SEROTONERGIC METAMODULATION OF mGLu2/3 RECEPTORS, A NEW MECHANISM FOR PAIN MANAGEMENT: THE CASE OF TRAZODONE.

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Summary

Trazodone (TZD) is commonly used for depression and insomnia and the likely effect accounting for its antidepressant activity is its antagonistic action at serotonergic receptors, particularly the 5-HT$_{2A}$ receptors. However, emerging evidence supports the application of TZD for the management of chronic pain. Several cellular events are potentially involved in its antalgic activity, and, among them, there is the metamodulation linking 5-HT$_{2A}$ and mGlu2/3 receptors in spinal cord glutamatergic nerve endings. The term “metamodulation” refers to the integration of the neuronal signal involving colocalized receptors functionally and even physically associated. These receptors can influence each other, finely tuning the efficiency of synaptic transmission.

The aim of this review is to analyze the data from both in vitro and in vivo preclinical studies proving the existence and the functional interaction linking 5-HT$_{2A}$ and mGlu2/3 receptors. The review then focuses on the maladaptation of this metamodulatory interaction in the spinal cord of animals suffering from sciatic ligation and finally describes the impact of TZD on the 5-HT$_{2A}$-mGlu2/3 metamodulation in both physiological and pathological conditions.

In this context, new unpublished results from in vivo preclinical studies in a chronic constriction injury rat model of neuropathic pain are provided which confirm the analgesic effect of TZD on mechanical hyperalgesia and spontaneous pain in these rats.

In conclusion, in a whole the data unveil a cellular mechanism that could subserve the analgesic activity of TZD and support its use in clinic for pain management.
Key words
Neuropathic pain; 5-HT$_{2A}$ receptor; mGlu2/3 receptor; metamodulation; trazodone

Impact statement
The 5-HT$_{2A}$ antagonist trazodone is a potent metamodulator of the spinal mGlu2/3 autoreceptors and recovers their efficiency in controlling glutamate exocytosis, supporting its use for the management of pain.

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**Introduction**

Since its introduction in therapy in the early 1970s, trazodone (TZD) hydrochloride has been used for the treatment of depression and often administered in association with other antidepressants to patients with insomnia. The drug is well tolerated, with a favorable pharmacokinetic profile and convenient pharmacodynamic characteristics [1-6]. It is commonly referred to as a serotonin (5-HT) receptor antagonist and serotonin reuptake inhibitor (SARI) [7], although its potency to block the 5-HT reuptake is lower than that of other specific serotonin reuptake inhibitors (SSRIs), such as citalopram and fluoxetine [8]. The most likely effect accounting for its antidepressant activity is the antagonistic action at serotonergic receptors, particularly the 5-HT₂A and the 5-HT₂C receptors [1,6,9]. The simultaneous inhibition of serotonin transporters (SERTs) and of the 5-HT₂A/5-HT₂C receptors leads to a synergistic action which potentiates the antidepressant activity and improves the tolerability, reducing some of the side effects exerted by classic antidepressants, including insomnia and anxiety [2,6]. TZD also antagonizes the 5-HT₂A receptors, although less efficiently than the 5-HT₂A ones and has a partial agonistic activity towards the 5-HT₁A receptors [1]. Furthermore, it interacts with noradrenergic α₁ receptors, but has minimal antagonistic activity on cholinergic and histaminergic H₁ receptors [6].

Despite the use in practice of TZD preferentially relates to the cure of major depressive disorder, evidence is emerging supporting its application for the management of other pathological conditions, including chronic pain [10]. The present review reports recent literature and unpublished data concerning the role of TZD as a “metamodulator” of the mGlu2/3 autoreceptors, which would support its application for the treatment of neuropathic pain.
The 5-HT$_{2A}$ receptors, which represent a preferential target of TZD [11], are G protein-coupled receptors (GPCRs) which bind Gq/Gi proteins to control phospholipase C (PLC)-protein kinase C (PKC)-dependent intraterminal pathway(s), that ultimately mobilize(s) calcium ions from the endoplasmic reticulum, then promoting cell functions. 5-HT$_{2A}$ receptors have a widespread distribution in the central nervous system (CNS), where they locate both presynaptically and postsynaptically to control the strength of synaptic connections, particularly at excitatory synapses [12-15]. In 1997, Aghajanian and Marek provided evidence describing the existence of 5-HT$_{2A}$ receptors having a preferential postsynaptic distribution, whose activation significantly increases the frequencies and the amplitude of the postsynaptic excitatory spontaneous currents at pyramidal cells of the V cortical layer [13]. Postsynaptic 5-HT$_{2A}$ receptors were also proposed to exist in GABAergic neurons in the spinal cord [14,16-17] and to exert at this level an indirect tonic inhibition of glutamatergic innervation useful for analgesic activity (as discussed below, see paragraph 7 [14,18]).

A decade later, functional evidence also unveiled the presynaptic distribution of the 5-HT$_{2A}$ receptors in cerebellar [19], cortical [12] and spinal cord glutamatergic terminals [15]. These presynaptic release-regulating 5-HT$_{2A}$ heteroreceptors shared a common pharmacological profile and were typified by a huge affinity for TZD that behaved in all regions as a receptor antagonist. Differently from the 5-HT$_{2A}$ receptors located postsynaptically, the presynaptic 5-HT$_{2A}$ receptors preferentially inhibited, instead of potentiating, glutamate exocytosis. A negative control of PLC/PKC intraterminal pathways reducing in this case the gating of Voltage-Operated Calcium Channels in plasma membranes was proposed to subserve the 5-HT$_{2A}$ receptor-mediated inhibitory effect [12].

The term “metamodulation” refers to a particular mechanism of integration of the neuronal signal which dramatically enhances the complexity of the synaptic network. It involves colocalized...
receptors functionally and even physically associated in plasma membranes that, beside their ability
to control directly the neuronal activity, participate to an intramembrane integrated interaction which
assures a receptor-receptor cross-talk whereby a neuromodulator acting at one receptor indirectly
affects the activity of a second neuromodulator acting at the colocalized receptor [20-22].

Central 5-HT$_{2A}$ receptors recently emerged to the interest of pharmacologists for their ability to
couple the group II metabotropic glutamate receptors subtype 2 and 3 (mGlu2/3; [23-30]; figure 1).
This functional cross-talk was proposed on the basis of in vivo observations [31-36] and then
definitively proven by in vitro results confirming their physical and functional association in the
cortex of mammals [24-30,32,37-41]. The mGlu2/3 receptors, particularly the mGlu2 receptor
proteins, were reported to physically connect the 5-HT$_{2A}$ receptor proteins by means of specific
aminoacid(s) within the intraterminal COOH tail [43]. This structural interaction is allosteric in
nature, but, differently from most of the receptor-receptor cross-talk described in the literature (A2-
D2 receptors [42,43]; sst5-NMDA receptors [22,44]; nicotine and NMDA receptors [45-51]) is an
antagonist-like cross-talk, since the activation of the mGlu2/3 receptors reduces the functional
responses elicited by the colocalized 5-HT$_{2A}$ receptors [25,28,40].

As far as the cortex is concerned, the 5-HT$_{2A}$-mGlu2/3 receptor-receptor cross-talk was preferentially
investigated unidirectionally, by analyzing the impact of the mGlu2/3 ligands on the 5-HT$_{2A}$ receptor-
evoked responses [36,52-55], giving scarce attention to the reciprocal relationship, i.e. whether and
how 5-HT$_{2A}$ receptors ligands influence the mGlu2/3 receptor-mediated signals [25,30,41].

The mGlu2/3 receptors have a wide-spread distribution in the CNS, and exist presynaptically on
glutamatergic nerve endings, in different regions of the mammal CNS, including the spinal cord,
where they inhibit glutamate release [15,21,56-59]. Spinal cord glutamatergic nerve endings also
possess inhibitory presynaptic 5-HT$_{2A}$ heteroreceptors, whose activation efficiently hampers
glutamate release as well [15]. Biochemical and functional studies unveiled that also in spinal cord
glutamatergic isolated nerve endings (we refer to as synaptosomes) the mGlu2/3 and the 5-HT2A receptors colocalize and functionally couple to control glutamate exocytosis (Figure 2).

The colocalization of the two receptors emerged in confocal microscopy analysis, which showed the presence of both receptors in spinal cord glutamatergic synaptosomes. Then, immunoprecipitation studies demonstrated that the mGlu2/3 receptor protein immuno-precipitates from synaptosomal lysates were also immunopositive for the 5-HT2A receptor protein, consistent with the physical association of the two receptor proteins in nerve endings [15]. Finally, functional studies carried out with the “up-down superfusion technique” [60-62] demonstrated that, beside controlling directly glutamate exocytosis, the two receptors were functionally coupled to dynamically metamodulate their releasing activity at glutamatergic terminals [15].

Differently from the cortex, the study of mGlu2/3-5-HT2A receptor-receptor metamodulation in spinal cord glutamatergic nerve endings preferentially focused on the serotonergic tuning of the mGlu2/3 autoreceptors. It emerged that 5-HT2A antagonists, including TZD, behaved as an “indirect positive allosteric modulator” (IPAMs) of the presynaptic release-regulating mGlu2/3 autoreceptors, since blockade of the 5-HT2A receptors reinforced the mGlu2/3-mediated presynaptic inhibition of glutamate release and, conversely, activation of the 5-HT2A receptor nulled the mGlu2/3-mediated control of glutamate exocytosis [15]. In particular, low (1-3 pM) concentrations of the mGlu2/3 receptors agonist LY379268, unable to inhibit on their own the glutamate exocytosis, became active in synaptosomes that were concomitantly exposed to TZD or other 5-HT2A antagonists. An increased insertion of the mGlu2/3 receptor proteins into the synaptosomal membranes subserved the TZD-mediated reinforcement of the mGlu2/3 receptors-mediated effect, unveiling a quick (within minutes) trafficking of the mGlu2/3 receptor proteins from a cytosolic compartment to the synaptic membranes. This event is efficiently “metamodulated” by the colocalized 5-HT2A receptors that by dictating the membrane distribution of the glutamatergic autoreceptors tunes their desensitization state (Figure 3) [15].
5-HT₂A-mGlu2/3 metamodulation in the central nervous system: from physiology to pathology.

The metamodulation unveils a huge, unexpected complexity of the central neuronal communication, that increases even more when shifting from physiological to pathological conditions. In general, a receptor-receptor interaction is first analyzed in physiological conditions, to deepen the knowledge of the mechanisms by which one receptor influences the colocalized one [47, 51, 62, 63]. Afterwards, the study often turns to pathological conditions, to investigate whether and how the receptor-receptor cross-talk is modified during the course of disease or, conversely, if conditions impairing the metamodulation subserve the onset of a central disorder. In this case, the study can be further extended to verify whether receptor ligands can recover the metamodulation to physiological levels, paving the road for the use of these drugs for new therapeutic purposes [21, 45, 50, 51].

As far as the pathogenetic role of the mGlu2/3-5-HT₂A receptor-receptor interaction in the cortex is concerned, changes in the mGlu2/3 receptors mediated control of the 5-HT₂A receptors were associated to the development of psychosis, as well as to altered responsiveness of schizophrenic patients to antipsychotics [25, 36, 52, 53, 64]. These observations led to hypothesize that modulators of the mGlu2/3-5-HT₂A receptors interaction could provide new therapeutic approaches for the management of schizophrenia [40, 55, 65, 66]. According to the hypothesis, mGlu2/3 receptor agonists, or even better mGlu2/3 positive allosteric modulators (PAMs), were analyzed for the cure of psychiatric illness and still represent promising approaches, as recently reviewed by Ferdinando Nicoletti and colleagues [67], despite the heterogeneity of the results from preclinical studies and the negative results of some of the clinical studies carried out so far (reviewed by [68], but see also [69]).

Recently, we proposed that the mGlu2/3-5-HT₂A receptors functional crosstalk could also have a pathogenic role in spinal pathologies, particularly in nociception [70]. The hypothesis originated from the observations of functional adaptations of both the mGlu2/3 and the 5-HT₂A receptors in spinal cord glutamatergic nerve endings isolated from animal suffering from sciatic nerve ligation. In these
animals it emerged that i) the efficiency of glutamate exocytosis is impaired, being significantly increased when compared to healthy animals, ii) the expression of presynaptic release-regulating mGlu2/3 autoreceptors is significantly reduced at the acute stage of disease, while that of the 5-HT$_{2A}$ receptors is largely conserved and, last but not least, iii) the presynaptic releasing-regulating activities of both the mGlu2/3 and the 5-HT$_{2A}$ receptors are modified, the two receptors undergoing opposite functional adaptations (as described in paragraphs 5 and 6, see [70]). Before describing the results supporting our hypothesis, the role of glutamate and serotonin (and of the mGlu2/3 and the 5-HT$_{2A}$ receptors) in nociception and analgesia will be briefly resumed.

**Glutamate and mGlu2/3 receptors in neuropathic pain.**

Hyperalgesia and allodynia elicited by acute nerve injury, inflammation or thermal/mechanical overstimulation rely on a cascade of events that mainly involves the spinal glutamatergic innervation. A continuous self-amplifying raise of extracellular glutamate occurs in the spinal cord of animal suffering from neuropathic pain [71-73] that is sustained by concomitant maladaptive adaptations including: i) impaired glutamate uptake efficiency, ii) increased efficiency of glutamate exocytosis from nerve terminals (and possibly astrocytes); ii) loss of function of receptors, particularly of the mGlu2/3 autoreceptors, which affects glutamate release and ultimately favours the central neuronal sensitization.

The excitatory amino acid transporters type 1 and 2 (EAAT1 and EAAT2), which mainly account for the uptake of glutamate in astrocytes (EAAT1, GLAST) and nerve terminals (EAAT2, GLT1) [74], control pain perception, as suggested by the finding that the intrathecal administration of EAAT inhibitors causes nociceptive behaviour in animals [75]. In particular, the reduced expression of EAATs in the spinal cord of injured animals is an early mark of neuropathic pain [76,77], which becomes evident soon after (7 days) the peripheral nerve injury [12, 78-80], but rapidly recovers during pain sensitization [75]. A concomitant amplification of glutamate exocytosis [70,81] parallels
the EAAT1/2 malfunctioning and contributes to the altered glutamate homeostasis in the injured animals [73,82]. The scenario is even more worsened by the desensitization of the presynaptic inhibitory mGlu2/3 autoreceptors, which definitively supports the hyper-glutamatergicity [70,83-84].

The loss of functions of the mGlu2/3 autoreceptor strictly depends on the gravity and the timing of the injury, being detectable shortly after the lesion (at the seventh day after the injury), but largely recovering at the chronic stage of sensitization (about at the twentieth day after the injury, see [70,84-85]. The dynamic adaptability of the mGlu2/3 receptors suggested these proteins as suitable targets for therapeutic treatments to manage nociception [86-89] and the pharmacological approaches so far proposed include:

i) the administration of orthosteric and allosteric agonists which directly potentiate the mGlu2/3 receptor-mediated signaling (reviewed by [89]) and of enzyme inhibitors that indirectly modulate the receptors by controlling the production of endogenous agonists (i.e., the inhibitors of the glutamate carboxypeptidases II and III, which control the availability of N-acetyl-aspartyl-glutamate, the endogenous mGlu3 agonist, [90].

ii) the administration of modulators of the glutamate transporters to indirectly force the activation of the mGlu2/3 autoreceptors. It is the case of N-acetyl cysteine (NAc), which dictates the bioavailability of endogenous glutamate by tuning the glial glutamate-cystine membrane exchange. NAc reduces the transmission of responses to nociceptive reflexes in rodents and humans through a mechanism involving the mGlu2/3 receptors [91-92].

iii) the epigenetic control of the mGlu2 receptor expression with acetyl donors (i.e., L-acetylcarnitine, already marketed for the management of neuropathic pain) or histone deacetylase inhibitors [87].

Their use is limited by the onset of side-effects, i.e., the tolerance after repeated administration (in the case of the drugs for the former approach [89,91-92] or the lack of region-specificity leading to unwanted harmful reactions (for drugs belonging to the second and the third groups).
Serotonin and 5-HT2A receptors in neuropathic pain

The main role of 5-HT in controlling nociception was definitively highlighted by using the Lmx1b conditional knock-out mice (Lmx1bf/f/p), which lack central serotonergic neurons and show enhanced inflammatory pain, that largely recovers following the intracerebroventricular injection of 5-HT [93]. The analgesic effect of 5-HT is also observed in injured animals administered the neurotransmitter precursor 5-hydroxytryptophan, which reduces nociception (revised by [94]). The analgesic activity of the neurotransmitter was proposed to reside on its pro-regenerative activity, which would favour locally the synaptic plasticity and the regrowth of axons (as discussed by [94]), also exerting anti-inflammatory properties which reduce pain sensitization [93].

The therapeutic activity of 5-HT involves the 5-HT receptors, that represent therefore specific targets for the therapeutic management of neuropathic pain (see for an exhaustive review [95]). In particular, the role of 5-HT2A receptors in controlling spinal algia was highlighted in 2007 by Okamoto and colleagues [96] and ten years later by Lopez-Alvarez and colleagues [97]. These authors reported an altered expression of the 5-HT2A receptors in the spinal cord dorsal horn following sciatic nerve transection in rats during the first 2 weeks after injury. Other authors [98-99] provided evidence showing that the 5-HT2A subtype receptors are upregulated and tonically active in the dorsal horn of animals suffering from the spinal nerve ligation. Furthermore, an efficient reduction of pain perception and analgesia was observed following both systemic and local administration of 5-HT2A receptor antagonists (including ketanserin or M100907, revised by [95]), well consistent with the efficacy of these drugs to reduce the feed-back mechanisms of auto-control of 5-HT release in nerve endings.

To investigate the molecular mechanism(s) accounting for the analgesic properties of the 5-HT2A antagonists, Pichon and colleagues in 2010 focussed on the postsynaptic 5-HT2A receptors located on spinal GABAergic interneurons [14]. The authors demonstrated that the expression of these receptors
is not modified in the spinal cord of diabetic rats suffering from neuropathic pain, but rather that they functionally desensitize, losing their analgesic properties. The mechanism accounting for the receptor desensitization was proposed to involve the postsynaptic density protein-95 (PSD-95), which is a marker of the postsynaptic component of synapsis, that is upregulated in the spinal cord of diabetic rats suffering from neuropathic pain. PSD95 contains PDZ motifs that permit the physical and functional interaction with colocalized proteins, including selected intraterminal sequences of the 5-HT_{2A} receptors. By using a peptide that disrupt the interaction between the PDZ motif of PSD95 and the 5-HT_{2A} receptor C-terminus, the authors demonstrated that PSD95 silences the serotonergic counterpart, then impeding its activity as modulator of GABA innervation indirectly affecting pain perception [14,17,18].

More recently, we focussed on the 5-HT_{2A} receptors located presynaptically in spinal cord glutamatergic nerve endings and found that also their expression is largely conserved in injured animals [70]. Differently from the postsynaptic ones, however, these receptors did not desensitize but rather became more active in inhibiting glutamate exocytosis (as a matter of fact, concentration of 5-HT_{2A} agonist one-fold lower than that activating the receptors in physiological condition was required to significantly reduce the glutamate exocytosis in injured rats), while the colocalized mGlu2/3 autoreceptors lost their inhibitory activity and their density decreased. In a whole, it emerged that the pathological conditions associated to neuropathic pain impair the presynaptic 5-HT_{2A}-mGlu2/3 metamodulation in these terminals, the serotonergic tuning prevailing on the glutamatergic one.

Because of the dynamic nature of the metamodulation, we hypothesized that the \textit{in vivo} administration of 5-HT_{2A} antagonists, i.e., TZD, would reprogram the 5-HT_{2A}-mGlu2/3 cross-talk, re-equilibrating it to physiological levels. The hypothesis was verified in \textit{in vivo} and in \textit{ex-vivo, in vitro} studies and the results (which are discussed below on paragraph 7) provided the rationale to the use of TZD for analgesic purposes in individuals suffering from neuropathic pain.
Trazodone for the cure of neuropathic pain: preclinical and clinical evidence.

The data in the literature concerning the analgesic activity of TZD have been recently reviewed by Belinskaja and colleagues in 2019 [100]. The results from *in vivo* preclinical studies indicated that TZD reduced the responsiveness of mice in the hot plate test [101], decreased the number of writhes in the mice writhing test [102] and slowed the responsiveness in the formalin test in mice [103]. Inasmuch, TZD tested in the chronic constriction injury (CCI) rat model of neuropathic pain [104,105] showed a significant anti-hyperalgesic effect [106]. Furthermore, TZD was proven to exert a significant and dose-dependent (0.3-3 mg/kg) analgesic effect on mechanical hyperalgesia and spontaneous pain in CCI rats [102].

More recently, Dr. Garrone and co-workers also investigated the impact of TZD on pain threshold and weight bearing deficits in rats suffering from sciatic nerve ligation. In these set of experiments the chronic constriction injury, the evaluation of mechanical hyperalgesia and weight bearing, and the *in vivo* pharmacological treatments were performed according to [102]. These experiments were carried out in accordance with the European Communities Council Directive guidelines (Directive 2010/63/EU of 22 September 2010) and approved by the National Council on Animal Care of the Italian Ministry of Health (Authorization n. 59/2013-B). All efforts were made to minimize animal suffering and to use the minimal number of animals required to produce reliable results.

Briefly, injured rats acutely administered with TZD (by gavage, dose as indicated in the x axis, Figures 4 and 5) at the seventh day after the injury showed a significant increase of the pain threshold (here used as a measure of analgesia) and a reduction of the weight bearing deficit (a condition that reflects aspects of spontaneous pain).

Interestingly, the TZD-induced beneficial effects that emerged in *in vivo* experiments in injured rats administered with the lowest dose of TZD (0.3 mg/Kg) were paralleled by a significant amelioration of the *ex vivo, in vitro* synaptic defects in spinal cord glutamatergic nerve endings (previously described in paragraph 4). In particular, in the TZD-treated injured rats i) the glutamate exocytosis
recovered to levels consistent with those observed in sham operated rats; ii) the density of mGlu2/3 receptor proteins increased significantly and iii) the efficiency of the mGlu2/3 autoreceptors was largely restored [70]. These data confirm the hypothesis that the antagonism at the serotonergic receptors could re-balance the mGlu2/3-5-HT2A receptors metamodulation to an almost physiological level, recovering the presynaptic efficiency of the mGlu2/3 autoreceptors and favouring analgesia [70].

To confirm whether the mGlu2/3 receptors could have had a role in the TZD analgesic effect in CCI rats, in vivo experiments were carried out to verify whether LY341495, which is a potent and systemically active mGlu2/3 receptor antagonist, could prevent the analgesic activity of TZD. To this purpose, TZD (10 mg/kg) was given at 7 days following surgery, alone or in combination with LY341495 (intraperitoneally given at 1 mg/kg, 30 minutes before TZD). Mechanical hyperalgesia was measured 1 h post-treatment as the hind paw withdrawal threshold to a noxious mechanical stimulus and was assessed using the paw pressure technique. Thereafter, the reduction in static weight bearing was also measured using an incapacitance tester, a device that measures the weight distributed to each hind paw individually. In these experiments, TZD confirmed its anti-hyperalgesic effect that however was prevented by LY341495 pre-treatment as indicated by the levels of mechanical hyperalgesia (Figure 6) and weight bearing deficit (Figure 7), definitively proving the main role of mGlu2/3 receptors in the TZD-induced analgesic activity.

TZD has been already used in human for neuropathic pain. Low-doses of TZD are recommended as an effective treatment option for painful diabetic neuropathy based on data showing that patients receiving this drug experience a complete pain relief with relatively minor side effects [107-108]. In addition, in patients with fibromyalgia, TZD did not markedly improve pain intensity, but had a significant effect on pain interference with daily activities [109,110].

Very recently Lipone and colleagues [111] carried out a phase II clinical study to collect data on the efficacy and the safety of low doses of TZD in patients affected by painful diabetic neuropathy. The
patients were allocated to groups receiving different doses of TZD alone (30 mg/day or 60 mg/day) together with gabapentin as background therapy. The primary endpoint of the study was to verify changes from baseline by using the Brief Pain Inventory Short Form item 5 after 8 weeks of TZD administration. Secondary endpoints included the responses to other Brief Pain Inventory Short Form items together with the assessment of anxiety, sleep, quality of life, patient’s improvement, and safety of the treatment. Although the study failed to fulfill the first endpoint, the results unveiled a better performance for the patients receiving the TZD (30 mg/day), with a favorable safety profile, confirming the efficacy of low doses of TZD for treating painful diabetic neuropathy.

**Conclusion**

The concept of metamodulation is innovative because it hugely amplifies the complexity of the synaptic and non-synaptic transmission in the CNS, but also because it paves the road to new unexpected approaches for pharmacological interventions to control the functions of receptors for which safe orthosteric/allosteric ligands are lacking or, if available, cannot be used in therapy due to the concomitant onset of unwanted side effects, tolerance or desensitization that limits their manageability [50].

Metamodulation also poses the basis for the repurposing of drugs for innovative therapeutic approaches as well as for potential clinical conditions that however lack the molecular/cellular rationale to support their use in clinic. It might be the case of TZD, that was reported to ameliorate, or even relieve, peripheral neuropathic pain in patients suffering from diabetes 1 and 2, but whose mechanism(s) of action still represents matter of debate. We have here revised preclinical findings that suggest the 5-HT$_{2A}$ receptor-mediated metamodulation of the mGlu2/3 autoreceptors in glutamatergic nerve endings of the spinal cord of mammals as a cellular mechanism underlying the analgesic activity of this drug. By this point of view, TZD might provide a manageable and safe
alternative to the use of mGlu2/3 ligands to control the activity of the mGlu2/3 autoreceptors in pathological conditions associated to spinal pain sensitization.

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Conflict of interests
BG, CM, FPDG are employees of Angelini Pharma S.p.A.. GO, AR, GV, AP declare that they have no conflict of interests.
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Figure legends

**Figure 1.** Glutamatergic mGlu2/3 receptors and serotonergic 5-HT2A receptors colocalize and associate in a heterodimeric assembly, metamodulating in an antagonist manner their functions.

**Figure 2.** The glutamatergic nerve endings isolated from the spinal cord of adult rats are endowed with both 5-HT2A and mGlu2/3 receptors that assembly in a receptor-receptor heterocomplex to metamodulate glutamate exocytosis.

**Figure 3.** Trazodone antagonizes the presynaptic 5-HT2A heteroreceptors in spinal cord glutamatergic nerve endings, favoring the insertion of mGlu2/3 receptor proteins in plasma membranes and reinforcing the inhibitory feed-back mechanism of auto-control of glutamate exocytosis. This would limit glutamate exocytosis interfering with the mechanisms of sensitization that support hyperalgesia and allodynia.

**Figure 4.** Effect of trazodone oral administration on mechanical hyperalgesia 7 days following rat sciatic nerve ligation. The results are expressed as pain threshold (in grams) recorded 1 h after treatment administration. ###P<0.001 vs naïve group; **P<0.01, ***P<0.001 vs vehicle CCI group. n=6 animals/group.

**Figure 5.** Effect of trazodone oral administration on weight bearing deficit 7 days following rat sciatic nerve ligation. The results are expressed as the difference between the contralateral and ipsilateral paws (in grams) recorded 1 h after treatment administration. #P<0.05 vs naïve group; *P<0.05, ***P<0.001 vs vehicle CCI group. n=5-6 animals/group.
Figure 6. Effect of LY341495 pre-treatment on trazodone oral administration on mechanical hyperalgesia 7 days following rat sciatic nerve ligation. The results are expressed as pain threshold (in grams) recorded 1 h after treatment administration. #P<0.05, ###P<0.001 vs naïve group; ***P<0.001 vs vehicle CCI group; §§P<0.01 vs trazodone CCI group. n=4-6 animals/group.

Figure 7. Effect of LY341495 pre-treatment on trazodone oral administration on weight bearing deficit 7 days following rat sciatic nerve ligation. The results are expressed as the difference between the contralateral and ipsilateral paws (in grams) recorded 1 h after treatment administration. ###P<0.01 vs naïve group; **P<0.01 vs vehicle CCI group. n=6-8 animals/group.
Functional antagonism

Glutamate

Serotonin

mGlu2/3 receptor

5-HT$_{2A}$ receptor

Spinal cord presynaptic terminal

Modulation of glutamate release

Functional antagonism