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CHAPTER FOUR

Restoring the Spinal Pain Gate: $GABA_A$ Receptors as Targets for Novel Analgesics

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Abstract

$GABA_A$ receptors ($GABA_ARs$) and glycine receptors are key elements of the spinal control of nociception and pain. Compromised functioning of these two transmitter systems contributes to chronic pain states. Restoring their proper function through positive allosteric modulators should constitute a rational approach to the treatment of chronic pain syndromes involving diminished inhibitory spinal pain control. Although classical benzodiazepines (i.e., full agonists at the benzodiazepine binding site of $GABA_ARs$) potentiate synaptic inhibition in spinal pain controlling circuits, they lack clinically relevant

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analgesic activity in humans. Recent data obtained from experiments in $GABA_AR$ point-mutated mice suggests dose-limiting sedative effects of classical nonspecific benzodiazepines as the underlying cause. Experiments in genetically engineered mice resistant to the sedative effects of classical benzodiazepines and studies with novel less sedating benzodiazepines have indeed shown that profound antihyperalgesia can be obtained at least in preclinical pain models. Present evidence suggests that compounds with high intrinsic activity at α2-$GABA_AR$ and minimal agonistic activity at α1-$GABA_AR$ should possess relevant antihyperalgesic activity without causing unwanted sedation. On-going preclinical studies in genetically engineered mice and clinical trials with more selective benzodiazepine site agonists should soon provide additional insights into this emerging topic.

ABBREVIATIONS

TPA023 7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine
TPA023B 6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-b][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile
MRK-409 7-cyclobutyl-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine
MK-0343 7-cyclobutyl-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine
L-838,417 7-*tert*-butyl-3-(2,5-difluorophenyl)-6-(2-methyl-2H-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine
HZ166 ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate
NS11394 3′-[5-(1-hydroxy-1-methylethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile
CCI chronic constriction injury
SNI spared nerve injury
SNL spinal nerve ligation
TNT tibial nerve transection
CFA complete Freund's adjuvant
$GABA_AR$ γ-aminobutyric acid type A receptor
α_x-$GABA_AR$ $GABA_A$ receptor containing the α_x subunit

1. INTRODUCTION

Chronic pain is a severe medical condition affecting millions of patients worldwide. It is almost generally accepted that neuronal and synaptic plasticity occurring at different levels of the neuraxis are major contributors to chronic pain (Luo, Kuner, & Kuner, 2014; Sandkühler, 2009; Zeilhofer, Witschi, & Johansson, 2009) (for a schematic illustration of the

pain pathway, see Fig. 1). Some of these neuroplastic changes occur already in the peripheral terminals of nociceptors, which sense noxious stimuli arriving at the skin or in other peripheral tissues and convey them to the central nervous system. The central terminals of these nociceptors innervate the substantia gelatinosa (lamina II) of the spinal dorsal horn, or the trigeminal nucleus of the brainstem in case of those nociceptors coming from the facial skin or the meninges. From there, signals are propagated through various relay stations in the brainstem, midbrain, and thalamus to several cortical areas which give rise to the conscious sensation of pain. One site that has attracted particular attention in pain-related neuroplasticity is the spinal dorsal horn, which constitutes as the first site of synaptic integration in the pain

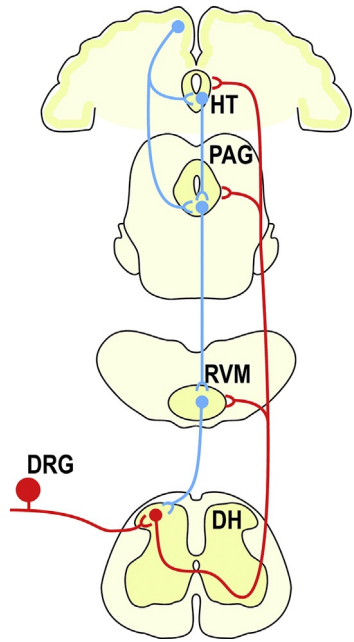

Figure 1 Schematic description of ascending pain pathways and descending antinociceptive fiber tracts. Nociceptive (painful) signals are conveyed by sensory fibers whose cell bodies reside in the dorsal root ganglia (DRGs). They reach the CNS at the level of the spinal dorsal horn (DH), where nociceptor terminals release glutamate to excite postsynaptic second-order neurons. These central neurons transmit the nociceptive information via the brainstem and the midbrain to cortical areas, where the conscious sensation of pain arises. Descending antinociceptive pathways are controlled by cortical areas, which contact the hypothalamus (HT) and the periaqueductal gray (PAG). The PAG in turn controls the rostral ventromedial medulla (RVM), which constitutes the main origin of descending antinociceptive fibers innervating the spinal cord.

pathway. Neurons located in the spinal dorsal horn integrate primary afferent sensory signals of painful and non-painful modalities with input from descending fiber tracts, which can either inhibit or facilitate pain. Inhibitory interneurons have been attributed a critical role in this process already in the gate-control-theory of pain (Fig. 2; Melzack & Wall, 1965). Although some of the synaptic connections proposed in the original scheme do apparently not exist, plenty of evidence indicates that compromising the function of inhibitory dorsal horn neurons induces symptoms reminiscent of chronic pain syndromes in humans. Animals develop an exaggerated sensitivity to painful stimuli (hyperalgesia), they respond with withdrawal responses upon exposure to stimuli, which are normally not felt as painful (allodynia), and they also show signs of spontaneous discomfort. Many lines of evidence indicate that typical causes of chronic pain such as inflammation or neuropathies compromise the function of inhibitory interneurons in the spinal dorsal horn through different mechanisms (for a review, see Zeilhofer, Benke, & Yévenes, 2012). According to this concept, a facilitation of inhibitory neurotransmission should be a rational strategy for the treatment of many chronic pain states. Yet, none of the established analgesics act- through a facilitation of inhibitory neurotransmission. In the following text, we will review mechanisms of pain-related spinal disinhibition and evidence supporting the concept that novel subtype-selective benzodiazepine agonists would be suitable for the treatment of chronic pain syndromes. In the context of this review, we use the term "benzodiazepine" for all agonists at the benzodiazepine binding site of γ-aminobutyric acid type A receptors

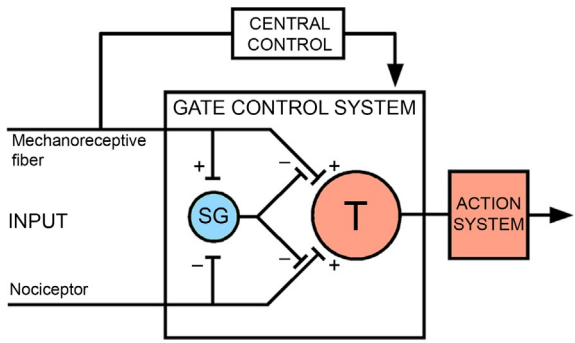

Figure 2 Gate-control theory of pain (Melzack & Wall, 1969). Inhibitory neurons located in the substantia gelatinosa (SG) control the spinal pain gate. According to the original concept these neurons are controlled by non-nociceptive input from mechanosensitive fibers and by nociceptive input in opposite directions. Their activation would in turn control the spinal output system (T).

($GABA_AR$s) independent of their chemical structure. It should also be mentioned here that $GABA_AR$s exist, which are resistant to modulation by classical benzodiazepines. These receptors contain α4 or α6 subunits instead of α1, α2, α3, or α5, or a γ1 or δ subunit instead of the γ2 subunit. These benzodiazepine-insensitive receptors are quite abundant in several brain regions (e.g., thalamus and cerebellum), but their expression in the spinal cord is sparse.

2. SYNAPTIC DISINHIBITION IN PATHOLOGICAL PAIN

Fast synaptic inhibition in the spinal dorsal horn is mediated by GABA and glycine acting respectively at $GABA_AR$ and strychnine-sensitive GlyRs. Plenty of evidence indicates that blockade of spinal $GABA_AR$s or GlyRs produces signs of allodynia and spontaneous pain (Beyer, Roberts, & Komisaruk, 1985; Miraucourt, Dallel, & Voisin, 2007; Roberts, Beyer, & Komisaruk, 1986). More recent studies provided insights into the mechanism of this sensitization on the level of dorsal horn neuronal circuits. The most consistent observation in these studies was a strong increase in polysynaptic input onto lamina II neurons after application of the $GABA_AR$ antagonist bicuculline (Baba et al., 2003). A second finding was related to the synaptic input of lamina I projection neurons, which express the neurokinin 1 receptor. These neurons serve an essential role in the relay of pathological pain, as their ablation strongly reduces hyperalgesia induced by inflammation and neuropathy (Nichols et al., 1999). Under normal conditions, these neurons receive sensory input almost exclusively from nociceptors (C and Aδ fibers). Blockade of $GABA_A$ and GlyRs however led to the *de novo* appearance polysynaptic responses from Aβ fibers (Torsney & MacDermott, 2006). These newly appearing polysynaptic connections likely underlie the allodynia seen *in vivo* after spinal application of bicuculline or strychnine. An increase in polysynaptic Aβ fiber input onto substantia gelatinosa (lamina II) may also occur as an endogenous process in chronic pain states (Baba, Doubell, & Woolf, 1999).

Several groups have identified signaling pathways that reduce inhibitory synaptic transmission in inflammatory or neuropathic pain states (Fig. 3). A prostaglandin E2-mediated phosphorylation of superficial dorsal horn GlyRs renders these receptors less responsive to glycine (Ahmadi, Lippross, Neuhuber, & Zeilhofer, 2002; Harvey et al., 2004; Reinold et al., 2005). Peripheral nerve damage leads to a downregulation of the GABA synthesizing enzyme GAD65 in the spinal cord (Moore et al.,

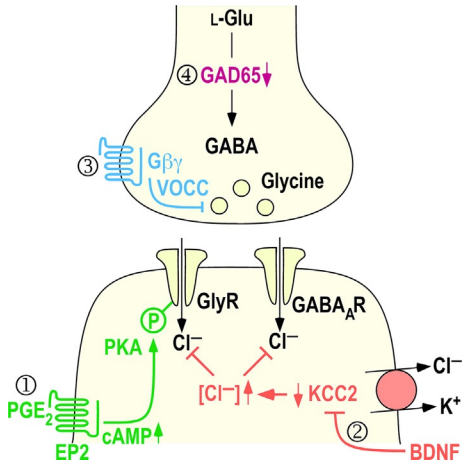

Figure 3 Four signaling pathways leading to spinal disinhibition in pathological pain states. (1) Prostaglandin E2 (PGE_2) produced in the spinal cord in response to peripheral inflammation increases cAMP production after activation EP2 receptors (EP2). The subsequent activation of protein kinase A (PKA) phosphorylates and inhibits GlyR of the superficial dorsal horn. (2) Peripheral nerve damage activates spinal microglia which releases brain-derived neurotrophic factor (BDNF). BDNF downregulates the expression of the potassium/chloride exporter KCC2 leading to an increase in intracellular chloride ($[Cl^-]_i$). As a consequence GABAergic and glycinergic input becomes less inhibitory (or even excitatory). (3) Several neuromodulators including endocannabinoids reduce presynaptic GABA and glycine release rendering dorsal horn neurons more excitable. (4) Peripheral nerve damage leads to the downregulation of the GABA synthesizing enzyme GAD65 and possible to reduced GABA content in inhibitory dorsal horn neurons.

2002), and both inflammation and nerve injury cause an epigenetic downregulation of the same enzyme in the brainstem (Zhang, Cai, Zou, Bie, & Pan, 2011). A large number of neuromodulators interfere with the release of GABA and glycine from inhibitory dorsal horn neurons via activation of G protein-coupled receptors and inhibition of Ca^{2+} channels (Zeilhofer, Wildner, & Yévenes, 2012). An endocannabinoid and CB1 receptor-mediated inhibition of glycine and/or GABA release contributes to spinal sensitization evoked by extensive nociceptive input to the dorsal horn (Pernia-Andrade et al., 2009). Microglia activated in the dorsal horn in response to peripheral nerve damage downregulates the expression of the potassium and chloride co-exporter KCC2 in superficial dorsal horn neurons, thereby shifting the reversal potential of GABA and glycine evoked chloride currents to more depolarized values. This shift renders glycinergic and GABAergic input less inhibitory (Coull et al., 2003, 2005; Keller, Beggs,

Salter, & De Koninck, 2007), or, if the shift is sufficiently large, glycinergic and GABAergic input may even become excitatory and trigger action potentials in postsynaptic neurons (Coull et al., 2003).

Pharmacological enhancement of GABAergic synaptic transmission in the dorsal horn should be able to reverse pathological pain states that result from reduced presynaptic GABA release or from reduced responsiveness of postsynaptic $GABA_AR$s. Some of the disinhibitory processes discussed above do specifically reduce glycinergic inhibition prompting the question whether a potentiation of GABAergic responses would be able to restore proper inhibition in these cases. Many inhibitory dorsal horn neurons, co-release GABA and glycine from the same terminals and even from the same vesicles (Bohlhalter, Möhler, & Fritschy, 1994; Colin, Rostaing, Augustin, & Triller, 1998; Feng et al., 2005; Todd & Sullivan, 1990; Todd, Watt, Spike, & Sieghart, 1996). In most dorsal horn neurons, inhibitory postsynaptic responses are mediated by $GABA_AR$ and GlyRs (Baccei & Fitzgerald, 2004; Yoshimura & Nishi, 1995) and even in cells, in which no GABAergic component is visible under normal conditions, a GABAergic IPSC component can be revealed with benzodiazepines and neurosteroids (Keller, Breton, Schlichter, & Poisbeau, 2004; Keller, Coull, Chery, Poisbeau, & De Koninck, 2001). It is thus conceivable that pharmacological enhancement of GABAergic neurotransmission would also compensate for reduced glycinergic transmission.

The situation is more complex in those cases where disinhibition results from changes in the transmembrane chloride gradient. As long as the activation of $GABA_AR$ or GlyRs remains below the threshold of action potential activation, potentiation of $GABA_AR$ or GlyR may still remain inhibitory. However, as soon as the chloride equilibrium potential reaches the action potential threshold, potentiation of $GABA_AR$ or GlyR would increase the risk of paradoxical GABAergic and glycinergic excitation (Prescott, Sejnowski, & De Koninck, 2006). We discuss this issue below in the context of preclinical studies on subtype-selective benzodiazepines.

3. SPINAL $GABA_AR$ SUBTYPES MEDIATING ANTIHYPERALGESIA: EVIDENCE FROM GENETICALLY ENGINEERED MICE

Analgesic or antihyperalgesic actions of benzodiazepines occur after local spinal injection suggesting that these effects are mediated by $GABA_AR$s expressed in the spinal cord. To identify the $GABA_AR$ subtypes responsible

for these antihyperalgesic effects, "knock-in" mice were investigated, in which the α1, α2, α3, or α5-$GABA_AR$ subunits had been rendered diazepam-insensitive through the introduction of a histamine to arginine (H/R) point mutation (Knabl et al., 2008; for information on the different point-mutated mouse strains, see Rudolph & Möhler, 2004). In wild-type mice, intrathecal diazepam strongly reduced hyperalgesia in models of inflammatory or neuropathic pain, but had no effects on acute nociceptive pain. This "antihyperalgesic" activity was unchanged in mice, which carried the H/R point mutation in the α1 subunit, but strongly reduced in mice, which carried the point mutation in the α2 subunit. Mice with point-mutated α3 or α5 subunits showed reduced antihyperalgesic activity in some but not in all tests. The different subtypes of benzodiazepine-sensitive $GABA_ARs$ contribute to spinal antihyperalgesia with the rank order $\alpha2 > \alpha3 \geq \alpha5 \gg \alpha1$. The $GABA_AR$ subtype-dependence of spinal antihyperalgesia hence matched well with the expression of the different α subunits in the superficial dorsal horn (Bohlhalter, Weinmann, Möhler, & Fritschy, 1996; Lorenzo et al., 2014; Paul, Zeilhofer, & Fritschy, 2012).

The lack of a contribution from α1-$GABA_ARs$, which mediate the sedative effects of diazepam (Rudolph et al., 1999), demonstrates that antihyperalgesia by benzodiazepines can be studied in the absence of confounding sedation as long as α1-$GABA_ARs$ are not activated. Such experiments were performed in mice carrying H/R point mutations in the α2, α3, or α5 subunits in addition to α1 (Knabl, Zeilhofer, Crestani, Rudolph, & Zeilhofer, 2009). These experiments showed that antihyperalgesia could also be obtained after systemic diazepam (and in the absence of sedation) and that α2 and α3-$GABA_AR$ subtypes were the most relevant subtypes also for antihyperalgesia following systemic administration.

So far, $GABA_AR$ point-mutated mice have been used to assess the antihyperalgesic properties of benzodiazepines in three pain models, i.e., against zymosan A-induced inflammatory hyperalgesia, against neuropathic hyperalgesia induced by chronic constriction injury (CCI) of the sciatic nerve and in the formalin test. $GABA_AR$ subtypes mediating antihyperalgesia are thus clearly different from those mediating sedation (Rudolph et al., 1999), amnesia (Rudolph et al., 1999), and the rewarding properties of classical benzodiazepines (Tan et al., 2010). In the case of the α2- and α3-$GABA_ARs$, there is a clear overlap with the receptors mediating anxiolysis (Löw et al., 2000) and muscle relaxation (Crestani et al., 2001), and, in case of α5-$GABA_AR$, possibly also with those responsible for benzodiazepine-induced cognitive impairment (Dawson et al., 2006). For

a comparison of the contribution of the different $GABA_AR$ subtypes to desired hyperalgesia and other effects, see Fig. 4E.

The antihyperalgesic efficacy of diazepam after systemic administration prompts two questions. First, how important are spinal versus supraspinal CNS areas for antihyperalgesia by systemic benzodiazepines? Second, do central effects such as a reversal of anxiety-induced hyperalgesia (Andre et al., 2005; Vidal & Jacob, 1982) indirectly contribute to the antihyperalgesia by systemic benzodiazepines? The latter question appears relevant in particular because α2-$GABA_ARs$ mediate not only antihyperalgesia but also anxiolysis (Löw et al., 2000). Both questions were addressed with conditional $GABA_AR$ deficient mice (*hoxb8*-$\alpha2^{-/-}$ mice), which lack the $GABA_AR$ α2 subunit specifically from the spinal cord and dorsal root ganglia (DRGs) (up to about segment C4). In these experiments, a recently developed benzodiazepine site agonists (HZ166; Rivas et al., 2009) was employed which exerts antihyperalgesic actions similar to systemic diazepam but with reduced sedative and muscle relaxant properties (Di Lio et al., 2011). Antihyperalgesia was assessed as the change in heat and pin-prick induced withdrawal responses. These withdrawal responses are under strong control from descending pain-modulating fiber tracts from various CNS areas (Carrasquillo & Gereau, 2007; Harris & Westbrook, 1995; Jasmin, Rabkin, Granato, Boudah, & Ohara, 2003; Tatsuo, Salgado, Yokoro, Duarte, & Francischi, 1999). Analysis of such withdrawal responses should

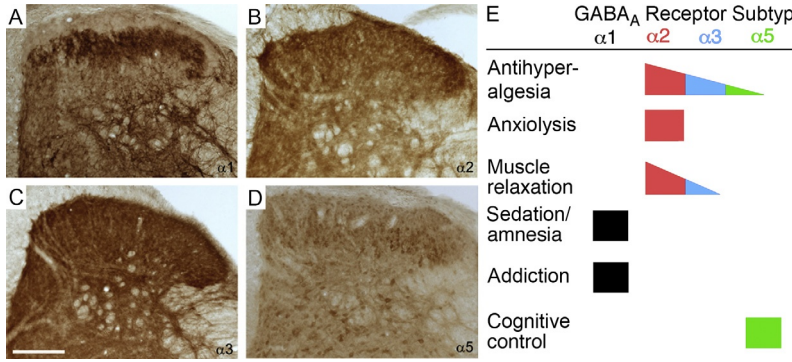

Figure 4 Distribution of α1, α2, α3, and α5-$GABA_ARs$ subunits in the lumbar spinal cord and contribution of the four subtypes of $GABA_ARs$ to antihyperalgesia. (A–D) Immunocytochemical analysis of the expression of $GABA_AR$ α subunits in the spinal dorsal horn of mice. Scale bar, 100 μm. (E) Contribution of the different $GABA_AR$ subtype to spinal antihyperalgesia and comparison with other behavioral effects of benzodiazepines. *Panels (A–D): reproduced from Paul et al. (2012); Panel E: modified from Zeilhofer, Möhler, and Di Lio (2009).*

hence reveal possible effects of descending (facilitating or inhibitory) pain modulation. Mice lacking the $GABA_AR$ α2 subunits specifically from the spinal cord showed virtually the same reduction in benzodiazepine-induced antihyperalgesia as global α2 (H/R) point-mutated mice confirming that the spinal cord was the most relevant site for the antihyperalgesic action of benzodiazepines also after systemic administration (Paul et al., 2014). This finding also largely ruled out that antihyperalgesia occurred secondary to other effects such as a reversal of anxiety-induced hyperalgesia. In this context, it should also be added that the spinal cords of *hoxb8*-$\alpha2^{-/-}$ mice completely lacked α2-$GABA_AR$s indicating that the spinal terminals of fibers descending from supraspinal CNS areas to the spinal cord do not express α2-$GABA_AR$s.

4. MECHANISMS OF SPINAL BENZODIAZEPINE-MEDIATED ANTIHYPERALGESIA

Immunohistochemistry studies have identified specific spinal distribution patterns of $GABA_AR$ subunits (Bohlhalter et al., 1996; Paul et al., 2012; Fig. 4A–D). These receptors are expressed on intrinsic dorsal horn neurons and on the central terminals of primary sensory nociceptors. Spinal antihyperalgesia may therefore originate either from classical postsynaptic inhibition mediated by $GABA_AR$s on intrinsic dorsal horn neurons or from $GABA_AR$s on nociceptor terminals which mediate presynaptic inhibition through so-called primary afferent depolarization. Both processes are illustrated in Fig. 5. The availability of a "floxed" α2-$GABA_AR$ allele for conditional gene deletion allowed experiments distinguishing between these two possibilities.

4.1. Contribution of presynaptic inhibition and primary afferent depolarization

To assess the contribution of presynaptic inhibition, the α2-$GABA_AR$ subunit was ablated specifically from nociceptor terminals using an sns::cre BAC transgenic mouse which expresses the cre recombinase under the transcriptional control of the *scn10a* (Nav1.8) gene (Agarwal, Offermanns, & Kuner, 2004). In the case of an inflammatory pain model, the degree of antihyperalgesia by spinally applied diazepam in the nociceptor-specific α2-$GABA_AR$ subunit-deficient (sns-$\alpha2^{-/-}$) mice fell between those measured in wild-type mice and in global α2-$GABA_AR$ point-mutated mice. In the inflammatory model, the partial loss of diazepam-induced antihyperalgesia

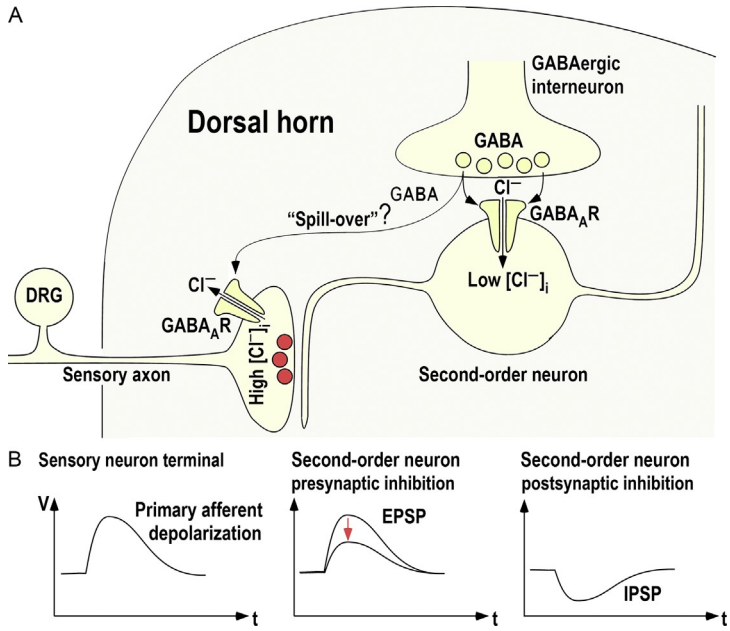

Figure 5 Mechanisms of the GABAergic control of spinal pain transmission. (A) Anatomical arrangement of pre- and postsynaptic inhibition. (B) Activation of $GABA_A$Rs on nociceptor terminals causes chloride efflux and primary afferent depolarization (left panel). This primary afferent depolarization causes presynaptic inhibition of glutamate release from nociceptor terminals (middle panel). Activation of $GABA_A$Rs on second-order neurons (intrinsic) dorsal horn neurons activates chloride influx and causes classical postsynaptic inhibition through hyperpolarization and dendritic shunting (right panel).

in sns-$\alpha2^{-/-}$ mice clearly indicated a contribution of presynaptic inhibition/primary afferent depolarization to antihyperalgesia by intrathecal diazepam. This was different in a neuropathy model in which all three genotypes responded with virtually identical antihyperalgesia (Witschi et al., 2011). This unaltered efficacy either indicates that antihyperalgesia in the neuropathy model was entirely due to postsynaptic inhibition of intrinsic dorsal horn neurons, or that antihyperalgesia occurred through inhibition of cre-negative (non-nociceptive) fibers. It has indeed been shown that inflammatory and neuropathic hyperalgesia depend on different classes of sensory fibers with Nav1.8 (sns) expressing sensory neurons being particularly important for inflammatory pain (Abrahamsen et al., 2008).

The results obtained in nociceptor-specific $\alpha2^{-/-}$ mice show that at least part of the antihyperalgesia originates from enhanced presynaptic inhibition.

The remaining α2-$GABA_A$R-mediated component may result either from inhibition of non-nociceptive fibers or from postsynaptic inhibition of intrinsic dorsal horn neurons. In both cases, the complete loss of α2-$GABA_A$R-mediated antihyperalgesia in *hoxb8*-$\alpha2^{-/-}$ mice unequivocally demonstrates that the major component of benzodiazepine-evoked antihyperalgesia is of spinal origin (Paul et al., 2014). This has not yet been formally proven for the α3- and α5-$GABA_A$R-mediated components. However, both subunits are also enriched in the dorsal horn and their antihyperalgesic actions may thus also come from the spinal cord.

4.1.1 Mechanisms of presynaptic inhibition

It is well established that the spinal terminals of primary sensory neurons carry functional benzodiazepine-sensitive $GABA_A$Rs. Activation of these presynaptic $GABA_A$Rs causes depolarization of sensory neurons rather than hyperpolarization because primary afferent sensory neurons lack an efficient chloride export mechanism (Kanaka et al., 2001; Price, Hargreaves, & Cervero, 2006). As a consequence, the intracellular chloride concentration in these neurons renders the chloride equilibrium potential more positive than the resting membrane potential. This depolarization is however still inhibitory probably because it leads to a voltage-dependent inactivation of Na^+ and Ca^{2+} channels in the axon and the axon terminal, respectively, and subsequently reduces transmitter release (Kullmann et al., 2005). This presynaptic inhibition can occur through axo-axonic synapses. Their existence is firmly established for non-nociceptive primary sensory fibers (Aβ and low threshold Aδ fibers; Ribeiro-da-Silva, 1995). Axo-axonic contacts have also been found in nociceptor terminals, but less frequently than in terminals of non-nociceptive fibers (Alvarez, Kavookjian, & Light, 1993; Ribeiro-Da-Silva, Castro-Lopes, & Coimbra, 1986; Ribeiro-da-Silva, Tagari, & Cuello, 1989). Two recent studies disagree on the presence of gephyrin clusters on nociceptor terminals (Lorenzo et al., 2014; Paul et al., 2012). Because gephyrin is required for postsynaptic clustering of inhibitory neurotransmitter receptors in central neurons, the presence or absence of gephyrin clusters from sensory fiber terminals may be taken as an argument in favor or against the presence of axo-axonic synapses between GABAergic interneurons and nociceptor terminals. Physiological studies have established that primary afferent depolarization and presynaptic inhibition exist also in nociceptors (Lin, Wu, & Willis, 1999; Lin, Zou, & Willis, 2000; Witschi et al., 2011). Nociceptor terminals lacking GABAergic axo-axonic synapses may be subject to presynaptic inhibition through $GABA_A$Rs

via "spill-over" of GABA from neighboring synapses and so-called volume transmission (Fig. 5; Rudomin & Schmidt, 1999, for a more recent review see also Zeilhofer, Wildner, et al., 2012).

5. ANTIHYPERALGESIC ACTION OF BENZODIAZEPINES WITH IMPROVED SUBTYPE SPECIFICITY: PRECLINICAL STUDIES

A number of benzodiazepines with reduced activity at α1-$GABA_A$Rs have been developed in the last two decades mainly in the quest for non-sedating anxiolytics (for a comprehensive list, see Rudolph & Knoflach, 2011). Because benzodiazepine-mediated anxiolysis and antihyperalgesia share a similar dependence on $GABA_A$R subtypes, some of these compounds were also tested in pain studies (Table 1). It should be mentioned here that α1-sparing compounds are sometimes referred to as "α2/3 selective" (e.g., Hofmann et al., 2012), this is however incorrect as all of them are also agonists at α5-$GABA_A$Rs. This additional activity at α5-$GABA_A$-Rs should not be forgotten when undesired effects of these compounds are discussed.

NS11394, which has very low activity at α1-$GABA_A$Rs (<10% relative to diazepam) and good partial agonistic activity at α2-, α3-, and α5-$GABA_A$Rs (26–78%, relative to diazepam) (Mirza et al., 2008), exhibited antinociceptive activity at nonsedating doses in several rodent pain models (Hofmann et al., 2012; Munro et al., 2008). The analgesic effect in the formalin test was blocked by the benzodiazepine site antagonist flumazenil (Munro et al., 2008) confirming that it occurred through $GABA_A$Rs. L-838,417, which completely lacks intrinsic activity at α1-$GABA_A$Rs and possesses partial agonistic activity at α2-, α3-, and α5-$GABA_A$Rs (15–32%, relative to diazepam; McKernan et al., 2000), was tested in several rodent pain models where it was not sedative but active against inflammatory and neuropathic hyperalgesia (Knabl et al., 2008; Nickolls et al., 2011) and formalin-induced nociception (Hofmann et al., 2012). In addition, L-838,417 was active against hyperalgesia evoked by skin incision, a model of postoperative hyperalgesia (Reichl et al., 2012), and against capsaicin-induced central pain sensitization (Hansen et al., 2012). Other benzodiazepines with low sedative propensities include HZ166 (Rivas et al., 2009) and TPA023 (Atack et al., 2006). HZ166, which exerts rather high intrinsic activity, was antihyperalgesic in inflammatory and neuropathic mouse models (Di Lio et al., 2011), while TPA023, which is a low

Table 1 Subtype-selective benzodiazepine site agonists in rodent pain models

Compound	Selectivity ratio α2/α1intrinsic activity at α2	Effects in pain models	Reference
Good selectivity and high intrinsic activity at α2			
HZ166[a]	3.1213%	Antihyperalgesic in mouse zymosan A and CCI[b]	Di Lio et al. (2011)
NS11394	6.273%	Antinociceptive in rat formalin and capsaicin test Antihyperalgesic in CFA[c] Inflammation, CCI, and SNI[d] reduced capsaicin-induced secondary hyperalgesia	Munro et al. (2008) and Hansen, Erichsen, Brown, Mirza, and Munro (2012)
Good selectivity and low intrinsic activity at α2			
L-838,417	28 42.7%	Antihyperalgesic in rat zymosan A and CCI Antiallodynic in rat SNL[e] but not TNT[f]Antihyperalgesic but not antiallodynic in rat CFAAntihyperalgesic against incisional pain	Knabl et al. (2008), Nickolls et al. (2011), and Reichl, Augustin, Zahn, and Pogatzki-Zahn (2012)
TPA023	1212%	Antiallodynic in rat SNL; no antihyperalgesia in rat CFALittle effect in rat formalin; hyperalgesic in rat carrageenan and CCI	Nickolls et al. (2011) and Munro, Erichsen, Rae, and Mirza (2011)
No selectivity toward α2			
Zolpidem	0.9210%	Antinociceptive in rat formalin and capsaicin, but only at sedative doses	Munro et al. (2008)
Bretazenil	0.560%	No antihyperalgesia in rat CCI and SNI at nonsedative doses	Munro et al. (2008)

[a]Compound 2 in Rivas et al. (2009).
[b]Chronic constriction injury.
[c]Complete Freund's adjuvant.
[d]Spared nerve injury.
[e]Spinal nerve ligation.
[f]Tibial nerve transection.
Modified from Zeilhofer, Benke, and Yévenes (2012).

intrinsic activity partial agonist, showed comparatively weak anti-allodynic or antihyperalgesic effects (Munro et al., 2011; Nickolls et al., 2011). Other compounds, which have higher intrinsic activities at α1-$GABA_A$Rs than at α2-, α3-, and α5-$GABA_A$Rs, did not exhibit antihyperalgesic activity at non-sedative doses. These results are fully consistent with the results obtained from H/R point-mutated mice.

Novel benzodiazepines for treatment of chronic pain would not only have to have good analgesic or antihyperalgesic efficacy and low, or at least weak, sedative properties, but would also have to avoid other typical side effects such as addiction and tolerance development (i.e., a loss of activity during prolonged treatment).

5.1. Addiction

Reinforcing (or addictive) properties were absent or reduced in the case of partial agonists which lack efficacy at α1-$GABA_A$Rs, including L-838,417 (Rowlett, Platt, Lelas, Atack, & Dawson, 2005) and TPA023 (Abe et al., 2009). Other partial agonists with higher efficacy at α1-$GABA_A$Rs such as TPA123 caused a withdrawal syndrome following upon cessation of self-administration, suggesting that the lack of agonistic activity at α1-$GABA_A$Rs was the relevant parameter rather than a general reduction in agonistic activity (Ator, Atack, Hargreaves, Burns, & Dawson, 2010). These results are consistent with studies in α1-$GABA_A$R point-mutated mice, in which no reinforcement was seen with the full agonist midazolam (Tan et al., 2010).

5.2. Tolerance development against antihyperalgesia

Analgesia by opioids shows a high liability to tolerance development and the same applies to many of the actions of classical benzodiazepines including antihyperalgesia in rats (Witschi & Zeilhofer, unpublished) and mice (Ralvenius, Benke, Rudolph, & Zeilhofer, 2013). Whether tolerance against antihyperalgesia occurs also with more selective or nonsedative benzodiazepines has been studied for three compounds. L-838,417 was devoid of tolerance developments against antihyperalgesic/analgesic actions during chronic 9-day treatment, while an equally effective dose of morphine completely lost its antihyperalgesic activity during the same time period (Knabl et al., 2008). Tolerance development was also not observed against antihyperalgesia by HZ166 (Di Lio et al., 2011), whereas NS11394 lost at least part of its analgesic activity against formalin-induced pain during

chronic 8-day treatment (Hofmann et al., 2012). It is at present unknown whether reduced tolerance liability of nonsedative or more selective benzodiazepines is due to their reduced intrinsic activity (partial agonism), to improved subtype-selectivity, or to pharmacokinetic differences. Mice carrying different combinations of point-mutated $GABA_AR$ α subunits should offer a tool to address these possibilities.

6. CLINICAL STUDIES ON ANTIHYPERALGESIA BY BENZODIAZEPINES

The preclinical studies discussed above performed in mice resistant to the sedative effects of benzodiazepines demonstrate that classical benzodiazepines do in principle exert profound antihyperalgesic actions but only at doses, which normally induce strong sedation. Less-sedating benzodiazepines exhibited antihyperalgesic efficacy at nonsedating doses also in wild-type mice.

In human patients, classical nonselective benzodiazepines do not exert relevant analgesic (or antihyperalgesic) actions at clinically used doses. The few publications that found positive evidence for efficacy in pain patients were pilot studies or open trials (e.g., Fishbain, Cutler, Rosomoff, & Rosomoff, 2000; Harkins, Linford, Cohen, Kramer, & Cueva, 1991), which provide only limited evidence. Possible explanations for this lack of analgesic or antihyperalgesic efficacy in humans include (1) species differences (rodents vs. humans), (2) biological differences between preclinical models of pain and pain in human patients, and (3) dose-limiting sedation after systemic administration of classical benzodiazepines. The authors of this review favor the latter possibility for several reasons.

A recent study investigated possible analgesic or antihyperalgesic effects of two classical benzodiazepines (clobazam and clonazepam) in a battery of pain tests in human volunteers (Vuilleumier, Besson, Desmeules, Arendt-Nielsen, & Curatolo, 2013). This study found of a (small) antihyperalgesic effect in several parameters including in the size of the hyperalgesic area induced by intracutaneous capsaicin injection and in several muscle pain-related read-outs. Non-selective full agonists such as diazepam, clonazepam, midazolam typically induce strong sedation or sleep already at receptor occupancies between 15% and 30% (Fujita et al., 1999; Malizia et al., 1995; Pauli, Farde, Halldin, & Sedvall, 1991; Shinotoh et al., 1989). Compounds with a better α2/α1-selectivity ratio should permit higher levels of

receptor occupancy and hence higher α2-$GABA_AR$ activation before reaching dose-limiting sedation.

The data available from the few benzodiazepines with improved selectivity profile that went into clinical trials provide some insight as to what degree of selectivity would be needed to avoid sedation in humans. One such study investigated MK-0343 (also known as MRK-409), which has very weak agonistic activity at α1-$GABA_AR$s (18% relative to the full agonist chlordiazepoxide; Atack et al., 2006). This compound was sedative in humans although previous preclinical tests in rodents had not shown any evidence for sedative properties (Atack et al., 2006; de Haas et al., 2008). A related compound (TPA023B) fully devoid of agonistic activity at α1-$GABA_AR$s did not produce sedation in man (Atack, Hallett, et al., 2011; Atack, Wafford, et al., 2011). These results suggest that the human brain is more susceptible to sedative actions of benzodiazepines than that of mice and rats (or, alternatively one might argue that our tests to assess sedation on rodents are less sensitive than those in man). In both cases, the available data indicate that sedation in humans can be avoided with compounds fully devoid of intrinsic activity at α1-$GABA_AR$s. Concert Pharmaceuticals is currently performing a phase 1 clinical trial on a deuterated version of L-838,417 (now called CTP-354), which has more favorable pharmacokinetics in humans than L-838,417 (http://www.concertpharma.com/CTP354Phase1Initiation.htm). It would be very informative to see whether CTP-354 or TPA023B, which has an intrinsic activity at α2-$GABA_AR$s even higher than that of L-838,417 and no agonistic activity at α1-$GABA_AR$s, possess antihyperalgesic or analgesic activity in human volunteers or pain patients.

7. OPEN QUESTIONS

7.1. Which $GABA_AR$ subtypes should be targeted for optimal analgesia with minimal side-effects?

Work in the $GABA_AR$ H/R point-mutated mice suggests that α2-, α3-, and α5-$GABA_AR$s contribute to spinal benzodiazepine antihyperalgesia. It is however not clear whether only one subtype (i.e., α2-$GABA_AR$s) should be targeted for optimal antihyperalgesia or whether simultaneous activity at more than one subunit would be advantageous. In the absence of fully selective subtype-specific drugs, investigations on mice carrying more than one point-mutated $GABA_AR$ subtype should be informative. When taking undesired effects into consideration, one would probably try to avoid

positive allosteric modulation of α5-$GABA_A$Rs as this receptor subtype might confer cognitive impairment. Another reason to avoid activity at α5-$GABA_A$Rs comes from a recent report suggesting that inverse agonistic activity at α5-$GABA_A$Rs could be analgesic (Munro et al., 2011). So far, one would not expect undesired effects from activity at α3-$GABA_A$Rs, however, it is at present unknown if development of tolerance against the α2-mediated antihyperalgesic activity can be avoided by sparing activity at other $GABA_A$R subtypes. This is again a question, which is potentially accessible to studies examining mutant mice carrying combinations of H/R point-mutated $GABA_A$R subunits.

The genetic approaches described in this review are very well suited for experiments addressing the function of precisely defined $GABA_A$R subtypes. It is however not clear whether structural differences in the benzodiazepine binding site of different $GABA_A$R subtypes are large enough to permit development of subtype-selective benzodiazepines for each subtype or subtype combination. In particular differences between α2 and α3 subunits may be too small to permit full subtype specificity. Searching for modulatory sites at $GABA_A$Rs different from the classical benzodiazepine binding site might offer an alternative very intriguing, yet so far largely unexplored, opportunity.

7.2. Mixed $GABA_A$Rs with more than one type of α subunit

One potential limitation of the H/R point mutation approach is the behavior of $GABA_A$Rs with more than one type of α subunit. More than 25% of α1 containing $GABA_A$Rs harbor a second α subunit different from α1 (mainly α2 or α3), and the majority of α2 and α3 $GABA_A$Rs are mixed receptors (Benke et al., 2004). Because only one of the two α subunits can interact with the γ subunit to form the high-affinity benzodiazepine binding site, the responses of these mixed receptors to fully subtype-selective compounds will be determined by the type of α subunit which associates with the γ subunit (Minier & Sigel, 2004). This association may occur randomly but biochemical data suggest a certain rank order of the α subunits for interaction with the γ2 subunit (Balic, Rudolph, Fritschy, Möhler, & Benke, 2009). Moreover, biochemical experiments suggest that the H/R point mutation in the α subunit not only abolishes modulation by diazepam but also impairs the interaction with the γ subunit. This may eventually lead to false negative results in experiments with H/R point-mutated mice and may contribute to some of the discrepancies between predictions made from

the H/R point-mutated mice and results obtained with subtype-selective agents (Sigel & Steinmann, 2012; Skolnick, 2012). Experiments comparing the phenotypes of single point-mutated mice and of triple point-mutated mice in which only a single subtype remains benzodiazepine-sensitive may provide additional insights also here.

8. CONCLUSION

There is compelling evidence from preclinical studies in rodents to support that non-sedative benzodiazepines with improved subtype specificity exert antihyperalgesic effects. Available clinical data are consistent with this view. Current knowledge suggests that robust antihyperalgesic activity with low sedative properties requires a high intrinsic activity at α2-$GABA_A$Rs (or possibly also at α3-/α5-$GABA_A$Rs) and very low activity at α1-$GABA_A$Rs. The optimal profile of such a drug in terms of $GABA_A$R subtype specificity is however still not known. On-going preclinical studies and clinical trials will hopefully soon provide additional insights.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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