Metabotropic receptors for glutamate and gaba in nociceptive transmission and pain regulation

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INTRODUCTION : CLASS C G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors constitute a large family of membrane proteins responsible for transduction of various external signals into intracellular responses through heterotrimeric G-proteins. As a result, GPCRs are involved in the regulation of many physiological or pathological processes and are the target of about a quarter of the drugs available on the market [Overington *et al*., 2006].
Based on genetic analysis, five main classes of GPCRs have been identified [Bockaert and Pin, 1999]. Interestingly, mGluRs and $GABA_B$ belong to the same class. They are members of class-C GPCRs (formerly known as family 3 GPCRs), which also includes the calcium-sensing receptor, the receptors for sweet and umami taste plus several pheromone and orphan receptors [Brauner-Osborne *et al*., 2007].
Class C GPCRs possess two remarkable features that are important for their regulations and function. The first structural particularity of most class-C receptors (except pheromone receptors) is the presence of a large bilobate extracellular domain where natural ligands bind. This domain is juxtaposed to a core transmembrane domain composed of seven helices, a feature common to all GPCRs and responsible of G-protein coupling (Fig.1). This domain is important for the pharmacology of these receptors [Pin *et al*., 2004b]. In the late 90's, a new class of class C GPCRs ligands has been identified; the allosteric modulators. These ligands can be either positive (PAM) or negative allosteric modulators (NAM) according to whether they enhance or inhibit the agonist-induced activity of the receptor, respectively. Contrary to orthosteric compounds that bind within the extracellular binding pocket of natural ligands, these modulators bind within the transmembrane domain (Fig. 1), where they affect the stability of the active conformation [Goudet *et al*., 2004b]. Interestingly, while the site of glutamate binding is highly conserved across phyla suggesting intense evolutionary pressure, the binding pocket of allosteric modulators is more variable and thus allows the discovery of subtype selective ligands for mGluRs. Also of interest, most of these compounds are devoid of activity by themselves, but modulate the system when it is activated. Thus, these compounds should present relatively fewer side effects and PAM should lead to less desensitization than agonists and are thus very promising as potential therapeutic drugs (see [Goudet *et al*., 2004a, Kew, 2004] for review). For these reasons, pharmaceutical companies are intensively searching for new allosteric modulators.
The second specific feature of class-C GPCRs is their constitutive dimeric nature (Fig.1) [Pin *et al*., 2004b]. mGluRs are homodimers crosslinked by a disulfide bridge across the extracellular domains of each subunit. Interestingly, mGluR dimers function asymmetrically with one subunit responsible for x and the second responsible for y [Goudet *et al*., 2005, Hlavackova *et al*., 2005, Kniazeff *et al*., 2004]. In contrast, $GABA_B$ are heterodimeric receptors, composed of two subunits named $GABA_{B1}$ and $GABA_{B2}$. $GABA_{B1}$ is responsible for ligand recognition but is unable to reach the cell surface by itself or to activate G-proteins [Filippov *et al*., 2000, Galvez *et al*., 2001, Margeta-Mitrovic *et al*., 2001a, Margeta-Mitrovic *et al*., 2001b]. Conversely, $GABA_{B2}$ is unable to bind GABA but is responsible for G-protein coupling [Duthey *et al*., 2002, Havlickova *et al*., 2002, Kniazeff *et al*., 2002]. Moreover,

$GABA_{B2}$ masks the retention signal located on the C terminus of $GABA_{B1}$ and allows the expression of the heterodimers at the cell surface [Brock *et al*., 2005a]. Thus, to get a functional receptor, $GABA_{B1}$ and $GABA_{B2}$ need to be associated [Pin *et al*., 2004a].
Moreover, to better understand the functioning of $GABA_B$ it is important to note that at least two isoforms of $GABA_{B1}$ exists, named 1a and 1b, which differ mainly by the presence of a pair of sushi (explain) domains in the N-terminal extracellular domain of $GABA_{B1a}$. Sushi domains are known to be involved in the interaction between proteins. The two isoforms display thus different localization in neurons and are involved in different synaptic functions (Fig. 2) [Bettler and Tiao, 2006].

METABOTROPIC GLUTAMATE RECEPTORS AND PAIN

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and plays an important role in pain transmission. It exerts its action through ionotropic and metabotropic receptors. Metabotropic glutamate receptors (mGluRs) belong to the superfamily of G-protein coupled receptors. These receptors are widely distributed throughout the central nervous system where they regulate cell excitability and synaptic transmission.

The mGluRs family

The mGluRs family is composed of 8 members that can be subdivided into 3 groups based on sequence identity, pharmacological profile and signal transduction. Group-I mGluRs (mGlu1 and mGlu5) are mainly coupled to Gq proteins and as such activate the phospholipase C pathway and generate intracellular calcium signals. Group-II (mGlu2 and mGlu3) and group-III (mGlu4, 6, 7 and 8) mGluRs are coupled to Gi/o, therefore inhibiting adenylyl cyclase or regulating the activity of various ion channels. Postsynaptically localized mGluRs (mostly of group I) often play an important role in upregulating neuronal excitability and in regulating currents through ionotropic glutamate receptors. Group II and III mGluRs are mostly presynaptic receptors and their activation tends to reduces synaptic transmission and neuronal excitability. As autoreceptors, they are involved in reducing transmission at glutamatergic synapses but they are also present in GABAergic terminals where they play the role of heteroreceptors reducing GABA release at inhibitory synapses (Fig. 2).

Localization of mGluRs within the nociceptive circuit

The different mGluRs are distributed throughout the peripheral and central nervous system and regulate nociceptive processes at different levels of the pain neuraxis.
Group I mGluRs (mGlu1 and 5) are predominantly found in postsynaptic elements. Group II mGluRs (mGlu2 and 3) are found in both postsynaptic and presynaptic elements. Group III mGluRs (mGlu4, 6, 7 and 8) are presynaptic receptors, located close to or in the active zone of the synapse (Fig. 2).
In the peripheral nervous system, group I and II mGluRs can be found in the peripheral terminals and in the soma of dorsal root ganglia neurons (DRGs) [Bhave *et al*., 2001, Carlton and Hargett, 2007, Valerio *et al*., 1997, Walker *et al*., 2001]. mGlu4 and mGlu7 are found in the presynaptic terminal of sensory neurons in the dorsal horn of spinal cord [Azkue *et al*., 2001, Li *et al*., 1997, Ohishi *et al*., 1995b]. mGlu8 is located in the soma of DRG neurons [Carlton and Hargett, 2007]. Of note, mGlu6 is highly expressed in retina but its expression in other nervous system areas is very low. Almost all mGluRs are expressed in the spinal cord [Azkue *et al*., 2001, Jia *et al*., 1999, Li *et al*., 1997, Ohishi *et al*., 1995a, Ohishi *et al*., 1995b],

excepting two of the four group III mGluRs: mGlu6 and mGlu8 [Berthele *et al*., 1999, Valerio *et al*., 1997].
The main ascending projections from dorsal horn neurons are directed toward the thalamus, via the spinothalamic tract, and the midbrain periaqueductal grey region (PAG). Thalamus shows an intense labeling for almost all mGluRs, as revealed by *in situ* hybridization [Lourenco Neto *et al*., 2000] and further confirmed by immunohistochemistry [Duvoisin *et al*., 1995, Petralia *et al*., 1996, Petralia *et al*., 1997, Romano *et al*., 1995, Shigemoto *et al*., 1993, Tamaru *et al*., 2001]. PAG is an important center for the processing of nociceptive informations and is an important source for descending modulatory circuitry. Expression of mGlu3 and mGlu5 has been reported in this center [Azkue *et al*., 1997, Tamaru *et al*., 2001] and modulation of descending pain pathway is observed following group III mGluRs activation [Marabese *et al*., 2005, Marabese *et al*., 2007a].
Chronic pain is often associated with affective disorders. Affective and emotional components are processed in amygdala. Nociceptive neurons are particularly abundant in the central nucleus of the amygdala. *In vivo* electrophysiological and pharmacological studies revealed that activation group I and group III mGluRs activation modulates noxious stimulation – induced synaptic plasticity in this area (see [Neugebauer, 2007a, Neugebauer *et al*., 2004] for review).
Besides being expressed in neurons, mGluRs have also been detected in astrocytes, oligodendrocytes, and microglia. Glial cells are active regulators and protectors of nervous system and as such may play a role in neuropathic pain [Saab *et al*., 2007] but this subject won't be reviewed here. Expression and function of mGluRs in glia have been reviewed elsewhere (see [D'Antoni *et al*., 2008, Pocock and Kettenmann, 2007] for recent review)

Roles of mGluRs in transmission of noxious stimuli

Group I mGluRs

A large body of research over the past several years has led to the general consensus that group I mGluRs serve a primarily pro-nociceptive role. Specific roles for mGlu1/5 in modulating nociception have been identified in the periphery, spinal cord, brainstem, thalamus, cortex, and amygdala (see [Varney and Gereau, 2002] for review).
The first suggestion that group I mGluRs might regulate nociceptive behaviors came from the work of Neugebauer *et al*. in 1994. The authors of this study showed that enhanced excitability of spinal dorsal horn neurons is reversed by L-AP3, a relatively weak and nonselective group I mGluR antagonist. This initial finding has been born out by subsequent studies using more specific and potent antagonists in reptiles, rodents and primates [Morisset and Nagy, 1996, Neugebauer *et al*., 1999, Russo *et al*., 1997].
Subsequent cellular studies have demonstrated that mGlu5 activation leads to ERK activation in spinal dorsal horn neurons, and this ERK activation underlies central sensitization in the context of persistent inflammation by reducing A-type K+ currents (due to ERK phosphorylation of Kv4.2) and thus increasing excitability of spinal dorsal horn neurons [Hu *et al*. 2007, Hu *et al*., 2006, Karim *et al*., 2001b].
Important roles for group I mGluRs have also been demonstrated in various forms of synaptic plasticity thought to underlie various components of pain observed following tissue injury [Chen and Sandkuhler, 2000, Derjean *et al*., 2003, Jung *et al*., 2006]. Thus, a convincing data set exists indicating that spinal group I mGluRs are critical for spinal central sensitization.
In addition to the large body of work demonstrating effects of mGlu1/5 in the spinal cord, the expression of these receptors in sensory neurons was noted by several groups. Work from

Katherine Walker's group and Robert Gereau's group first demonstrated that group I mGluRs are functionally expressed in nociceptor terminals in the periphery, and that activation of these receptors produces hyperalgesia while blocking these receptors reduces hyperalgesia in inflammatory pain models [Bhave *et al*., 2001, Walker *et al*., 2001]. Dogrul *et al*. (2000) had also shown that peripherally administered antagonists of mGlu1/5 can also reduce neuropathic hypersensitivity in rats. A similar role for peripheral mGlu5 in postoperative pain has also been proposed [Zhu *et al*., 2005]. These pioneering studies demonstrated a functional role for group I mGluRs in the periphery in mediating pain sensitization, and opened the door for the potential development of peripherally restricted group I mGluR antagonists for the treatment of pain [Karim *et al*., 2001a, Neugebauer *et al*., 2002],

Group I mGluRs have also been shown to have important roles in modulating nociceptive transmission in higher brain centers. For example, mGlu1 and 5 are involved in the firing of neurons in the ventrobasal thalamus induced by noxious stimuli [Salt and Binns, 2000]. Pro-nociceptive actions of mGlu1/5 have also been demonstrated in the central nucleus of the amygdale [Han and Neugebauer, 2005].

Collectively, there is substantial evidence suggesting that mGlu1/5 activation leads to sensitization at multiple levels of the nociceptive circuit. A large body of literature supports the idea that mGlu5 antagonists, in particular, could be very useful for the treatment of chronic pain conditions. The next few years of clinical studies should provide a clear picture as to whether mGlu5 antagonists are viable as a novel class of analgesic medications. Indeed, there are current clinical trials that are testing the efficacy of mGlu5 antagonists in migraine.

Group II mGluRs

Group II mGluRs are located primarily in presynaptic terminals where they serve as to regulate the synaptic release. There are a number of studies suggesting that activation of mGlu2/3 can be analgesic. For example, systemically administered mGlu2/3 agonists reduce inflammation-induced hypersensitivity to mechanical or thermal stimuli [Sharpe *et al*., 2002, Simmons *et al*., 2002]. Furthermore, antinociceptive actions of mGlu2/3 agonists have been demonstrated in the periphery, spinal cord and amygdala [Dolan and Nolan, 2002, Li and Neugebauer, 2006, Yang and Gereau, 2003].

N-acetylaspartylglutamate (NAAG) is an endogenous peptide agonist of mGlu3 [Wroblewska, 2006]. Interestingly, there is mounting evidence that NAAG may be involved in nociception. For example, intrathecal administration of NAAG or of NAAG peptidase inhibitors (which prevent the breakdown of endogenous NAAG by inhibiting the peptidase), has been shown to have analgesic effects in rat models of inflammatory and neuropathic pain [Nagel *et al*., 2006, Yamamoto *et al*., 2004, Yamamoto *et al*., 2001].

In the context of nociception, it is also notable that mGlu2/3 receptors are expressed on the peripheral terminals of primary afferent neurons [Carlton *et al*., 2001]. Activation of peripheral group II mGluRs was shown to reduce inflammation-induced thermal and mechanical hypersensitivity [Yang and Gereau, 2002, Yang and Gereau, 2003]. Interestingly, it was shown that endogenous activation of mGlu2/3 on primary afferent terminals actually contributes to the recovery from mechanical hypersensitivity following carrageenan-induced inflammation, suggesting that these receptors mediate endogenous anti-allodynia in the context of inflammation [Yang and Gereau, 2003]. This suggest the exciting possibility that drugs acting to enhance endogenous activation of peripheral group II mGluRs might be useful for the treatment of inflammatory pain.

The expression of group II mGluRs can be dramatically altered by experience, and recent studies have suggested that his is particularly important in the context of pain. For example, mGlu3 expression is enhanced in the cortex after UV irradiation and in a model of

monoarthritis in rats [Boxall *et al*., 1998, Neto *et al*., 2001], and in the spinal cord after persistent inflammation [Dolan *et al*., 2003]. The role of this up regulation in pain behavior is not presently known. While mGlu3 up regulation is observed in the context of injury, mGlu2 expression has been shown to be enhanced by analgesic drugs. Specifically, Chiechio *et al*. (2002) demonstrated that mGlu2 expression was increased following treatment with L-acetylcarnitine (LAC), and that the analgesia produced by LAC in neuropathic rats was reversed by the mGlu2/3 antagonist, LY341495. This up regulation was subsequently demonstrated to be dependent on regulation of NF-kappaB transcription factors, possibly via regulation of acetylation [Chiechio *et al*., 2006a]. The fact that LAC is analgesic in both animal and human neuropathic pain conditions suggests that LAC-induced up regulation of mGlu2 expression might be a novel mechanism for the treatment of neuropathic pain [Chiechio *et al*., 2006b].
Taken together, the studies discussed here suggest that group II mGluRs represent a potentially exciting target for the development of novel analgesic drugs. One potential roadblock to this path is the reported tolerance to mGlu2/3 agonists that develops with repeated dosing, possibly reducing the viability of this approach [Jones *et al*., 2005].

Group III mGluRs

Like the Group II mGluRs, Group III mGluRs (mGlu4, 6, 7 and 8) are presynaptic receptors. They are located close to or in the active zone of the synapse where they induce inhibition of synaptic transmission and neurotransmitter release (Fig. 2).
Group III mGluRs are coupled to heterotrimeric Gi/o proteins, leading to the inhibition adenylyl cyclase production of cAMP via Gαi subunit. The inhibition of neurotransmitters release by group III mGluRs is mediated both by a direct inhibition of exocytosis [Chavis *et al*., 1998] and by an inhibition of voltage-gated Ca^{2+} channels via Gβγ [Capogna, 2004, Guo and Ikeda, 2005, Perroy *et al*., 2000, Takahashi *et al*., 1996]. Activation of group III mGluRs also induces an increase of K+ exit via G-protein activated Inward Rectifying K channels (GIRK) which in turn decreases the neuronal excitability [Dutar *et al*., 1999]. Other studies suggest that group III mGluRs may activate background K channels such as TREK1 and TREK2 [Cain *et al*., 2008, Lesage *et al*., 2000]. This mechanism may also contribute to the decrease of neuronal activity.
Whereas, the involvement of group I and II mGluRs in nociceptive processing is well documented, the specific role of group III mGluRs is still poorly understood due in part to the lack of specific pharmacology [Neugebauer, 2007b, Varney and Gereau, 2002]. Group III mGluRs are abundant all along the nociceptive pathways in particular mGlu4 and mGlu7, which are highly enriched in the terminals of primary afferent fibers in the dorsal horn of the spinal cord, a key localization for the regulation of ascending information [Azkue *et al*., 2001, Li *et al*., 1997]. The role of spinal group III mGluRs in nociceptive processing has been assessed by several electrophysiological, behavioral and pharmacological studies. Activation of group III mGluRs following infusion in dorsal horn of the group III mGluRs agonist L-AP4 inhibits ascending information in spinothalamic tracts resulting from mechanical noxious stimuli and reduces capsaicin induced central sensitization [Neugebauer *et al*., 2000]. Moreover, in spinal cord slices, L-AP4 application decreases postsynaptic excitatory potentials occurring from afferent A fibers [Gerber *et al*., 2000]. Acute thermal and mechanical nociceptive thresholds are unchanged in healthy rats following group III mGluRs activation after intrathecal administration of a group III mGluR agonist [Chen and Pan, 2005, Goudet *et al*., 2008]. However, activation of these receptors inhibits hyperalgesia associated to inflammation or neuropathy. Spinal group III mGluRs activation following intrathecal administration of the group III's agonists L-AP4 or ACPT-I reduces the nociceptive behavior

of rats in the formalin model [Fisher and Coderre, 1996, Goudet *et al*., 2008]. Activation of group III mGluRs also reverses mechanical hyperalgesia observed in carrageenan-treated rats and in monoarthritic rats [Goudet *et al*., 2008]. These receptors also inhibit the capsaicin-induced central sensitization [Neugebauer *et al*., 2000]. Tactile allodynia and mechanical hyperalgesia associated to neuropathic pain in the chronic constriction injury model in rat are reversed following activation of spinal group III mGluRs in parallel with a decrease of hyperactivity of ascending dorsal horn neurons [Chen and Pan, 2005, Goudet *et al*., 2008]. A reversal of mechanical hyperalgesia following spinal group III mGluRs activation is also observed in a model of vincristine-induced nociceptive peripheral neuropathy [Authier *et al*., 2003, Goudet *et al*., 2008].

Descending modulatory circuitry originating in the PAG is influenced by group III mGluRs. PAG infusion mGlu8 agonists are analgesic while mGlu7 agonists facilitate inflammatory and neuropathic pain in mice [Marabese *et al*., 2005, Marabese *et al*., 2007a]. These opposite effects on pain perception of mGlu7 and mGlu8 correlate with their autoreceptor or heteroreceptor roles on glutamatergic or GABAergic terminals respectively in PAG [Marabese *et al*., 2007b] (see [Palazzos *et al*., 2006] for review).

The amygdala plays a key role in the modulation of pain, especially the emotional/affective component (see [Neugebauer, 2007a, Neugebauer *et al*., 2004] for review). Several studies suggest that group III mGluRs are involved in the regulation of pain-related activity in the amygdala. Activation of group III mGluRs by L-AP4 reduced pain – related synaptic plasticity in the central nucleus of the amygdala (CeA) in a model of arthritis [Han *et al*., 2004]. A recent study of the relative contribution of mGlu7 and mGlu8 in CeA on nocifensive and affective pain responses and on pain-related anxiety-like behavior suggests that mGlu7 is pro-nociceptive under normal conditions while mGlu8 inhibits nocifensive and affective behaviors and anxiety in a model of arthritic pain [Palazzo *et al*., 2008].

Taken together, these studies suggest that group III mGluRs should represent promising new targets to develop new analgesics. However, further studies are needed to better understand the role of these particular receptors.

$GABA_B$ RECEPTORS AND PAIN

γ-aminobutiric acid (GABA) mediates most inhibitory actions in the central and peripheral nervous system (PNS), being involved also in the processing of nociceptive information. These effects are mediated through activation of ionotropic (i.e. $GABA_A$ and $GABA_C$) and metabotropic (i.e. $GABA_B$) receptors.

$GABA_B$ receptors in pain circuitries

The importance of $GABA_B$ receptors in nociceptive processing was well documented in the early 80's in a series of preclinical studies in which the $GABA_B$ agonist baclofen proved to be antinociceptive in models of acute [Malcangio *et al*., 1991] and chronic pain [Dirig and Yaksh, 1995, Patel *et al*., 2001, Potes *et al*., 2006, Smith *et al*., 1994]. However, in rat models of chronic pain, Baclofen effects are likely to be only partial [Franek *et al*., 2004, Gwak *et al*., 2006, Wang *et al*., 2007]. These effects are likely mediated by spinal and supraspinal $GABA_B$ receptors. The supraspinal effects appear to reflect depression of ascending adrenergic and dopaminergic pathways, and facilitation of descending noradrenergic input to the spinal cord dorsal horn [Sawynok, 1984]. The baclofen-induced antinociception at spinal cord level is attributed, at least partly, to the activation of presynaptic $GABA_B$ receptors localized on the terminals of peptidergic primary afferents fibers [Price *et al*., 1984]. At presynaptic sites, the $GABA_B$ receptor activation leads to inhibition of high voltage-gated Ca channel activity

[Bowery, 2006]. In the substantia gelatinosa of the spinal cord baclofen has a greater effect on C-fiber than Aδ-fiber-evoked glutamate release, suggesting a preferential expression of $GABA_B$ in C fibers afferent terminals [Ataka *et al*., 2000]. Baclofen also inhibits the electrically evoked release of calcitonin gene-related peptide (CGRP) [Malcangio and Bowery, 1995] and substance P from rat spinal cord slices. The inhibition of glutamatergic EPSPs [Iyadomi *et al*., 2000] and the regulation of intrinsic neuronal properties [Derjean *et al*., 2003] suggest not only a pre-synaptic but also an additional postsynaptic site for the baclofen action on pain. $GABA_B$ receptors are also involved in the inhibitory effects of other neurotransmitters on glutamatergic synaptic transmission and pain: e.g. ACh via muscarinic receptors, mu-opioid receptors, endocannabinoids via CB1 receptors and adenosine via A1 receptors [Chen and Pan, 2004, Naderi *et al*., 2005, Suzuki *et al*., 2005].

Localization of $GABA_B$ receptors

The presence of $GABA_{B1a}$, -$_{B1b}$, and -$_{B2}$ isoforms in the cerebral cortex and thalamus [Margeta-Mitrovic *et al*., 1999, Princivalle *et al*., 2001], lamina I-III of the spinal dorsal horn [Charles *et al*., 2001, Malcangio and Bowery, 1996, Price *et al*., 1984] and dorsal root ganglia [Charles *et al*., 2001, Engle *et al*., 2006, Tombler *et al*., 2006] provide the anatomical basis for the antinociceptive actions of baclofen. A fully functional $GABA_B$ receptor, indeed, must be composed of a heterodimer of $GABA_{B1}$ and $GABA_{B2}$ subunits [Bettler and Tiao, 2006, Price *et al*., 1984]. The cellular and subcellular localization as well as the mechanisms of action of the different $GABA_B$ receptor isoforms in pain pathways were recently investigated. In particular, $GABA_{B1a}$ and $GABA_{B1b}$ splice variants display differential distribution. Though not the rule, only one isoform is predominantly expressed in some areas. For instance, the expression of $GABA_{B1b}$ in the thalamus of rat and humans was several times higher than that of $GABA_{B1a}$ [Bischoff *et al*., 1999, Calver *et al*., 2000]. Conversely, more than 90% of the $GABA_{B1}$ subunit mRNA is $GABA_{B1a}$ in dorsal root ganglia cell bodies giving rise to primary Aδ and C afferent fibers. In contrast, both splice variants are expressed in spinal cord superficial laminae [Towers *et al*., 2000]. These data match other results suggesting a differential pre- and post-synaptic distribution of $GABA_{B1a}$ and $GABA_{B1b}$ subunits in various brain areas.

$GABA_{B1a}$ and $GABA_{B1b}$ appear to be associated with pre- and post-synaptic elements, respectively, both in rat or human cerebellum [Billinton *et al*., 1999], and at glutamatergic synapses in the dorsal cochlear nucleus of the rat [Lujan, 2007]. The subcellular localization has been best illustrated in the rat hippocampus [Bettler and Tiao, 2006, Kulik *et al*., 2003, Ulrich and Bettler, 2007], and showed the selective presence of $GABA_{B1a}$ at glutamatergic terminals, whereas both $GABA_{B1a}$ and $GABA_{B1b}$ are present at GABAergic terminals. By using different sets of complementary approaches, the authors showed that $GABA_{B1a}$-containing heterodimers mainly control pre-synaptic release of glutamate, whereas receptors involving $GABA_{B1b}$ subunits predominantly mediate post-synaptic inhibition (Fig. 2).

It is to be noticed that both the expression [Fritschy *et al*., 1999] and the subcellular localization [Lujan, 2007, Lujan and Shigemoto, 2006] of $GABA_{B1}$ subunit is plastic in the cerebellum and may be modulated in a developmental context. B1a subunit is predominant during the prenatal period. Its expression is down-regulated after birth and B1b becomes predominant at P10 [Fritschy *et al*., 1999]. Moreover, at the surface of Purkinje cells, $GABA_{B1}$ subunits undergo changes in their localization, from dendritic shafts to the dendritic spines, along with the establishment and the maturation of excitatory synapses [Lujan, 2007, Lujan and Shigemoto, 2006]. Whether this plasticity also manifests in the spinal cord, and,

more importantly, whether ontogenetic mechanisms of plasticity can be reactivated after nerve injury, remains to be investigated.

Different roles of $GABA_B$ receptors in pain transmission

The contribution of $GABA_B$ receptors to nociceptive processing was further explored with $GABA_{B1}$ knock-out mice [Schuler *et al*., 2001]. Acute thermal thresholds has assessed with hotplate and tail flick tests, were reduced in knock-out animals. These animals also showed a reduced paw withdrawal threshold to mechanical stimuli. Thus, tonic $GABA_B$ receptor activation appears to contribute to the establishment of nociceptive threshold.
In addition to their localization, the native function of the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms were recently dissected by comparing genetically modified mice, with selective knock-out of either splice variant [Ulrich and Bettler, 2007]. Morphological and electrophysiological analysis of these mutant mice provides convincing evidence suggesting non-redundant functions for $GABA_{B1a}$ and $GABA_{B1b}$ at glutamatergic synapses [Vigot *et al*., 2006].
$GABA_{B1}$ knock-out mice have recently been used to assess the peripheral contribution of this subunit to nociceptive processing [Magnaghi *et al*., 2008]. The thermal hyperalgesia, as measured with the plantar test, was more pronounced in knock-out mice that showed a lower latency of paw withdrawal. Conversely, the knock-out mice presented a higher mechanical threshold to Von Frey filament testing without sign of allodynia [Magnaghi *et al*., 2008]. Several morphological and morphometric studies of the PNS were performed in order to explain the different pain behavior of the $GABA_{B1}$ knock-out mice. These mice presented a higher number of small myelinated fibers and small neurons of the lumbar dorsal root ganglia [Magnaghi *et al*., 2008]. Given the presence of $GABA_B$ receptors also in the Schwann cells [Magnaghi *et al*., 2004], these results strengthen the hypothesis of a $GABA_B$-mediated, control of the peripheral myelination [Magnaghi, 2007]. In this context, co-regulation between $GABA_A$ and $GABA_B$ receptors has been hypothesized, accounting for GABAergic-mediated control of Schwann cell physiology [Magnaghi, 2007]. A similar speculation of a physical cross-interaction between GABA-A and $GABA_B$ receptors has been suggested to explain the decrease in antinociceptive potency of baclofen in mice lacking the $GABA_A$ $\alpha 3$ subunit [Ugarte *et al*., 2000]. In a model of diabetic neuropathy, bearing peripheral demyelination and neuropathic pain, the increased glutamatergic input from primary afferents to dorsal horn neurons results from a reduction in presynaptic $GABA_B$ receptors [Wang *et al*., 2007]. However, in $GABA_{B1}$ knock-out mice not only the peripheral myelin but also the PNS neuronal compartments are modified (Magnaghi *et al*., 2008). Electron microscopy analysis of the lumbar dorsal root ganglia, where the somata of the sensory neurons that synapse the lamina I-IV of the spinal dorsal horn are located, showed an increase in small neurons [Magnaghi *et al*., 2008]. Collectively, the altered PNS morphological findings in the $GABA_{B1}$ knock-out mice might be ascribed to an increase in Aδ or C primary afferents. The intraepidermal nerve fiber density and the immunolabeling of peripheral nerves for specific neuropeptides (e.g. CGRP) or neurofilaments (i.e. NF200), however, suggest that Aδ-sensory fibers are primarily increased in the $GABA_{B1}$ knock-out mice.
Post-synaptic roles for $GABA_B$ receptors in nociception have been also demonstrated at the spinal level. Post-synaptic effects of $GABA_{B1b}$-containing receptors have been demonstrated in somatosensory neurons where $GABA_{B2}/GABA_{B1b}$ dimers specifically induce post-synaptic, long-lasting inhibition of dendritic Ca2+ spikes [Perez-Garci *et al*., 2006].
The involvement of calcium channels in $GABA_B$-mediated control of neuronal activity has been documented in the spinal cord. Post-synaptic $GABA_B$ receptors modulate intrinsic firing properties of spinal neurons, from tonic to plateau or endogenous bursting properties. With

regards to its physiological significance, this property results in a switch between three functional meta-stable states that depend on L-type calcium channels activity upon mGluR and GABAB metabotropic modulation and are capable of modifying information transfer to dorsal horn neurons [Derjean *et al*., 2003]. $GABA_B$ receptors exert a sustained basal inhibition that is challenged by a tonic excitatory control mediated through group I mGluRs, thus resulting in a dynamic balance of inhibitory and excitatory inputs on dorsal horn neurons [Derjean *et al*., 2003]. Both $GABA_B$ receptors and group I mGluRs modulate endogenous neuronal properties through the regulation of inwardly rectifying potassium (Kir) channels. $GABA_B$ receptors also directly modify calcium influx through L-type voltage-gated calcium channels [Voisin and Nagy, 2001]. L-type channels are responsible for the expression of plateau potentials, an intrinsic mechanism for input-output amplification that occurs also in spinal neurons [Morisset and Nagy, 1999]. Whatever the mechanisms involved, $GABA_B$ receptors modulate endogenous membrane properties expressed by dorsal horn neurons and contribute to the complexity of nociceptive signaling in these neurons. Moreover, the internalization and trafficking of another type of voltage-gated calcium channels, the N-type, has been recently shown to be triggered by $GABA_B$ receptors [Tombler *et al*., 2006]. It is thus plausible that $GABA_B$ receptors inhibit the expression of intrinsic membrane properties by removing calcium channels from the plasma membrane, therefore reducing neuronal excitability. Preliminary anatomical data (Landry *et al*., 2002) show that the intracellular targets of post-synaptic $GABA_B$ receptors are mainly located at the level of the cell bodies and proximal dendrites and receive GABAergic inputs (Fig.3 a – c). These data also demonstrate the colocalization of $GABA_B$ receptors, L-type calcium channels, and Kir3 channels (Fig. 3d and e). Finally, they provide anatomical support for $GABA_B$ post-synaptic signaling to ionic channels in the soma and proximal dendrites of spinal neurons.

Desensitization and impairment of the $GABA_B$ receptors

The best known antinociceptive effect of GABA activation relies on the suppression of transmitter release from primary afferent transmitter [Ataka *et al*., 2000, Bowery, 2006]. However, as described above, post-synaptic mechanisms are most probably involved as well. Despite its analgesic properties, the use of Baclofen has been limited in clinics due to rapid tolerance and adverse effects after systemic administration [Bowery, 2006], but also to its limited effectiveness even after intrathecal injection [Becker *et al*., 2000, Loubser and Akman, 1996]. Limited effects of Baclofen may be due to desensitization of $GABA_B$ receptors. However, $GABA_B$ desensitization processes are very complex. Moreover, pre- and post-synaptic $GABA_B$ responses exhibit profound differences in their desensitization properties [Bettler and Tiao, 2006]. The possible internalization of the $GABA_B$ receptor in response to its agonist has been controversial. In neuronal models, $GABA_B$ only exhibits little, if any, basal or agonist-activated internalization [Fairfax *et al*., 2004, Perroy *et al*., 2003]. In a co-culture model of DRG and spinal cord, clathrin-dependent internalization of post-synaptic receptors, followed by a rapid recycling to the plasma membrane has been demonstrated [Laffray *et al*., 2007]. However, in conditions of chronic stimulation of primary sensory afferents, the receptor may not remain fully functional, although present at the plasma membrane, therefore suggesting a lack of correlation between $GABA_B$ protein expression and signaling. Another set of data showing discrepancies between the expression levels of $GABA_B$ subunits and the receptor signaling further supports the existence of non-genomics mechanisms leading to $GABA_B$ impairment [Sands *et al*., 2003]. A novel FRET-based approach recently provided evidence for $GABA_B$ desensitization through an original mechanism based on the heterodimer dissociation [Laffray *et al*., 2007]. GTPγS binding studies further showed that the $GABA_B$ responsiveness to the endogenous ligand is altered

under these conditions. This is in agreement with lack of signaling associated with the presence of single monomers at the plasma membrane [Engle *et al*., 2006]. Chronic stimulation of sensory afferent fibers is thus likely to modulate receptor oligomerization, providing additional levels of control of $GABA_B$ signaling in spinal cord nociceptive networks. This mechanism may provide a clue to explain limited analgesic effects of the Baclofen (see supra) while the agonist is efficiently used to control muscle spasticity in locomotor networks of spinal cord injury patients.
Changes in lateral diffusion of the receptors subunits represent another putative non-genomic mechanism of $GABA_B$ signaling regulation. It was demonstrated that the C-Terminus of the $GABA_{B2}$ subunit is involved in regulating lateral diffusion in the plasma membrane [Pooler and McIlhinney, 2007]. Restriction of the lateral diffusion would confine the receptor to specific membrane compartments, e.g. lipid raft enriched domains. It is proposed that localizing the $GABA_B$ receptor to lipid rafts provides a mechanism for receptor inhibition [Bettler and Tiao, 2006]. It may also prevent the receptor to reach its proper subcellular localization and to associate to its intracellular targets.
An additional level of complexity in receptor trafficking or activation derives from $GABA_B$-associated proteins. These proteins may be involved in increasing the receptor diversity in vivo inasmuch as they modify receptor activity and pharmacology, or receptor trafficking and membrane-anchoring. In line with this hypothesis, 14-3-3 proteins, a family of chaperone proteins, have been showed to associate with the $GABA_{B1}$ subunit [Couve *et al*., 2001]. It has been proposed that 14-3-3 compete for the binding site of the coat protein I complex (COPI) at the coiled-coil C-Terminus of $GABA_{B1}$ to regulate its export and/or recycling to the endoplasmic reticulum [Bettler and Tiao, 2006]. However, 14-3-3 association does not affect receptor trafficking [Brock *et al*., 2005b]. Moreover, in the spinal cord, 14-3-3 proteins have been found outside the endoplasmic reticulum, in close association with membrane domains (Laffray *et al*., unpublished data) where they may be enriched in lipid rafts [Couve *et al*., 2001]. Therefore, the functional significance of 14-3-3 association remains unclear. Another potential binding partner is the extra-cellular matrix protein Fibulin 2 that was identified in a yeast-two-hybrid screening [Blein *et al*., 2004]. Its specific binding to $GABA_{B1a}$ sushi repeats makes this binding potentially interesting. However, the role of Fibulin 2 as a regulator of membrane trafficking or activity of the specific $GABA_{B1a}/GABA_{B2}$ heterodimer, is not yet demonstrated. Finally, the physiological significance of $GABA_B$ association with binding partners, and its relevance in regulating nociceptive transmission, remains to be investigated in different cell types.

In conclusion, the $GABA_B$ receptors exhibit a high diversity of mechanisms modulating pain transmission in the spinal cord. Using $GABA_{B1}$ knock-out mice, we showed that peripheral $GABA_{B1}$ receptors participate to pain transmission and contribute to the hyperalgesic phenotype and altered pain perception.. Beside pre-synaptic functions, the $GABA_B$ receptors also exert a post-synaptic inhibitory control on spinal neurons activity. In chronic pain conditions, GABA- receptors may be desensitized and their signaling altered through, in part, changes in their association to other neurotransmitter receptors and/or other binding partner proteins.

Figure 1 : Structure of mGluRs and $GABA_B$

A. mGluRs are constituted of 1) a large bilobate extracellular domain (ECD) in which the natural ligand glutamate and other orthosteric ligands (agonists or competitive antagonists) bind, 2) a cysteine rich domain (CRD) constituted of about seventy amino acids which contains 9 cysteines and 3) a transmembrane domain (TMD) constituted of seven α helixes

spanning the lipid membrane which is common to all GPCRs and is involved in G protein binding and activation. Allosteric ligands able to positively or negatively modulate receptor activation are binding within the TMD. Moreover, mGluRs are obligatory homodimers crosslinked by a disulfide bridge across the lobe 2 of their ECD. B. $GABA_B$ is an obligatory heterodimer, constituted of two subunits $GABA_{B1}$ (GB1) and $GABA_{B2}$ (GB2). Each subunit is composed of an extracellular domain (ECD) and a transmembrane domain (TMD). Of note, two isoforms of $GABA_{B1}$ exist that differ mainly by the presence in $GABA_{B1a}$ of two extracellular sushi domains. $GABA_{B1}$ is responsible of GABA and other orthosteric ligands recognition but is unable to bind and activate G-proteins and is unable to reach the cell surface by itself due to the presence of a retention site KRR in its C-terminus tail. $GABA_{B2}$ is unable to bind GABA but allows the expression of the heterodimer at the cell surface by masking the retention signal of $GABA_{B1}$ and is responsible of G-protein coupling of the dimer. Moreover positive allosteric modulators binding within $GABA_{B2}$ TMD and able to enhance the receptor activity have been identified.

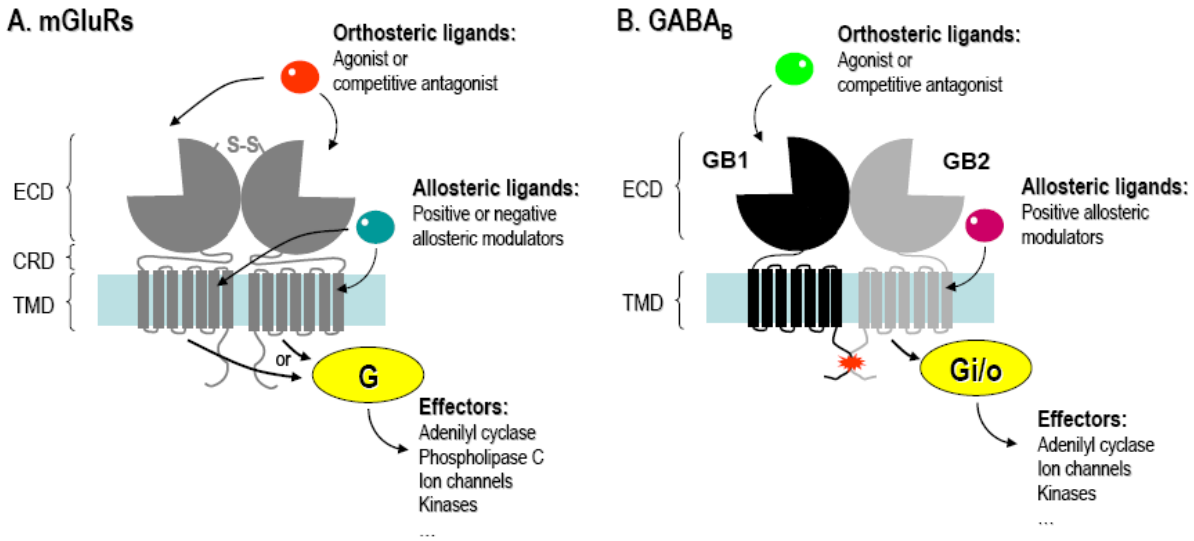


Figure 2 : Synaptic localization of mGluRs and $GABA_B$ receptors

Group I mGluRs are predominantly localized in the postsynapse where they potentiate NMDA activity. Postsynaptic group II mGluRs decrease synaptic activity via activation of GIRK channels. Group II and III mGluRs autoreceptors localized in glutamatergic terminals modulate glutamate release mainly through inhibition of N or P/Q type voltage gated calcium channels or direct interaction with the release machinery. Group III mGluRs heteroreceptors are found in GABAergic terminals where they modulate GABA release. $GABA_{B1a}$-$GABA_{B2}$ and $GABA_{B1b}$-$GABA_{B2}$ are coexpressed in GABAergic terminals where they modulate GABA release via the inhibition of voltage gated calcium channels. $GABA_B$ heteroreceptors are found in glutamatergic terminals (and also in other neurotransmitter terminals) and are activated by spillover or ambient GABA. $GABA_{B1a}$-$GABA_{B2}$ is predominantly associated with glutamatergic terminals where it modulates glutamate release through voltage gated calcium channels. Postsynaptic $GABA_B$ (mainly $GABA_{B1b}$) decrease neuronal activity through K channels activation for example. $GABA_{B1a}$-$GABA_{B2}$ and $GABA_{B1b}$-$GABA_{B2}$ are also found in the dendritic shaft and the spine neck. They are activated by GABA spillover from adjacent GABAergic terminals and regulate neuronal activity through activation of GIRK channels.

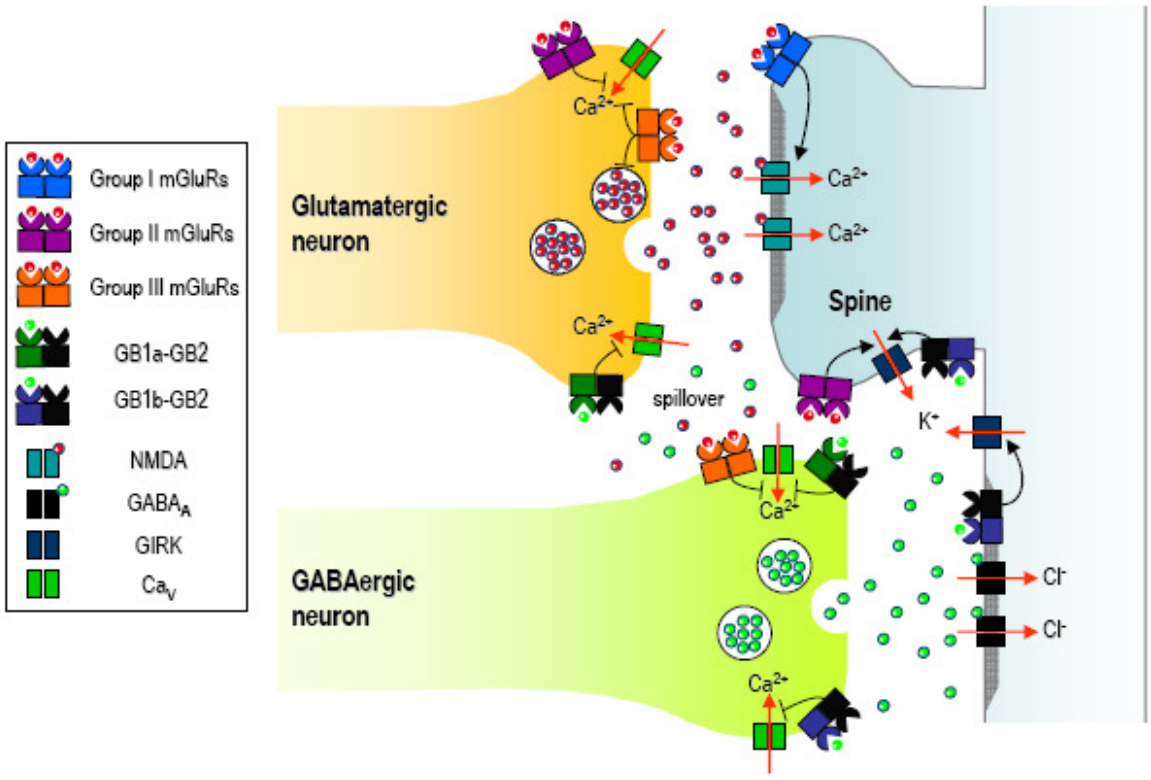
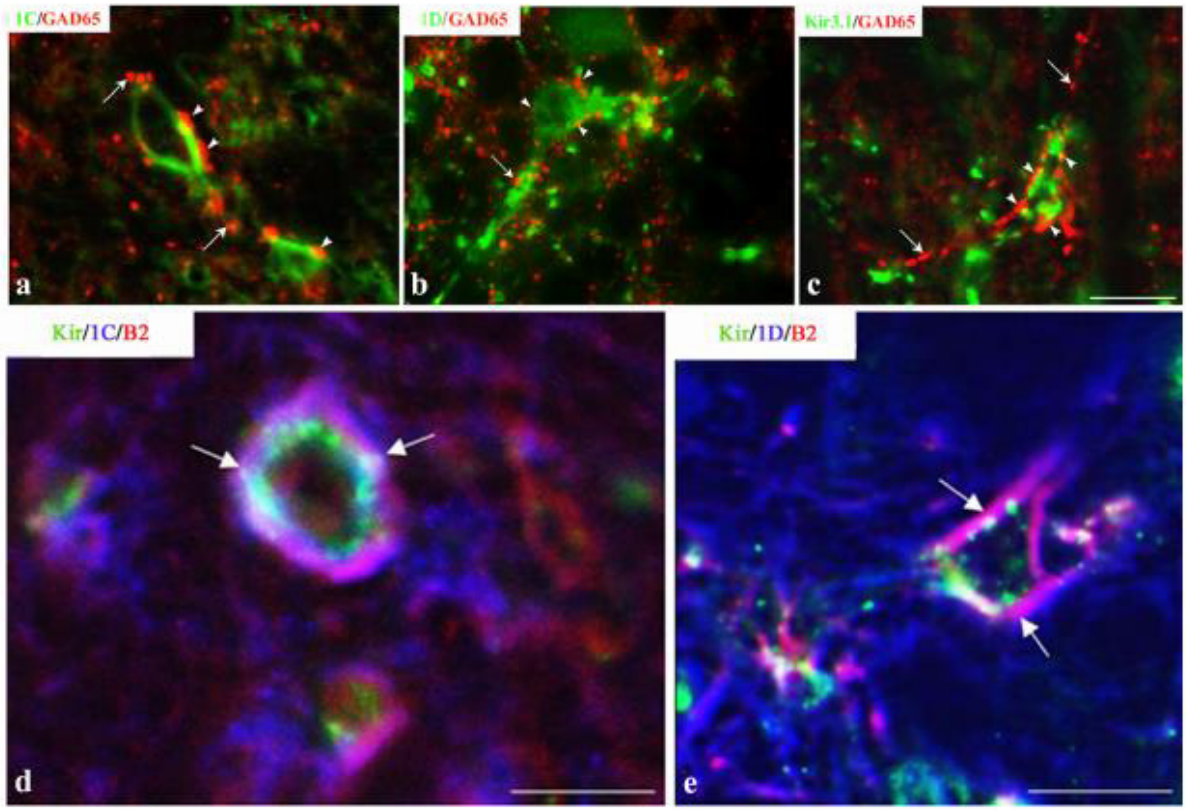


Figure 3 : Immunohistochemical analysis of the GABAergic/GABA$_B$ contacts in dorsal horn neurons of naïve rat spinal cord

Fibers containing GAD65, a GABA synthesising enzyme, make appositions onto soma (arrowheads in a to c) and proximal dendrites arrows in a to c) of dorsal horn neurons also expressing GABA$_{B\text{-}B}$ intracellular targets, i.e. Kir3 channels (a), or one or the other of the two alpha 1 subunits of L-type calcium channels (alpha 1C in b, alpha 1D in c). Dorsal horn soma co-express at the plasma membrane (arrows in d and e) the GABA$_{B2}$ subunit (red), and its intracellular targets, Kir channels (green) and alpha 1C (blue in d) or alpha 1D (blue in e) subunits of L-type calcium channels. Bars: 20 µm



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