Glycine Transport Inhibitors for the Treatment of Pain

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Key Words

Glycine transporter 2, Neuropathic Pain, Glycine Transport Inhibitors

Highlights

1. Many patients suffering from chronic pain do not find relief from current analgesics.
2. Glycine neurotransmission in the spinal cord is altered in chronic pain states.
3. Glycine transport inhibitors can restore glycine neurotransmission and are analgesic
4. This review discusses progress in the development of glycine transporter 2 inhibitors

Abstract

Opioids, local anaesthetics, anticonvulsant drugs, antidepressants and non-steroidal anti-inflammatory drugs (NSAIDs) are used to provide pain relief, but they do not provide adequate pain relief in a large proportion of chronic pain patients and are often associated with unacceptable side effects. Inhibitory glycinergic neurotransmission is impaired in chronic pain states, which provides a novel target for drug development. Inhibitors of the glycine transporter 2 (GlyT2) enhance inhibitory neurotransmission and show particular promise for the treatment of neuropathic pain. N-Arachidonyl-glycine is an endogenous lipid that inhibits glycine transport by GlyT2 and also shows potential as an analgesic, which may be further exploited in drug development. In this review we will discuss the role of glycine neurotransmission in chronic pain and the future prospects for the use of glycine transport inhibitors in the treatment of pain.

Glycine Neurotransmission and Chronic Pain

A normal response to peripheral tissue injury or inflammation involves activation of nociceptive (pain) neurons that produce pain. Acute, reversible, adaptive changes in the sensory nervous system also lead to sensory hypersensitivity that serves a protective function while the damaged tissue is healing. However, nerve injury in the peripheral or central nervous system (CNS) can lead to chronic pain that persists well beyond the period of tissue damage, lasting for more than 3-6 months and is characterized by spontaneous pain, hyperalgesia (an exaggerated response to noxious stimuli), and allodynia in thermal and mechanical modalities (the perception of normally innocuous stimuli such as mild cooling or light touch as painful) [1]. Although currently available analgesics effectively treat many acute nociceptive and inflammatory pain conditions, chronic pain symptoms are very

difficult to manage. A large body of evidence in animal models and humans suggest that chronic pain states involve persistent pathological adaptations in excitatory neurotransmission, neuron-glial interactions and inhibitory neurotransmission (reviewed by [1]). Drugs that can enhance inhibitory neurotransmission, such as inhibitors of the glycine transporters (GlyTs), reverse signs of chronic pain in animal models, strongly suggesting potential efficacy for managing chronic pain.

Glycine is an inhibitory neurotransmitter in the spinal cord and brain stem where it binds and activates glycine receptors (GlyRs) to cause hyperpolarization [2, 3]. Glycine is also an excitatory neurotransmitter throughout the CNS where it acts as a co-agonist with glutamate at the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors to cause depolarization [4]. There are two types of GlyTs which regulate synaptic glycine concentrations to control GlyR and NMDAR activity [5-7]. The GlyT1 subtype of transporters is expressed by astroctyes at both inhibitory and excitatory synapses as well as a subset of glutamatergic neurons, whereas the expression of GlyT2 is predominantly expressed in presynaptic terminals of inhibitory glycinergic neurons [6-8] (Figure 1).

Inhibitory glycinergic neurons and receptors are found throughout the CNS, but are most abundant in the dorsal horn of the spinal cord, particularly in lamina III [9-11]. GlyT1, GlyT2 and GlyRs are also abundant in the dorsal horn, with GlyT2 most strongly enriched in lamina III [6, 12, 13]. These glycinergic neurons contribute to inhibition of nociceptive signalling and have important roles in segregating nociceptive and non-noxious information pathways [1, 11] (see Figure 2). Dysfunctions of glycinergic systems, together with GABAergic systems [14-19], contribute to neuropathic and inflammatory pain.

Inhibition of glycine receptors in the spinal cord with strychnine has long been established to induce mechanical hyperalgesia and allodynia at lower doses than those that influence thermal pain [19]. Conversely, enhancing glycinergic neurotransmission in the spinal cord with GlyT1 or GlyT2 inhibitors reduces mechanical pain (see below for more details). As outlined in Figure 2, lamina III glycinergic neurons prevent non-nociceptive, mechanical information from reaching pain projection neurons in lamina I of the dorsal horn [14, 18]. Impairment of this inhibitory mechanism has been directly demonstrated in neuropathic pain models [14], which is likely to contribute to mechanical hyperalgesia and allodynia, and perhaps spontaneous pain states [14, 18]. The ability of GlyT2 inhibitors to reverse mechanical allodynia is likely to be due, at least in part, to restoration of these inhibitory mechanisms.

The second major source of glycinergic innervation of the dorsal spinal cord arises from supraspinal areas. Stimulation of these descending inhibitory networks inhibits nociception [20]. Anatomical tracing methods combined with immunohistochemistry have established that a large proportion of neurons in the rostral ventromedial medulla (RVM) that project to lamina I-IIo and IV-V of the spinal cord are GABAergic/glycinergic [21, 22]. Kato *et al.* [23] used *in vivo* patch recording to demonstrate that partially overlapping, monosynaptic GABAergic and glycinergic pathways from RVM to lamina II inhibit mechanical nociceptive responses. There is strong evidence that these descending inhibitory mechanisms are suppressed in chronic pain states, which may contribute to various symptoms of chronic pain [1, 20]. GlyT2 inhibitors could relieve many aspects of chronic pain by elevating synaptic glycine concentrations and thereby restoring glycinergic descending inhibition.

Compromised glycinergic neurotransmission has been reported in specific regions of the dorsal horn in both inflammatory and neuropathic pain. In an inflammatory pain model, prostaglandin type E_2 (PGE_2) selectively blocks glycinergic transmission through activation of Protein Kinase A [24]. Inhibition of the α3β subtypes of GlyRs by PGE_2 has also been shown to occur in inflammatory pain states and this modulation is likely to contribute to mechanical hypersensitivity [25]. These processes contribute to pain pathology by increasing excitability in nociceptive pathways that project to the brain. In trigeminal mechanical allodynia induced by strychnine microinfusion, glycine signalling is decreased through a local PKCγ-mediated excitatory NMDA circuit [26]. This was further studied in lumbar dorsal horn neurons in a neuropathic pain model [14], which revealed suppression of a feed-forward glycinergic circuit that gates mechanical allodynia (Figure 2). Additionally, in neuropathic pain, a shift in the anion gradient develops exclusively in dorsal horn lamina I pain transmission neurons [27]. This is due to a reduction in the activity of the potassium chloride co-transporter (KCC2) [27] that leads to an increase in intracellular chloride, so activation of GABA or glycine receptors causes depolarization and a pronociceptive excitation of these pain transmission neurons. However, GlyT inhibitors would not be expected to strongly exacerbate this pronociceptive adaptation because inhibitory neurotransmission in lamina I of the dorsal horn is predominantly GABAergic [28] and GlyT2 expression is relatively sparse in this region [9-11].

These studies provide compelling evidence that reduced glycinergic signalling produces mechanical hyperalgesia and allodynia, and failure of glycinergic signalling in the dorsal horn develops in chronic neuropathic and inflammatory pain states. Increasing glycinergic

transmission is therefore likely to be a good therapeutic strategy for treating pain. One way of increasing available glycine is to inhibit glycine transporters. This increases glycine concentrations in the synaptic cleft and enhances neurotransmission through GlyR in the spinal cord, reducing pain signals.

The Roles of GlyTs in Regulating Glycinergic Neurotransmission

The two glycine transporters, GlyT1 and GlyT2, differ in their functional properties, which have a direct bearing on their concentrating capacities and their influence on glycine neurotransmission. Glycine transport by GlyT1 is coupled to the co-transport of 2 Na^+ ions and 1 Cl^- ion, whereas glycine transport by GlyT2 is coupled to the co-transport of 3 Na^+ ions and 1 Cl^- ion [29, 30] (see Box 1 for more details). The extra Na^+ ion coupled to transport by GlyT2 creates a more powerful transporter and allows it to operate over a wider glycine concentration range than GlyT1, which serves two purposes at inhibitory synapses. First, the transporter is able to reduce the synaptic glycine concentration to the low nM range, under equilibrium conditions, to prevent low level GlyR activity, and second, the extra concentrating capacity allows the transporter to maintain the intracellular glycine concentration at approximately 10-20 mM, which is necessary to provide sufficient glycine for transport into synaptic vesicles via the vesicular inhibitory amino acid transporter (VIAAT) [30] (Box 1). VIAAT is non-selective for glycine over GABA and it will accumulate either neurotransmitter which is partly dependent on the neurotransmitter concentration in the presynaptic terminal [31]. Thus, for a glycinergic neuron it is important for the intracellular glycine concentration to be maintained at levels that allow glycine loading of vesicles [31]. In interneurones of the spinal cord, both glycine and GABA can be present in the same synaptic vesicles, which is presumably due to the presence of both GlyT2 and a

GABA transporter capable of maintaining the intracellular GABA concentration at similar levels as glycine [31, 32]. However, segregation of GABAergic and glycinergic neurotransmission in dorsal spinal cord is extensive [11, 19, 28], with GABAergic synapses dominating in laminae I and IIo, and glycinergic transmission dominating in laminae Iii and III. This selectivity is presumably generated by cell type selective expression of GlyT2, GABA transporters and subsynaptic receptor types [11]. In the spinal cord, GlyT1 is expressed by astrocytes and neurons [6-8] and serves different purposes at inhibitory and excitatory synapses (Figure 1). At inhibitory synapses, GlyT1, together with GlyT2, contributes to the clearance of glycine after presynaptic release to terminate glycine transmission. At excitatory synapses, glycine is not released from presynaptic vesicles, but rather glycine concentrations are maintained at a relatively steady state in the mid-high nM range [30]. The higher resting glycine concentration at excitatory synapses is due to the lower concentrating capacity of GlyT1 compared to GlyT2, and the transporter operates close to its equilibrium capacity. Mid-high nM glycine is sufficient for partial occupancy of the glycine binding site of NMDAR [30, 33], whilst keeping the intracellular glycine concentration at approximately 1-2 mM [30]. It has been suggested that small fluctuations in the driving forces for glycine transport, such as a reduction in the electrochemical gradient across the membrane that may occur with AMPA receptor activation, will be sufficient to allow reverse glycine transport leading to an elevation in synaptic glycine concentrations and stimulation of NMDAR activity [30, 34]. It is important to note that the stoichiometry of ion flux coupling determines the equilibrium glycine concentration gradient (see Box 1), whereas the rate at which equilibrium is attained is determined by the number of transporters and the kinetics of the transport mechanism. Therefore, if an inhibitor acts by reducing the number of available transporters, this will slow down the rate at which equilibrium can be attained, but

not the final gradient achieved. This is an important distinction for understanding the effects of GlyT inhibitors on the dynamics of neurotransmission and also the loading capacity of the transporters. For example, 50% inhibition of GlyT2 may slow the clearance of glycine from the synapse and prolong the time course of synaptic transmission but it should not influence the equilibrium or resting intracellular glycine concentration and loading of presynaptic vesicles.

GlyT knockouts and knockdowns reveal different roles for GlyT1 and GlyT2.

GlyT1 and GlyT2 knockouts have been generated and they show quite different phenotypes [5, 35, 36]. At birth, both knockout mice appear to be relatively normal, but analysis of inhibitory glycinergic neurotransmission shows distinct differences. In recordings of hypoglossal motor neurons of the brain stem of GlyT1 knockout mice, larger chloride conductances are observed that are consistent with elevated glycine levels leading to greater GlyR activity. This suggests that the remaining glycine transporter, GlyT2, cannot fully compensate for the lack of GlyT1 and is unable to clear sufficient glycine to regulate synaptic transmission. Thus, it may be concluded that GlyT1 plays an important role in regulating synaptic glycine concentrations. In contrast, the GlyT2 knockout mice show greatly diminished inhibitory glycine neurotransmission. The absence of GlyT2 prevents the accumulation of intracellular glycine and loading of synaptic vesicles and thus glycine cannot be released for neurotransmission. The lack of inhibitory glycinergic neurotransmission leads to motor disturbances and death shortly after birth. The phenotypes of these knockout mice initially deterred the development of GlyT2 selective inhibitors as analgesics because of the expectation of considerable side effects, such as loss of motor control. However, GlyT knockdowns, with incomplete knockdown of the transporter, show more

promising results and suggest that GlyT2 inhibitors have considerable potential as treatments of chronic pain. GlyT2 knockdowns, generated using siRNAs in a sciatic nerve injury model for neuropathic pain, show reduced allodynia with a time course that correlates with reductions in GlyT2 immunoreactivity, suggesting that reduced GlyT2 activity may alleviate pain [37, 38]. Furthermore, the GlyT2 knockdown mice show no adverse motor or respiratory effects, alleviating concerns that GlyT2 inhibitors may generate adverse side effects. Similarly, GlyT1 knockdown mice also show similar reductions in allodynia without any overt behavioural changes. Importantly, the partial reductions in GlyT1 and GlyT2 expression generated by the siRNAs (25% of normal) appears to be sufficient to slow the clearance of glycine from synapses and thereby enhance glycinergic inhibitory tone, but still allow sufficient re-uptake of glycine into presynaptic terminals for repackaging into synaptic vesicles to maintain glycine neurotransmission. Presumably, for the GlyT1 knockdowns, the remaining GlyT1 is also sufficient to allow adequate regulation of glycine concentrations at excitatory synapses. These GlyT knockdown studies provide a rational basis for the application of GlyT inhibitors for the treatment of pain.

GlyT2 Inhibitors

Two GlyT2 inhibitors, ALX1393 and ORG25543 (Figure 3), have been the most extensively studied and in the following section we will review research highlighting their effectiveness and also some limitations in their use. We will also discuss the actions of some lipid compounds that inhibit GlyT2 and show promise as analgesics for the treatment of chronic pain.

ALX1393

ALX1393 has been studied in animal models of pain using either intravenous or intrathecal injections [38-42]. Although intrathecal ALX1393 inhibits acute thermal and mechanical pain responses, it is considerably more potent against sensitized responses (tonic phase of the formalin test) [38, 40, 42]. A single intravenous injection of 0.01 mg/kg ALX1393 reduces mechanical allodynia over a 4 hour period in a neuropathic pain model. These effects remain for up to 24 hours and then return to pre-drug injection levels over the course of 4 days. Intrathecal injections of 10 ng of ALX1393 show similar reductions in mechanical allodynia caused by nerve ligation injury, the streptozotocin-induced diabetic pain model, and also the Complete Freud's Adjuvant model for inflammatory pain. These effects last for 48-72 hours and can be reversed by intrathecal strychnine injections to block glycine receptors [38]. Furthermore, no effects on locomotor activity, motor behaviour in the rotorod test, or the righting reflex were observed in these studies [38]. ALX1393 has also been studied in a mouse model of allodynia caused by herpetic and postherpetic pain induced by infection with herpes simplex virus [41]. Intrathecal ALX1393 injections alleviate allodynia within 60 minutes after injection, but return to baseline pain states after 24 hours.

In a more recent study, Mingorance Le-Meur *et al*. [40] found that only 5% of ALX1393 crossed the blood-brain barrier after 60 minutes when given intravenously, and furthermore, that ALX1393 was only 40-fold selective for GlyT2 over GlyT1. Although ALX1393 demonstrates clear analgesic effects and minimal side effects, this pharmacokinetic and selectivity profile may limit its usefulness as a therapy for chronic pain.

Chronic exposure of cultured glycinergic neurons to ALX1393 alters the ratio of glycinergic and GABAergic neurotransmission [42]. Presumably this arises due to complete and prolonged block of GlyT2 causing reduced cytoplasmic glycine concentrations and thereby allowing preferential loading of synaptic vesicles with GABA instead of glycine (see discussion above). This observation is in apparent contradiction to that of Morita and colleagues [38], whose results suggest that repeated injections of GlyT2 inhibitors enhance glycine neurotransmission rather than cause a switch to GABAergic neurotransmission. A possible explanation for the apparent discrepancy is that the intrathecal injections of GlyT2 inhibitors may not lead to complete inhibition of glycine transport compared to the cell culture experiments where the high concentrations of ALX1393 would completely block transport. Partial inhibition due to limited brain uptake may be sufficient to enhance glycine neurotransmission, but also allow for sufficient re-uptake into presynaptic boutons to provide glycine for transport into synaptic vesicles via the VIAAT.

ORG25543 and related compounds

In general, the analgesic actions of ORG25543 are similar to ALX1393 [38]. Intrathecal and intravenous injections of ORG25543 reduce allodynia associated with nerve ligation injury, streptozotocin-induced diabetic pain model, and CFA-induced inflammatory pain. The acute actions of high doses of ORG25543 in lamina X neurons of mouse spinal cord slices show clear changes in the dynamics of glycinergic neurotransmission [43]. Exposure of slices to 10 µM ORG25543 initially caused a reduction in baseline current and increase in current noise, due to elevation of glycine concentrations and stimulation of glycine receptors. Furthermore, ORG25543 caused a three-fold increase in the decay constant of miniature inhibitory postsynaptic currents (IPSCs) and electrically evoked IPSCs without affecting the

amplitude of the currents. These observations suggest that ORG25543 inhibits the GlyT2-mediated clearance of glycine from the synapse. However, after 10 minutes exposure to the high concentration of ORG25543, glycinergic IPSCs were greatly diminished [43]. These observations are consistent with the proposal that prolonged complete inhibition of GlyT2 will initially enhance glycine neurotransmission, but subsequently cause depletion of cytosolic glycine concentrations and prevent glycine loading of presynaptic vesicles. This phenotype correlates closely with the GlyT2 knockout mice [36]. ORG25543 differs from ALX1393 in its mechanism of inhibition of GlyT2. In contrast to ALX1393, ORG25543 is an irreversible inhibitor of GlyT2 [40] and it was hypothesized that this may make this inhibitor more prone to generating side effects with prolonged exposure. An irreversible GlyT2 inhibitor will have greater propensity to cause long term changes in cytosolic glycine concentrations and greater potential for motor side effects, which is analogous to the phenotype of GlyT2 knockouts. A modified version of ORG25543 has been developed by Mingorance Le-Meur *et al*. [40] (Figure 3), which has lower affinity for GlyT2 but is also reversible. This new compound shows similar blood-brain penetration as ORG25543 and analgesic properties, but fewer motor side effects at high transporter occupancy.

N-Arachidonyl Glycine – An endogenous analgesic?

N-Arachidonyl glycine (NAGly) is an endogenous fatty acid that is found in highest concentrations in the spinal cord and lower concentrations in many other organs [44, 45]. NAGly is structurally related to the endocannabinoid, anandamide (Figure 3), which prompted investigations into its *in vivo* effects and its potential for modulating neurotransmission.

In animal models of formalin-induced pain, co-injection of NAGly with formalin suppresses the tonic pain phase [44]. Furthermore, intrathecal injection of NAGly reduces both mechanical allodynia and thermal hyperalgesia in a model of inflammatory pain [46] and reduces mechanical allodynia in a nerve-injury induced animal model of neuropathic pain [47]. The synthetic cannabinoid receptor agonist, HU-210, also alleviates pain, but in contrast to NAGly, the actions of HU-210 are blocked by cannabinoid receptor antagonists [46, 47]. Thus, the analgesic actions of NAGly are unlikely to be mediated by cannabinoid receptors. The dose dependence of NAGly activity and the lack of effect by the degradation products, glycine and arachidonic acid, imply that the analgesic activity of NAGly is mediated by modulation of a specific protein.

Our group has tested the effects of NAGly on glycine and GABA transporters and found that NAGly selectively inhibits GlyT2 over GlyT1 and the GABA transporter, GAT1 [48]. Other reported actions of NAGly include: both stimulation and inhibition of glycine receptors [49]; inhibition of cyclooxygenase-2 [50]; inhibition of fatty acid amide hydrolase [44, 51]; and stimulation of the orphan G-protein coupled receptors GPR18, GPR92 [52, 53]. Most of these effects of NAGly are apparent in the low μM range. The effects of NAGly on glycinergic synaptic neurotransmission have also been investigated [47]. In lamina II neurons of the superficial dorsal horn, NAGly prolongs the time course of glycine-evoked currents. β-alanine is an alternate GlyR agonist, but is not a substrate of GlyTs and in contrast to glycine, the time course of β-alanine-evoked currents is insensitive to NAGly, which suggests that NAGly mediates its effects at glycinergic synapses by inhibiting GlyTs. In this study, synaptic currents were also investigated, and although miniature inhibitory post-synaptic currents (IPSCs) were insensitive to NAGly, responses to trains of electrical impulses, to generate

multi-vesicular release of glycine, were enhanced by NAGly. The actions of NAGly are mimicked by the synthetic GlyT2 selective inhibitor, ALX-1393 [47], but not by the GlyT1 selective inhibitor N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS or ALX5407) [47, 54, 55]. Thus, NAGly enhances inhibitory glycinergic synaptic transmission by inhibiting GlyT2.

Structure Activity Studies of NAGly and potential for further drug development

A limited structure-activity profile of lipid inhibitors of GlyT2 has been conducted, which demonstrates that both the lipid head group and the hydrocarbon tail influence potency, reversibility, and the degree of maximal inhibition [48, 56-58]. Oleoyl-L-carnitine (Figure 3) is the most potent of the lipid inhibitors identified, but it also shows irreversible inhibition. N-Oleoyl-glycine is a more potent inhibitor than NAGly and it is also reversible. Another important feature of these lipid inhibitors is the critical micelle concentration. Many lipids have the capacity to form micelles and once in this form it is difficult to estimate the free concentration of the lipid that may access an inhibitory lipid binding site [56]. This physiochemical feature may also influence the degree of occupancy and level of inhibition that may be achieved. It may provide a mechanism to ensure that such compounds can only attain partial inhibition of transport and thus prevent unwanted "on-target" side effects associated with complete and prolonged inhibition.

GlyT1 Inhibitors

The GlyT1 inhibitors, ORG25935 and ALX5407 (NFPS), and the GlyT1 substrate, sarcosine have also been tested in mouse models of neuropathic pain [38, 54, 55, 59]. Reduction in GlyT1 activity would be expected to increase the activity of both GlyRs and NMDARs and

thus influence both inhibitory and excitatory neurotransmission. Both ORG25935 and sarcosine provide anti-allodynia effects, but in contrast to the GlyT2 inhibitors, a lag time of up to 4 days is observed before before anti-allodynia effects are observed [38]. The NMDA receptor antagonist L-701,324 was able to abolish this lag time, suggesting that the lag effect was due to transient activation of NMDA receptors induced by elevated levels of glycine generated by GlyT1 inhibition. No such lags are apparent for the GlyT2 inhibitor ALX1393 ([38], see above). ALX5407 also generates anti-allodynia effects in neuropathic pain, but it shows an unusual biphasic dose response. Low and high doses of ALX5407 provide analgesia, but medium doses exacerbate nociception, which may be related to elevations of glycine in both inhibitory and excitatory synapses. Activation of NMDA receptors might be expected to worsen pain symptoms because they are involved in spinal sensitization mechanisms in chronic pain states [1] and for these reasons further development of GlyT1 inhibitors for the treatment of pain may be limited.

Concluding Remarks

The use of GlyT2 inhibitors for the treatment of pain shows considerable potential. There is evidence that they reduce acute mechanical and thermal pain in animal models of chronic pain, and their mechanisms of action in the spinal cord suggest they may be particularly useful for managing allodynia associated with neuropathic pain. However, there are concerns with their use which must be appreciated and overcome if these inhibitors are to realize their potential. The comparison between GlyT2 knockouts and GlyT2 knockdowns provides an excellent illustration of both the potential and the problems of GlyT2 inhibitors. GlyT2 knockouts are lethal in the first week after birth, with greatly diminished glycine neurotransmission, whereas GlyT2 knockdowns show no overt adverse effects and are less

sensitive to pain. The key to further development of this field will be to understand the conditions that can lead to partial inhibition of GlyT2 to elevate glycine concentrations but not compromise filling of synaptic vesicles with glycine. Studies of the synaptic physiology of glycinergic synapses under both normal and pain states and how GlyT2 inhibitors influence the dynamics of glycine neurotransmission will be required if the goal of reliable, partial inhibition is to become an effective treatment for pain.

There are no high resolution crystal structures of glycine transporters available, but the structures of a growing number of related transporters in the SLC6 family of transporters have been determined, including a dopamine transporter from *Drosophila melanogaster* (dDAT) [60], a bacterial leucine transporter (LeuT) [61] and a bacterial betaine transporter (BetP) [62]. The various crystal structures represent different functional states of the transporters, and from these, it has been possible to develop structural models for the various conformational changes required for the transport process and to identify potential drug binding sites (Figure 4). The transporters consist of 12 TM α-helical domains arranged in a "shallow shot glass" like structure with the substrate and ion binding sites located halfway across the membrane at the bottom of an extracellular-facing cavity. The protein oscillates such that the substrate and ion binding sites are exposed to either the extracellular surface or the intracellular surface as part of the alternating access mechanism for transport [63, 64]. The crystal structure of the dopamine transporter of *Drosophila melanogaster* has been determined with the tricyclic antidepressant, nortriptyline, bound. Nortriptyline binds to the primary substrate binding site preventing substrate binding and also prevents the external cavity from closing (Figure 4), which is necessary for the transport process. This inhibitor bound complex may provide clues for how ALX1393 or ORG25543

bind to GlyT2, which may be investigated by molecular modeling and site directed mutagenesis studies to establish GlyT2 inhibitor binding sites on GlyT2.

Preliminary structure activity profiles of NAGly and OLCarn inhibition of GlyT2 have been conduced [57, 58]. Using chimeric transporters derived from GlyT2 and the closely related but lipid-insensitive GlyT1 transporter, extracellular loop 4 (EL4) appears to play a key role in determining sensitivity toward both NAGly and OLCarn. EL4 moves into the extracellular vestibule during the transport process [65], and it is possible that NAGly and OLCarn influence the movement of EL4 [57, 58] and indirectly regulate the rate of glycine transport. Further structure activity studies will be required to elucidate the structural basis for how NAGly, OLCarn and other lipids bind and inhibit GlyT2. It is also interesting to note that a cholesterol molecule is bound to the dDAT on an external membrane-exposed surface formed by TM5, TM7 and TM1a [60], (Figure 4). It has been demonstrated that cholesterol can stabilize the outward conformation of human DAT and thereby increase cocaine binding capacity. It has been postulated that cholesterol may bind to this membrane exposed surface and inhibit the conformational transitions required for transport. Thus, the cholesterol binding site may also provide clues as to how lipid-based inhibitors, such as NAGly, may bind and inhibit GlyT2.

GlyT2 inhibitors show considerable promise for the treatment of neuropathic pain, but there these is scope for further refinement of compounds required to optimize activity that will enhance glycine neurotransmission without motor side effects that may result from complete and prolonged inhibition. There is also further work required to better understand

how synaptic neurotransmission in the dorsal horn is disturbed in neuropathic pain states and how GlyT2 inhibitors may help to restore normal signaling.

BOX 1

Stoichometry of Ion Flux Coupling of Glycine Transporters

For a secondary active electrogenic transporter, the direction of transport can be driven by both the electrical potential across the membrane and the gradients of the coupled ions. For a glycine transporter that is coupled to n Na+ ions and m Cl- ions the membrane potential at which the transport reverses direction (Erev) is given by:

$$Erev = \frac{2.3RT}{(n-m)F} \log([Na]_e^n [Cl]_e^m [Gly]_e) / ([Na]_i^n [Cl]_i^m [Gly]_i)$$

Where R, T and F have their usual meanings, $[Na]_e$ and $[Na]_i$ are the extracellular and intracellular Na^+ concentrations and similarly for Cl^- concentrations. For GlyT1, n=2 and m=1 and for GlyT2, n=3 and m=1. Insertion of these values and ion concentrations into the equations will give a ratio of extracellular/intracellular glycine concentrations supported by the transporter. If equilibrium is reached, the neuronal GlyT2 will be able to support a concentration gradient of 6 orders of magnitude, such that if the extracellular [gly] is 10-20 nM, the intracellular [gly] will be ~10-20 mM. For astrocytes expressing GlyT1, a concentration gradient of only 4 orders of magnitude can be maintained, such that if the extracellular [gly] is 100-200 nM, the intracellular [gly] will be 1-2 mM[29, 30].

GLOSSARY BOX

Allodynia: the perception of normally innocuous stimuli such as mild cooling or light touch as painful

GlyR: Inhibitory glycine receptor. Consist of 5 subunits usually with 3 α subunits and 2 β subunits. Three different α subtypes have been described.

GlyT1: glycine transporter subtype 1 that is predominantly expressed by astrocytes

GlyT2: glycine transporter subtype 2 that is predominantly expressed glycinergic neurons

Hyperalgesia: an exaggerated response to noxious stimuli

NAGly: N-arachidonyl-glycine is a glycine conjugate of arachidonic acid that is produced in highest concentrations in the spinal cord and has analgesic properties

Neuropathic pain: Pain that is caused by damage or diseases that affect the sensory system. It may have continuous and/or episodic components. Common qualities include burning or coldness, “pins and needles”

NMDAR: N-methyl D-aspartate subtype of ionotropic glutamate receptors

Nociception: stimulation of sensory neurons called nociceptors, or pain receptors

OLCarn: Oleoyl-L-Carnitine (an endogenous lipid molecule)

streptozotocin-induced diabetic pain: Streptozocin (STZ) is toxic to insulin producing β cells of the pancreas and produces animal models of diabetes and the associated pain.

Figure Legends

Figure 1: Schematic diagram of glycine neurotransmission at inhibitory and excitatory synapses. At inhibitory synapses, high cytosolic glycine concentrations are maintained by the presynaptic glycine transporter, GlyT2. Upon stimulation of an inhibitory glycinergic neuron, glycine is released into the synapse and will activate postsynaptic GlyR. Glycine is then cleared from the synapses by a combination of diffusion, and uptake by GlyT1 expressed on surrounding astrocytes and GlyT2 in the presynaptic terminal [6, 7]. At excitatory synapses, glycine is not released in the classical presynaptic mechanism, but rather glycine levels are maintained close to an equilibrium concentration that is set by the activity of the glycine transporter, GlyT1. GlyT1 is expressed by both astrocytes and in some glutamatergic neurones [6-8], and will influence NMDA receptor activity. Glycine may also diffuse from inhibitory glycinergic synapses.

Figure 2. Nociceptive and non-noxious pathways in the dorsal horn of the spinal cord.

In normal physiological states, excitatory Aβ (light touch) sensory inputs converge onto excitatory interneurons (green cells; E, some of which are PKCγ-positive) in lamina II of superficial dorsal horn, as well as glycinergic neurons in lamina III (blue cells; Gly) [1, 11, 14, 19]. Coincident activation of these glycinergic interneurons produces strong feed-forward inhibition of lamina II excitatory interneurons (green; E) that prevents non-noxious sensory information from invading pain transmission neurons in lamina I (red cell; NK1 receptor positive) [14, 19]. This feed-forward inhibition is suppressed following peripheral nerve injury, or pharmacological glycine receptor inhibition, allowing non-noxious information to invade lamina I neurons [14, 16-19]. Thus non-noxious light touch information is perceived

as painful (allodynia). Descending inputs from the brain also utilise GABA/glycine to produce ongoing modulation of nociception [20-22]. There is good evidence that descending inhibition is blunted in chronic pain states but the precise dysfunction of glycinergic projections is uncertain [18].

Figure 3: Chemical structures of GlyT2 inhibitors: ALX1393, ORG25543, Compound 1 from Mingorance Le-Meur et al (2013), NAGly and OLCarn.

Figure 4: Structure of the *Drosophila melanogaster* dopamine transporter, dDAT. dDAT (PDB 4M48) is shown in cartoon representation and viewed in the plane of the membrane. The anti-depressant Nortriptyline (cyan) and cholesterol (yellow) are shown in sphere representation. Nortriptyline is shown bound to the primary binding site while cholesterol is bound at the protein:lipid interface. Extracellular loop 4 (EL4) is shown in dark blue and lines the external vestibule which is indicated by a red circle. Bound Na1 (purple) and Cl (green) are also shown as spheres. TM6a has been removed for viewing the substrate binding site and Na2 is not visible in this orientation. Figure was made and rendered using PyMol [66].

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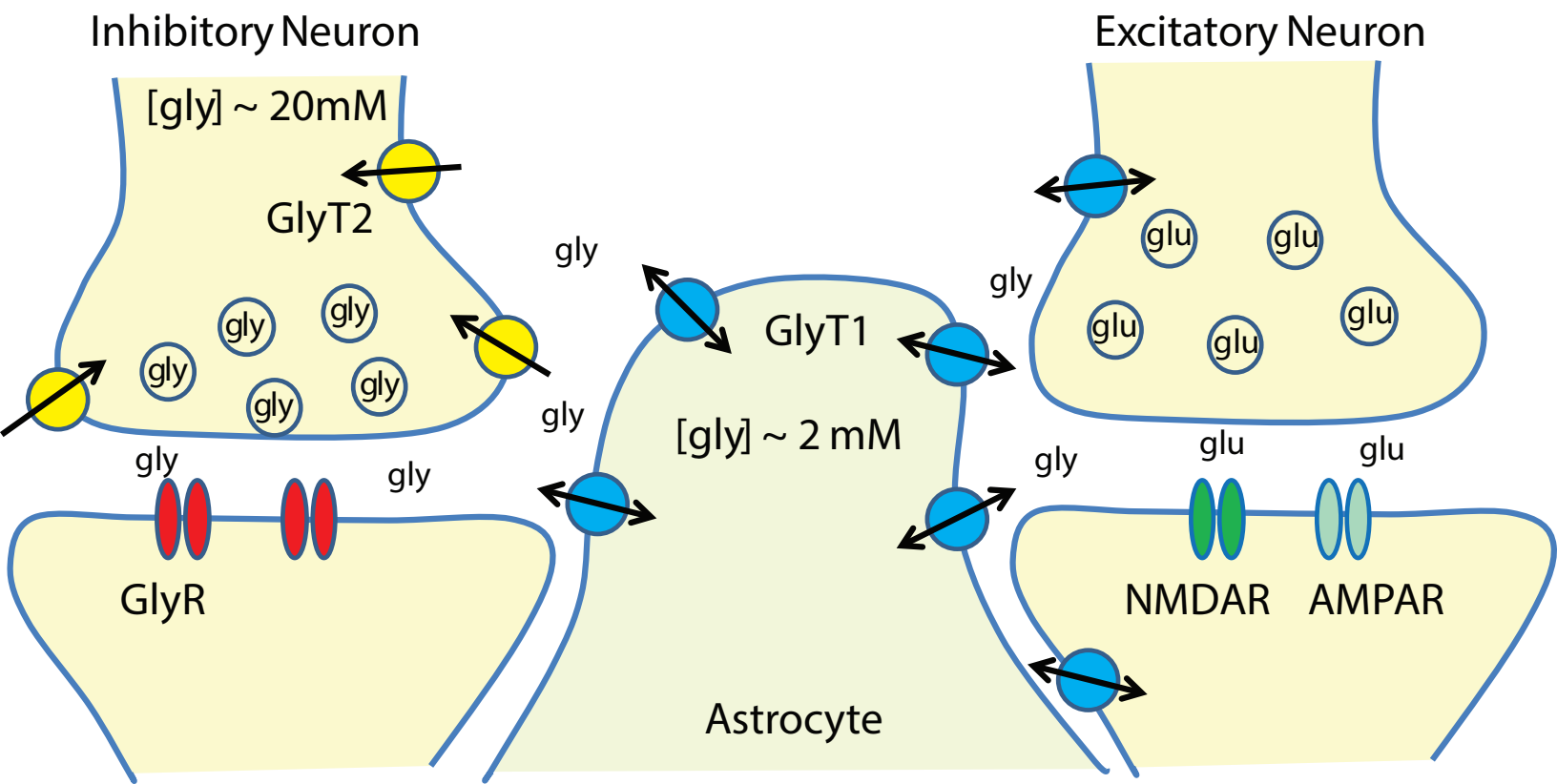
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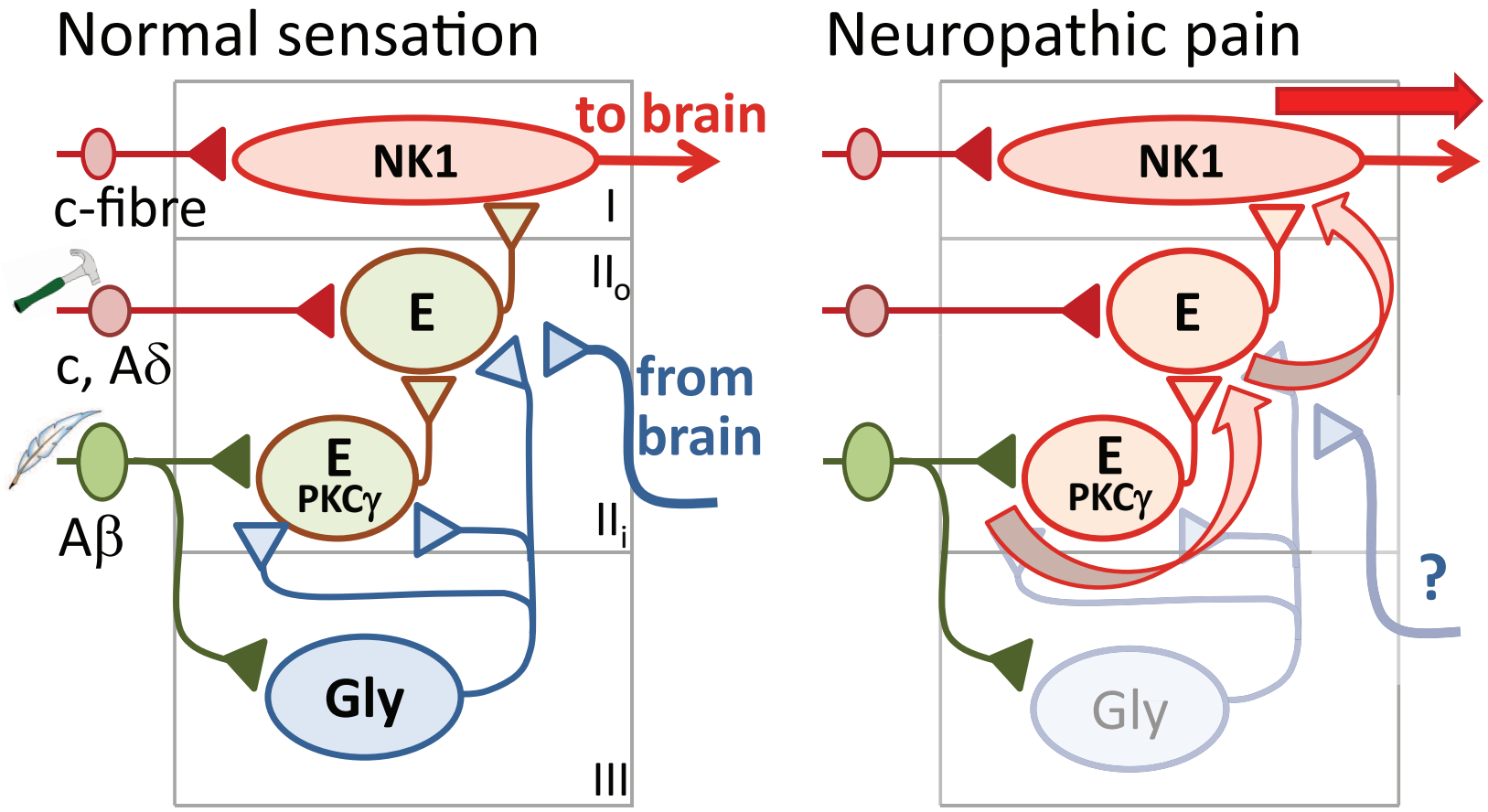
Normal sensation
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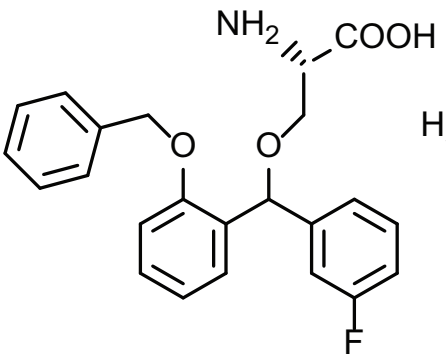

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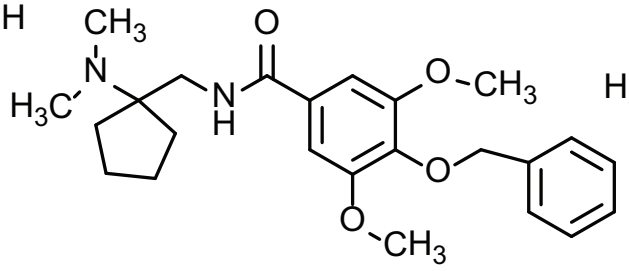

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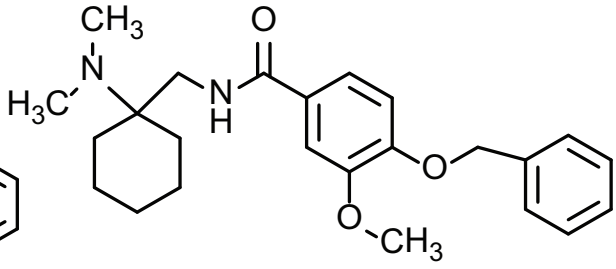

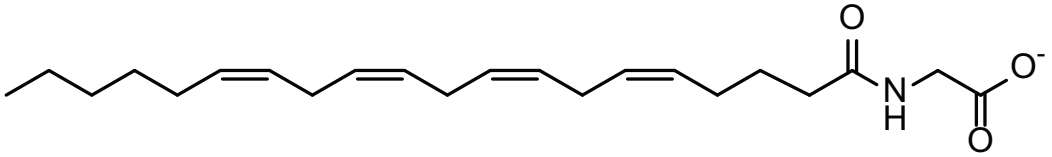

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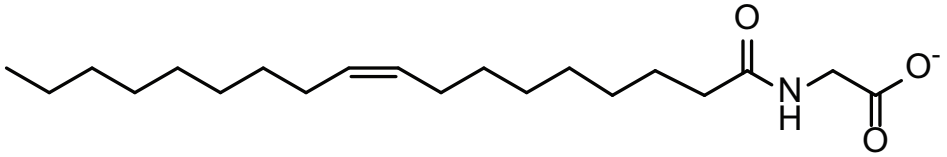

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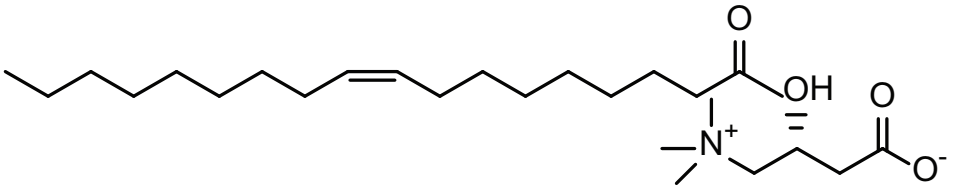

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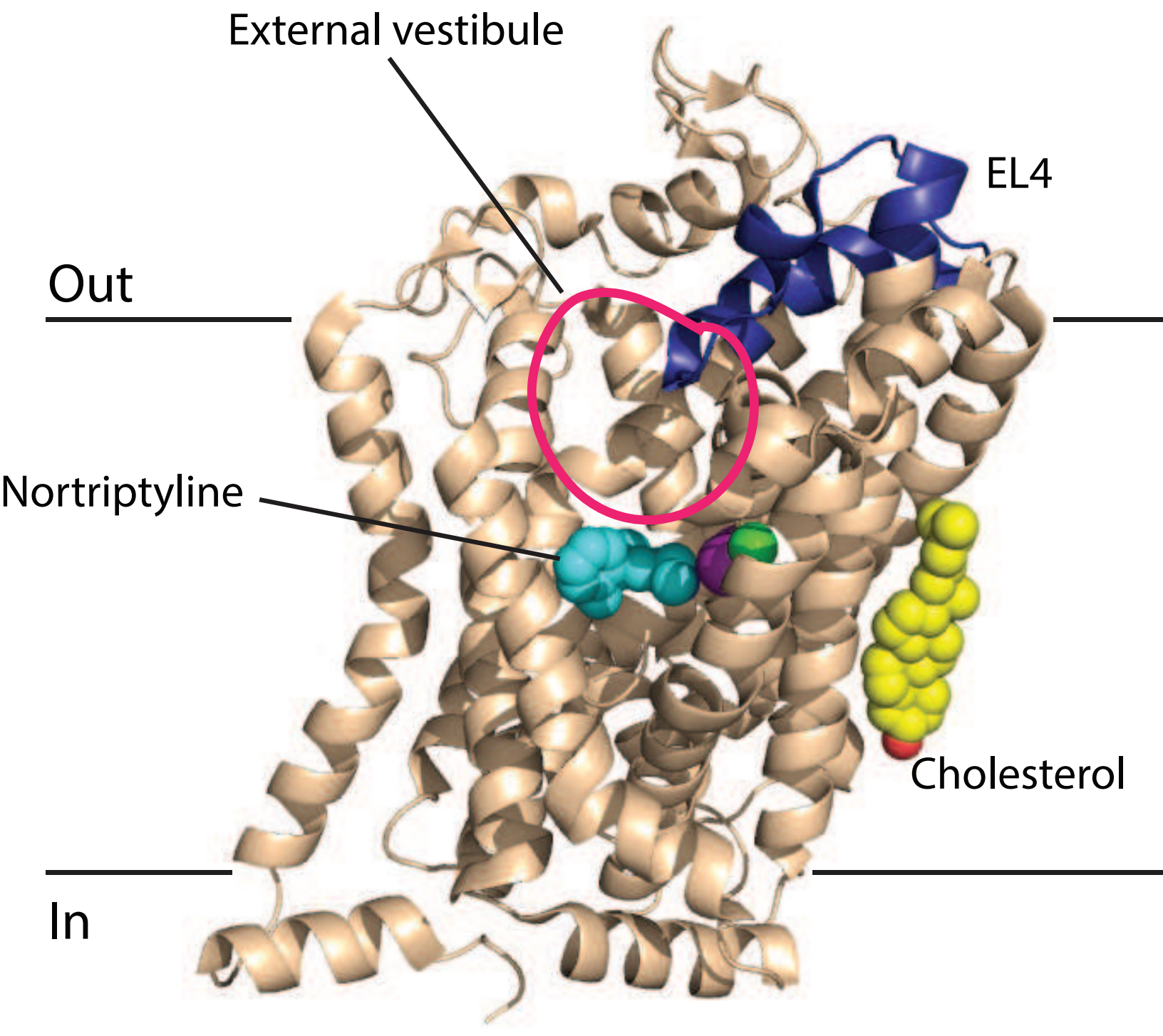
External vestibule
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Nortriptyline
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In

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