

Ph.D. THESIS

**THE NEUROMODULATOR AND
NEUROTRANSMITTER ROLE OF GLUTATHIONE,
S-NITROSOGLUTATHIONE AND CYSTEINE IN THE
CENTRAL NERVOUS SYSTEM**

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INTRODUCTION

Electrical stimuli spread through intercellular connections, synapses. We can distinguish chemical, electrical and combined synapses. In the case of chemical synapses, a presynaptic neuron releases chemical signal compounds, neurotransmitters. These transmitters interact with specific receptor proteins located at the surface of post-synaptic neurons and hence induce cellular responses. The neurotransmitters can be classified into two groups, excitatory and inhibitory, depending on the nature of effects elicited in the target cells. The well-known transmitters include acetylcholine, biogenic amines, adenosine and certain amino acids and peptides. Less conventional transmitters are gaseous nitrogen monoxide (NO) and carbon monoxide (CO). In the central nervous system (CNS) of mammals, the main neurotransmitters are amino acids. Amino acids act as excitatory (e.g., glutamate and aspartate) or inhibitory [e.g., γ -aminobutyrate (GABA) and glycine] transmitters. GABA is the main inhibitory and glutamate the main excitatory transmitter. In the medulla oblongata and spinal cord glycine is inhibitory, whereas in the higher brain regions predominantly excitatory, potentiating the effects of glutamate. Glutamate released into the synaptic clefts upon depolarisation stimulates postsynaptic glutamate receptors and alters the excitation state of the target neurons either by evoking a rapid and transient increase in the cation permeability of the receptor-coupled ion channels or by affecting intracellular metabolism via the second messenger systems. Based on pharmacological and electrophysiological studies, glutamate receptors are divided into two families: ionotropic and metabotropic. The ionotropic receptors can be further divided into classes named after their specific ligands, N-methyl-D-aspartate (NMDA), kainate and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). Activation of these receptors in most cases induces depolarisation, i.e., a change in the electrical potential of the neuronal plasma membrane. The metabotropic receptors are coupled to intracellular GTP-binding proteins (G-proteins) and evoke cellular responses via changes in the inositolphospholipid metabolism or in the synthesis of intracellular cyclic nucleotides. Glutamate is involved in all regulatory processes in the brain, e.g., in differentiation and growth of neurons and glial cells, formation of interneuronal connections, maintenance of general electric activity, processing of sensory information, nociception, regulation of motion and muscle tone, learning and memory acquisition, and emotional reactions such as anxiety and stress. It is thus obvious that any pathological alteration in glutamatergic neurotransmission can disturb vital functions. The activity of neurons is determined mainly by the combined effects of glutamate and GABA. A disturbance in this balance between excitation and inhibition results in a pathological state and eventual cell death. An excessive increase in extracellular glutamate induces excitotoxicity by overexcitation (e.g., in acute ischemia or hypoglycemia). Glutamate-induced toxicity is also apparently involved in the pathogenesis of many neurological and neuropsychiatric disorders, such as epilepsy, schizophrenia, Alzheimer's dementia, Parkinson disease and Huntington's chorea, olivopontocerebellar atrophy, AIDS-encephalopathy and amyotrophic lateral sclerosis (ALS).

Compounds regulating glutamatergic transmission

Nitric oxide

Nitric oxide is an unconventional neuromodulator/neurotransmitter, which is not packaged in vesicles but rather diffuses from its site of production without any specialized release machinery. In addition, it can bypass normal signal transduction routes and does not have any specialized receptors. NO can modulate almost all processes of neurotransmission, e.g. the release and uptake of neurotransmitters, synaptic signal transduction, brain development, formation and functional strengthening of synapses, and synaptic plasticity. It participates in the long-term potentiation (LTP) and long-term depression (LTD) which has been proven to be a powerful system for learning. NO is synthesized from L-arginine by neural nitric oxide synthase (nNOS) in the nerves. In the same brain region the nerves containing nNOS possess more NMDA receptor subunits than those which do not. In the postsynaptic cells, nNOS is located predominantly near the NMDA receptors in the synaptic region. After NMDA receptor activation, biosynthesis of NO is enhanced in brain tissue. All this supports a close functional connection of NMDA receptors with nNOS. The effect of NO is very complicated at the NMDA receptors and highly depends on the redox state of receptors. Furthermore, NO exists in three various redox states endogenously in brain tissue. In the case of nitrosonium ion (NO^+), the mechanism often involves S-nitrosylation (transfer NO group to a cysteine sulfhydryl). Nitroxyl anion (NO^-) in a triplet state plays a role in toxic processes, while $\text{NO}\bullet$ can react with thiols only under specific circumstances. Hence, NO and NO equivalents can interact directly with the NMDA receptors through its modulatory sites and receptor ionophores. However, it also acts through Ca^{2+} homeostasis and other signal transduction mechanisms. In a cell, the major target of NO is guanylyl cyclase. This interaction increases the cGMP levels and hence affects ion channels or activates cGMP-dependent protein kinase. NO can have a role in excitotoxic processes in the brain mainly by producing highly reactive free radicals and mediating neurotoxic effects of glutamate. On the other hand, it has beneficial effects due to nitrosylation processes and its potent antioxidant property.

Glutathione

In the CNS, peptides with a relatively small molecular weight, e.g., endorphins, vasointestinal peptide (VIP), neuropeptide Y, cholecystochinin (CCK) and peptide-type releasing hormones also have regulatory effects. These peptide mediators are located in the same neurons with the conventional transmitters but stored in different vesicles. The peptides, released upon depolarisation, can exert their effects on the target cells as cotransmitters with another transmitter or as neuromodulators. There are also a number of di- and tripeptides isolated from brain tissue. Their functions are not fully understood, even though their structures are known.

γ -Glutamylcysteinylglycine (GSH) is a phylogenetically ancient tripeptide ubiquitous in living organisms. GSH is the most abundant thiol-containing peptide in the CNS. The total level of glutathione in the CNS is within the range of 1.4-3.4 mM. The greater part of glutathione (about 95%) is in reduced form. Glutathione is synthesized from its constituent amino acids and broken down via the γ -glutamyl cycle. The synthesis of GSH is catalyzed by γ -glutamylcysteine synthetase and glutathione synthetase, the former being the rate-limiting enzyme. The activity of both enzymes is

under substrate and product (feed-back) control. The breakdown of glutathione is catalyzed by γ -glutamyl transferase.

The redox functions of glutathione are well known; for example, the molecule can scavenge free radicals as an antioxidant, protecting cells membranes against oxidative stress, DNA against radiation and ultra violet light, and the whole cell against xenobiotics. Glutathione also serves as a cofactor or substrate for various enzymes in the regulation of the cell cycle and in cellular metabolism. Since the constituent amino acids are neuroactive, glutathione may also have neuromodulatory and neurotransmitter properties in the CNS.

Neuromodulatory role of glutathione

GSH interacts both with the agonist and antagonist binding sites of NMDA receptors. Since GSH and its S-alkyl derivatives are effective displacers of NMDA receptor ligands, the γ -glutamyl moiety is involved in the binding. Based on functional tests, GSH, depending on its concentration, has either agonist or antagonist effects on these receptors. The NMDA-activated Ca^{2+} influx is influenced by GSH in a concentration-dependent biphasic manner. At micromolar concentrations, GSH reduces the influx by inhibiting antagonist binding, while at millimolar concentrations GSH increases the influx by reducing functional thiol groups. Among non-NMDA receptors, GSH binds with high affinity mainly to AMPA receptors. By activating AMPA receptors GSH facilitates ion currents. It can thus be involved in the fine tuning of neurotransmission. GSH as a neuromodulator can influence glutamatergic neurotransmission. It can affect the release of excitatory neurotransmitters, their glial and neuronal uptake, and alter cellular responses via pre- and postsynaptic receptors.

Possible neurotransmitter role of glutathione

In rat cerebral cortical slices, glutathione elicits concentration-dependent excitatory field potentials which are not blocked by any antagonist of ionotropic glutamate receptors. GSH presumably produces an effect on some receptors which are not glutamate receptors. In addition, GSH has been reported to possess binding sites in the CNS, which cannot be separated with certainty from glutamate binding sites based on this findings.

S-Nitrosoglutathione

As a neuromodulator or neurotransmitter, NO is released from the synthesis place diffuses through the plasma membranes due to its concentration gradients. NO can directly interact with the cysteinyl side chains of peptides and proteins, iron-containing heme groups, superoxide anion and oxygen. Since the life-span of NO^\bullet is only few seconds it is easily inactivated. It has been suggested that some intermediate compounds, for example S-nitrosothiols may act as a carrier system to stabilize, target and to preserve its biological activity. S-nitrosothiols are readily formed from glutathione and NO. Since neurons and glial cells contain GSH at high concentrations, there is a good chance for the reaction to occur. There is indeed evidence that GSNO is synthesized in the CNS.

S-nitrosothiols have a wide range of effects: e. g., antimicrobial effect, inhibition of platelet aggregation, bronchodilation and vasodilation. However, GSNO has also been

suggested to exert its biological action without producing NO. GSNO may have a beneficial effect in neurons due to its antioxidant properties and it can inhibit some toxic processes that cause neurodegeneration.

Cysteine

L-Cysteine is an endogenous component of proteins, a precursor of a number of peptides such as glutathione. It provides inorganic sulfate for detoxification reactions. Therefore it may thus be involved in neuroprotection. Cysteine also protects neurons by forestalling the entry of heavy metal ions across the blood-brain barrier. Since cysteine is released from brain slices upon depolarisation in a Ca^{2+} -dependent manner, it can also excite neuronal processes. An excess of L-cysteine has proved to be neurotoxic in vivo, however, in vivo in developing animals with a still immature blood-brain barrier. Upon cerebral ischemia, a big amount of L-cysteine is released mainly from glial cells to the extracellular space. The increased efflux of L-cysteine could originate from glutathione breakdown rather than enhanced release of cysteine alone. In this case, L-cysteine can evoke rapid neuronal damage in a great number of brain regions. This neurotoxic effect of L-cysteine is likely to manifest itself NMDA receptors. In addition, L-cysteine can chelate metal ions. These complexes then catalyze generation of free oxygen radicals and hydrogen peroxide. Similarly to GSH, cysteine also can interact with NO, generating cysteine-NO (Cys-NO). Cys-NO can liberate NO that reacts with free radicals (e.g., superoxide) and creates toxic peroxynitrite (OONO^{\cdot}) under pathological circumstances. Cys-NO can have protective effects in the presence of superoxide dismutase (SOD), however, since it inhibits the NMDA-evoked influx of Ca^{2+} . In this case, can Cys-NO transfer nitrosonium ion (NO^+) to thiol(s) in the redox modulatory site(s) of NMDA receptors resulting in a nitrosothiol derivative. This process downregulates NMDA receptor activity possibly through facilitation of disulfide formation. Excitotoxic actions of L-cysteine have been implicated in the pathogenesis of numerous neurological disorders, e.g., amyotrophic lateral sclerosis, and Parkinson's, Alzheimer's and Hallervorden-Spatz diseases and in hypoxic and ischemic brain damage.

AIMS OF THE STUDY

GSH has an important cell-protecting function by scavenging free radicals and participating in several redox and detoxification reactions. In addition, glutathione may interact with ionotropic glutamate receptors in the CNS and through this modification of intercellular signal transduction it can regulate a numerous physiological processes. GSH can react with the gaseous odd neuromodulator and neurotransmitter NO, forming nitrosoglutathione (GSNO). In this manner the lifetime of NO can be prolonged. It has been suggested that, similarly to GSH, GSNO can bind to glutamatergic receptors and release NO to a specific site in the receptor. To support this suggestion, it needs to prove that GSNO interacts with glutamate receptors. Moreover, experimental data show that GSNO is able to react not only with glutamate receptors but presumably with receptors of its own and can modify the activity of neurons through these own receptors. The existence of a GSH receptor, however, is not fully proved. The structure and function of these putative glutathione receptors is not understood. The characterization of interactions of the putative

receptor protein with the GSH ligand is unsatisfactory so far. It is not clarified how and why the cysteine exerts toxic effects in the CNS.

The aim was,

1. to elucidate whether GSNO plays a role in the regulation of ionotropic glutamate receptors,
2. to characterize the binding of GSH to its putative receptors,
3. to study whether the ionotropic and metabotropic receptor ligands interact with the putative GSH receptors,
4. to assess how different amino-acid side chains of the plasma membrane are involved in the binding of GSH, and
5. to establish how L-cysteine causes neuronal degeneration.

METHODS

Preparative methods

For the binding assays, we used synaptic plasma membranes prepared from the pig cerebral cortex. For the Ca^{2+} uptake and intracellular Ca^{2+} measurements cerebellar granule cells were used cultured from 7 day old Wistar rats. Crude synaptic plasma membrane fraction (P2) was prepared from the cortex homogenisate. Postsynaptic plasma membranes were prepared from the P2 fraction using sucrose gradient centrifugation. In order to determine which membrane side chains are involved in GSH binding, chemical modification was carried out on appropriate side chains. The cysteinyl side chains were modify with 5,5-dithio-nitrobensoic acid (DTNB), 4,4-dithiodipyridyl, N-ethylmaleimide (NEM) and phenylisothiocyanate. For the reduction of disulfide bridges dithiothreitol were used. The arginyl side chains of synaptic plasma membranes were modified with phenylglyoxale (PGO), while the glutamyl and aspartyl side chains were treated with ethyl-dimethylaminopropylkarbodimiide (EDC).

Binding assays

In binding assays interactions of receptor-ionophores and their specific ligands is characterized. In these experiments, the binding of specific ligands labeled with tritium are influenced by the studied compounds. First, it can be concluded whether or not the investigated compound is a ligand of the receptors. Furthermore, under certain conditions the activating or inhibiting properties of a compound can be determined. In properly planned experiments an inference can be drawn whether or not the inhibition is competitive in nature.

For the identification of amino acid residues involved in ligand binding to receptor proteins, chemical modifications of amino-acid side chains were carried out. The binding assays were made after covalent modification of plasma membranes.

In this work, binding assays were carried out on synaptic plasma membranes prepared from the pig cerebral cortex. To proof the interaction between GSNO and synaptic ionotropic glutamate receptors we studied the effects of GSNO on the binding of different specific radioligands, e.g., [³H]glutamate, [³H]kainate, [³H]fluorowillardine, [³H]CPP and [³H]dizocilpine.

To distinguish between putative GSH and ionotropic glutamate receptors we tested the effects of glutamate, glutamate derivatives, cysteine, cysteine derivatives, sulfhydryl compounds, dipeptides, GSH analogs and inhibitory transmitters on the binding of [³H]GSH.

Ca²⁺ uptake and intracellular free Ca²⁺ measurements

We used ⁴⁵Ca²⁺ for measuring the uptake of Ca²⁺ evoked by glutamate and NMDA to cerebellar granular cells. Intracellular free Ca²⁺ affected by glutamate was analyzed with using acetoxymethylester of Fura 2.

RESULTS

The effect of GSNO on the binding of different radioligands to ionotropic glutamate receptors

1. GSNO displaced ionotropic glutamate receptor radioligands [³H]glutamate, [³H]CPP and [³H]kainate.
2. The effect of GSNO was competitive in nature on the binding of [³H]CPP and [³H]kainate.
3. GSNO increased the binding of non-competitive NMDA-antagonist [³H]dizocilpine in a concentration dependent manner.
4. GSNO binds to the glutamate receptors via its γ -glutamyl moiety.

Characterization of the specific binding sites of GSH

We characterized the high-affinity, Na⁺-independent specific binding of GSH. GSH binds to one high-affinity, low-capacity, and to one a low-affinity high-capacity receptor population in the plasma membrane fraction.

Effects of glutamate derivatives and glutamate receptor ligands on the binding of [³H]GSH

1. Of the glutamate derivatives, L-glutamate, kynurenate and pyroglutamate slightly but significantly inhibited the binding.
2. Of the NMDA receptor ligands, only quinolinate had a significant displacing effect.
3. Of the ligands effective on glycine-coactivatory site in the NMDA receptor, only D-serine inhibited the binding.
4. Non-NMDA receptor ligands were ineffective.

Effects of cysteine derivatives and sulfhydryl compounds on the binding of [³H]GSH

1. L-cysteine, L-cystamine, L-cysteamine and dithiothreitol inhibited the binding.
2. L-Homocysteinate and aminomethanesulfonate exhibited moderate efficacy as displacers.
3. Thiokynurenate had a concentration-dependent activatory effect.

Effects of dipeptides and glutathione derivatives on the binding of [³H]GSH

1. γ -Glutamylcysteine and L-cysteinyglycine were fairly strong displacers while γ -D-glutamylglycine and γ -glutamylphenylalanine were weak displacers.
2. γ -L-Glutamyl-GABA and γ -L-glutamylleucine enhance the binding in a concentration dependent manner.
3. All glutathione derivatives were effective in the binding, of them GSH and GSNO were the most potent displacers.

Effects of glycine and GABA receptor ligands on the binding of [³H]GSH

These ligands had no effect on the binding.

Effects of chemical modification of the amino acid residues in plasma membrane on the binding of [³H]GSH

Effects of compounds modifying cysteinyl residues and reducing disulfide bonds

1. The oxidising 5,5 dithionitrobenzoate (DTNB) and 4,4-dithiodipyridyl (DDP) strongly enhanced the binding of [³H]GSH in a concentration-dependent manner.
2. Treatment of the plasma membrane fraction with DTNB enhanced the maximal capacity of binding while the affinity of binding was not altered.
3. Phenylisothiocyanate (PITC) and N-ethylmaleimide (NEM) reduced the binding and their effects were additive.

Effects of modification of arginyl residues on the binding of [³H]GSH

1. Modification arginyl residues of plasma membranes with phenylglyoxale (PGO) did not alter the binding.

Effects of modification of glutamyl and aspartyl residues on the binding of [³H]GSH

1. Modification of these side chains in the membranes with EDC had no effect.

Effects of L-cysteine on the glutamate and NMDA-evoked Ca²⁺ uptake and on the intracellular level of free Ca²⁺

1. L-Cysteine alone increased ⁴⁵Ca²⁺ uptake to cerebellar granular cells in a concentration-dependent manner. It also potentiated the Ca²⁺ uptake evoked by glutamate and NMDA.
2. When granular cells were pretreated with ZnCl₂ or when it was administrated together with cysteine the Ca²⁺ efflux stopped.
3. L-Cysteine alone slightly elevated the intracellular level of free Ca²⁺ and potentiated the increase in Ca²⁺ level evoked by glutamate.

DISCUSSION AND CONCLUSIONS

We showed that endogenous GSNO interacts with ionotropic glutamate receptors. We verified that GSNO effectively displaces specific NMDA and kainate receptor ligands and the inhibition is competitive in nature. GSNO directly influences the functions of glutamate receptors. It is suggested that nitrosylation and glutathiolation of receptor proteins evolve from the release of nitrosonium ion (NO^+) and glutathionyl radicals from the GSNO. Hence, GSNO can act as a neuromodulator.

We showed that GSH has receptors of its own in cortical synaptic plasma membranes, consisting of a high- and a low-affinity population. The cysteinyl moiety of the molecule is essential for the binding of GSH to this receptor. According to our results, these specific binding sites for GSH differ from any known excitatory or inhibitory receptor.

On the ground of results from chemical modification of amino-acid side chains in the receptor we assume that glutathione binds to disulfides and free thiol groups in synaptic plasma membranes. The arginyl and lysyl side chains are probably involved in the binding of GSH. We have clearly contributed to the elucidation of the neurotransmitter role of GSH by characterizing the GSH receptors and the interactions of GSH and with these specific binding sites.

We have shown that cysteine increases Ca^{2+} uptake evoked by glutamate and NMDA into cerebellar granular cells. It relieves the block of NMDA receptors by Zn^+ . L-Cysteine alone slightly elevates intracellular Ca^{2+} levels but strongly potentiates the increasing effect of glutamate on the intracellular Ca^{2+} level.

This thesis based on the following original communications.

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