 37°C. In order to stop the enzyme activity, the tissue was washed two times with ice-cold tyrode solution. Thereafter, a single cell suspension was obtained by triturating the tissue in high glucose (4.5 g/l) DMEM containing 5% hiFCS and 10 mg/l insulin, 25 000 U/l penicillin, 25 mg/l streptomycin and 50 µg/l human β-nerve growth factor. For trituration, three fire-polished Pasteur pipettes with decreasing tip diameter were used. ~50 000 cells were plated into micro-chambers created by glass rings (inner diameter 10 mm) placed in the center of 35 mm culture dishes (Nunc, Roskilde, Denmark) coated with poly D-lysine 0.1 mg/mL and containing 2 ml FCS-free culture medium. After 2-3 hours, the rings were carefully removed and 5% hiFCS was added to the medium. The next day, medium was changed to 3 ml penicillin- and streptomycin-free but hiFCS containing culture medium.

Cultures were kept in a water-saturated, 5% CO₂ atmosphere at 37°C and used for measurements after 2-4 days.
5.1.3 Cell line and transfection

**tsA 201 cells (a subclone of human embryonic kidney (HEK) 293 cells stably expressing the SV40 large T-antigen)** were cultured in DMEM containing 1 g/l glucose and l-glutamine, supplemented with 10 % FCS and 25,000 IU/l penicillin plus 25 mg/l streptomycin. For patch-clamp experiments, cells were plated in 35 mm culture dishes coated with poly-D-lysine. Cells were transfected with human Kv7.2 and Kv7.3 (kindly provided by M.S. Shapiro, San Antonio, Tx, USA) using ExGen 500 (Fermentas; St.Leon-Rot, Germany) according to the manufacturers' recommendations. To identify successfully transfected cells, the pEYFP-N1 vector (Becton Dickinson, Heidelberg, Germany) was cotransfected, and patch clamp recordings were carried out with fluorescent cells 24 to 48 h after transfection.

5.2 Electrophysiological recordings

5.2.1 General considerations

The patch clamp technique is used for electrophysiological recordings of ion currents across the membrane of excitable cells. The technique was developed in the late 1970s and early 1980s, by the German physiologists Erwin Neher and Bert Sakmann, who were able to record for the first time the currents of single ion channel (Neher and Sakmann, 1976). Figure 5.1 shows a schematic representation of different patch clamp configurations. Patch clamp recording uses, as an electrode, a glass capillary that has an open tip diameter of about 1 µm. This electrode is sealed onto the surface of the cell membrane (the "patch") that often contains just one or a few ion channel molecules. Therefore, the pipette tip is attached to the cell membrane and by caution suction, an electric high resistance seal of more than 1 gigaohm (so called “gigaseal”) is formed between the glass capillary and the cell membrane. In this situation, the membrane patch is electrically connected to the electrode (**cell-attached mode**), allowing the recording of ion flow across the membrane patch. Now, a brief suction can be applied in order to rupture the patch and thus, allow electrical recordings of the whole cell membrane (**whole-cell configuration**). This configuration leads to dialysis of the cytoplasm, i.e. that soluble contents of the cell interior will slowly be replaced by the contents of the electrode, since the volume of the electrode
is larger than that of the cell. Thus, any properties of the cell that depend on soluble intra- 
cellular contents will be altered. Alternatively, chemicals that insert ion-permeable pores 
into the cell membrane and thus provide electrical access to the cell interior (such as am-
photericin-B), can be added to the solution inside the patch electrode (perforated configu-
ration). That way, electrical recordings of the whole cell membrane can be made, without 
loss of soluble cytoplasmic components. The interior of the pipette is filled with a solution 
matching the ionic composition of the cytosol and connected to a chloride silver wire that 
conducts electric current to the amplifier. A second electrode (reference electrode) is 
placed in the bath solution and this is used in order to set the zero current level. Keeping 
the voltage constant, changes in current can be observed (voltage-clamped configuration), 
or, alternatively, changes in membrane voltage can be monitored, by keeping the current 
constant (current-clamped configuration).

5.2.2 Patch clamp set up

Recorded cells were visualized under an inverted microscope (Nikon). Patch elec-
trodes were pulled with a Flaming-Brown puller (Sutter Instruments, Novato, CA, USA) 
from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) to yield 
tip resistances of 2-4 MΩ. The electrodes were filled with an isotonic buffer solution that 
mimics intracellular conditions and electrically connected to the amplifier via an AgCl 
coated wire.

The standard bathing solution consisted (in mM) of: NaCl (140), KCl (6), CaCl₂ 
(3), MgCl₂ (2), glucose (20) and HEPES (10), adjusted to pH 7.35 with NaOH. During 
recordings, cells were continuously superfused with this solution containing test drugs as 
appropriate. Superfusion was performed with a DAD-12 (Adams & List, Westbury, NY, 
USA) drug application system, which permits complete exchange of solutions within less 
than 100 ms (Boehm and Betz, 1997). Pipettes were filled with a standard solution contain-
ing (in mM): KCl (140), CaCl₂ (1.6), EGTA (10) and HEPES (10) adjusted to pH 7.2 with 
KOH. When perforated patch clamp was performed, pipettes were front-filled with the 
standard solution and then back-filled with the same solution containing freshly dissolved 
amphotericin-B in a final concentration of 250 µg/mL. Differences in ion concentrations 
between the solutions resulted in a calculated liquid junction potential below ±10 mV that 
was not taken into further consideration during experimentation or data evaluation.
All currents were registered using an Axopatch 200B amplifier, Digidata 1322A digitizer (Axon Instruments) and the PCLAMP 8.0 hard- and software (Molecular Devices, Sunnyvale, CA, USA). Currents were low-pass filtered at 2-10 kHz, digitized at 5-20 kHz and stored on an IBM compatible computer. Traces were analysed off-line using the CLAMPFIT 10.2 program (Molecular Devices).

Figure 5.1. Schematic representation of different patch clamp configurations. Cell attached, outside-out and inside-out configurations are used for single channel recordings, while whole-cell configuration is used to study the whole ion channel population of a cell (Hamill et al., 1981).
5.2.3 Recording and analysis of autaptic currents

Autaptic currents were recorded from 10-16 days old hippocampal microisland cultures. In such cultures, isolated hippocampal neurons grow on islands of one or few glial cells. Since building synapses is the nature of neuronal cells, those isolated neurons will build synapses to themselves, called autapses; and the current recorded from an autaptic neuron, is called autaptic current. The advantage of these measurements over recordings in mass culture is that one is able to stimulate and record the very same neuron and the number of activated synapses remains constant with repeated stimulations.

Currents were recorded in the whole-cell configuration of the patch-clamp technique at room temperature (20-24°C). The recorded neurons were clamped at a holding potential of -70 mV and depolarised for 1 ms to voltages between 0 and 30 mV at a frequency of 1 Hz. When a functional autapse was present, a typical postsynaptic current was observed. The origin of the current could be identified by blockage either with 10 µM cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) (excitatory autaptic current) or with 30 µM bicuculline methionide (BMI) (inhibitory autaptic current).

Autaptic currents were quantified as described before (Dorostkar & Boehm, 2007). Autaptic inhibitory (aIPSC) or excitatory (aEPSC) postsynaptic currents were analysed for their total charge transfer recorded in the presence or absence of flupirtine. Calculated values are expressed as percentage of the arithmetic means of the total charge transfer of the first and the last current recorded within a time series (relative transferred charge). Using the GraphPad Prism 4.0 software, the results obtained from all recorded cells were fitted into a sigmoidal dose-response curve and plotted against the logarithmic concentration for flupirtine.

5.2.4 Recording currents through ligand-gated ion channels

Currents through ligand-gated ion channels were recorded in the perforated patch clamp mode from 10-20 days old hippocampal and dorsal horn cultures or 2-4 days old dorsal root ganglia cultures. Currents were evoked by application for 3-5 s of the agonist to neurons clamped at -70 mV. All measurements were conducted in presence of the Na\(^+\) channel blocker tetrodotoxin (TTX; 0.1 µM). In addition, CNQX (10 µM) was used for GABA- and glycine- evoked currents to block ionotropic glutamate receptors, while BMI (30 µM) was added when glutamate-evoked currents were measured in order to block GABAA receptors (Boehm et al., 1997). For NMDA-evoked currents, the solution lacked
MgCl₂, but contained glycine (10 µM), strychnine (0.1 µM) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoline-7-sulfonamide (NBQX) (10 µM) to facilitate the gating of NMDA receptors, but block inhibitory glycine receptors (Boehm et al., 1997). Currents were quantified by determining peak current amplitudes.

5.2.5 Recording of miniature inhibitory postsynaptic currents (mIPSCs)

mIPSCs were recorded in the whole-cell configuration of the patch clamp technique. 10-20 days old hippocampal neurons grown in mass cultures were clamped at -70 mV and measured in standard internal and external solutions, the latter containing 0.1 µM TTX. In order to isolate mIPSCs from miniature excitatory postsynaptic currents (mEPSCs), 10 µM CNQX was added to the bath solution. This way, all recorded events corresponded to spontaneous release of one or more vesicles with inhibitory (GABAergic) neurotransmitter. Flupirtine or other drugs were applied for 60 seconds. Before (A) and after (C) application of any substance (B), cells were washed with control solution for 60 seconds. mIPSCs were analysed with the MINIANALYSIS 6.0.3 software (Synapto-soft, Inc., Decatur, GA, USA). All events recorded during 60 seconds washing or drug application period, were analysed for 10-90% rise time, amplitude and decay time. The mean value for all events in one 60 seconds recording period was calculated for each parameter. Control values were calculated as C/A and the effect of flupirtine or midazolam was calculated with the equation: 2*B/(A+C).

5.2.6 Recording currents through KCNQ/Kv7 channels (Iₘ)

Currents through KCNQ/Kv7 channels (Iₘ) were determined in the perforated patch clamp mode. The standard external (bathing) solution was used, while the internal solution contained (in mM) K₂SO₄ (75), KCl (55), MgCl₂ (8), and HEPES (10), adjusted to pH 7.3 with KOH. To quantify Iₘ, non-inactivating outward currents were activated by depolarizing the cell membrane to -30 mV. Once every 10 s, cells were hyperpolarized to -55 mV for 1 s periods in order to let the channels close; the deactivation current observed during these 1 s hyperpolarisations is specific for the Kv7 channels (Boehm, 1998). Increasing concentrations of flupirtine were applied for one minute period each, with one minute washing periods in between and currents were quantified by measuring amplitudes observed at -30 mV.
5.2.7 Calculations and statistics

To evaluate the effects of flupirtine on various types of currents, chosen drug concentrations and the appropriate solvent were applied alternately; current amplitudes obtained in the presence of either flupirtine or solvent were then normalized to the arithmetic mean of control current amplitudes recorded before and after the application of both, drug and solvent (normalized current amplitudes). For statistical purposes, amplitudes in the presence of flupirtine were compared with those in the presence of solvent. Significances of differences were evaluated using a Kruskal-Wallis analysis of variance followed by a Dunn’s multiple comparison test; for pairwise comparisons, a Mann-Whitney test was used instead.

For concentrations response curves of GABA induced currents in the presence of solvent or flupirtine, current amplitudes evoked by different GABA concentrations in solvent or flupirtine were normalized to those evoked by 30 µM GABA in solvent in the very same neuron.

5.3 Western blot analysis

5.3.1 Principle of Western blot analysis

The Western blot is a method to detect specific proteins in a given sample of tissue homogenate or extract. It uses Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in order to separate denatured proteins by the length of the polypeptide. The proteins are first denatured and linearized using denaturing reagent (dithiothreitol or β-mercaptoethanol) and sodium dodecyl sulphate (SDS). SDS imparts negative charge to the linearized proteins, which are then separated based on their molecular weight using an electric field on SDS-PAGE gels. After the protein components have been sufficiently separated by electrophoresis, they can be transferred to a polyvinylidene fluoride (PVDF) or nitrocellulose membrane. The transfer process uses the same principle as SDS-PAGE – this time the electric current is applied at 90 degrees to the gel and the proteins migrate out of the gel onto the membrane. The proteins of interest are then detected using specific antibodies directed against the target protein.
5.3.2 Membrane protein preparation

Total amount of membrane protein was prepared from hippocampi, DRGs or dorsal horns of 10-14 days old rats. All steps were carried out on ice or at 4°C. Isolated tissue was homogenized in homogenizing buffer (HB) containing (in mM): HEPES (10), ethylenediaminetetraacetic acid (EDTA) (1) and sucrose (300) and supplemented with EDTA-free Protease Inhibitors. After two steps of homogenization followed by centrifugation at 4°C and 20000 rpm for 30 min, the pellet was re-suspended in sucrose-free HB and subsequently re-centrifuged at 20,000 rpm. Pellets were stored at -20°C until usage.

Determination of protein concentration was carried out using the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer’s instructions. This method uses the reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of Cu⁺¹ using a unique reagent containing bicinchoninic acid (BCA). Subsequently, protein concentration was detected using the KC Junior software, by measuring the absorbance at 562 nm wavelength.

5.3.3 Preparation of the polyacrylamide gels and electrophoresis

For western blot experiments, polyacrylamide gels consisting of 5% stacking and 10% resolving gel were used. The percentage of the acrylamide within the resolving gel was chosen according to the expected size of the GABA_A receptor subunits (~50 kDa). 5% stacking polyacrylamide gel contains 125 mM bis(2-hydroxyethyl)aminotris(hydroxymethyl)methan (Bis-Tris) buffer (pH 6.4), 5% Acrylamide/Bis, 0.1% SDS, 0.1% tetramethylethylendiamin (TEMED) and 0.15% ammonium persulfate (APS). 10% resolving polyacrylamide gel contains 375 mM Bis-Tris (pH 6.4), 10% Acrylamide/Bis (BioRad), 0.1% SDS, 0.1% TEMED and 0.1% APS.

The resolving gel was prepared on ice and filled in 1 mm Plastic Cassettes (Invitrogen). Subsequently, gels were covered with 0.1% SDS and let polymerise at room temperature for at least four hours for subsequent use or for at least one hour at room temperature and then stored at 4°C until usage. Before usage, stacking gel solution was prepared on ice and was filled in the gel cassette on top of the resolving gel, after this was rinsed 3x with 125 mM Bis-Tris buffer (pH 6.4). A fifteen-lane plastic comb was inserted and the gel polymerised at room temperature for at least 45 minutes. After polymerization, two gel cassettes were placed in a XCell SureLock™ Mini-Cell vertical electrophoresis unit (Invitro-
gen), which was then filled with 3-(N-morpholino)propanesulfonic acid (MOPS) buffer containing (in mM): MOPS (50), Tris (50), EDTA (1) and 0.1% SDS, adjusted to pH 7.7 with HCl. The core chamber between the cassettes was filled with the same buffer, containing 0.25% NuPage antioxidants. Before loading, the samples were dissolved in Laemmli sample buffer to yield concentrations of 1 μg/μl protein, denatured at 70°C for 10 min and subsequently centrifuged for 2 min at 13.000 rpm. In each lane, equal amount of protein (8 μg) was loaded. As a standard for molecular weight, the 'MagicMark™ XP Western Standards’ (Invitrogen, Carlsbad, CA, USA) was used on a separate lane. The electrophoresis was carried out at a voltage of 200 V (constant) and a current of 125 mA per gel for 45 min. After the electrophoresis, the stacking gel was removed and the resolving gel was swaying in transfer buffer containing (in mM) Tris (47.9), glycine (38.6), SDS (1.7) and 10% MeOH for at least 15 minutes before blotting.

5.3.4 Blotting and immunostaining

Blotting was carried out using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad Laboratories, Hercules, CA, USA). Extra thick blot paper Protean® XL Size (BioRad Laboratories, Hercules, CA, USA) and 0.45 μm ImmobilonTM PVDF transfer membrane (Millipore) were cut in the size of the gel (6 x 8 cm) and soaked in transfer buffer for at least 10 minutes before blotting. Previous to this procedure, PVDF membranes were equilibrated for 20 seconds in methanol absolute and immediately rinsed with double-distilled H2O. Blotting was carried out for 1 h at a constant voltage of 20 V and adjusted current to 58 mA per gel. After transfer was completed, the membranes were incubated at room temperature for 1 h in blocking buffer containing 1.5% milk powder and 0.1% Tween20 in PBS (containing in mM: NaCl 137, KCl 2.7, KH2PO4 1.8, Na2HPO4 10, pH 7.4).

After blocking, the membranes were incubated overnight at 4°C with one of the following antibodies directed against specific GABA_A receptor subunits: α1(amino acid residues 328-382); α2(416-424), α3(338-385), α4(379-421), α5(337-388), β1(350-404), β2(351-405), β3(345-408), γ2(1-33) or δ(1-44) (Poltl et al., 2003). All antibodies directed against one of the GABA_A receptor subunit were produced in rabbits and used at concentration of 1 μg/ml. Next day, the membranes were washed in blocking buffer three times for 20 min and then incubated with the secondary antibody for 1 h at room temperature. As a secondary antibody, a goat anti-rabbit antibody linked to alkaline phosphatase (Alkaline Phos-
phatase-conjugated Goat Anti-Rabbit IgG F(ab′)2 fragment, Jackson Immuno Research Laboratories, West Grove, PA, USA) at a dilution of 1:2000 was used. Subsequently, the membranes were washed in blocking buffer 3x for 20 min each. Before detection, the membranes were equilibrated by rinsing two buffers, for 5 and 10 min, with assay buffer containing 1 mM MgCl₂ and 25 mM diethanolamine adjusted to pH 10.0 with HCl. For detection of alkaline phosphatase, CDP-Star® Chemiluminescent Substrate (Applied Biosystems, Bedford, MA, USA) was used at the dilution of 1:1000 in assay buffer. Finally, detection was performed by using the quantitative imaging system Fluor-STM MultiImager (BioRad Laboratories, Hercules, CA, USA). Quantification of the intensity of the bands was performed with Quantity One® quantification software (BioRad Laboratories, Hercules, CA, USA).

Two lanes in each blot were also stained for α₁ subunits and all images were taken after 10 minutes. The experiments were performed three times and each subunit was detected on the same blot in duplicates. Relative amounts of GABAₓ receptors proteins in the various tissues were estimated by comparison with staining of the α₁ subunit within the same tissue and the same blot. Thereafter, these normalized densities were compared for the three tissues investigated.

5.4 Materials

Flupirtine, GABA, BMI, kynurenic acid, CNQX, glutamate, glycine, NMDA, midazolam strychnine, NBQX, putrescine, progesterone, poly-D-lysine, cytosine arabinoside, amphotericin B, Penicillin G, collagenase as well as the bulk chemicals were obtained from Sigma-Aldrich (Vienna, Austria). Insulin, transferrin, Na-selenite and dispase were obtained from Roche (Mannheim, Germany); Dulbecco's modified Eagle's medium (DMEM), Leibovitz L-15 medium, penicillin, streptomycin and l-glutamine were purchased from PAA Laboratories (Pasching, Austria); papain and trypsin were bought from Worthington (Lakewood, NJ, USA); nerve growth factor from R&D Systems Inc. (Minneapolis, MN, USA); heat-inactivated fetal calf serum was obtained from Invitrogen (Lofer, Austria), linopirdine from Tocris bioscience (Austria) and tetrodotoxin (TTX) from Latoxan (Rosans, France).

Acrylamide/Bis was obtained from BioRad Laboratories (Hercules, CA, USA), Bis-Tris, TEMED, Tween-20, diethanolamine from Sigma-Aldrich (St. Louis, MO) and
SDS from Applichem (Darmstadt, Germany). Protease Inhibitors were purchased from Roche (Vienna, Austria); APS form Amersham Biosciences (Buckinghamshire, UK) and NuPage antioxidants from Invitrogen (Carlsbad, CA, USA). MOPS was bought from Avantor Performance Materials (Center Valley, PA) and Tris-aminomethan and Sucrose from Merck (Whitehouse Station, NJ).
6. RESULTS

6.1 Flupirtine enhances currents through Kv7 channels

M-type K⁺ channels were first described in sympathetic ganglion neurons (Brown and Adams, 1980; Constanti and Brown, 1981) and its name relates to the inhibition through stimulation of muscarinic acetylcholine receptors (Delmas and Brown, 2005). Functionally, M-current is a slowly activating and non-inactivating voltage dependent K⁺ current with a threshold voltage near -60 mV. The functional channel is composed of heteromeric or homomeric assemblies of subunits belonging to the Kv7/KCNQ family. In total, five members of this family are known (Kv7.1-Kv7.5). Sensory neurons predominantly express Kv7.2 and Kv7.3 subunits and only small amounts of Kv7.5 (Rose et al., 2011). Therefore, Kv7.2 and Kv7.3 were co-expressed in tsA cells, and currents were elicited by slow voltage ramps from -100 to -20 mV. The resulting outward rectifying currents were shifted to more negative potentials in a concentration-dependent manner in the presence of either 3 or 30 µM flupirtine (Figure 6.1a).

![Fig. 6.1. Effects of flupirtine on heterologously expressed Kv7 channels.](image)

Human Kv7.2 and 7.3 were co-expressed in tsA cells. Currents were evoked by ramp hyperpolarizations from -25 to -100 mV during periods of 1 s. (a) shows representative current traces in the absence or presence of 3 or 30 µM flupirtine. (b) Current amplitudes were determined at -30 mV in the absence or presence of increasing concentrations of flupirtine; the average of normalized current amplitudes plotted against the logarithmic concentration of flupirtine is shown for 7 cells.
To obtain full concentration response curves, non-inactivating outward currents were measured at –30 mV, and their enhancement in the presence of 0.1 to 100 µM flupirtine was determined. The analgesic augmented outward current amplitudes by up to 70.9 ± 8.8 % with half maximal effects at 4.6 ± 1.2 µM (Figure 6.1b). Virtually identical results were obtained with currents through Kv7 channels endogenously expressed in rat superior cervical ganglion neurons: 30 µM flupirtine shifted the current voltage relation by about 20 mV in a hyperpolarizing direction (not shown), and currents at -30 mV were enhanced by up to 70.9 ± 6.8 % with half maximal effects occurring at 4.6 ± 3.9 µM flupirtine (n = 6).

6.2 Flupirtine enhances inhibitory, but not excitatory, autaptic currents in hippocampal microculture

Recently, Martire et al. provided evidence for a major regulatory role of the KCNQ2 subunit of M-type K⁺ channels in neurotransmitter release from rat hippocampal nerve endings (Martire et al., 2004). Thus, we next wanted to investigate whether flupirtine has an effect on the neurotransmitter release at the presynaptic site of hippocampal neurons. Therefore, we used hippocampal microisland cultures and measured the effect of flupirtine on autaptic currents. Autaptic currents were elicited by depolarising the neurons from a holding potential of -70 mV to voltages of 10 mV for a time period of 1 ms at a frequency of 1 Hz. When a current was recorded, its origin regarding the type of neurotransmitter was identified by application of typical blockers. Thus, inhibitory currents, mediated by GABA_A receptors, could be blocked by 30 µM BMI, while excitatory currents, mediated by ionotropic non-NMDA glutamate receptors, could be blocked by 10 µM CNQX (Fig. 6.2a). Application of flupirtine prolonged the decay time of inhibitory (aIPSC), but did not alter the excitatory (aEPSC) autaptic currents (Fig. 6.2a). Fig. 6.2b shows that flupirtine dose-dependently potentiated aIPSC, with a maximal effect of more than 400% and an EC₅₀ value of 12 µM.
Figure 6.2. Flupirtine enhances inhibitory, but not excitatory autaptic currents. Autaptic inhibitory (aIPSC) or excitatory (aEPSC) postsynaptic currents were elicited by 1 ms depolarization to 10 mV and recorded at a holding potential of -70 mV in control solution, in the presence of flupirtine, and in the presence of BMI (30 µM) or CNQX (10 µM), for inhibitory and excitatory aPSC, respectively. (a) shows representative current traces for both inhibitory (aIPSC) and excitatory (aEPSC) autaptic currents. aIPSC and aEPSC could be blocked by BMI (30 µM) and CNQX (10 µM), respectively. (b) shows the concentration-response curves for the effect of flupirtine on aIPSCs as determined via the relative charge transfer. For each cell (n = 6), the values were calculated as percentage of the control and plotted against the logarithmic concentrations for flupirtine. EC50 value was calculated to be 12 µM.

6.3 GABA<sub>A</sub> receptors, but no other ligand-gated ion channels are modulated by low micromolar flupirtine concentrations

Next, we investigated whether flupirtine might have a direct effect on postsynaptic transmitter-gated ion channels. Amongst all transmitter-gated ion channels, GABA<sub>A</sub>, glycine and ionotropic glutamate receptors are of utmost importance in pain perception (Basbaum et al., 2009). Therefore, the effects of flupirtine on these receptors were investigated using primary rat hippocampal neurons. To be able to identify potential effects whether these synaptic receptors are just partially or fully activated, just suprathreshold and near saturating concentrations of agonists were applied as follows: 3 and 100 µM glutamate (in the presence of 2 mM Mg<sup>2+</sup> to block NMDA receptors) to activate non-NMDA glutamate receptors, 10 and 100 µM NMDA in the continuous presence of 10 µM glycine to activate NMDA receptors, 3 and 100 µM GABA to activate GABA<sub>A</sub> receptors, and 30 and 300 µM glycine to activate inhibitory glycine receptors. These agonist concentrations were applied either in solvent or together with 10 or 30 µM flupirtine. For glutamate and
glycine, current amplitudes determined in the presence of either flupirtine concentration were not different from those in solvent (Figure 6.3a and 6.3d). However, NMDA induced current amplitudes, irrespective of the agonist concentration employed, were significantly reduced by 30 µM flupirtine as compared with solvent (Figure 6.3b). In contrast, amplitudes of currents evoked by 3 µM GABA were significantly enhanced by 10 as well as 30 µM flupirtine, whereas the currents evoked by 100 µM GABA were not affected (Figure 6.3c).

Fig. 6.3. Effects of flupirtine on ligand-gated ion channels in hippocampal neurons. Hippocampal neurons were clamped at a voltage of -70 mV and currents through different ligand-gated ion channels were evoked by above threshold or near saturating concentrations of agonists in either the absence or presence of 10 µM (black bars) or 30 µM (white bars) flupirtine. Normalized amplitudes of currents evoked by application of 3 µM (n=9) or 100 µM (n=12) glutamate (a), 10 (n=10) or 100 µM (n=10) NMDA (b), 3 µM (n=6) or 100 µM (n=8) GABA (c) and 30 µM (n=6) or 300 µM (n=6) glycine (d) are shown. * and ** indicate statistically significant differences at p<0.05 and p<0.01, respectively, as determined by a Kruskal-Wallis analysis of variance followed by a Dunn’s multiple comparison test.
6.4 Flupirtine acts as an allosteric modulator on GABA$_A$ receptors in hippocampal neurons

As flupirtine effects on GABA$_A$ receptors depended on the concentration of the receptor agonist used to induce currents, concentration response curves for GABA-evoked currents were determined in hippocampal mass cultures in the presence and absence of flupirtine. Therefore, neurons were clamped at -70 mV and different concentrations of GABA were applied for 3 or 5 sec (Fig. 6.4a). When flupirtine was co-applied, cells were washed with the same concentration flupirtine for 10 sec before and after application of GABA. To exclude false results due to receptors desensitisation or opening/closing of the cell during recordings, the same concentration of GABA was applied before (A) and after (C) co-application with flupirtine and only currents where the ratio C/A was <1.2 and >0.8 were used for further analysis.

![Concentration response curves for GABA-evoked currents in the presence and absence of flupirtine.](image)

Figure 5.4. Flupirtine acts as an un-competitive antagonist at the postsynaptic GABA$_A$ receptors in hippocampal neurons. For generation of concentration-response curves, cells were held at a holding potential of -70 mV and currents were evoked by the direct application for 3-5 sec of various concentrations of GABA in presence and absence of flupirtine 30 µM. (a) shows representative traces of currents evoked by 3, 30, and 300 µM GABA in either the absence (control) or presence of flupirtine (30 µM). (b) shows concentration-response curve for GABA-induced currents in the absence (full circles) and presence (empty circles) of 30 µM flupirtine, respectively (n = 20). For each cell, current...
amplitudes were normalized to that evoked by 30 µM GABA in the absence of flupirtine and plotted against the logarithmic concentrations of GABA. Calculated EC₅₀ values were 5.9 µM and 2.4 µM in the absence and presence of flupirtine, respectively. Flupirtine reduces the maximal effect by 8.3%.

In resulting concentration-response curves for GABA-induced currents in hippocampal neurons, flupirtine (30 µM) shifted the concentration-response curve to the left and at the same time decreased the maximal effect (Fig. 6.4b). EC₅₀ values were decreased 2.4-fold, from 5.9 µM in the absence, to 2.4 µM in the presence of flupirtine and the maximal effect was significantly reduced by 8.3% (p < 0.05). Thus, flupirtine acts as an uncompetitive antagonist at GABAₐ receptors. We further generated the concentration-response curve for flupirtine at low (3 µM) GABA concentrations (Fig. 6.5). Flupirtine increased currents induced by 3 µM GABA in hippocampal neurons, with a maximal effect of 1.8-fold and an EC₅₀ value of 17.3 µM.

These results show that flupirtine acts as an allosteric modulator at the postsynaptic GABAₐ receptors in hippocampal neurons.

Figure 6.5. Flupirtine concentration-dependently potentiates the current induced by 3 µM GABA in hippocampal neurons. (a) shows representative traces of currents evoked by application for 3 sec of 3 µM GABA alone (control) and in the presence of flupirtine 3, 30, and 100 µM. (b) shows concentration-response curve for flupirtine on current evoked by 3 µM GABA (n = 4). For each cell, current amplitudes were normalized to that evoked by 3 µM GABA in the absence of flupirtine. The calculated EC₅₀ value was 17.3 µM and the maximal effect (Emax) was 1.8-fold increase of the GABA (3 µM)–induced current.
6.5 Flupirtine enhances currents through tonically-active GABA<sub>A</sub> receptors but does not alter synaptic transmission

Previous to co-application of flupirtine with GABA, cells were washed with flupirtine alone for 10 s. During this time we observed, in case of higher concentration of flupirtine, a shift in the holding current towards more negative values. Thus, we further investigated whether flupirtine alone may induce an inward current in hippocampal neurons. Therefore, we clamped the cells again at -70 mV and applied flupirtine for a period of 10 sec. As shown in Fig. 6.6, flupirtine indeed, concentration dependently, induced an inward current and this current was mediated by GABA<sub>A</sub> receptors, since it could be blocked by 30 µM BMI. However, since no GABA was present in the solution, these results suggest that flupirtine may modulate tonically active GABA<sub>A</sub> receptors.

![Figure 6.6](image)

**Figure 6.6.** In hippocampal neurons, flupirtine induces an inward current mediated by GABA<sub>A</sub> receptors. Different concentrations of flupirtine alone were applied to hippocampal neurons clamped at a holding potential of -70 mV. Bar graphs show the current amplitudes (in pA) induced by flupirtine 3, 30 and 100 µM (n = 6, each). This current could be block by co-application of BMI 30 µM, indicating that it is mediated by GABA<sub>A</sub> receptors.

Regarding their subcellular localization, GABA<sub>A</sub> receptors can be divided in two different groups: synaptic and extrasynaptic receptors. They mediate GABAergic transmission via different mechanisms, with synaptic receptors being responsible for the synaptic – or “phasic” – GABA-mediated inhibition, while extrasynaptic GABA<sub>A</sub> receptors generate a persistent or tonic inhibition (Mody and Pearce, 2004). Synaptic transmission mediates the rapid and precise translation of presynaptic activity into a postsynaptic signal. When an action potential arrives at the presynaptic terminal, a local calcium influx triggers the release of GABA from multiple presynaptic vesicles into the synaptic cleft, with GABA achieving concentrations in the millimolar range (Mody et al., 1994). This leads to simultaneous opening of all (synaptic and extrasynaptic) GABA<sub>A</sub> receptors and thus, to the gen-
eration of hyperpolarizing inhibitory postsynaptic potential (IPSP) on the postsynaptic neuron. Even in the absence of action potentials, spontaneous release of a single GABA-containing presynaptic vesicle can occur. In this case, only those synaptic GABA_A receptors that are clustered in the postsynaptic membrane immediately beneath the release site will get activated, generating the so-called miniature inhibitory postsynaptic current (mIPSC). Extrasynaptic GABA_A receptors present a much higher affinity to GABA and get activated by near-micromolar levels of the agonist (Lerma et al., 1986; Tossman et al., 1986). Although it has been shown that some recombinant (Maksay et al., 2003; Sigel et al., 1989) and native (Birmir et al., 2000) GABA_A receptors are able to open spontaneously in the absence of GABA, it is generally considered that binding of the agonist is required for opening of extrasynaptic GABA_A receptors. Presence of GABA molecules in the extrasynaptic regions can arrive from GABA spillover from the synaptic cleft following vesicle release (Rossi and Hamann, 1998). It has been shown that tonic activity of GABA_A receptors can be reduced by enhanced activity of specific GABA transporters (Rossi et al., 2003), which are normally responsible for removing GABA from the extracellular space. These findings were supported by reverse experiments, where pharmacological blockage of GABA transporters increased the magnitude of the tonic current (Semyanov et al., 2003; Wall and Usowicz, 1997).

Thus, we wanted to investigate whether flupirtine exerts different effects on synaptic and extrasynaptic GABA_A receptors. Therefore, we measured mIPSCs in hippocampal neurons, by clamping the cells at -70 mV and blocking action potential generation via TTX (0.5 µM) and glutamatergic transmission via CNQX (10 µM). By using BMI (30 µM) as a blocker for GABA_A receptors, we could make sure that we measured inhibitory, GABAergic synaptic activity (Fig. 6.7a). Upon application of BMI, not only synaptic events, but also the current mediated through tonically-active GABA_A receptors could be blocked. The latter is observed by the upward shift of the baseline current during the presence of BMI. The effect of BMI could be easily washed out and application of flupirtine resulted in a great potentiation of the tonic current (downward shift of the baseline in Fig. 6.7a). However, as shown by the single synaptic event illustrated in Fig. 6.7b, flupirtine does not seem to alter GABAergic transmission through synaptic GABA_A receptors. We used midazolam (3 µM) as a positive control, since benzodiazepines are known to increase GABAergic synaptic transmission (Poncer et al., 1996). All events were analysed for amplitude, 10-90% rise time and decay time using the MINIANALYSIS 6.0.3 software (Synaptosoft, Inc., Decatur, GA, USA). For each of these parameters, we plotted all control values and
values calculated for flupirtine or midazolam, against the cumulative probability for all events in one cell (Fig. 6.8a). In the case of flupirtine, there was no difference between the values measured in presence or absence of the drug. In contrast, in presence of midazolam (3 µM), the correlation was altered for the decay time and apparently slightly for the amplitude values, but not for the 10-90% rise time values. However, in the statistical analysis for all cells (n = 6), midazolam significantly increased the decay time, but did not alter the amplitude or rise time of the mIPSCs and none of the parameters were changed by flupirtine (Fig. 6.8b).

These results indicate that flupirtine only potentiates currents through extrasynaptic GABA<sub>A</sub> receptors, but does not alter the synaptic GABAergic transmission.

**Figure 6.7. Flupirtine enhances GABA<sub>A</sub> receptors –mediated tonic current, but does not alter synaptic transmission in hippocampal neurons.** Miniature inhibitory postsynaptic currents (mIPSCs) were measured in order to distinguish between synaptic and extrasynaptic GABA<sub>A</sub> receptors. Cells were clamped at -70 mV and superfused with standard control solution containing 10 µM CNQX and 0.1 µM TTX. (a) Representative trace showing spontaneous synaptic activity recorded during washing steps and application of BMI (30 µM), flupirtine (30 µM) or midazolam (3 µM) (as indicated by the bars) for 60 seconds each. (b) shows representative single events in control condition and in the presence of flupirtine (30 µM) or midazolam (3 µM). Scaled graphs show that flupirtine does not alter, while midazolam enhances, GABA<sub>A</sub> receptors –mediated synaptic transmission.
Figure 6.8. Flupirtine does not alter any of the mIPSC parameters in hippocampal neurons (a) current amplitude, 10-90% rise time and decay time were plotted against the cumulative probability for all events recorded in one cell under control condition (full circles) and application of 30 µM flupirtine (upper row) or 3 µM midazolam (lower row) (empty circles). (b) bar graphs represent amplitude, rise time and decay time calculated for all cells (n = 6). Values were normalized to the relative control values obtained under control conditions before and after drug application. * p<0.05 in one-way ANOVA, Kruskal-Wallis test
6.6 When synaptic GABA$_{A}$ receptors are blocked by Penicillin G 5 mM, flupirtine acts as a pure potentiator on GABA$_{A}$ receptors in hippocampal neurons

Further, we wanted to investigate the effect of flupirtine selectively on the synaptic and/or extrasynaptic GABA$_{A}$ receptors. Therefore, we made use of the different pharmacological properties of these two groups of receptors. Penicillin G in high concentrations (5 mM) it has been shown to selectively block synaptic GABA$_{A}$ receptors (Bai et al., 2001). Thus, we used this drug to investigate the effect of flupirtine on (pharmacologically isolated) extrasynaptic GABA$_{A}$ receptors. First, we generated the concentration-response curve for GABA in absence and presence of Penicillin G 5 mM in hippocampal neurons. Here, Penicillin G acts as a non-competitive antagonist at GABA$_{A}$ receptors, reducing the EC$_{50}$ value 2.9–fold and the maximal amplitude by 70% (Fig. 6.9a). The original trace in fig. 6.9a shows that 5 mM Penicillin G selectively blocks the synaptic, but not the extrasynaptic GBAA$_{A}$ receptors. Upon application of Penicillin G, all synaptic events are abolished, while the holding current is not affected. However, the presence of Penicillin G did not preclude the effect of flupirtine on GABA$_{A}$ receptor-mediated tonic current, since co-application of flupirtine increased the current through extrasynaptic GABA$_{A}$ receptors (trace in fig. 6.9a).

Further, we generated a concentration-response curve for GABA in presence of Penicillin G 5 mM and in presence or absence of flupirtine (30 µM). In these conditions, flupirtine acts as a pure potentiator at the extrasynaptic GABA$_{A}$ receptors by enhancing the maximal effect by 43% and reducing the EC$_{50}$ values only slightly (1.5–fold).
Figure 6.9. When synaptic GABA receptors are blocked by Penicillin G 5 mM, flupirtine acts as a pure potentiator on GABA-mediated currents in hippocampal neurons. Synaptic GABA receptors were selectively blocked by application of Penicillin G 5 mM and the effect of flupirtine on electrophysiologically isolated extrasynaptic GABA receptors was investigated. (a) concentration-response curve for GABA in absence (full circles) and presence (empty circles) of Penicillin G 5 mM was measured in hippocampal neurons (n = 7). For each cell, current amplitudes were normalized to that evoked by GABA 30 µM in absence of Penicillin G. Penicillin G decreased EC50 value and maximal effect by 2.9-fold and 70%, respectively. Representative trace shows the blockage of synaptic events upon application of Penicillin G (5 mM), while tonic current (baseline) remains unaffected and is still enhanced upon co-application of flupirtine (30 µM). (b) shows concentration-response curve for GABA in presence of Penicillin G alone (full circles) and together with 30 µM flupirtine (empty circles) (n = 4, each). In these conditions, flupirtine (30 µM) potentiated GABA-induced currents by increasing the maximal effect by 43% and reduces only slightly (1.5-fold) the EC50 value. For each cell, current amplitudes were normalized to that evoked by GABA 30 µM in presence of Penicillin G.

6.7 Effects of flupirtine on GABA receptors in pain-related and non-related neurons

As the effect of flupirtine on GABA receptors depended on the receptor subtype, we further investigated its effect also on different types of neurons. GABA receptors are found to be expressed not only in the central, but also in the peripheral nervous system (Labrakakis et al., 2003; Magnaghi et al., 2006). Thus, we compared the effect of flupirtine in hippocampal neurons, versus neurons contained in the pain pathway, like DRG neurons and neurons found in the dorsal horn of the spinal cord.

Using the same measuring conditions, concentration-response curves for GABA-evoked currents were determined in DRG and dorsal horn neurons. As we know from previous experiments (see fig. 6.4), the GABA concentration required to achieve half-maximal activation in hippocampal neurons was 5.4 ± 0.8 µM. In DRG and dorsal horn neurons, however, the GABA concentration required to achieve the same result, was much higher: 30.0 ± 11.4 µM for dorsal horn and 32.5 ± 3.5 µM for DRG neurons (Fig. 6.10). In the presence of 30 µM flupirtine, the concentration response curves obtained in each of the three types of neurons were shifted to the left, but the extent of leftward shift was more pronounced in DRG (4.1-fold shift) and dorsal horn (3.7-fold shift) than in hippocampal (2.5-fold shift) neurons (Fig. 6.10). Furthermore, the effect of 30 µM flupirtine on maximal amplitudes of currents induced by high (≥ 100 µM) GABA concentrations was much stronger in DRG then in dorsal horn and hippocampal neurons. Hence, effects of flupirtine on GABA receptors are clearly different in the three types of neurons investigated.
Figure 6.10. Comparison of the effects of flupirtine on GABA_\text{A} receptors in hippocampal, DRG and dorsal horn neurons. Neurons were clamped at -70 mV and increasing GABA concentrations were applied for 3 to 5 s in the presence of either solvent (empty circles) or 30 µM flupirtine (filled circles). (a) to (c) show original traces evoked by 3 µM GABA in hippocampal, DRG, and dorsal horn neurons, respectively. (d) to (f) show concentration response curves obtained in hippocampal (n = 5 to 11), DRG (n = 5 to 15), and dorsal horn (n = 6 to 16) neurons. Amplitudes evoked by different GABA concentrations in solvent or flupirtine were normalized to those evoked by 30 µM GABA in solvent in the very same neuron.
6.8 Differences in GABA<sub>A</sub> receptor subunits in pain-related and non-related neurons

Unfortunately, the subunit composition of the receptors expressed in different regions of the nervous system remains unknown. In order to correlate the differences in the effects of flupirtine on GABA-evoked currents in the three types of neurons with the expression pattern of GABA<sub>A</sub> receptor subunits, membrane preparations from hippocampi, dorsal horns or DRGs of 10-14 day old rats were subjected to an immunoblot analysis using a series of 10 antibodies directed against the following proteins: α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, α<sub>4</sub>, α<sub>5</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and δ subunits of GABA<sub>A</sub> receptors (Figure 6.11a). The individual protein bands stained by the subunit-specific antibodies exhibited the appropriate molecular masses as determined previously (Poltl et al., 2003). Only in cases of low abundance of a subunit, due to the longer exposure times required, additional crossreactive protein bands became more prominent relative to the subunit and were thus detected (one additional protein band of 62 kDa labeled by anti-α<sub>1</sub> antibodies in DRG, one additional protein band of 57 kDa labeled by anti-α<sub>4</sub> antibodies in DRG, two additional protein bands of 58 and 61 kDa stained by the anti-α<sub>5</sub> antibody in DRG and DH, two additional bands labeled by anti-δ antibodies at 65 and 45 kDa in hippocampus, DRG, and DH).

Since different antibodies were used for individual GABA<sub>A</sub> receptor subunits, and since staining intensities of antibodies depend on the number of epitopes recognized, their avidities for the individual epitopes, their interactions with secondary antibodies, the time of incubation with the alkaline phosphatase substrate, as well as the abundance of the subunits in the membranes, the data cannot be used to estimate the absolute amounts of GABA<sub>A</sub> receptor subunits in different tissues. However, as compared to the positive controls employed for each of these antibodies, it is obvious that there is much more GABA<sub>A</sub> receptor subunit expression in hippocampal and dorsal horn neurons than in DRG neurons. As far as the relative amounts of subunits in each type of neuron are concerned (normalized to the staining of α<sub>1</sub> subunits in the same tissue), a densitometric analysis of expression levels determined as shown in figure 6 revealed the following rank orders of predominating subunits: in hippocampus α<sub>5</sub> > β<sub>3</sub> > γ<sub>2</sub> > β<sub>1</sub>; in dorsal horn β<sub>3</sub> > α<sub>3</sub> > α<sub>5</sub> > γ<sub>2</sub>; in DRG α<sub>2</sub> > γ<sub>2</sub> > β<sub>3</sub> > α<sub>3</sub> (figure 6.11b).
Fig. 6.11. Comparison of the expression pattern of GABA_A receptor subunits in hippocampal, DRG, and dorsal horn neurons. Hippocampus (hipp), dorsal horn (DH) and DRGs were collected from 10-14 days old rats and membrane proteins were prepared. The proteins were separated on 10% polyacrylamid minigels and transferred to membranes which were then incubated in one of 10 different antibodies directed against various GABA_A receptor subunits. As a positive control (pos ctl), the same amount of membrane protein from whole mouse brain was used for the antibodies against the α_1, α_2, α_3, α_4, β_1, β_3, and γ_2 subunits. For the antibody against α_5, membrane proteins from mouse hippocampus were used, while mouse cerebellum was used as positive controls for the antibodies directed against the β_2 and δ subunits. (a) shows the various bands obtained with the antibodies in membrane preparations of the three different tissues, as observed in one experiment. (b) The densities of all bands were normalized to that of the α_1 band within the same tissues obtained in the same experiments; the results show the average values for three independent experiments.
6.9 Effects of flupirtine on Kv7 channels in pain-related and non-related neurons

So far, flupirtine at therapeutic concentrations (i.e. 10 µM or below; see (Hummel et al., 1991)) had been found to affect Kv7 channels and GABA_A receptors. Therefore, our further experiments focused on these and employed DRG, dorsal horn and hippocampal neurons, to compare effects in neurons involved and not involved in pain sensation, respectively. To obtain an estimate as to how much Kv7 channels contribute to outward currents in the near threshold voltage range, neurons were depolarized to -30 mV and shortly hyperpolarized to -55 mV to see the deactivation of these channels; using this voltage protocol, 30 µM linopirdine which blocks currents through Kv7 channels in sensory neurons by more than 90% (Passmore et al., 2003) was used to quantify the contribution of these channels to the overall currents. The inhibition of standing outward currents at -30 mV by 30 µM linopirdine was similar for the three types of neurons and ranged between 30 and 60% (figure 6.12 a and b). In the presence of flupirtine, the outward currents seen at -30 mV were enhanced in a concentration-dependent manner, but this enhancement was more pronounced in hippocampal than in DRG or dorsal horn neurons (Figure 6.12 a and c).

Fig. 6.12. Comparison of the effects of flupirtine on Kv7 channels in hippocampal, DRG and dorsal horn neurons. Cultured hippocampal, DRG, or dorsal horn (DH) neurons were clamped at -30 mV and hyperpolarized to -55 mV for 1 s periods every 10 seconds in order to deactivate Kv7 channels. (a) shows representative traces measured in a DRG neuron in the presence of solvent, 30 µM flupirtine or 30 µM linopirdine. The shift in
the outward current at -30 mV is indicated by the arrows. (b) normalized amplitudes of outward currents at -30 mV were measured in presence of solvent or 30 µM linopirdine in hippocampal (n = 5), DRG (n = 6) and dorsal horn (n = 4) neurons. (c) Normalized amplitudes of currents measured at -30 mV in the absence and presence of increasing concentrations of flupirtine were determined in hippocampal (circles, n = 9), DRG (squares, n = 10) and dorsal horn (triangles, n = 8) neurons, respectively. EC50 values were 6.1, 4.4 and 5.4 µM in hippocampal, DRG and dorsal horn neurons, respectively. The maximal effects observed in hippocampal neurons were significantly different from those in DRG or dorsal horn neurons at p < 0.01.

6.10 Concentration dependence of the effects of flupirtine on GABA<sub>A</sub> receptors in pain-related and non-related neurons; comparison with the effects on Kv7

To obtain information about the flupirtine concentrations required to enhance currents evoked by low GABA concentrations, concentration response curves for the analgesic were constructed for each neuron type using GABA concentrations equivalent to the EC5 value: this was 0.4 µM in hippocampal, 1 µM in DRG, and 3 µM in dorsal horn neurons (Fig. 6.13a). The results show that flupirtine acted more potently on GABA induced currents in DRG neurons (half maximal effects at 22 ± 3 µM) than in dorsal horn (half maximal effects at 53 ± 10 µM; p < 0.01) or hippocampal (half maximal effects at 65 ± 20 µM; p < 0.01) neurons. Moreover, maximal enhancement of GABA induced currents by flupirtine was more pronounced in dorsal horn (15-fold increase) than in DRG neurons (6-fold increase; p < 0.001), but was not significantly different from that in hippocampal neurons (7-fold increase; p > 0.05). At a concentration of 30 µM flupirtine, the enhancement of GABA induced currents in hippocampal neurons was significantly different from that observed in either DRG or dorsal horn neurons (p < 0.05).

When comparing the effects of flupirtine on Kv7 channels (Fig. 3.12) with those on GABA<sub>A</sub> receptors (figure 3.13a), it is obvious that the analgesic is more potent in enhancing currents through Kv7 channels (half maximal effects: 5 µM vs. >20 µM), but more efficacious in augmenting GABA induced currents (maximal increases: < 2-fold vs. >6-fold). To directly compare the effects of flupirtine at therapeutic concentrations, outward currents at -30 mV and currents induced by GABA EC5 concentrations were determined in the presence of either 3 µM flupirtine or solvent. In dorsal horn and DRG neurons, currents through Kv7 channels and GABA<sub>A</sub> receptors were enhanced to about the same extent. In hippocampal neurons, in contrast, the enhancement of Kv7 currents was much more pro-
nounced than the enhancement of GABA induced currents (figure 6.13b). This indicates that therapeutic concentrations of flupirtine act equally well on Kv7 channels and GABA<sub>A</sub> receptors involved in pain sensation.

**Figure 6.13. Comparison of the effects of therapeutic flupirtine concentrations in hippocampal, DRG and dorsal horn neurons.** (a) The concentration dependence of the effects of flupirtine on GABA<sub>A</sub> receptors was investigated in hippocampal (n = 6), DRG (n = 6 to 8), and dorsal horn (n = 5 to 8) neurons clamped at -70 mV using GABA concentrations corresponding to the calculated EC<sub>5</sub> values to induce currents; these EC<sub>5</sub> values were 0.4, 3, and 1 µM for hippocampal (HC), DRG and dorsal horn (DH) neuron, respectively. * indicates a significant difference vs. the corresponding result in DRG and dorsal horn neurons at p < 0.05. (b) Outward currents at -30 mV or currents induced by GABA at the above EC<sub>5</sub> concentrations were determined in hippocampal (n = 6), DRG (n = 6 to 8), and dorsal horn (n = 4 to 5) neurons in the presence of either solvent or 3 µM flupirtine. The graph shows normalized current amplitudes in the presence of flupirtine; *** indicates a significant difference between the effects on the two types of currents as determined by a Kruskal-Wallis analysis of variance followed by a Dunn’s multiple comparison test.
7. DISCUSSION

Flupirtine has been used as an analgesic for more than 20 years and is marketed as a SNEPCO (Selective NEuronal Potassium Channel Opener) (Kornhuber et al., 1999b). This characterization reflects the fact that this drug has been suggested to activate Kir 3 channels (Jakob and Krieglstein, 1997). Later on, flupirtine was shown to facilitate the gating of Kv7 channels (Wladyka and Kunze, 2006) which further supports the characterization as SNEPCO. The present results confirm the action on Kv7 channels and reveal, for the first time, that GABA_A receptors are modulated by flupirtine in a neuron-specific manner.

One K+ channel family that has been proposed to be opened by flupirtine is the Kv7 family: the voltage-dependence of recombinant Kv7.2 channels (Martire et al., 2004) as well as native Kv7 channels in nodose ganglion neurons (Wladyka and Kunze, 2006) was found to be shifted in a hyperpolarizing direction. This is confirmed here using Kv7.2/Kv7.3 heteromers expressed in tsA cells and Kv7 channels endogenously expressed in superior cervical ganglion, DRG, dorsal horn and hippocampal neurons of the rat; in all these systems, effects of flupirtine were half maximal at about 5 µM, and outward currents determined at -30 mV were enhanced by up to 90%. The maximal potentiation of currents through Kv7 channels by high flupirtine concentrations was more pronounced in hippocampal than in DRG or dorsal horn neurons.

With respect to the presynaptic localization of M-channels and its consequential role in tuning neurotransmitter release, the literature is contradictory. This issue has been repeatedly investigated in functional studies using different specific activators and blockers of the M-channels. Thus, activation of M-channels by flupirtine or retigabine resulted in an inhibition of depolarisation-induced release of various transmitters, including noradrenaline, aspartate and GABA, from rat hippocampal (Martire et al., 2004) and of dopamine in rat striatal (Martire et al., 2007) synaptosomes. In contrast, retigabine was found to enhance inhibitory postsynaptic currents (IPSCs), but not excitatory postsynaptic currents (EPSCs), and the former effect appeared to arise post-synaptically (Otto et al., 2002). The M-channel blocker, linopirdine, however, was reported to increase the frequency of miniature EPSCs in hippocampal neurons, and NH6, a newly synthesized molecule that opens M-channels, exerted an opposite effect (Peretz et al., 2007). In peripheral sympathetic neurons, linopirdine also triggered noradrenaline release, but this effect was abolished by tetrodotoxin and was thus not mediated by a presynaptic site of action (Kristufek et al., 1999;
Lechner et al., 2003). Likewise, inhibition of M-currents via M1 receptors triggered noradrenalin release in a tetrodotoxin-sensitive manner (Lechner et al., 2003). Conversely, retigabine was reported not to affect spontaneous or electrically evoked release of $[^{3}H]$noradrenaline from sympathetic neurons. In contrast to these results, retigabine and XE991, a potent blocker of M-channels, were found to enhance and reduce the release of noradrenaline from sympathetic neurons respectively, as determined by microamperometry (Hernandez et al., 2008). Additionally, the M-channel subunit Kv7.2 has been shown to colocalize with syntaxin 1A at the presynapse of cultured dissociated hippocampal neurons (Regev et al., 2009). In immunostaining experiments on unfixed rat brain slices, however, no obvious co-localization of Kv7.2 or Kv7.3 subunits with the synaptic marker, synaptophysin, was observed (Klinger et al., 2011).

Taken together, contradictory results were obtained so far with respect to the presynaptic localization and the functional role of M-channels in neurotransmitter release. Our experiments on evoked autaptic currents conducted in hippocampal microiland cultures, revealed flupirtine to increase inhibitory, but not to affect excitatory transmission. The potentiating effect of flupirtine on the inhibitory autaptic currents cannot be explained by its activation of M-channels, since activation of M-channels induces neuronal hyperpolarisation, which in turn, leads to inhibition rather than enhancement of neurotransmission. Hence, flupirtine might have a direct effect on the postsynaptic GABA_A receptors. Indeed, in our subsequent experiments, we showed for the first time that flupirtine does modulate currents through postsynaptic GABA_A receptors by increasing the potency of GABA to induce currents without enhancing maximal current amplitudes. Although such an effect has never been reported for flupirtine itself, another Kv7 channel opener with a highly similar structure, retigabine, has been found to potentiate GABA induced currents (Rundfeldt and Netzer, 2000) as well as inhibitory synaptic currents (Otto et al., 2002) in cortical and hippocampal neurons, respectively. However, retigabine is not used as analgesic, but has been marketed recently as adjunctive treatment of partial-onset seizures (Stafstrom et al., 2011).

Our results show that the potentiation of GABAergic transmission by flupirtine is greater when GABA is present in low concentrations and this effect is also known to be true for other GABA_A receptor modulators, like anesthetics and benzodiazepines (Harris et al., 1995). GABA_A receptors are very diverse regarding their subunit composition and different GABA_A receptors have been shown to posses different physiological and pharmacological characteristics (Korpi and Sinkkonen, 2006; Ranna et al., 2006). While $\alpha$ sub-
units are important for the activation by GABA, other subunits contribute to the binding of allosteric ligands, such as benzodiazepines and barbiturates (Korpi et al., 2002; Rudolph and Mohler, 2004).

GABA_A receptors with different subunit composition are targeted to different subcellular regions. Receptors composed of α_1_, α_2_ or α_3_ subunits together with β_2/3_ and γ subunits, are largely synaptically located, while α_4_, α_5_, α_6_, β_1_ and δ subunits containing receptors localize preferentially at the extrasynaptic regions of the neurons (Brunig et al., 2002; Fritschy et al., 1998; Jacob et al., 2008; Mangan et al., 2005). In patch clamp experiments, it has been shown that the extrasynaptic GABA_A receptors in hippocampal neurons exhibit different functional and pharmacological properties compared to those mediating quantal synaptic currents. Here, both, the mIPSCs and the tonic current, could be blocked by the GABA_A receptor antagonists, bicuculline and picrotoxin. In contrast, gabazine (SR-95531) inhibited mIPSCs, but not the tonic current in hippocampal neurons (Bai et al., 2001). In our experiments, we demonstrate that flupirtine is able to differentiate between tonic and phasic GABA-induced currents. As shown in recordings of mIPSCs (figure 6.7), flupirtine clearly potentiates GABAergic transmission through tonically-active GABA_A receptors, but does not alter mIPSCs. These results appear contradictory to the results obtained in the recordings of evoked autaptic currents (see above) where flupirtine enhanced current amplitudes during late phases of the autaptic responses.

IPSCs are known to show a biphasic decay time with two time constants, a fast and a slow one (Puia et al., 1994; Roepstorff and Lambert, 1994). The current component with the fast time most likely involves synaptic receptors, as it is prolonged by benzodiazepines which do not affect the slow decay (Puia et al., 1994). In contrast, the GABA uptake inhibitor, tiagabine, did not alter the fast, but increased the component of stimulus-evoked IPSCs with the longer time constant (Roepstorff and Lambert, 1994). Similarly, SKF-89976A, another GABA uptake inhibitor, had no effect on the initial decay phase, but strongly prolonged the late phase of IPSCs in CA1 hippocampal neurons. In these neurons, spontaneous IPSCs or IPSCs evoked by weak stimuli were not affected by SKF-89976A, which can be explained by insufficient GABA spillover from the synaptic cleft into the regions containing extrasynaptic GABA_A receptors (Isaacson et al., 1993). In concordance with these results, IPSCs have been shown to decay significantly slower in cerebellar granule cells of wild type mice than in mice lacking GABA_A receptor δ subunits (δ^-/-) (Wei et al., 2003), these being known to localize predominantly extrasynaptically (Mangan et al., 2005; Nusser et al., 1998). Moreover, NO711, another specific GABA uptake inhibitor
failed to affect IPSCs in granule neurons from δ−/− mice, but significantly prolonged the slow decay time of evoked IPSCs in wild type neurons (Wei et al., 2003). Taken together, this indicates that synaptic GABA_A receptors contribute to the fast component, while extrasynaptically located GABA_A receptors are mainly responsible for the slow component of evoked IPSCs. Accordingly, the selective enhancement of late phases of autaptic currents by flupirtine confirms that this drug acts preferentially on extrasynaptic GABA_A receptors.

As mentioned above, different GABA_A receptor subunits are known to localize predominantly at the extrasynaptic site of the neurons. However, a variety of subunits combination may result and recording in native neurons makes it impossible to differentiate between the different receptor subtypes. In electrophysiological experiments using recombinant GABA_A receptors composed of subunits specific for synaptic and extrasynaptic receptor isoforms, penicillin G was reported to exert a context-dependent, but not a isoform-specific modulation of GABA_A receptors (Feng et al., 2009). Here, penicillin G blocked both, synaptic and extrasynaptic, GABA_A receptor isoforms when they were stimulated by brief application of nearly saturating GABA concentrations (corresponding to the phasic activation), but failed to inhibit GABA-mediated currents evoked by prolonged application of sub-saturating GABA concentration (corresponding to tonic activation). Therefore, we found appropriate to use penicillin G as a selective blocker of phasic GABA_A receptors, rather than blocking GABA_A receptors containing specific subunits. In our experiments, in presence of penicillin G, flupirtine acted as a pure potentiator on GABA-mediated currents in hippocampal neurons. This could be an indication that flupirtine might modulate GABA_A receptors depending on their phasic or tonic activity, but not on their subunit composition.

GABA_A receptors are found to be expressed not only in the central, but also in the peripheral nervous system (Labrakakis et al., 2003; Magnaghi et al., 2006). When the effect of flupirtine was compared for DRG, dorsal horn and hippocampal neurons, it became obvious that its action was tissue-specific with respect to the shift of the concentration response curves for GABA and to the changes in maximum current amplitudes. Moreover, at half maximal flupirtine concentrations the potentiation of GABA induced currents was significantly greater in DRG and dorsal horn than in hippocampal neurons.

The expression pattern of GABA_A receptor subunits has been reported to be different in different regions of the central and peripheral nervous system. Although the exact subunit composition of the receptors remains unknown, some subunits are known to be predominately expressed in certain regions. For example, in hippocampal neurons, most
predominant subunits that are found to be expressed are $\alpha_1$, $\alpha_5$, $\beta_2$ and $\gamma_2$ (Sieghart, 2005), in DRG neurons $\alpha_2$, $\alpha_3$, $\beta_3$ and $\gamma_2$ are most abundant (Persohn et al., 1991), while $\alpha_2$, $\alpha_3$, $\beta_3$ and $\gamma_2$ have been shown to be expressed at significant levels in the rat spinal cord (Wisden et al., 1991). The present semiquantitative analysis using immunoblots of membranes derived from these tissues in principle confirmed these previous findings and revealed that the expression level of all the subunits investigated is much lower in DRG than in the central regions. Amongst the $\alpha$ subunits, $\alpha_5$ displayed the highest levels in the hippocampus, $\alpha_2$ in DRGs and $\alpha_3$ in the dorsal horn. For $\beta$ and $\gamma$ subunits, $\beta_2$ and $\gamma_3$ had the highest expression levels in the three tissues investigated. Thus, these results help to explain why the effects of flupirtine on GABA$\Lambda$ receptors was different in the three types of neurons investigated and suggest that flupirtine may modulate GABA$\Lambda$ receptors in an $\alpha$ subunit-specific manner. Nevertheless, this remains to be confirmed in experiments using a variety of recombinant receptors.

Considering that flupirtine concentrations of 10 $\mu$M affected only Kv7 channels and GABA$\Lambda$ receptors, but no other ligand-gated ion channel (figure 6.3), the question remains as to whether both of these effects may contribute to the analgesic action. In fact, an enhancement in the activities of both, Kv7 channels (Passmore et al., 2003) and GABA$\Lambda$ receptors (Zeilhofer et al., 2009), is known to provide analgesic effects. However, therapeutic flupirtine plasma concentrations are in the range of about 5 $\mu$M only (Devulder, 2010; Hummel et al., 1991). In DRG and dorsal horn neurons, 3 $\mu$M flupirtine enhanced currents through Kv7 channels and GABA$\Lambda$ receptors to a similar extent; in hippocampal neurons, for comparison, the effects on Kv7 channels were much more pronounced than those on GABA$\Lambda$ receptors. Thus, in neurons involved in pain perception, Kv7 channels and GABA$\Lambda$ receptors are equally affected by this analgesic.

In conclusion, the present results reveal GABA$\Lambda$ receptors as novel sites of action for the non-opioid analgesic flupirtine. Thus, flupirtine is not a SNEPCO as inferred up to now, but rather exerts its analgesic actions by combining two therapeutic principles, the potentiation of both, GABA$\Lambda$ receptors and Kv7 channels.
8. REFERENCES


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