SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Morphological and neurochemical characteristics, and synaptic targets of glycinergic neurons in laminae I-IV of the spinal dorsal horn

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Introduction and Aims

According to the definition of the International Association for the Study of Pain, nociceptive pain arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors. The term is used to describe pain occurring with a normally functioning somatosensory nervous system to contrast with the abnormal function seen in neuropathic pain. The term pain is going to be used in this thesis according to this definition.

As the definitions define, damages to non-neuronal tissue as a set of stimuli act on membrane receptors of axon terminals of nociceptors, representing peripheral axon terminals of C and Aδ primary sensory fibers with cell bodies in the dorsal root ganglia. The activation of the membrane receptors results in membrane depolarization which is converted into action potentials at the first node of Ranvier along the axons. The action potentials will be conducted to the nocireceptive zone of the spinal dorsal horn. In the dorsal horn, spinal neurons will be activated, the nerve impulses will be processed by neuronal circuits, then the nerve signals will be transmitted to various brain stem and diencephalic nuclei by projection neurons. The brain stem and diencephalic nuclei forward the nerve activities to several cortical areas where a complex sensation called pain will be generated.

As described in the previous paragraph, the first relay station of pain processing is the spinal dorsal horn, which presents a complex neural apparatus that can attenuate, or even block, the incoming

nociceptive signals, or enhance and forward them to higher brain centers. Thus, although spinal cord neural circuits cannot generate pain sensation, they play essential roles in pain modulation. For this reason, the investigation of spinal neural circuits underlying nociceptive/pain processing is in the forefront of pain studies.

Fast inhibitory neurotransmission mediated by GABA and/or glycine plays essential roles in shaping up all functional states of nociceptive/pain processing neural circuits of the spinal dorsal horn. The relative contribution of GABA and glycine to the inhibitory events is, however, under continuous debate. Recent physiological and pharmacological observations indicate that although GABAergic transmission is very important, glycine seems to be the neurotransmitter which mediates most of the fast inhibitory neurotransmission in the spinal nociceptive/pain processing neuronal circuits. Results of morphological studies, however, strongly emphasize the primary importance of GABA-mediated inhibition over glycine-mediated inhibition. One of the main reasons of this contradiction is that our knowledge about the morphology of glycinergic neurons, and about the way how they contribute to the formation of nociceptive/pain processing neuronal circuits in the spinal dorsal horn is very limited.

To fill up this gap in our knowledge and to advance our understanding about the contribution of glycinergic inhibition to spinal pain processing we intended to explore the distribution,

morphology, neurochemical characteristics, and synaptic relations of glycinergic neurons in Rexed laminae I-IV of the spinal dorsal horn.

Material and Methods

Animals

The experiments were performed on adult male mice. The Animal Care and Protection Committee at the University of Debrecen approved all animal experimental protocols (2/2017/DEMÁB) which were conducted by following the European Community Council Directives.

The following types of mice were used for the experiments:

1) wild-type B6,

2) transgenic mice

a. GlyT2::CreERT2-,

b. Tg(PAX2-Cre)1Akg/Mmnc-,

c. Prkcgtm2/Cre/ERT2/Ddg/J-,

d. 007914-B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J).

The Cre or CreERT2 lines were crossed with the tdTomato reporter mice, and a postnatal intraperitoneal tamoxifen injection was given to the offspring of the CreERT2 lines to induce the tdTomato expression.

All the offspring were genotyped for the tdTomato expression, and for GlyT2 expression in the GlyT2::CreERT2, PAX2 expression in the Tg(PAX2-Cre)1Akg/Mmnc, and PKCγ expression in the Prkcgtm2/Cre/ERT2/Ddg/J transgenes at the 18th to 20th postnatal days. The animals that expressed both genes were used for the experiments at the age of 3 to 5 months.

Hemisection

Animals used for the investigation of the synaptic targets of GlyT2 positive axon terminals underwent a laminectomy at the level of the 9th and 10th thoracic vertebrae under deep sodium pentobarbital anaesthesia. One side of the spinal cord at the level of the 11th and 12th thoracic spinal segment was transected, whereas the other side remained intact. Further experiments were performed on the hemisected side at the level of the L4 and L5 segments of the spinal cord after 3 to 4 weeks of the hemisection.

Immunohistochemistry

The animals were anesthetized with sodium pentobarbital, and perfused transcardially with Tyrode's solution, followed by a fixative containing 4.0% paraformaldehyde for light microscopy, or 2.5% paraformaldehyde and 0.5% glutaraldehyde for electron microscopy. The lumbar segments of the spinal cord were sectioned at 50 or 100 μm on a vibratome and washed several times. Sections which were fixed for electron microscopy were treated with 0.1% H2O2 and 1% sodium borohydride.

Immunohistochemistry for light microscopy

In general, the free-floating sections were incubated with 10% normal goat or rabbit serum, followed by the primary antibodies for 2 days at 4^{o}C. Then, the sections were transferred into a solution containing the respective secondary antibodies for 5 to 6 hours at room temperature. Finally, the sections were affixed on glass slides and covered with Vectashield mounting medium.

Sections from wild-type B6 animals were single immunostained with GlyT2, and double immunostained with GlyT2 and CGRP, b-IB4, VGLUT1 or VGLUT2. Sections from GlyT2-CreERT2-tdTomato animals were single immunostained with PAX2, nNOS, NPY, PV, GAL, and CR, and double immunostained with GlyT2 and GAD65/67. Sections from PAX2-Cre-tdTomato were double immunostained with GlyT2 and nNOS, PV, GAL, CR, CaB or PKCγ. Sections from PKCγ-CreERT2-tdTomato animals were triple immunostained with GlyT2, PAX2, and b-IB4 or VGLUT1.

Immunohistochemistry for electron microscopy

Sections were placed into normal goat serum, followed by guinea pig anti-GlyT2 primary antibody for 2 days at 4ºC. Then, the sections were transferred into biotinylated goat anti-guinea pig IgG for 5-6 hours, followed by avidin biotinylated horseradish peroxidase solution (ABC) for overnight. The immunostaining was visualized with nickel-intensified diaminobenzidine (NiDAB) chromogen solution. The NiDAB end-product was intensified with silver precipitation, which was stabilized with gold. After this, the sections were treated with a mixture of 0.5% OsO_4 and 1.5% ferricyanide, followed by dehydration. Finally, the sections were embedded in Durcupan. Ultrathin sections were cut at 50-60 nm thickness, collected onto Formwar-coated single-slot grids, and counterstained with 2% lead citrate. They were analysed with a JEOL1010 transmission electron microscope.

In situ hybridization

Animals were deeply anesthetized with sodium pentobarbital. The lumbar segments of the spinal cord were removed and frozen on liquid nitrogen. Sections (16 μm thick) were cut from the frozen spinal cord at a cryostat and affixed on glass sides.

To revel colocalization between tdTomato and RET or RORβ mRNAs, the hybridization was performed following the RNAScope® Multiplex Fluorescent Assay detection protocols with appropriate mRNA probes.

To reveal colocation between GlyT2 and GAD65/67 mRNAs in tdTomato mRNA expressing neurons, the hybridization was performed on PAX2-Cre-tdTomato mice spinal cord sections, following the same Assay with appropriate mRNA probes.

Confocal microscopy

Serial optical sections with 1 μm thickness and 0.5 μm overlap were obtained in an Olympus FV3000 confocal microscope using a 40x oil-immersion lens (numerical aperture: 1.3). The images were evaluated at Adobe Photoshop CS5 software.

Neurolucida reconstruction

Neurolucida system was used for the 3D reconstruction of the cell bodies and dendrites of neurons, as well as axon terminals making contacts with the labelled dendrites and cell bodies.

Results

Specificity of GlyT2::CreERT2-tdTomato transgenic mice

After crossing GlyT2::CreERT2 mice with tdTomato reporter animals, first we tested the specificity of tdTomato expression in the GlyT2::CreERT2-tdTomato mice for glycinergic neurons. We first analysed the expression of PAX2, a transcription factor that is an accurate marker of inhibitory neurons in the spinal cord, in the tdTomato labelled neurons, and found that 97.35±2.38% of them were stained for PAX2. To confirm if these inhibitory neurons were glycinergic, we detected GlyT2 and tdTomato mRNAs using multiplex FISH, and all of the td Tomato labelled neurons showed positive signal also for GlyT2 mRNA in laminae I-IV, indicating that the tdTomato labelling in the GlyT2::CreERT2-tdTomato transgenic mice is selective for glycinergic neurons.

Distribution of glycinergic neurons in laminae I-IV

Studying tdTomato labelling in the lumbar spinal dorsal horn of GlyT2::CreERT2-tdTomato animals, strong labelling was observed in laminae I to IV. Although labelled cell bodies were rare in lamina II and only slightly more numerous in lamina I, punctate labelling was remarkably strong in these laminae. The number of labelled neurons was higher in lamina III, but the highest density was observed in lamina IV.

Morphology of glycinergic neurons

We studied the dendritic morphology of glycinergic neurons in laminae I to IV of the L4-L5 spinal segments.

Laminae I-II

Considering the shape and size of the cell bodies and dendritic morphology, tdTomato-labelled neurons in laminae I and II were classified into 3 groups.

(1) Type 1. These neurons had small rostro-caudally oriented fusiform cell bodies. The cell bodies' two ends gave rise to dendrites, which spread out in opposing rostro-caudal directions to form a short, weakly arborizing dendritic tree.

(2) Type 2. These neurons were also fusiform, but their cell bodies were twice as large as the cell bodies of type 1 neurons. The origin and orientation of their dendrites were similar to type 1 neurons.

(3) Type 3. The cell bodies of these neurons were multipolar. They had 3 or 4 stem dendrites, some of which extended rostro-caudally, others turned ventrally and run in an oblique direction.

Laminae III-IV

Deeper, in laminae III and IV, the cell morphology was more diverse, and the tdTomato-labelled neurons were divided into 6 groups:

(1) Type 1. Most of the neurons were classified into this group. These neurons had small fusiform cell bodies similar in shape and size to type 1 neurons in laminae I and II. Some neurons presented poorly arborizing, others richly arborizing dendritic tree.

(2) Type 2. Their cell bodies were multipolar with 4 to 5 stem dendrites that extended rostro-caudally forming a rich arborization.

(3) Type 3. The cell bodies were like type 2 neurons, but the dendritic tree was different. Although some of the dendrites were running rostro-caudally, others extended ventrally.

(4) Type 4. These neurons presented elongated cell bodies with dendrites arising from the poles of the cell body. The dendrites extended into the rostral-caudal and dorsal direction.

(5) Type 5. They had pyramidal-shaped cell bodies with 3 stem dendrites: 2 arising from the base and 1 arising from the apex. The basal dendrites extended rostro-caudally, whereas the apical dendrite run dorsally and entered more superficial grey matter regions.

(6) Type 6. We grouped the neurons into this category that we could not classify into the other groups.

Neurochemical markers of glycinergic neurons

Galanin and Calretinin

In the regions where GAL and CR immunostained neurons were detected, we found 49 and 45 tdTomato-labelled cells, respectively, but none of them were positive for GAL or CR.

Neuropeptide Y

We found double labelled axon terminals of NPY and tdTomato only occasionally and in very low numbers.

Neuronal nitric oxide synthase

We found 46 and 231 tdTomato-labelled neurons in laminae I-II and in laminae I-IV from which 13 (28,2%) and 15 (6.5%) were also positive for nNOS, respectively.

Parvalbumin

A total of 367 tdTomato-labelled neurons were counted in laminae IIi-III-IV, from which 150 (40.8%) were also positive for PV.

Tyrosine kinase RET

We detected 27 and 139 tdTomato-labelled neurons in laminae I–II and laminae III-IV, of which 15 (55.5%) and 79 (56.8%) presented positive hybridization signal for RET, respectively.

Nuclear orphan beta receptor RORβ

In laminae I-II and III-IV we found 21 and 104 tdTomato-positive neurons, of which 5 (23.8%) and 13 (12.5%) also expressed RORβ, respectively.

Colocalization of GlyT2 and GAD65/67 mRNA in neurons within laminae I-III and IV

By using multiple fluorescence *in situ* hybridization, we investigated whether the glycinergic neurons are also GABAergic, or some of them can be regarded as glycine-only neurons. We investigated the GlyT2 and GAD65/67 mRNA expression in Pax2:Cre-tdTomato transgenic animals in which tdTomato mRNA identifies PAX2 expressing inhibitory neurons. Since most of the GlyT2-containing neurons were found in lamina IV, we performed this part of the study in a way that neurons in laminae I-III were pooled in one group and neurons in lamina IV were separated in another group.

We identified 389 neurons in laminae I–III that were positive for tdTomato mRNA and also expressed GlyT2 and/or GAD 65/67 mRNA. We found that 261 (67.1%) of these neurons expressed both GlyT2 and GAD65/67 mRNA, while 128 (32.9%) of them were only positive for GAD65/67 mRNA. The intensity of the signals in neurons that expressed both GlyT2 and GAD65/67 mRNA varied greatly from weak to strong along a continuous scale, and the intensity of signals for GlyT2 and GAD65/67 changed

independently. No cells were found to be GlyT2 mRNA positive while being GAD65/67 mRNA negative.

Even though GlyT2 mRNA labelling predominated in lamina IV neurons, only 24 (3.7%) of the 656 cells that presented positive signal for GlyT2 and/or GAD 65/67 mRNAs expressed only GlyT2 mRNA. The percentage of these neurons was lower than that of neurons that expressed only GAD 65/67 mRNA; 43 (6.5%). In the 589 neurons that expressed both GlyT2 and GAD65/67 mRNAs (89.8%), the intensity of the signals altered independently for GlyT2 and GAD65/67, and it was very variable from weak to strong along a continuous scale, likewise in laminae I-III. GlyT2 mRNA predominated in most of the neurons in lamina IV, in contrast to what was seen in laminae I–III, while GAD65/67 mRNA labelling was, with a few exceptions, typically weak or moderate in these neurons.

Colocalization of GlyT2 and GAD65/67 in axon terminals within laminae I-II

To explore the GlyT2 expression and the colocalization between GlyT2 and GAD65/67 in axon terminals within laminae I-III we performed double immunostaining for GlyT2 and GAD 65/67 in sections obtained from GlyT2::CreERT2-tdTomato animals. Because of the relatively high densities of dendrites that may bias our results in lamina III, we analysed the immunostaining only in laminae I-II.

In the investigated sections, 105 tdTomato-labelled puncta were found, of which 68 were also immunostained for GlyT2 (62.12 ± 3.94%), and 31 of the GlyT2 immunoreactive boutons were also immunostained for GAD65/67 (45.49 ± 3.84%).

To confirm the presence of glycine-only axon terminals in laminae I-II, we investigated the GAD65/67 immunostaining in GlyT2-positive axon terminals regardless of whether they were labelled with tdTomato. We found 123 axon terminals immunostained for GlyT2 in our sample, and 56 of them were also immunostained for GAD65/67 (46.02 ± 4.22%).

These findings indicate that glycine-only inhibition may exist in laminae I-II of the spinal dorsal horn, but glycine only axon terminals may arise primarily, if not exclusively, from glycinergic neurons in lamina IV.

Spinal vs supraspinal glycinergic innervation of laminae I-III of the spinal dorsal horn

Besides spinal neurons, glycinergic axons descending from brain stem nuclei may also substantially contribute to the glycinergic innervation of the spinal dorsal horn by. Because we intended to identify glycinergic axon terminals of spinal origin, we removed the descending glycinergic fibres, performing hemisection of the spinal cord at the level of thoracic segments 11 and 12, and the remaining glycinergic axon terminals in the lumbar spinal cord ipsilateral to the hemisection were considered as terminals of spinal origin.

The numbers of GlyT2 immunoreactive axon terminals were counted both in laminae I-II and lamina III. In lamina I-II, in total 230 and 156 immunostained axon terminals were counted in non-operated mice and mice that underwent hemisection, respectively. In lamina III, in total 715 and 432 immunostained axon terminals were counted in non-operated mice and mice that underwent hemisection, respectively. According to these results, we can conclude that at the level of L4-L5 spinal segments roughly 68% and 60% of GlyT2 immunoreactive axon terminals were of spinal origin in laminae I–II and lamina III, respectively, while the rest can be considered as brainstem-origin. These findings also indicate that low thoracic spinal cord hemisection is obligatory for the analysis of glycinergic axon terminals of spinal origin in the superficial lumbar spinal dorsal horn. In the following experiments only mice that underwent hemisection were used.

Synapses made by GlyT2-IR axon terminals in laminae I-III of the spinal dorsal horn

In total, 138 boutons labelled for GlyT2 were collected from which 104 (75.4%) formed axo-dendritic, 16 (11.6%) axo-somatic, and 18 (13.0%) axo-axonic synaptic contacts. From the 18 labelled boutons that formed axo-axonic contacts, 7 were found in non-glomerular and 11 in glomerular synaptic arrangements.

Neurons receiving axo-somatic inputs from glycinergic axon terminals in laminae I-III

To identify the types of interneurons that are targets of glycinergic inhibition, we used immunostaining for GlyT2 and several cellular markers, such as CaB, PKCγ, CR, GAL, nNOS, and PV, which have already been identified as markers of inhibitory and excitatory neurons in laminae I-III of the spinal dorsal horn. Because we wanted to find out whether glycinergic innervation is received by inhibitory or excitatory neurons, we carried out the immunostaining on sections taken from Pax2:Cre-tdTomato transgenic mice.

Our main finding was that almost all groups of interneurons that we studied formed close axo-somatic appositions with GlyT2 immunostained axon terminals. Several axo-somatic contacts were identified on CaB-, CR-, nNOS-, and PV-positive excitatory neurons. Only few contacts were detected between GlyT2 immunostained axon terminals and the cell bodies of PKCγ-positive excitatory neurons, instead, we found contacts on their proximal dendrites. We couldn't detect any contacts between the cell bodies and GlyT2-positive axons terminals of excitatory GAL-containing neurons. The somata of PKCγ-, CR-, GAL-, nNOS-, and PV-positive inhibitory neurons were likewise in contact with GlyT2 immunostained axon terminals in addition to excitatory neurons. Only CaB positive inhibitory neurons were not found to receive contacts from GlyT2 immunostained boutons. These findings imply that glycinergic postsynaptic inhibition has a significant and variable impact on the activity of almost all populations of excitatory and inhibitory interneurons in laminae I–III of the spinal dorsal horn.

Axon terminals receiving axo-axonic inputs from glycinergic axon terminals in laminae I-III

To identify the types of axon terminals that can be the targets of glycinergic presynaptic inhibition in laminae I-III of the spinal dorsal horn, we combined GlyT2 immunostaining with IB4-binding and immunolabelling for CGRP, VGLUT1 and VGLUT2, markers of non-peptidergic nociceptive afferents, peptidergic nociceptive afferents, and non-nociceptive primary afferents, and axon terminals of intrinsic excitatory spinal neurons, respectively.

We found that GlyT2-positive boutons did not form axo-axonic contacts with CGRP- or VGLUT2-expressing axon terminals. However, we found close appositions between GlyT2 and VGLUT1 immunoreactive as well as IB4-binding axon terminals. All axo-axonic close appositions were found in inner lamina II and lamina III, according to the distribution of the postsynaptic axon terminals.

Distribution of glycinergic axon terminals in post- and presynaptic positions along the dendrites of PKCγ- containing neurons

To analyse how axo-dendritic glycinergic synapses are distributed along the dendritic tree, we chose the PKCγ-containing neurons because they are known to receive significant excitatory inputs from primary afferents and are strongly inhibited by glycinergic neurons. Considering that 13.2% of PKCγ-positive interneurons were

identified as inhibitory, and that inhibitory neurons can be under different glycinergic inhibitory control than excitatory neurons, we carried out triple immunostaining of sections from Prkcgtm2/cre/ERT2-tdTomato animals for GlyT2, PAX2 and IB4-binding or VGLUT1.

We studied 47 and 59 tdTomato-positive PKCγ-containing neurons that received contacts from IB4-binding or VGLUT1 immunoreactive primary afferents, respectively. Nine and 19 of the PKCγ-positive cells contacted by IB4-binding or VGLUT1 immunoreactive axon terminals, respectively, were also positive for PAX2.

For the cells stained for GlyT2 and IB4-binding, 1520 and 1306 contacts made by GlyT2 immunoreactive and IB4-binding axon terminals, respectively, were identified on the dendrites. In the case of the cells stained for GlyT2 and VGLUT1, 1763 and 1019 close appositions made by GlyT2 immunoreactive and VGLUT1 immunoreactive axon terminals, respectively, were counted on the dendrites. Independent of the markers, the contacts were uniformly dispersed along the dendrites and did not exhibit any signs of aggregation. Between PAX positive and PAX negative neurons, there were no appreciable variations in these parameters.

For the neurons on which there were GlyT2 terminals making axo-axonic contacts, we found that 101 (9.2%) of the 1095 IB4-binding axon terminals making close appositions with the labelled dendrites were contacted by GlyT2 immunoreactive terminals. On the other

hand, 126 (16.4%) of the 765 VGLUT1 immunostained axon terminals making contacts with the labelled dendrites were contacted by GlyT2-positive terminals.

Summary

We studied the morphological and neurochemical properties, as well as the synaptic targets of glycinergic neurons in laminae I-IV of the mouse spinal dorsal horn by using transgenic technologies, immunohistochemistry, in situ hybridization, light and electron microscopy.

We showed that although cell bodies of glycinergic neurons were rare in lamina II and only a slightly more numerous in lamina I, axon terminals were densely distributed in these superficial laminae, indicating that glycinergic neurons in the deeper layers of the dorsal horn send their axons into laminae I-II. The number of labelled neurons was higher in lamina III, but the highest density was observed in lamina IV.

We demonstrated that there are at least 3 and 6 subtypes of glycinergic neurons showing distinct morphology in laminae I-II and III-IV, respectively. All the labelled glycinergic neurons in laminae I-II and many of them in laminae III-IV were identified as islet, central and vertical neurons. Cell morphologies in laminae III-IV, however, were more diverse; some labelled neurons in laminae II-IV resembled inverted stalked cells and pyramidal cells, while the morphologies of others did not correspond to any of the previously identified cell types.

We showed that distinct populations of glycinergic neurons in laminae I-IV express neuronal markers like PV, nNOS, RET and RORβ.

First in the literature, we provided experimental evidence that there are glycinergic axon terminals in abundant numbers in laminae I-II that do not express GABA, and these glycine-only axon terminals may arise primarily, if not exclusively, from neurons in lamina IV. Thus, glycinergic neurons with cell bodies in lamina IV may contribute substantially to spinal pain processing.

Our findings suggest that glycine-mediated postsynaptic inhibition must be significantly more dominant than glycinergic presynaptic inhibition in laminae I-III.

Our results indicate that glycinergic postsynaptic inhibition, including glycinergic inhibition of inhibitory interneurons must be a common functional characteristic of neural circuits underlying spinal pain processing. On the other hand, it is possible to hypothesize that glycinergic presynaptic inhibition, even though can be weak at the cellular level, may be crucial for locally targeting functionally distinct subpopulations of primary afferent inputs.

List of publications

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Registry number: DEENK/261/2023.PL
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Candidate: Camila De Oliveira Miranda
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List of publications related to the dissertation

1. **De Oliveira Miranda, C.**, Hegedüs, K., Kis, G., Antal, M.: Synaptic Targets of Glycinergic Neurons in Laminae I-III of the Spinal Dorsal Horn.
Int. J. Mol. Sci. 24 (8), 1-26, 2023.
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2. **De Oliveira Miranda, C.**, Hegedüs, K., Wildner, H., Zeilhofer, H. U., Antal, M.: Morphological and neurochemical characterization of glycinergic neurons in laminae of the mouse spinal dorsal horn.
J. Comp. Neurol. 530 (3), 607-626, 2022.
DOI: http://dx.doi.org/10.1002/cne.25232
IF: 3.028 (2021)

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List of other publications

3. Javdani, F., Hegedűs, K., **De Oliveira Miranda, C.**, Hegyi, Z., Holló, K., Antal, M.: Differential expression of Na+/K+/Cl- cotransporter 1 in neurons and glial cells within the superficial spinal dorsal horn of rodents.
   Sci. Rep. 10 (1), 11715-11728, 2020.
   DOI: http://dx.doi.org/10.1038/s41598-020-68638-3
   IF: 4.379

4. Soares Romeiro, L. A., da Costa Nunes, J. L., **De Oliveira Miranda, C.**, Simões Heyn Roth Cardoso, G., de Oliveira, A. S., Gandini, A., Kobrlova, T., Soukup, O., Rossi, M., Senger, J., Jung, M., Gervasoni, S., Vistoli, G., Petralla, S., Massenzio, F., Monti, B., Bolognesi, M. L.: Novel Sustainable-by-Design HDAC Inhibitors for the Treatment of Alzheimer's Disease.
   ACS Med. Chem. Lett. 10 (4), 671-676, 2019.
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