ANDRÁS HERMANN

The Neuromodulatory Roles of Glutathione, S-Nitrosoglutathione and Cysteine in the Central Nervous System

Neurotransmitter Characteristics of Glutathione

■

ACADEMIC DISSERTATION

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CONTENTS

CONTENTS **4**

LIST OF ORIGINAL COMMUNICATIONS **7**

ABBREVIATIONS **8**

ABSTRACT **10**

INTRODUCTION **12**

REVIEW OF THE LITERATURE **13**

1 Glutamate receptor classes **13**
1.1 Ionotropic receptors **13**
1.1.1 N-Methyl-D-aspartate (NMDA) receptors 13
1.1.1.1 Structure and cloning *13*
1.1.1.2 Pharmacology and location *14*
1.1.1.3 Functions of NMDA receptors *16*
1.1.2 2-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors 17
1.1.2.1 Structure and cloning *17*
1.1.2.2 Pharmacology and location *17*
1.1.2.3 Functions of AMPA receptors *18*
1.1.3 Kainate receptors 19
1.1.3.1 Structure and cloning *19*
1.1.3.2 Pharmacology and location *21*
1.1.3.3 Functions of kainate receptors *21*
1.2 Metabotropic receptors **23**
2 Dopamine **25**
3 Glutathione **26**
3.1 Metabolism and functions of glutathione **26**
3.2 Neuromodulatory role of glutathione **27**
3.2.1 Effects of glutathione on ionotropic glutamate receptors 27
3.2.1.1 Effects on NMDA receptors *27*
3.2.1.2 Effects on non-NMDA receptors *28*
3.2.2 Effects of glutathione on the release and uptake of neurotransmitters 29
3.3 Glutathione as a putative neurotransmitter **29**
4 Nitric oxide **30**
4.1 Formation of nitric oxide **31**
4.2 Nitric oxide targets **32**
4.3 Interaction of N-methyl-D-aspartate receptors and nitric oxide **32**
4.4 Role of nitric oxide in neurotoxicity **33**
4.5 Neuroprotective effects of nitric oxide **34**
4.6 Nitrosoglutathione **35**

5 Cysteine 36

AIMS OF THE STUDY 38

MATERIAL AND METHODS 39

1 Preparative methods 39
1.1 Experimental animals 39
1.2 Preparation of synaptic plasma membranes 39
1.3 Cultures of cerebellar granular cells 40
1.4 Chemical modification of amino acid residues in membrane proteins 40
1.4.1 Modification of cysteinyl and lysyl residues 40
1.4.2 Disruption of disulfide bonds 42
1.4.3 Modification of arginyl residues 43
1.4.4 Modification of glutamyl and aspartyl residues 44
2 Superfusion experiments on striatal and hippocampal slices 44
3 Binding assays 44
3.1 [^{3}H]Glutamate binding 44
3.2 [^{3}H]Kainate binding 45
3.3 [^{3}H]Fluorowillardiine binding 45
3.4 [^{3}H]CPP binding 45
3.5 [^{3}H]Dizocilpine binding 45
3.6 [^{3}H]Glutathione binding 46
4 Ca^{2+} uptake experiments 46
5 Measurements of intracellular free Ca^{2+} 46
6 Calculations 47

RESULTS 48

1 Effects of glutamate receptor agonists and glutathione on dopamine release 48
2 Effects of nitrosoglutathione on the binding of ligands to ionotropic glutamate receptors 48
3 Properties of [^{3}H]glutathione binding 49
4 Effects of glutamate derivatives and glutamate receptor ligands on the binding of [^{3}H]glutathione 49
5 Effects of glycine and GABA receptor ligands on the binding of [^{3}H]glutathione 49
6 Effects of cysteine, cysteine derivatives and sulfhydryl compounds on the binding of [^{3}H]glutathione 49
7 Effects of some dipeptides and glutathione derivatives on the binding of [^{3}H]glutathione 50
8 Effects of chemical modification of cysteinyl residues and disulfide bridges on the binding of [^{3}H]glutathione 50
9 Effects of modification of arginyl residues on the binding of [^{3}H]glutathione 51
10 Effects of modification of glutamyl and aspartyl residues on the binding of [^{3}H]glutathione 51
11 Effect of cysteine on the neuronal $^{45}Ca^{2+}$ influx and the level evoked by glutamate and N-methyl-D-aspartate 51
12 Effect of cysteine on intracellular free Ca^{2+} 52

13 Effect of cysteine on the release of D-aspartate from rat hippocampal slices 52

DISCUSSION 52

1 Methodological considerations 52
1.1 Dopamine and glutamate release 52
1.2 Binding experiments 53
1.3 Chemical modification of receptor proteins 53
2 Regulation of dopamine release by glutamate receptor ligands and glutathione 54
3 Interactions of nitrosoglutathione with ionotropic glutamate receptors 54
4 Binding of glutathione to synaptic plasma membranes 56
5 Role of amino-acid side-chains in glutathione binding 58
6 Effect of cysteine on Ca^{2+} influx, intracellular free Ca^{2+} and glutamate release 59

SUMMARY 61

ACKNOWLEDGEMENTS 62

REFERENCES 63

ORIGINAL COMMUNICATIONS 87

LIST OF ORIGINAL COMMUNICATIONS

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II. Hermann, A., Varga, V., Janáky, R., Dohovics, R., Saransaari, P. and Oja, S.S. (2000) Interference of S-nitrosoglutathione with the binding of ligands to ionotropic glutamate receptors in pig cerebral cortical synaptic membranes. Neurochem. Res. 25:1119-1124.

III. Janáky, R., Shaw, C.A., Varga, V., Hermann, A. Dohovics, R., Saransaari, P. and Oja, S.S. (2000) Specific glutathione binding sites in pig cerebral cortical synaptic membranes. Neuroscience 95:617-624.

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V. Hermann, A., Janáky, R., Dohovics, R., Saransaari, P., Oja, S.S. and Varga, V. (1999) Potentiation by L-cysteine of N-methyl-D-aspartate receptor effects on intracellular free Ca^{2+} in cultured cerebellar granule cells. Proc. West. Pharmacol. Soc. 42:25-26.

VI. Janáky, R., Varga, V., Hermann, A., Saransaari, P. and Oja, S.S. (2000) Mechanisms of L-cysteine neurotoxicity. Neurochem. Res. 25:1397-1405.

ABBREVIATIONS

ADP	adenosinediphosphate
ACPT-I	(1S,3R,4R)-1-aminocyclopentane-1,3,4-tricarboxylate
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APV	DL-2-amino-5-phosphonovalerate
BSA	bovine serum albumin
CBPG	2-(3'-carboxybicyclo[1.1.1]pentyl)glycine
[^{3}H]CGP	DL-(E)-2-[^{3}H]amino-4-propyl-5-phosphono-3-pentanoate
cGMP	cyclic guanosine-3',5'-monophosphate
CHPG	(RS)-2-doro-5-hydroxyphenylglycine
4C3HPG	4-carboxy-3-hydroxyphenylglycine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPP	3-[(R,S)-carboxypiperazin-4-yl]propyl-1-phosphonoate
CPPG	α-cyclopropyl-4-phophonophenylglycine
Cys-NO	S-nitrosocysteine
DAG	diacylglycerol
D-AP5	D-2-amino-5-phosphonovalerate
DCG-IV	(2S,S'R,3'R)-2-(2,3-dicarboxcycloprpyl)glycine
DCQX	6,7-dinitroquinoxaline-2,3-dione
DDP	4,4-dithiodipyridine
3,5-DHPG	3,5-dihydroxyphenylglycine
DMSO	dimethylsulfoxide
DNQX	6,7-dinitroquinoxaline-2,3-dione
DTNB	5'5-dithio-bis-2-nitrobenzoate
DTT	D,L-dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodimiide
EDTA	ethylenediaminetetra-acetate
EPSP	excitatory postsynaptic potential
FAD	flavin adeninedinucleotide
FMN	flavin mononucleotide
[^{3}H]FWD	(S)-5-fluoro[^{3}H]willardiine
FWD	(S)-5-trifluoromethyl-willardiine
GABA	γ-aminobutyric acid
GSA	glutathionesulphonate
GTP	guanosine triphosphate
iGluR	ionotropic glutamate receptor
IP_3	inositol-1,4,5-triphosphate
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	oxidized glutathione
GYKI53655	1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5 H -2,3-benzodiazepine
HA-966	1-hydroxy-3-amino-2-pyrrolidone
HEK293	human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate
KRH	Krebs-Ringer-HEPES

L-AP3	L(+)-2-amino-3-phosphonopropionate
L-AP4	L(+)-2-amino-4-phosphonobutyrate
L-AP5	L(+)-2-amino-5-phosphonovalerate
L-SOP	L-serine-O-phosphate
LTD	long-term depression
LTP	long-term potentiation
LY367385	(S)-2-methyl-4-carboxyphenylglycine
LY379268	1R,4R,5S,6R)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate
LY215490	racemic decahydroisoquinoline
LY294486	((3SR, 4aRS, 6SR, 8aRS)-6-({[(1H-tetrazol-5-yl)methyl]oxy} methyl)1,2,3,4,4a, 5,6,7,8,8a decahydroisoquinoline-3-carboxylate
LY307452	(2S, 4S)-2-amino-4-(4,4-diphenylbut-1-yl)pentane-1,5-dioate
LY341495	2S-2-amino-2-(1S,2S-2-carboxycycloprop-1-yl)-3-xanth-9-yl) propanoate
LY354740	(1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate
LY393675	α-thioxanthylmethyl-3-carboxycyclobutylglycine
MAP4	α-methyl-AP4
MCCG-I	α-methyl-L-CCG-I
MNQX	5,6-dinitroquinoxaline-2,3-dione
MK-801	dizocilpine, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate
MPEP	2-methyl-6-(phenylethynyl)pyridine
NAAG	spagutamate, N-acetyl-L-aspartyl-L-glutamate
NADPH	nicotinamide adenine dinucleotide phosphate
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzol[f]quinoxaline-7-sulfonamide
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PCP	phencyclidine
PGO	phenylglyoxale
PITC	phenylisothiocyanate
PKC	protein kinase C
PPG	phosphonophenylglycine
SBG	S-butylglutathione
SEG	S-ethylglutathione
SIN-1	3-morpholinosydnonimine
SMG	S-methylglutathione
SNAP	S-nitroso-N-acetylpenicillamine
SOD	superoxide dismutase
SPG	S-propylglutathione
SpeG	S-pentylglutathione
SYM 2081	(2S-4R)-4-methylglutamate
TES	2-[3-hydroxy-1,1-bis(hydroxymethyl)ethyl]-aminoethanesulphonate
trans-ACPD	trans-1-aminocyclopentane-1,3-dicarboxylate

ABSTRACT

Glutathione (γ-glutamylcysteinylglycine, reduced glutathione, GSH) is the most abundant thiol-containing peptide in the central nervous system. It is an ancient multifunctional molecule, e.g. a redox agent and free radical scavenger. Since the three constituent amino acids of the glutathione molecule are neuroactive (of them glutamate and glycine are neurotransmitters) it was reasonable to investigate its possible role in glutamate neurotransmission. Glutathione may act as a neuromodulator and neurotransmitter.

Among other actions glutathione interferes with glutamatergic neurotransmission and also affects the release of different neurotransmitters. The cerebral cortex is joined to the neostriatum through the corticostriatal pathway, which exercises complex control over dopamine release. Glutathione indeed directly facilitates the release of dopamine in the mouse striatum via activation of ionotropic glutamate receptors at the nigrostriatal dopaminergic nerve endings.

GSH has been shown to elicit concentration-dependently excitatory field potentials in a cortical wedge preparation, these not being blocked by any antagonist of ionotropic glutamate receptors. This has led to the hypothesis that GSH has receptors of its own. To prove that GSH in fact possesses receptors which differ from any glutamate receptor, and to shed light on structure-function relationships, a detailed pharmacological characterization of [^{3}H]GSH binding was undertaken using specific ligands of glutamate receptors, γ-glutamyl and other dipeptides, cysteine, derivatives of cysteine and analogs of GSH. GSH indeed proved to possess receptors of its own with high and low affinity in cortical synaptic plasma membranes. Of the compounds tested, mainly those containing cysteine were effective in displacing [^{3}H]GSH. This group is thus essential for the binding. According to our results these specific binding sites of GSH differ from any known excitatory or inhibitory glutamate receptor.

In order to determine which amino-acid side-chains of plasma membranes are involved in the binding of glutathione, cysteinyl, arginyl, lysyl, carboxyl and aspartyl residues in pig synaptic membranes were chemically modified and then subjected to radioactive ligand binding assays. The cysteinyl side-chains of plasma membranes were treated with 5-dithio-bis-2-nitrobenzoate and 4,4-dithiodipyridine, which enhanced the binding of [^{3}H]GSH, while N-ethylmaleimide and phenylisothiocyanate reduced it. The binding of [^{3}H]GSH was decreased when disulfide bonds were broken with dithiothreitol. Treatment of arginyl side-chains with phenylglyoxale reduced the binding, whereas modification of carboxyl and aspartyl residues were without effect. It seems that disulfides, cysteinyl and probably lysyl and arginyl side-chains in the plasma membranes are involved in the binding of GSH.

GSH can react with the gaseous odd messenger nitric oxide (NO) to form S-nitrosoglutathione (GSNO). The packaging of NO in nitrosothiol form may prolong its actions and facilitate its delivery to the specific target proteins. However, liberation of NO may not account for all the biological activities of nitrosothiols. We therefore studied the effects of GSNO on the binding of ligands to different ionotropic glutamate receptors in pig cerebral cortical synaptic membranes. GSNO interacted with these receptors by means of its γ-glutamyl

moiety and displaced specific ligands of the N-methyl-D-aspartate (NMDA) and kainate receptors.

So far, little has been known regarding the action mechanism(s) of the third constituent amino acid in glutathione, i.e., cysteine, which is toxic in excess. We therefore estimated how cysteine affects the NMDA-receptor-dependent Ca^{2+} fluxes across plasma membranes and the intracellular levels of free Ca^{2+} in cerebellar granule cells in culture. Cysteine increased the Ca^{2+} flux evoked by glutamate and NMDA. It also elevated the levels of free intracellular Ca^{2+} and potentiated the increase evoked by glutamate. In addition, the effect of cysteine was investigated on the release of glutamate from hippocampal slices. It evoked glutamate release and potentiated the release of glutamate evoked by KCl. Cysteine toxicity probably evolves from these mechanisms.

INTRODUCTION

Communication between neurons and neurons and glia in the brain occurs primarily through synapses made onto elaborate treelike dendrites. The varying morphology and electrical and chemical properties of dendrites make possible a spectrum of local and long-range signaling, defining the input-output relationship of neurons and the rules for induction of synaptic plasticity (Hausser et al., 2000). The signals between neurons and glia include ion fluxes, neurotrasmitters, cell adhesion molecules and specialized signaling molecules released from synaptic and non-synaptic regions of the neuron. By releasing neurotransmitters and other extracellular signaling molecules, both glia and neurons can affect neuronal excitability and synaptic transmission (Fields et al., 2002).

In the central nervous system (CNS) the excitatory and inhibitory neurotransmission underlie communication between different cells. Transmitters are released into the synaptic cleft, developing their adequate responses on the post- or presynaptic membrane.

The main neurotransmitters are amino acids, being excitatory (e.g., glutamate and aspartate) or inhibitory [e.g., γ-aminobutyrate (GABA) and glycine] (Oja et al., 1977; Monaghan et al., 1989). Glutamate is the main excitatory transmitter (Roberts et al., 1976). In the medulla oblongata and spinal cord glycine is inhibitory, whereas in the higher brain regions it is predominantly excitatory, potentiating the effects of glutamate. Based on pharmacological and electrophysiological studies, glutamate receptors are divided into two families: ionotropic N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors, and metabotropic receptors.

Endogenous chemical compounds and numerous other factors exist in the brain interacting with these receptors and possibly modulating the neurotransmission or playing roles in up- or downregulation of intercellular interactions. These neuromodulators can be ions, gaseous molecules, amino acids or peptides.

γ-Glutamylcysteinylglycine (GSH) is a phylogenetically ancient tripeptide ubiquitous in living organisms. It is the most abundant thiol-containing peptide in the CNS (Orlowski and Karkowsky, 1976), and best known as a redox agent, free radical scavenger and antioxidant. It protects cell membranes against oxidative stress, DNA against radiation and ultraviolet light and the whole cell against xenobiotics (Meister and Anderson, 1983). The three constituent amino acids of the glutathione molecule are neuroactive. Two of them, glutamate and glycine, are neurotransmitters in the CNS, whereas cysteine in excess is excitotoxic. Due to its chemical structure, GSH may function both as a neuromodulator and as a neurotransmitter of its own. However, very little is known of these actions. One of its neuromodulatory roles consists in its capacity to react with the gaseous messenger nitric oxide (NO). The result of this reaction is S-nitrosoglutathione (GSNO) (Stamler et al., 1992), the function of which has so far remained totally unknown.

The aim of this study was to characterize the neuromodulatory and putative neurotransmitter roles of glutathione in neurotransmission. In addition, the implication is proposed of the constituent amino acid L-cysteine of the glutathione molecule in neurotoxicity.

REVIEW OF THE LITERATURE

1 Glutamate receptor classes

In the mammalian brain several pharmacologically and structurally different groups of receptors for acidic amino acids have been identified. The common endogenous ligand of these receptors is L-glutamate, the most important excitatory amino acid (Collingridge and Lester, 1989; Nakanishi, 1992). Glutamate receptors are divided into two families: *ionotropic* and *metabotropic*. The former are integral proteins of the neuronal plasma membrane. They consist of a number of subunits and form cation-selective ionophores. These receptors belong to the following three classes: N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate, named after their selective synthetic agonists (Monaghan, 1989; Watkins et al., 1990; Récasens et al., 1992). The AMPA and kainate receptors are also referred to as non-NMDA receptors. The metabotropic receptors, for their part, are coupled to intracellular GTP-binding (G) proteins and evoke cellular responses via regulation of the inositolphospholipid metabolism (Conn and Desai, 1991) or intracellular cyclic nucleotide synthesis (Nakanishi, 1992; Monaghan and Wenthold, 1997).

1.1 Ionotropic receptors

1.1.1 N-Methyl-D-aspartate (NMDA) receptors

1.1.1.1 Structure and cloning

The native NMDA receptor is a heteromultimeric channel comprising the ubiquitous NMDAR1 subunit and at least one regionally localized NMDAR2 subunit. The NMDAR3A subunit is related to the NMDA receptor subunits and functions as a regulatory subunit of the NMDAR channels, and is involved in the development of dendritic spines (Das et al., 1998)**.** These subunits are coded by three gene families which have been cloned from the mouse, rat and man (Moriyoshi et al., 1991; Ikeda et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Le Bourdallés et al., 1994; Hollmann and Heinemann, 1994, Dingledine, 1999). The NMDAR1 subunit is encoded by one gene**.** Eight variants of this subunit (1 a/b to 4 a/b) are known, which differ in the amino-acid sequences of the extracellular N-terminal and the intracellular C-terminal. Four genes encode the NMDAR2A-D subunits. The common feature of these latter subunits is a long intracellular C-terminal domain which contains potential phosphorylation sites (Chen and Huang, 1992, Hollmann and Heinemann, 1994). Therefore certain protein kinases (e.g. protein kinase C), the Ca^{2+}-calmodulin kinase II and some phosphatases (e.g. calcineurine) are involved in synaptic plasticity processes such as long-term potentiation (LTP) or depression (LTD) (Wan and Feng, 1992). Depending on the stimulation frequency and the intracellular Ca^{2+} level present upon stimulation, Ca^{2+} entering through the NMDA receptors can initiate both of these processes (Mulkey and Malenka, 1992). A high postsynaptic intracellular Ca^{2+} concentration promotes potentiation

while a lower concentration evokes depression. In the ionotropic glutamate receptor subunits, the extracellular N-terminal and intracellular C-terminal consist of three membrane-spanning hydrophobic domains (M1, M3, M4) and a membrane-associated domain (M2) (Henley, 1994; Hollmann and Heinemann, 1994; Wo et al., 1995; Hirai et al., 1996; Sutcliffe et al., 1996; Paas, 1998). The M2 domain forms a hairpin loop which enters the membrane from the cytoplasmic side, turns around in the plane of the membrane, and returns to the cytoplasmic side. It appears to form a pore in a manner similar to the P segment of the voltage-dependent potassium and cyclic nucleotide-gated channels (Bennett and Dingledine, 1995; Kuner et al., 1996). The segment of the extracellular N-terminal immediately preceding the M1 domain (S1 region) and the loop between the M3 and M4 domains contains the side-chains responsible for the binding of ligands (O'Hara et al., 1993; Kuusinen et al., 1995; Tygesen et al., 1995; Chen and Gouaux, 1997). The site-directed mutagenesis of some amino acids in these segments abolishes the binding of ligands (Kuryatov et al., 1994; Hirahi et al., 1996; Laube et al., 1997). The M2 region of all ionotropic glutamate receptors shares the same so-called Q/R/N (glutamine/arginine/asparagine) site. In each NMDA receptor this common site is occupied by asparagine, which determines the Ca^{2+} permeability of the ionophore (Hume et al., 1991). Substitution of this asparagine by glutamine in the NMDAR1 subunits reduces Ca^{2+} permeability. Similar substitution in the NMDAR2 subunits alleviates Mg^{2+} blockage of the channel (Burnashev et al., 1992).

1.1.1.2 Pharmacology and location

The NMDA receptor subunits form a complex comprising an ion channel which is selective for Ca^{2+}. However, Na^{+} and K^{+} also enter through NMDA receptor channels, albeit at a rather low rate. The complex contains several binding sites. (1) An agonist binding site for excitatory amino acids which specifically binds endogenous L-glutamate, L-aspartate, L-homocysteate, L-cysteinesulfinate and quinolinate as well as synthetic NMDA (Collingridge and Lester, 1989, Récasens et al., 1992; Stone, 1993). The competitive antagonists bind to the same site, e.g. L-2-amino-5-phosphonovalerate (L-AP5) and 3-[(R)-carboxypiperazine-4-yl]propyl-1-phosphonate (CPP). (2) A strychnine-insensitive glycine coactivatory site which binds D-serine and D-alanine in addition to glycine. D-serine is a predominant endogenous ligand for this site. D-serine is synthesized by means of the serine racemase enzyme and released from glial cells by non-NMDA receptor agonists (Wolosker et al., 1999). This site can be blocked by the glycine antagonist 1-hydroxy-3-amino-2-pyrrolidone (HA-966) and kynurenate, 7-chlorokynurenate and 6,8-dinitroquinoxalinedione (MNQX) (Table 1). (3) Inside the channel, close to the intracellular surface, an Mg^{2+} binding site is located which plays a role in the voltage-dependent block of the ion channel (Nowak et al., 1984). (4) A phencyclidine (PCP) binding site likewise located inside the channel. In addition to PCP, other non-competitive NMDA-antagonistic dissociative anesthetics bind to this site, e.g. ketamine, dextrophane, desipramine and (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine hydrogen maleate (MK-801, dizocilpine). These compounds bind to the open channel, indicating its activation (Anis et al., 1983; Collingridge and Lester, 1989; Lodge and Johnson, 1990). (5) A Zn^{2+}-binding site which mediates the

voltage-independent channel block (Peters et al., 1987). (6) A polyamine-binding site, to which is bound putrescine, the endogenous activatory spermine and spermidine, and the polyamine-antagonist neuroprotective ifenprodil (Ransom and Stec, 1988; Carter et al., 1989, Williams et al., 1989; Lodge and Johnson, 1990) (Fig 1A). (7) Proton (H^+) binding sites in which the binding of protons produces an allosteric block of the receptor (Aizenman et al., 1989; Yoneda et al., 1994). Ifenprodil enhances the potency of ambient protons to block the NMDA receptors (Mott et al., 1998).

In addition to the above, a number of other compounds may affect the function of NMDA receptors (e.g., ethanol (Lovinger et al., 1989, 1990; Buller et al., 1995), gangliosides, insulin (Liu et al., 1995) and histamine (Vorobjev et al., 1993)), although the exact sites of their effects are not known. The two last-mentioned are stimulatory. Moreover, NMDA receptors contain several redox modulatory sites. Köhr and associates (1994) carried out a detailed characterization of the effects of redox agents on the recombinant NMDA receptors expressed in the human embryonic kidney (HEK 293) cells. They discovered pronounced subunit-dependent differences in the actions of thiol compounds on these receptors. The effects of dithiothreitol (DTT) on NR1/NR2A receptors had rapidly developing and slowly developing components. For the slow component two cysteine (cys744 and cys798) residues were responsible in the NR1 subunit and for the rapid component cys370 in the N-terminal of the NMDA receptor. The receptors of NR1/NR2B, NR1/NR2C and NR1/NR2D subunit compositions possess only the slow redox modulatory site (Paoletti et. al. 1997). Choi and co-workers (2000) inferred that DTT exerts its potentiating effects not only through reduction of the rapid redox modulatory site of the subunit, but also by chelation of Zn^{2+}. The activity of the NMDA receptors is also influenced by free radicals and thiol-redox agents, e.g. nitric oxide (NO) or glutathione (Aizenman et al., 1989, 1990; Levy et al., 1990; Sucher and Lipton, 1991; Lipton et al., 1993; Sullivan et al., 1994). Several interactions between the above binding sites are possible. The agonist and antagonist binding sites co-operate positively and the co-agonist glycine and the agonist glutamate mutually facilitate their bindings (Monaghan et al., 1988). Similarly, the partial glycine agonist HA-966 activates binding of the antagonist CPP (Compton et al., 1990). The antagonist of one site, in turn, inhibits association of the agonist with the other site (Monaghan et al., 1988; Kaplita and Ferkany, 1990; Monahan et al., 1990). By reason of the heterogeneity of NMDA receptors their pharmacology is very complex. During development, the distribution of NMDA receptor binding sites is altered, being in the adult brain considerably different. The receptors in different brain regions show different affinities for CPP (van Amsterdam et al., 1992), glycine (Sakurai et al., 1993) and the stimulating effects of polyamines are likewise dissimilar (McGonigle et al., 1991). Based on the above, it is plausible to suggest that there exist agonist- and antagonist-preferring sub-populations or conformational states of NMDA receptors (Monaghan et al., 1988), whose distribution differs from one brain region to another.

The NMDAR1 is the subunit in each NMDA receptor which is found in all brain regions (Moriyoshi et al., 1991; Meguro et al., 1992; Shigemoto et al., 1992), whereas the NMDAR2A-D subunits show characteristic distributions. The NMDAR2A subunit is present predominantly in the forebrain (cortex), hippocampus and cerebellum and corresponds to the antagonist-preferring sub-population (Meguro et al., 1992; Monyer et al., 1992). The NMDAR2B is most

abundant in the forebrain, NMDAR2C in the cerebellum and NMDAR2D in the diencephalon and lower brain regions (Kutsuwada et al., 1992; Meguro et al., 1992: Monyer et al., 1992). In the striatum the presence of only NMDAR2A and B subunits has been demonstrated (Nakanishi et al., 1992). The characteristics of the recombinant heteromeric NMDA channels were originally addressed by Stern and associates (1992, 1994). Heteromeric NMDA channels can be primarily differentiated into two types: high- and low-conductance forms. High-conductance types of NMDA channels include NR1/NR2A and NR1/NR2B, whereas the NR1/NR2C and NR1/NR2D subunits are low-conducdance.

1.1.1.3 Functions of NMDA receptors

The combined effects of the agonist glutamate and the co-activators glycine and D-serine activate the NMDA receptor-ion channel complex. The consequent membrane depolarization alleviates the voltage-dependent block, the ionophore opens and Ca^{2+} enters the cell. This Ca^{2+} influx triggers a number of intracellular events: (1) Activation of phospholipase A2 (PLA2) which generates arachidonic acid (Dumuis et al., 1988). This enhances the presynaptic release and inhibits the glial uptake of glutamate (Chan et al., 1983). Hence the postsynaptic Ca^{2+} influx increases (Miller et al., 1992). (2) The increase in the intracellular Ca^{2+} level activates nitric oxide synthase, which generates NO. This in turn activates the synthesis of cyclic guanosine-3',5'-monophosphate (cGMP) in the nerve endings and evokes glutamate uptake (Garthwaite et al., 1988). (3) Activation of ornithine decarboxylase results in the production of polyamines which enhance Ca^{2+} influx through the NMDA receptors (Porcella et al., 1992). (4) Activated protein kinase C (PKC) phosphorylates the receptor protein and alleviates its Mg^{2+} block (Chen and Huang, 1992; Tingley et al., 1993; Roche et al., 1994). (5) Immediate early genes are expressed, the transcripts of which regulate target gene expression. Among the potential target genes are those inducing apoptosis (Chen et al., 1995).

In the mammalian brain, the NMDA receptors play a pivotal role in normal neurotransmission and induction of activity-dependent synaptic plasticity such as LTP and LTD. Synaptic plasticity is determined by morphological and functional changes in synapses, which are the basis for storing information and modulating behavior. The NMDA receptor-dependent LTP requires two simultaneous signals for its induction: presynaptically released glutamate has to bind to the NMDA receptors and the postsynaptic membrane must be depolarized (Malgaroli, 1994; Kullmann et al., 2000). The postsynaptic Ca^{2+} accumulation is a key event in LTP induction. The activation of Ca^{2+}-dependent protein kinases modulates LTP induction.

Recent findings, however, would indicate the involvement of a complex multiprotein machinery in producing NMDA signaling at postsynaptic dendritic spines. The NMDA receptors are only a part of this machinery, while the AMPA receptors are present in separate complexes. In this new kind of signaling machinery, several proteins are involved: cell-adhesion proteins, adaptor molecules, cytoskeletal proteins and many downstream signaling molecules (kinases, phosphatases) (Grant et al., 2001).

In addition to these, several molecules function as retrograde signals during LTP, e.g. nitric oxide (Zhuo et al., 1993; Schuman and Madison, 1994), carbon monoxide (Stevens and Wang, 1993) and arachidonic acid (Williams et al., 1993).

The NMDA receptors may be regarded as molecular coincidence detectors (Seeburg, 1993), which function upon the sustained depolarization evoked by tetanic stimulation of AMPA/kainate receptors. The NMDA receptors generate the delayed postsynaptic excitatory potential (EPSP). In this way they serve as sensors of the synchronous activity of pre- and postsynaptic neurons. Ca^{2+} influx through the activated NMDA receptors may evoke the release of other neurotransmitters, e.g. acetylcholine, dopamine (Cai et al., 1991) and noradrenaline (Wang et al., 1992).

1.1.2 2-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors

1.1.2.1 Structure and cloning

According to our present knowledge, the AMPA receptors consist of four subunits (iGluR1-4 or iGluRA-D). These are encoded by four different genes cloned from the mouse, rat and human (see for review Hollmann and Heinemann, 1994). The homology of other subunits in these three species is 68-73%, but in the case of iGluR1 96-97% (Hollmann and Heinemann, 1994). All subunits include a section close to the end of the C-terminal which regulates the desensitization of AMPA receptors. The subunits possess two different primary sequences in this section, which are generated by the alternative splicing of mRNA. These two splice variants are called flip and flop (Sommer et al., 1990). There are three membrane-spanning domains (M1, M3, M4) and a membrane-associated hairpin-like M2 domain. Similarly to the NMDA and kainate receptors, the AMPA receptors are heteromeric receptor-channel complexes comprising four subunits. Comparison of the amino acid sequences in the subunits revealed that in the M2 domain of iGluR2 there is exists an arginine (R) residue, while the M2 domain in other iGluR subunits contains glutamine (Q). Arginine is not in fact encoded in the gene of the iGluR2 subunit; the original glutamine is replaced by arginine by the editing of mRNA (Sommer et al., 1991). It alters the permeability of the ionophore and renders it less permeable to divalent cations. iGluR2 seems to be the dominant subunit determining the gating, ion conductance and permeability properties, which are similar to those of native AMPA receptors. The AMPA receptor-ionophores containing iGluR2 subunits are highly permeable to Na^+ and K^+ but much less so to Ca^{2+} (Hollmann et al., 1994). Recombinant subunits may form functional channels independently of the heteromeric or homomeric construction of the receptor (Hollmann and Heinemann, 1994). The topology of the AMPA receptors is similar to that of the NMDA receptors.

1.1.2.2 Pharmacology and location

The endogenous agonist ligands for AMPA receptors are L-glutamate and L-aspartate. Glutamate is a more potent agonist in the iGluRA/B than in the iGluRB/D subunit combination. AMPA receptor-selective agonists are AMPA, some AMPA derivatives (Krogsgaard-Larsen et al., 1980), natural willardiine and its derivatives (Watkins et al., 1990; Hawkins et al., 1995) and plant-derived β-N-oxalylamino-L-alanine and β-N-methylamino-L-alanine (Gill and Lodge, 1997).

In addition, the AMPA receptors can also be activated by domoate, kainate and quisqualate. Cyclothiazide only functions as an allosteric modulator of AMPA receptors and suppresses the desensitization of these receptors, thus enhancing their responses (Yamada and Turetsky, 1996). The competitive antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) are approximately five times more selective for AMPA than for kainate receptors (Honoré et al., 1988). Competitive antagonists are also 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzol[f]quinoxaline-7-sulfonamide (NBQX), with 30 times higher efficacy at AMPA than at kainate receptors (Lodge et al., 1991), and racemic decahydroisoquinoline (LY215490) (Gill and Lodge, 1997). GYKI53655 is a selective non-competitive antagonist (Fig. 1B, Table 1). In addition, barbiturates, e.g. methohexiton, pento- and phenobarbital, are also non-competitive antagonists of AMPA receptors (Gill and Lodge, 1977). Another non-competitive antagonist, agriotoxin, is selective for iGluR1, 3 and 4 subunits. It blocks the open AMPA receptor-ionophore in a voltage-sensitive manner (Blaschke et al., 1993; Herlitze et al., 1993). This toxin, isolated from the Joro spider, affects the Q/R/N site of the M2 subunit, which is responsible for Ca^{2+} selectivity. It does not block the edited (R) version and thus may help in the identification of nerves which contain Ca^{2+}-selective AMPA receptors.

The intracellular C-terminal region in AMPA receptors contains potential phosphorylation sequences. The iGluR1 subunit is phosphorylated at two sites by cyclic adenosine 3',5'-monophosphate (cAMP) -dependent PKC and at one site by protein kinase A (PKA) (Roche et al., 1996). By means of phosphorylation at these sites, the opening time and opening probability of the receptor-associated channel is altered. There are five glycosylation sites in the iGluR2 and iGluR3 and six in the iGluR1 and iGluR4 subunits (Hullebroeck and Hampson, 1992). They may regulate the assembly of subunits and maintain receptor conformation for the binding of ligands (Kawamoto et al., 1994, 1995). The iGluR1-4 subunits are found mainly postsynaptically (Petralia and Wenthold, 1992), but the presynaptic presence of iGluR1 and iGluR3 subunits has also been observed in the spinal cord (Ye and Westlund, 1996). There are characteristic differences in the distribution of the four subunits and their flip and flop variants in the CNS. Cerebellar granule cells express mainly the iGluR2 and iGluR4 subunits and Bergman glial cells only iGluR2 and iGluR4. The hippocampal CA1 region is characterized by strong and the CA3 region by weak iGluR4 expression (Keinänen et al., 1990).

1.1.2.3 Functions of AMPA receptors

The AMPA receptors containing iGluR2 subunits are primarily responsible for fast excitatory glutamate transmission (Monaghan et. al. 1989). Upon their activation the channels open and monovalent cations flow into the intracellular space down to their concentration gradients, this evoking rapid postsynaptic depolarization. Na^+ and K^+ conductances induce monosynaptic fast EPSPs. The co-location of NMDA and AMPA receptors (Nicoll et al., 1990) may be responsible for the repetitive activation of NMDA receptors and the generation of LTP. The AMPA receptor complexes lacking iGluR2 subunits may play a direct role in LTP generation due to their Ca^{2+} permeability, or glutamate can trigger neurotransmitter release from these cells (Iino et al., 1990).

The memory-enhancing effects of allosteric potentiators have given impetus to suggestions that such compounds may relieve memory deficits (Stäubli et al., 1994). AMPA receptor antagonists protect against the cell death caused by global and local ischemia (Sheardown et al., 1990) and may help in the prevention of brain damage by other pathologic processes or traumas. Antagonists may also be useful as anticonvulsants. Since AMPA receptors may serve in the greater part of fast excitatory neurotransmission, it is probably undesirable to block every AMPA receptor in the CNS. The invention of drugs selectively targeting particular AMPA receptor subtypes is therefore an important goal.

1.1.3 Kainate receptors

1.1.3.1 Structure and cloning

Five different subunits of kainate receptors have so far been cloned: iGluR5 (Bettler et al., 1990; Sommer et al., 1992), iGluR6 (Egebjerg et al., 1991) and iGluR7 (Bettler et al., 1992), which are combined to the KA1 (Werner et al., 1991) and KA2 (Herb et al., 1992; Sakimura et al., 1992) subunits. The subunits are encoded by two gene families (see for review Hollmann and Heinemann, 1994; Korczak et al., 1995).

The sequence homology between the members of the two families is 43%. Co-expression of iGluR5 to 7 with the KA1 or KA2 subunits leads to the assembly of functional heteromeric channel complexes. In addition, iGluR5, 6 and 7 may form homomeric complexes whose properties are very similar to those of native receptors (Schiffer et al., 1997). However, the KA1 and KA2 subunits can form neither homomeric nor heteromeric channels with each other (Seeburg et al., 1993).

Kainate receptors are believed to share the same transmembrane topology and stoichiometry as AMPA and NMDA receptors (Laube et al., 1998; Rosenmund et al., 1998). They are thus thought to be tetramers in which each monomer carries its own ligand binding site and contributes with a specific amino acid stretch to the formation of the channel lumen (M2 segment) composed of hydrophobic residues which dip into the membrane, forming a hairpin-like structure. The number of isoforms derived from alternative splicing has revealed that the structural repertoire of the kainate receptor subtype is not limited to the above five proteins. Another source of the structural variability of kainate receptors is mRNA editing. The glutamine/arginine site of the M2 domain in iGluR6 and iGluR7 can undergo mRNA editing, which modifies ion permeability (Sommer et al., 1992). In iGluR6 subunits two further amino acids of M1 also can undergo RNA editing: one isoleucine for valine and one tyrosine for cysteine (Köhler et al., 1993). The ion selectivity of the homomeric iGluR6 channels is determined by RNA editing in a manner opposite to that of the AMPA receptors. When both amino acids are edited in M1, the channels with genomic M2 conduct Na^+ and K^+, but the editing in M2 (arginine at the Q/R site) results in Ca^{2+}-selective channels (Egebjerg and Heinemann, 1993). There are still further sites in the subunits of kainate receptors which modify their functions, e.g. a glycosylation site in the extracellular domains (Wo et al., 1995), and the intracellular C-terminal segment possesses consensual phosphorylation sites for PKA (Wang et al., 1993).

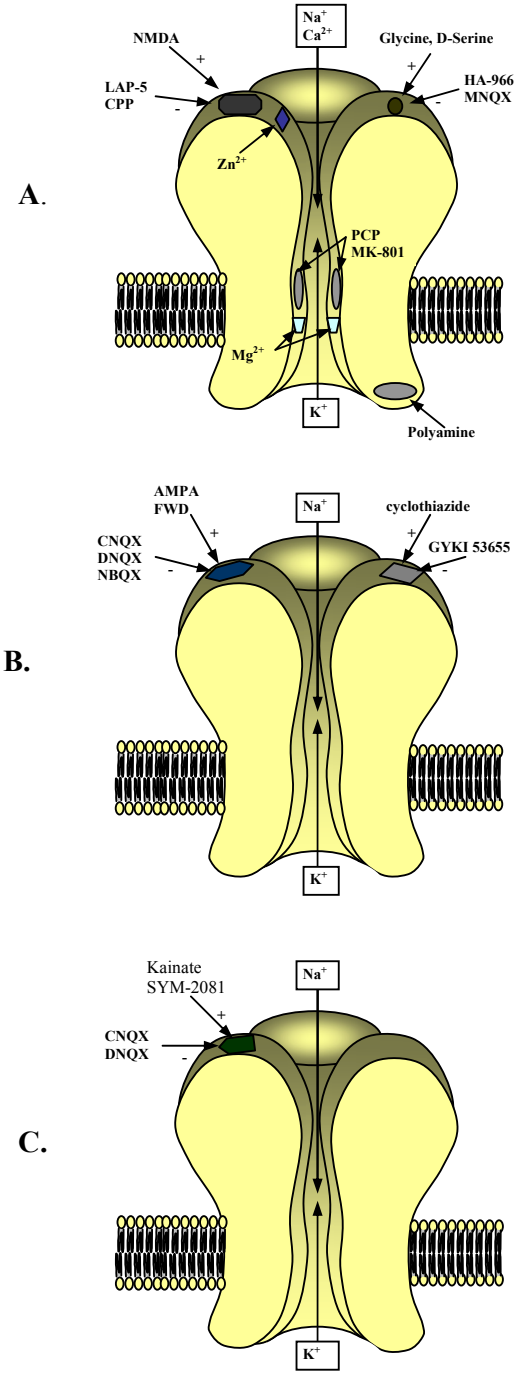

Fig. 1. (A) Pharmacological properties of NMDA receptors. The NMDA receptor gates a cation channel which is permeable to Na^+, Ca^{2+} and K^+ and blocked by Mg^{2+} in a voltage-dependent manner. The NMDA receptor channel is blocked by MK-801, PCP and regulated at the glycine, Zn^{2+} and polyamine modulatory sites. CPP and LAP-5 are competitive antagonists, whereas HA-966 and MNQX are antagonists at the modulatory site. (B) Pharmacological properties of AMPA receptors. The AMPA receptors gate cation conductances, which underlie fast depolarizing responses at most excitatory synapses. AMPA and FWD are agonists while CNQX, DNQX and NBQX are competitive antagonists. Cyclothiazide, one of the allosteric modulators, and GYKI53655 are effective allosteric inhibitors. (C) Pharmacological properties of kainate receptors. Kainate receptors mediate rapidly desensitizing currents. Kainate and SYM-2081 are the most effective agonists while CNQX and DNQX are specific antagonists.

1.1.3.2 Pharmacology and location

Kainate can activate different ionotropic receptors, including AMPA (Patneau and Meyer, 1991), kainate receptors (Agrawal and Evans, 1986; Huettner, 1990) and small-molecular-weight kainate binding-proteins in some animal species (Henly, 1994). Although kainate activates these receptors with a lesser efficacy than AMPA, glutamate or quisqualate, it evokes a stronger ion current than these agonists (Patneau and Meyer, 1990). Kainate and domoate (Egebjerg et al., 1990; Sommer and Seeburg, 1992) and (S)-5-trifluoromethyl-willardiine (FWD) (Wang et al., 1993) are selective agonists of the kainate receptors (Fig. 1C). In addition, (2S-4R)-4-methylglutamate (SYM 2081) shows strong selectivity for kainate receptors (100-1000 fold compared to AMPA receptors) (Donevan et al., 1998). CNQX and DNQX are non-selective competitive antagonists (Table 1). The pharmacological properties of the kainate receptors are determined by their subunit composition. The expressed homomeric iGluR5 and iGluR6 receptor complexes cannot be activated by AMPA, though it stimulates a heteromeric receptor assembled from the iGluR6 and KA2 subunits (Sakimura et al., 1992, Wenthold et al., 1994). Homomeric iGluR7 receptors display very low sensitivity to glutamate and insensitivity to domoate (Schiffer et al., 1997). LY293558, which is a non-competitive antagonist of AMPA receptors, also blocks iGluR5-mediated responses without any effect on iGluR6. A related derivative, LY294486, has a significantly higher selectivity for iGluR5 than for the AMPA receptors and no effect on the iGluR6 and 7 homomeric channels (Clarke et al., 1997). Desensitization of kainate receptors is completely suppressed by concanavalin A (Patnau et al., 1994).

The distribution of the kainate receptor subunits has a specific pattern in the CNS, often corresponding to the vulnerability of brain regions in certain neurodegenerative diseases (Bettler et al., 1992). These receptors are prominently expressed in the striatum, reticular thalamic nuclei, hypothalamus, the deep layers of the cerebral cortex, the granule cell layer of the cerebellum, and the dentate gyrus and stratum lucidum of area CA3 in the hippocampus. Over the years, both presynaptic (Coyle, 1983; Represa, 1987, Huntley, 1993, Petralia, 1994) and postsynaptic (Greenamyre and Young, 1989; Petralia, 1994) localization of kainate receptors have been proposed. Moreover, some groups have suggested a putative extrasynaptic location of these receptors (Lerma, 1997; Mayer, 1997).

1.1.3.3 Functions of kainate receptors

The physiological role of kainate in the CNS is not fully understood. In the newborn rat, the mRNA level encoding different kainate receptor subunits is already very high, bespeaking their involvement in the development and plasticity of the brain (Bahn et al., 1994). Furthermore, there is a dynamic switch which governs the number of synapses of each class during development (Kidd and Isaac, 1999). According to recent observations, the iGluR5 and iGluR6 subunits can form heteromeric kainate receptors which mediate synaptic currents (Cui and Mayer, 1999; Paternain et al., 2000). These receptors at the mossy fiber pathway are involved in the long-term potentiation of transmission (Bartolotto et al., 1999). Postsynaptic kainate receptors are commonly assumed to coexist with other glutamate receptor subtypes, in particular with AMPA receptors (Frerking et al.,

1998). However, despite co-expression of both AMPA and kainate receptors in a given cortical neuron, they never seem to be co-localized at the same synaptic site (Kidd and Isaac, 1999). In addition to the postsynaptic role of the kainate receptors, kainate has been found to modulate transmitter release by a presynaptic mechanism. In particular, activation of iGluR6 kainate receptors markedly depresses the release of glutamate at the mossy fibers (Contractor et al., 2001b; Kamiya and Ozawa, 2000; Schmitz et al., 2000), where they have been postulated to play a significant role in synaptic plasticity (Bartolotto et al., 1999; Contractor et al., 2001a). Similarly, kainate has been found to suppress the excitatory postsynaptic currents evoked by dorsal root fiber stimulation (Kerchner et al., 2001), indicating that presynaptic kainate receptors may also regulate primary afferent sensory transmission. The modulation of GABA release by the activation of kainate receptors has also been reported in the hippocampus (Rodriguez-Moreno et al., 1997; Min et al., 1999) and the hypothalamus (Liu et al., 1999). Kainate reduces the efficacy of inhibitory connections in the hippocampus. In the genesis of this effect, activation of kainate receptors located at the GABAergic terminals is also involved. Blockage of the action of kainate by LY294486 (Clarke et al., 1997) and the large number of presumptive interneurons expressing GluR5 mRNA (Paternain et al., 2000) point to the involvement of this subunit in the phenomenon. On the other hand, the evoked inhibitory postsynaptic currents and the increase in interneuronal firing are still reduced in mice lacking the GluR6 subunit (Bureau et al., 1999). Apparently this subunit does not participate in the presynaptic modulation of GABAergic neurotransmission.

Table 1. Pharmacology of ionotropic glutamate receptors

	NMDA	**AMPA**	**Kainate**
Selective agonists	NMDA	AMPA	Kainate
Other agonists	L-glutamate, L-aspartate, L-homocysteate, L-cysteinesulfinate, quinolinate	L-glutamate, L-aspartate, quisqualate, kainate, domoate, FWD	SYM 2081, domoate
Competitive antagonists	L-AP5, CPP	CNQX, DNQX, NBQX, LY215490	CNQX, DNQX, LY294486
Allosteric modulators	D-serine, Glycine, D-alanine, spermine, spermidine, putrescine, ifenprodil	cyclothiazide	concanavalin A
Antagonist at modulatory site	HA-966, kynurenate, 7-chlorokynurenate, MNQX	GYKI53655,	
Ion channel inhibitors	MK-801, PCP, ketamine, dextrophane, desipramine	barbiturates, metohexiton, pento- and phenobarbital, agriotoxin	

Selective and non-selective agonists, competitive and non-competitive antagonists, allosteric modulators and ion-channel inhibitors. For abbreviations see the list on pages 8-9.

1.2 Metabotropic receptors

Metabotropic glutamate receptors are linked to GTP-binding proteins. Eight receptors (mGluR1-8) have so far been cloned, their structures resembling each other. They have a long extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal domain. The ligands are bound by the N-terminal domain, which contains sequences homologous to those found in bacterial periplasmic amino-acid binding proteins (O'Hara et al., 1993). The metabotropic receptors are classified into three groups with respect to their homology and intracellular signaling pathways (Conn and Pin, 1997; Corti et al., 1998).

Group I comprise mGluR1 and mGluR5 receptors. These activate phospholipase C and generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacting with its own receptors releases Ca^{2+} from intracellular pools while DAG activates PKC (Conn and Desai, 1991). Group II ($mGluR_2$ and mGluR3) and Group III (mGluR4, 6, 7, 8) can inhibit adenylyl cyclase activity. Many of these receptors exist in various isoforms with different intracellular carboxy termini generated by alternative splicing of their pre-messenger RNAs (Conn and Pin, 1997; Corti et al., 1998). These receptors have been localized either pre- or postsynaptically at most (if not all) glutamatergic synapses and at some GABAergic synapses. In most instances, group I mGluR receptors increase cell excitability by inhibiting the activity of K^+ channels (Conn and Pin, 1997), but some inhibitory actions resulting from the activation of Ca^{2+}-activated K^+ channels have also been reported (Fagni et al., 1991; Fiorillo and Williams, 1998). Although group I mGluRs are mostly located in postsynaptic elements, presynaptic actions of these receptors have also been observed (Manzoni et al., 1995). In most cases, they have been shown to reduce neurotransmitter release, probably by inhibiting Ca^{2+} channels (Conn and Pin, 1997), but potentiation of glutamate release has also been reported (Herrero et al., 1998). Group I mGluRs have been shown to facilitate glutamate-induced neurotoxicity and to participate in pain sensitivity (Conn and Pin, 1997). Group II and group III mGluRs are mostly located on glutamatergic terminals and inhibit the release processes (Conn and Pin, 1997). Accordingly, agonists for these receptor types are expected to have potential therapeutic applications in inhibiting glutamatergic excitotoxicity (Bond et al., 1998), and in the treatment of anxiety (Helton et al., 1998), Parkinson's disease (Konieczny et al., 1998), schizophrenia (Moghaddam and Adams, 1998) and drug addiction (Helton et al., 1998).

Group I receptors are activated by L-glutamate, quisqualate and trans-1-aminocyclopentane-trans-1,3-dicarboxylate (trans-ACPD). The most selective group I agonist is 3,5-dihydroxyphenylglycine (3,5-DHPG) (Conn and Pin, 1997). A group I antagonist is α-thioxanthylmethyl-3-carboxycyclobutylglycine (LY393675), which has a similar high potency on both mGluR1 and mGluR5 receptors. Most competitive group I antagonists so far characterized are fairly specific for the mGluR1 receptors. These inhibitors are 4-carboxy-3-hydroxyphenylglycine (4C3HPG), 2-(3'-carboxybicyclo[1.1.1]pentyl)glycine (CBPG) (Pellicciari et al., 1996) and (S)-2-methyl-4-carboxyphenylglycine (LY367385) (Clark et al., 1997). The most potent, selective, non-competitive antagonist for mGluR5 is 2-methyl-6-(phenylethynyl)pyridine (MPEP).

The group II receptors can be activated by L-glutamate, trans-ACPD and 2S,S'R,3'R)-2-(2,3-dicarboxcycloprpyl)glycine (DCG-IV), but the most potent agonists are 1S,3S-ACPD, (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) (Schoepp et al., 1997) and its derivative (1R,4R,5S,6R)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) which is even more potent (Monn et al., 1998). N-Acetylaspartyl-glutamate is a potent agonist for the mGluR3 suptype. The first selective group II antagonists identified were α-methyl-L-CCG-I (MCCG-I) (Conn and Pin, 1997) and its phenyl derivative (Thomsen et al., 1996). Highly potent and competitive group II antagonists are (2S,4S)-2-amino-4-(4,4-diphenylbut-1-yl)pentane-1,5-dioate (LY307452) and 2S-2-amino-2-[(1S,2S-2-carboxycycloprop-1-yl)-3-xanth-9-yl]propanoate (LY341495).

The most potent group III agonists are L-AP4, L-serine-O-phosphate (L-SOP), (1S,3R,4R)-1-aminocyclopentane-1,3,4-tricarboxylate (ACPT-I) and 4-phosphonophenylglycine (PPG) (Gasparini et al., 1999a). Their specific antagonists are α-methyl-AP4 (MAP4), α-cyclopropyl-4-phosphonophenyl-glycine (CPPG) and DCG-IV (see for review, Pin et al., 1999) (Table 2).

Table 2. Pharmacology of metabotropic glutamate receptors

Group	Suptypes	Group selective agonists	Subtype selective agonists	Group selective antagonists	Suptype selective antagonists
I	**mGluR1**	quisqualate, trans-ACPD, 3,5-DHPG		LY393675	CBPG, 4C3HPG, LY367385
	mGluR5		CHPG		MPEP
II	**mGluR2**	trans-ACPD, DCG-IV, 1S,3S-ACPD, LY354740, LY379268		MCCG-I, LY307452, LY341495	
	mGluR3		NAAG		
III	**mGluR4**	LAP-4, L-SOP, ACPT-I, PPG		CPPG, DCG-IV, MAP4	
	mGluR6				
	mGluR7				
	mGluR8				

The various mGluR receptor subtypes, their classification in three groups and their selective agonists and antagonists. Abbreviations are defined on pages 8-9.

While group II mGluRs (particularly mGluR2) are located at both sides of the synaptic cleft, group I and group III receptors are localized post- and presynaptically, respectively. Only mGluRs are situated in the active zone of the presynaptic terminal. Group II and group III receptors exist mainly presynaptically, whereas mGluR2 and possibly mGluR3 are located away from the site of neurotransmitter release (Cartmell and Schoepp, 2000). The last two presynaptic receptors are inhibitory autoreceptors at the glutamate synapse. Upon activation, they diminish the intracellular levels of cAMP and cGMP by inhibiting adenylyl and guanylyl-cyclases but they can also exert their actions via G-protein-

mediated Ca^{2+} channels. Presynaptic receptors thus inhibit the release of glutamate and reduce the activity of cells. Different types of mGluRs can also be found in glial cells (Mineff and Valtschanoff, 1999). Glial ionotropic AMPA and kainate and mGluR1, 5, 2, 3, 4 and 7 receptors are likely to be involved in the regulation of neuronal-glial intercommunication (Pin and Duvoisin, 1995; Nicoletti et al., 1996; Riedel, 1996).

2 Dopamine

Dopamine is an important neurotransmitter in the mammalian brain. It is synthesized mainly in the presynaptic parts of a neuron and stored in vesicles in the nerve endings. Dopamine can be released from the vesicular pool and newly synthesized dopamine also from the intracellular pool by depolarizing stimuli (McMillen and Shore, 1980; Kelly, 1993). Dopamine is known to control a wide range of physiological functions. It participates in motor coordination (Missale et al., 1998; Emilien et al., 1999) and regulates the functions of other neurotransmitter (glutamate, GABA, acetylcholine, noradrenaline) systems (Johnson, 1998; Missale et al., 1998).

The activity of dopaminergic neurons can also be regulated by the other above-mentioned neurotransmitters (Grace et al. 1998). In addition, dopamine systems are involved in processing reward responses and learning behavior (Schultz, 1998). As the unpredicted occurrence of the reward responses is pivotal for learning, they allow dopamine neurons to play an important role in reward-driven learning (Everitt and Robbins, 1997).

Dysfunction of the dopaminergic system underlies Parkinson's disease, due to lesions in the nigrostriatal system with a loss of dopaminergic neurons. Huntington's chorea is also associated with the dopaminergic system via the acetylcholinergic system. Dopamine may also act on the adrenergic systems (Missale et al., 1998) and may affect endocrinological control (Missale et al., 1998). Dopamine stimulates the synthesis of cyclic adenosine monophosphate (cAMP), has a calming action, dilates blood vessels in the brain, kidneys, heart and intestines, increases the stroke and minute volumes of the heart and potentiates peripheral catecholamine release (Missale et al., 1998; Emilien et al., 1999).

There are three main central dopaminergic systems: (1) The nigrostriatal pathway, joining the substantia nigra (A9 region) to the striatum, which is involved in extrapyramidal motor functions. The effects of the dopaminergic system motor functions are connected with dopaminergic-glutamatergic interactions in the nigrostriatal pathway. Konradi (1998) has shown that stimulation of the D_1 receptor mediates c-fos expression, which is dependent on the NMDA receptor function as shown by inhibition of the glutamate agonist dizocilpine. Dopamine acting at D_1-like receptors potentiates the postsynaptic glutamate responses mediated by the NMDA receptors, while D_2 agonists induce glutamatergic excitation of striatal neurons mediated by non-NMDA receptors (Carlsson, 1993; Adriani et al., 1998; Capeda and Levine 1998; Grace et al., 1998). (2) The mesolimbic/mesocortical pathway from the ventral tagmental area (A10 region) to the cortical structures is responsible for the cognitive functions

and motivation, and indirectly for motor activation (Missale et al., 1989; Alexander et al., 1990; Emilien et al., 1999). (3) The tubular/infundibular pathway from the hypothalamus (A12) to the hypophysis regulates neuroendocrine functions (Missale et al., 1998; Emilien et al., 1999). In addition to the above main pathways, dopaminergic neurons join the frontal cerebral cortex with the septum, striatum and nucleus accumbens (Hantraye, 1998).

3 Glutathione

3.1 Metabolism and functions of glutathione

Glutathione (γ-glutamylcysteinylglycine, GSH, reduced glutathione) is a phylogenetically ancient molecule which is ubiquitous in organisms and the most abundant peptide in the CNS (Orlowski and Karkowsky, 1976). 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 (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). The activity of both enzymes is under substrate feedback control. The breakdown of glutathione is catalyzed by γ-glutamyl transferase (EC 2.3.2.2), a membrane-bound enzyme which catalyzes the transfer of the γ-glutamyl moiety to free amino acids (Meister and Anderson, 1983; Deneke and Fanburg, 1989). The cellular and regional distribution of the enzymes involved in glutathione metabolism parallels that of glutathione (Philbert et al., 1991). The redox functions of GSH are well known; for example, the molecule can scavenge free radicals as an antioxidant, protecting cell membranes against oxidative stress, DNA against radiation and UV light, and the whole cell against xenobiotics (Meister and Anderson, 1983). In addition, GSH serves as a cofactor or substrate for various enzymes and is involved in the regulation of the cell cycle and cellular metabolism (Kosower and Kosower, 1978; Meister, 1988; Max, 1989).

Glutathione (Reichelt and Fonnum, 1969; Slivka et al, 1987a,b; Kirstein et al., 1991), S-methylglutathione (SMG) (Kanazawa et al., 1965), and glutathione sulfonate (GSA) (Li et al., 1993b) are endogenous constituents of brain tissue. The total level of glutathione in the CNS is within the range of 1-4 mM. The greater part of glutathione (about 95%) is in reduced form (Slivka et al., 1987a,b), but under oxidizing conditions the oxygenated form (GSSG) may exist at higher concentrations (Folbergrova et al., 1979). Glutathione is present in both intra- and extracellular spaces (Orlowski and Karkowsky, 1976). The concentration of GSH in cerebrospinal fluid is in the micromolar range (Rehncrona and Siesjö, 1979). Cellularly, glutathione has been histochemically located in non-neuronal elements (epithelial and glial cells) and neurons (in axons and nerve terminals, but not in perikarya except for cerebellar granule cells) (Slivka et al., 1987a; Philbert et al., 1991; Hjelle et al., 1994). The glutathione levels are much higher in cultured differentiated astrocytes than in undifferentiated astrocytes or cultured neurons (Raps et al., 1989).

In cultured astrocytes, the intracellular glutathione level in mitochondria is 8-20 mM (Yudkoff et al., 1990; Jain et al., 1991). Immunohistochemically, GSH has been located in the CNS in both neurons and glia (Hjelle et al., 1998).

Glutathione is rapidly released from astrocytes, but whether or not a specific efflux system exists for it is not known (Yudkoff et al., 1990; Zängerle et al., 1992). Glutathione transport between neurons and glial cell is assumed to occur. Wallin and co-workers (1999) have shown that brain ischemia enhances the efflux and metabolism and thus reduces the tissue concentration of glutathione. The efflux is dependent on extracellular Ca^{2+} and enhanced by the blockage of γ-glutamyl transpeptidase (EC 2.3.3.2). This would suggest that an increase in glutathione could be a factor which initiates nerve cell death due to a change in the intracellular redox potential (Wallin et al., 1999). In addition, there are metabolic interactions between astrocytes and neurons in GSH metabolism (Dringen et al., 1999).

3.2 Neuromodulatory role of glutathione

3.2.1 Effects of glutathione on ionotropic glutamate receptors

Although the molecular weight of GSH is greater than that of glutamate and the molecule is less flexible than glutamate, GSH can interact with the glutamate receptors and glutamate transporter. In addition, cysteine in GSH affects the redox state of glutamate receptors and carriers (Lazarewicz et al., 1989; Aizenman et al., 1989; Sucher et al., 1990; Janáky et al., 1993; Köhr et al., 1994; Tang and Aizenman, 1993).

3.2.1.1 Effects on NMDA receptors

GSH and GSSG displace [^{3}H]glutamate from its binding sites in rat synaptic plasma membranes at temperatures > 30°C in the presence of either Cl^- or Na^+, which indicates that glutathione can interact with the binding sites of glutamate (Koller and Coyle, 1985; Ogita et al., 1986; Ogita and Yoneda, 1987; Yoneda et al., 1990; Ogita et al., 1995; Jenei et al., 1998; Janáky et al., 1998). However, such temperature-dependent [^{3}H]glutamate binding has been claimed to represent Cl^-/Ca^{2+}-dependent transport (Pin et al., 1984) and Na^+-dependent uptake (Ogita and Yoneda, 1986). GSH is more potent in displacing Na^+-independent than Na^+-dependent binding of [^{3}H]glutamate at 2°C (Ogita et al., 1986; Varga et al., 1989, 1997; Janáky et al., 1998, 1999; Jenei et al., 1998). In addition, GSH and GSSG also inhibit the binding of the ^{3}H-labeled radioligands 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate ([^{3}H]CPP) (Yoneda et al., 1990) and DL-(E)-2-amino4-propyl-5-phosphono-3-pentanoate ([^{3}H]CGP 39653) Ogita et. al., 1995; Pasqualotto et al., 1998) to the NMDA receptor domain in the NMDA receptor. Similarly to GSH and GSSG, the S-alkyl derivatives of glutathione, SMG, S-ethylglutathione (SEG), S-propylglutathione (SPG), S-butylglutathione (SBG), S-pentylglutathione (SPeG) and GSA inhibit the binding of [^{3}H]CPP and [^{3}H]glutamate (Jenei et al., 1998).

Since both GSH and its S-alkyl derivatives effectively inhibit the binding of the above NMDA receptor ligands, this interaction is likely to be independent of the thiol moiety of GSH. The γ-glutamyl residue is probably essential for their effects. None of this, however, gives an answer as to whether GSH and its

derivatives are agonists or antagonists at the NMDA receptors. Since the NMDA agonists appear to be more effective in inhibiting the binding of agonists than the binding of antagonists, it has been suggested that GSH may be an agonist at this receptor (Ogita et al., 1995). However, the nature of a ligand, agonist or antagonist, is concentration-dependent. GSH antagonizes NMDA actions at micromolar concentrations and enhances them at millimolar concentrations. GSH would thus appear to be an inverse agonist at the NMDA receptor (Janáky et al., 1993, 1998; Jenei et al., 1998). Such an assumption is supported by the findings that GSH, GSSG and S-alkyl derivatives of GSH potentiate dizocilpine binding (Jenei et al., 1998) in a concentration-dependent manner. However, at high concentrations GSH again reduces dizocilpine binding (Ogita et al., 1995; Varga et al., 1997, Jenei et al., 1998). The GSH-mediated potentiation is prevented by NMDA antagonists, e.g. by CGP and L-AP5, but not affected by NMDA agonists or glutamate dehydrogenase (Ogita et al., 1995). These results indicate that GSH potentiation is not attributable to free glutamate derived from the possible cleavage of GSH. Neither GSH nor its derivatives potentiate dizocilpine binding in the presence of glutamate, a circumstance which would support the assumption that GSH binds to the glutamate binding site in the NMDA receptor via its γ-glutamyl moiety (Ogita et al., 1986; Varga et al., 1989; Oja et al., 1995; Janáky et al., 1998, 1999).

GSH and its derivatives at micromolar concentrations displace [^{3}H]glycine and the glycine antagonist [^{3}H]kynurenate from the binding site of co-activatory glycine in NMDA receptors (Ogita et al., 1995; Varga et al., 1997; Jenei et al., 1998; Janáky et al., 1998, 1999). In addition, the effects of GSH and other thiol-containing compounds are discernible in the open NMDA receptor channel. In cultured rat cortical neurons, the NMDA receptor-mediated currents are substantially potentiated after exposure to reducing DTT (Leslie et al., 1992; Lipton, 1993; Lipton and Stamler, 1994), whereas the oxidizing compound 5,5'-dithio-bis-2-nitrobenzoate (DTNB) attenuates NMDA responses and abolishes DTT-mediated potentiation (Aizenman 1989). GSH has been reported to have a clear biphasic effect on the NMDA-evoked Ca^{2+} currents: at micromolar concentrations GSH inhibits and at millimolar concentrations increases them. The increasing effect can be prevented by L-AP5 and dizocilpine. At lower concentrations, GSH inhibits the binding of agonists, whereas at higher concentrations it reduces the functional SH groups in NMDA receptors, similarly to DTT (Janáky et al., 1993).

3.2.1.2 Effects on non-NMDA receptors

Many endogenous γ-glutamyl peptides inhibit the Na^+- and temperature-independent bindings of [^{3}H]AMPA and [^{3}H]kainate to synaptic plasma membranes isolated from the rodent brain (Varga et al., 1989, 1994). The affinities of both GSH and GSSG for AMPA receptors are higher than those for kainate receptors (Jenei et al., 1998). Similarly, S-alkyl derivatives of GSH bind with a relatively high affinity to AMPA receptors. The alkylation of cysteinyl residues in the GSH molecule does not alter these affinities. These residues have apparently no role in the binding of GSH to AMPA receptors. On the other hand, the alkylation of cysteinyl residues in GSH increases selectivity for AMPA

receptors in comparison with kainate receptors. The mechanism of action of GSH on AMPA receptors is complex and may also involve allosteric interactions. It appears that at physiological concentrations GSH displaces glutamate mainly from the AMPA class of non-NMDA receptors in an allosteric manner due to the γ-glutamyl moiety of GSH. This effect may play a role in the fine-tuning of glutamatergic neurotransmission to curtail the duration of AMPA-receptor-mediated currents. Since the AMPA and NMDA receptors are colocalized and co-operate at the postsynaptic membrane (Seeburg, 1993), the co-release of glutamate and GSH from presynaptic terminals (Hjelle et al., 1994, 1998) may have profound consequences for synaptic transmission.

3.2.2 Effects of glutathione on the release and uptake of neurotransmitters

Werman and co-workers (1971) report that GSH and GSSG evoke acetylcholine release from the neuromuscular junction. The neurotransmitter release from neurons is likewise affected. Both GSH and cysteine evoke the release of glutamate from cultured cerebellar granule cells (see references in Janáky et al., 1998). GSH and GSSG inhibit the Cl^-/Ca^{2+}-dependent, temperature-sensitive binding of glutamate to crude synaptic plasma membranes (Varga et al., 1994), this being thought to represent glutamate transport. However, these peptides enhance glutamate uptake by synaptosomes (Varga et al., 1994). Neither GSH nor GSSG have influenced the basal release of [^{3}H]GABA from hippocampal slices (Janáky et al., 1994). However, DTT enhances and prolongs the glutamate-activated [^{3}H]GABA release in a concentration-dependent manner. The activating effect of DTT is inhibited by dizocilpine (Janáky et al., 1993, 1998). In contrast to DTT, GSH and GSSG do not enhance the glutamate-agonist-induced [^{3}H]GABA release and block DTT-mediated release. These data suggest that at least in some cases GSH and GSSG can attenuate the potentially damaging release of some neurotransmitters evoked by strong reducing agents (Pittaluga and Raiteri, 1992). However, the possible involvement of GSH receptors (possibly as autoreceptors) in the presynaptic regulation of other neurotransmitters awaits future investigation.

According to the above results, GSH can modulate glutamatergic neurotransmission as a neuromodulator by interacting with glutamate receptors.

3.3 Glutathione as a putative neurotransmitter

The possibility has already emerged from the above that GSH has a role not only as a neuromodulator but also as a neurotransmitter in the CNS. Groups under Shaw (1996) and Pasqualotto (1998) have demonstrated that bath-applied GSH induces a dose-dependent depolarizing potential in the rat cerebral cortex, which resembles those evoked by AMPA or NMDA. This GSH response could not be diminished by the AMPA and NMDA receptor antagonists DNQX and L-AP5 or removal of all external Ca^{2+}. Removal of all Na^+ from the bathing medium reversibly eliminates the GSH-evoked response. Moreover, GSH depolarization can be partially reversed by co-application of the inhibitory neurotransmitter GABA. These data suggest that GSH binds to a site (or sites) which regulates

membrane depolarization. The GSH response does not however appear to depend on the activation of NMDA channels, since L-AP5 and MK-801 are unable to block the response to GSH. In addition, [^{3}H]glutathione binds with a high affinity to plasma membrane fractions isolated from different brain regions (Ogita and Yoneda, 1987, 1988, 1989; Ogita et al., 1988; Guo and Shaw, 1992; Lanius et al., 1993, 1994). However, these binding sites cannot be distinguished unambiguously from glutamate binding sites.

The above data from electrophysiological studies together with the results of binding studies show that GSH may bind to a receptor(s) which is possibly not identical with any glutamate receptor. In addition, the cysteine-peptide structure is mandatory for the binding of GSH. [^{3}H]GSH binding sites exhibit an uneven distribution in different brain regions. The highest levels are found in the retina, and then in decreasing order in the hypothalamus, striatum, spinal cord, midbrain, medulla-pons, hippocampus, cerebellum and cerebral cortex (Ogita and Yoneda, 1988). The regional sub-variations revealed by receptor autography have shown dense binding in the retinal photoreceptor and pigment epithelium layers, the dentate gyrus of the hippocampus, habenula, hypothalamus and spinal cord (Bains et al., 1997; Shaw, 1998). Some of these binding sites appear to be located on astrocytes (Guo and Shaw, 1992; Guo et al., 1992). [^{3}H]GSH binding is also detectable in peripheral tissues, including the pituitary and adrenal glands, liver, skeletal muscle and heart (Ogita and Yoneda, 1988).

GSH may act as a neurotransmitter in the CNS if and only if all the following general criteria are met:

- The chemical compound must be localized in presynaptic elements of neuronal tissue, probably with an uneven distribution in the brain.
- Its precursors and synthetizing enzymes must be present in neurons usually in the close vicinity of the site of action.
- Stimulation of afferents should cause release of the chemical compound in physiologically significant amounts.
- Effects evoked by direct application of the chemical compound to the synapse should be identical to those produced by stimulation of the afferents.
- Specific receptors which interact with the chemical compound should be present in close proximity to presynaptic structures.
- Interaction of the chemical compound with the receptor should induce changes in postsynaptic membrane permeability, leading to excitatory or inhibitory postsynaptic potentials.
- A specific inactivating mechanism must exist.

4 Nitric oxide

The gaseous nitric oxide (NO) is a neuronal messenger which carries out diverse signaling tasks in both central and peripheral nervous system. NO is an unconventional transmitter, not packaged in vesicles but rather diffusing from its site of production without any specialized secretion machinery. NO can be released from both pre- and postsynaptic terminals. Since NO is a gaseous molecule it readily penetrates plasma membranes and bypasses all normal signal

transduction routes involving interactions with synaptic membrane receptors (Schuman and Madison, 1994). NO can mediate the neuronal plasticity associated with both brain development and information storage. Indeed, a huge amount of evidence has accumulated indicating the involvement of NO in synaptic plasticity. At the cellular level, NO signaling is essential for two forms of neuronal plasticity: LTP in the hippocampus (Schuman and Madison, 1994) and LTD in the cerebellum (Shibuki and Okada, 1991). Moreover, NO appears to mediate synaptic plasticity by potentiating the release of neurotransmitters. In addition to the regulation of glutamate release, NO can also regulate the secretion of hormones and neuropeptides and affect behavior (Bredt, 1999). NO also has an additional physiological role in the relaxation of smooth muscles in the cerebral circulation and gastrointestinal, urogenital and respiratory tracts (Bredt, 1999).

4.1 Formation of nitric oxide

Nitric oxide is produced from L-arginine with a stochiometric generation of L-citrulline by NO synthase (NOS). In the past ten years, three NOS enzymes have been identified. NOS from endothelial cells (eNOS) (Janssens et al., 1992) and neurons (nNOS) (Bredt et al., 1991b) are both constitutively expressed enzymes which are activated by an increase in intracellular Ca^{2+}. Ca^{2+}-independent inducible NOS (iNOS) (Lyons et al., 1992) mediates immune functions. As NO cannot be stored, released or inactivated by conventional mechanisms, regulation by biosynthesis is more important for it than for other mediators. NO synthase is one of the most highly regulated enzymes. The amino acid sequences of different cloned NO enzymes are about 50% identical. All forms of NOS so far characterized require several electron donors, e.g. flavin adeninedinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin. nNOS was originally purified from the rat cerebellum (Bredt and Snyder 1990). It is a protein of 160 kD containing several recognition sites for required cofactors, including a basic amphipathic α-helix calmodulin binding site, a cAMP-dependent protein kinase phosphorylation consensus sequence, an NADPH-binding domain and potential binding sites for FMN and FAD (Bredt et al., 1991a,b).

The expression of iNOS is initiated by various cytokines and microbial products (Hibbs et al., 1987). Following induction, NO is produced in large quantities (nanomoles) for several hours. This form of NOS is activated by Ca^{2+} bound to calmodulin (Knowles et al., 1989, Bredt and Snyder, 1990). In the periphery, the primary source of Ca^{2+} may be agonist-induced phosphoinositide hydrolysis, resulting in IP_3-mediated release of Ca^{2+} from intracellular stores. Intracellular Ca^{2+} regulates the activity of eNOS and nNOS (Dawson et al., 1998). In the brain, an increase in the intracellular level of Ca^{2+} originates from the extracellular space by the activation of NMDA receptors and voltage-dependent Ca^{2+} channels or from intracellular stores after the activation of mGluR1 receptors.

nNOS is present in the nerves in both cytoplasmic and membrane-bound forms. The enzyme activity is high in the cerebellum, olfactory bulb and pedunculopontine tegmental area, hippocampus (dentate gyrus) and supraoptic

nucleus. Isolated NOS-containing neurons have also been observed in the cerebral cortex and corpus striatum (Bredt et al., 1991a; Dawson et al., 1991b).

4.2 Nitric oxide targets

The major effector in NO action is soluble guanylyl cyclase, identified in many tissues (Arnold et al., 1977; Miki et al., 1977; Murad et al., 1978). Soluble guanylyl cyclase is a heterodimer which contains a heme group, the region responsible for the NO-evoked activation of cyclase. When NO binds to Fe^{2+} in the porphyrin ring of heme, the interaction draws Fe^{2+} out of the plane of the porphyrin ring, resulting in a conformational change and activation of guanylyl cyclase (Wolin et al., 1982). The resulting increase in the cGMP levels then affects the activity of ion channels or phosphodiesterase, or activates a cGMP-dependent protein kinase. In smooth muscle cells, the NO-induced increase in cGMP may activate a cGMP-dependent protein kinase which is ultimately responsible for muscle relaxation (Rapoport et al., 1983). Alternatively, cGMP has been reported to lower the intracellular Ca^{2+} levels, which may also contribute to relaxation (Rashatwar et al., 1987). NO may also exert its effects by stimulating the adenosinediphosphate (ADP) -ribosylation of proteins (Dimmeler and Brune, 1992). In addition, NO can readily interact with various other molecules (superoxide anions, free radicals) and take part in a number of beneficial or pathophysiological processes.

4.3 Interaction of N-methyl-D-aspartate receptors and nitric oxide

One role of NO in the brain may be as a neuromodulatory substance. NO can influence ion currents through the NMDA receptor channel. Ca^{2+} enters the cells after activation of NMDA receptors. By means of modulation of the current flow through this channel, NO could potentially influence many Ca^{2+}-regulated neuronal processes which utilize the NMDA receptor, for example synaptic transmission, plasticity, neurotoxicity and certain processes during development. Hoyt and colleagues (1992) have suggested that NO may play a role as a feedback modulator. When the NMDA receptor is activated, the resulting entry of Ca^{2+} into the cell activates NOS, reducing subsequent currents. These NMDA-mediated currents could be influenced by the redox state of the receptor channel complex (Aizenman et al., 1989; Lazarewitz et al., 1989). A reduction of the redox site of the NMDA receptor complex by agents such as DTT increases the current flow through the channel, whereas oxidation by 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) diminishes the flow (Lei et al., 1992). However, the magnitude of potentiation by the reducing agent DTT has been sevenfold greater than the magnitude of inhibition by the oxidizing agent DTNB. This finding indicates that the native redox state of NMDA receptors is closer to the fully oxidized than to the fully reduced state (Aizenman et al., 1989). Alkylating agents such as N-ethylmaleimide (NEM) can block further effects of redox agents on the NMDA receptor, implying the existence of critical sulfhydryl groups in the receptor which mediate these effects. The effect of NO on the NMDA currents appears to be mediated through this redox site. It has been suggested that the free sulfhydryl

groups in the NMDA channel complex are oxidized in the presence of NO to form S-nitrosothiols (Lei et al., 1992). In concert with such a conception, the oxidant DTNB had no further significant effects after treatment with NO-donating compounds, whereas the reducing agent DTT could still substantially reverse the effect of NO. cGMP has apparently no role in these phenomena, since the application of cGMP had no effect on the currents (Kiedrowski et al., 1992).

The molecular mechanism of the action of NO is not yet entirely clear. Other research groups have reported that NO reduces NMDA receptor activity, Zn^{2+} being possibly involved (Manzoni et al., 1992b; Hoyt et al., 1992; Fagni et al., 1995). The discrepancies arise partly from the fact that different forms of NO [NO^+ (nitrosinium ion), $NO^\bullet$ (nitric oxide) and NO^- (nitroxyl ion)], each having an additional electron, have distinct mechanisms of action. These different redox forms of NO appear to exist endogenously and have important pharmacological and physiological roles (Choi and Lipton, 2000). The transfer of NO^+ equivalents occurs from one nitrosothiol to another in a reaction termed transnitrosylation (Arnelle et al., 1995; Hogg et al., 1996, Kluge et al., 1997). The NO^+ equivalent can react with a redox modulatory site in the NMDA receptor to downregulate the activity of the receptor, which produces neuroprotection (Choi and Lipton, 2000). NO^- can exist in a high (singlet) or low (triplet) energy state. In particular, singlet NO^- can react directly or indirectly with the thiol groups in the NMDA receptors and thus downregulate their activity (Kim et al., 1999), whereas triplet NO^- does not do so (Bonner et al., 1996). However, in the triplet state, NO^- may react with O_2 to form peroxynitrite ($ONOO^-$), which in turn may oxidize free thiols to disulfides (Kim et al., 1999). $NO^\bullet$ preferably reacts with superoxide $(O_2^\bullet)^-$ to form peroxynitrite, which causes neuronal destruction either by itself or via its breakdown products (Lipton et al., 1993; Dawson et al., 1993). Proteins (ion channels, receptors, enzymes and transcription factors) can undergo functional changes upon S-nitrosylation. Stamler and associates (1997) have suggested that there is a consensus motif for S-nitrosylation. S-Nitrosylation of cysteinyl groups or transfer of NO^+ may function as a signaling pathway, analogously to phosphorylation, and this mechanism could serve as a molecular switch for the regulation of protein functions (Stamler et al., 1997).

4.4 Role of nitric oxide in neurotoxicity

Excitotoxic injury is a major cause of neuronal cell death in the CNS in both acute and chronic neurological disorders (Choi, 1988; Meldrum and Garthwaite, 1990). There is a paradox here in that excitatory neurotransmitters are also potent neurotoxins. In particular, the role of NO in NMDA receptor-mediated neurotoxicity has been a controversial subject. A component of this neurotoxicity has been reported to be mediated by NO (Dawson et al., 1991). However, other authors have failed to demonstrate the role of NO in NMDA-receptor-mediated neurotoxicity in cortical cultures (Demerle-Pallardy, 1991; Hewett et al., 1993) or in vivo after stroke (Buchan et al., 1994). Mice lacking nNOS have had smaller infarctions in the middle cerebral artery occlusion model of stroke when compared to controls expressing this enzyme (Huang et al., 1994). Cultured neurons derived from nNOS-knockout mice are likewise resistant to glutamate toxicity, which establishes that NO derived from nNOS can be toxic (Dawson et al., 1996). These

studies, together with a number of other experiments on the effects of NO scavengers, inhibition of nNOS and elimination of nNOS-containing neurons, have convincingly demonstrated that NO indeed has an important role in excitotoxicity (Dawson et al., 1991a,b; 1993; Nowicki et al., 1991).

There are several factors apparently contributing to the variable expression of NO-mediated excitotoxicity. The expression of NOS varies in dissociated cultures and the cellular localization of the damaging insult may thus be important. The most vulnerable cellular site of excitotoxic injury has proved to be the dendrite, which finding is consistent with the fact that excitatory synapses are located on dendrites. Furthermore, morphological studies have shown that the first signs of injury in response to exposure to excitotoxins are found on dendrites (Park et al., 1996; Faddis et al., 1997).

This hypothesis is supported by evidence showing that NOS is localized in dendrites in close association with NMDA receptors (Aoki et al., 1997). However, Aizenman and colleagues (1998) report that nitric oxide generation is not necessary in the induction of NMDA-receptor-mediated neurotoxicity. This conception is supported by studies on excitotoxicity in mixed cultures of cortical neurons derived from mice with a disrupted NR1 gene and from wild-type mice (Tokita et al., 1996). The neurotoxic effect of NO may require simultaneous activation of the NMDA receptor. In addition, NO generation is not sufficient to cause damage, since superoxide dismutase blocks the toxicity of NO (Radi et al., 1991). These authors presumed that a reaction with superoxide is required for the formation of peroxynitrite. Although NOS-containing neurons are readily vulnerable to Ca^{2+}-dependent toxicity due to AMPA/kainate receptors (Weiss et al., 1993), kainate activation is not sufficient to trigger NO-mediated toxicity in surrounding (NOS negative) neurons (Dawson et al., 1993). The NMDA-receptor-mediated activation of superoxide production (Lafon-Casal et al., 1993) is possibly a necessary concomitant event in NO toxicity.

The neurotoxicity of NO very probably stems from its free radical character, which renders it reactive with certain proteins containing heme-iron prosthetic groups, iron sulfur clusters or reactive thiols (Stamler, 1994). Cellular energy depletion is a hallmark of neuronal cell death associated with ischemic injury. NO can attenuate oxidative phosphorylation by inhibiting mitochondrial iron-sulfur cluster enzymes, including NADH-ubiquinone oxidoreductase and the succinate-coenzyme Q oxidoreductase. NO can also inhibit glycolysis by means of reactions with aconitase (Drapier and Hibbs, 1996) and by competing with oxygen for cytochrome oxidase (Brown, 1995). In addition to direct reactions with protein prosthetic groups, NO also readily reacts with superoxide to produce peroxynitrite, which mediates much of NO neurotoxicity (Beckman, 1996). In addition, there is direct evidence from studies on transgenic animals that NO and superoxide conspire in neuronal toxicity. Overexpression of SOD in transgenic mice reduces the infarct volume in the middle cerebral artery occlusion model of focal ischemia when compared to the injuries in wild type mice (Kinouchi et al., 1991).

4.5 Neuroprotective effects of nitric oxide

Nitric oxide can directly nitrosylate ras proteins, altering their functions as GTP-GDP exchangers, resulting in activation of the MAP kinase pathway (Lander

et al., 1997). In addition to its prominent roles in the regulation of cerebral blood flow and the modulation of cell-to-cell communication in the brain, some recent in vivo studies indicate that NO is a potent antioxidant. NO terminates oxidative stress in the brain by suppressing iron-induced generation of hydroxyl radicals ($OH^{\bullet}$) via the Fenton reaction, interrupting the chain reaction of lipid peroxidation, augmenting the antioxidative potency of reduced glutathione and inhibiting cysteine proteases. In addition, the less reactive NO scavenges highly reactive oxygen species, i.e. superoxide anion or $OH^{\bullet}$, and converts them into nonradicals such as nitrates [$O_2^{\bullet-} + NO^{\bullet} = (ONOO^-) \rightarrow NO_3^-$] and nitrites [$OH^{\bullet} + NO^{\bullet} = (HONO) \rightarrow NO_2^-$]. Brain anion transporters rapidly eliminate these metabolites, but at millimolar concentrations they are highly toxic in cell cultures. Furthermore, short-lived NO can scavenge thiyl radicals such as $GS^{\bullet}$ and $CYS^{\bullet}$ by converting them to biological active S-nitrosothiols [$GS^{\bullet} + NO^{\bullet} = GSNO$], [peptide-CYS + $NO^{\bullet} \rightarrow$peptide-CYSNO], which have a much longer half-life in vivo (Chiueh and Rauhala, 1999; Chiueh, 1999).

4.6 Nitrosoglutathione

In view of the lability and fast inactivation of NO by heme iron, non-heme iron, superoxide anion, oxygen and other agents (Stamler et al., 1992), it has been suggested that some intermediate molecular species such as S-nitrosothiols serve in the stabilization and transfer of NO from the site of origin to targets (Stamler, 1994). The predominant bound forms of NO in human plasma are nitrosothiols, mostly S-nitrosoproteins (Stamler et al., 1992). S-Nitrosothiols (predominantly GSNO) have been reported to be present at nanomolar to micromolar concentrations in the human airways. Their levels vary in different pathophysiological states (Gaston et al., 1994). GSNO may also be formed intracellularly in human neutrophils upon exposure to NO gas and following cell activation (Clancy et al., 1994). There is evidence that NMDA receptor activation induces the release of NO from cerebellar cells (Garthwaite et al., 1988). Owing to the relatively high concentration of GSH (millimolar) intracellularly (Griffith and Meister, 1979) and extracellularly (micromolar) (Zängerle et al., 1992), it is highly likely that GSH and NO interact with each other. Thus, NMDA receptor activation can form GSNO both intracellularly and extracellularly. NO is able to react with GSH under both anaerobic and aerobic conditions. In these reactions GSH is oxidized to GSSG (Hogg et al., 1996). Indeed, Kluge and associates (1997) have detected GSNO in 10-day-old and adult rat cerebellum. When cerebellar granular cells are incubated with glutamate or NMDA the total level of GSNO increases. These data corroborate the assumption that GSNO, similarly to GSH, may be released from neurons and interacts with proteins extracellularly.

S-Nitrosothiols exhibit a wide range of biological activities, including antimicrobial effects (De Groote, 1995), vasodilatation (Kowaluk and Fung, 1990), platelet inhibition (Hirayama et al., 1999), bronchodilation (Bannenberg et al., 1995) and inhibition of intestinal motility (Slivka et al., 1994). GSNO inhibits iron-evoked OH^- generation and lipid peroxidation and protects brain dopamine neurons against oxidative damage (Rauhala et al., 1998). Oxidative brain injury is prevented by GSNO in low nanomolar doses in vivo. In addition, S-nitrosylated GSH or GSNO at micromolar concentrations possesses a greater antioxidative

potency than GSH. The neuroprotective properties of NO and GSNO may be physiologically significant, since oxidative stress depletes GSH, while the increasing formation of $GS^{\bullet}$ and $NO^{\bullet}$ in astroglial and endothelial cells generates the more potent antioxidant GSNO, providing additional neuroprotection at its micromolar concentration. This putative GSNO pathway ($GSH \rightarrow GS^{\bullet} \rightarrow GSNO \rightarrow NO^{\bullet} + GSSG \rightarrow GSH$) may be an important part of the endogenous antioxidative defense system, which could protect neurons and other brain cells against oxidative stress caused by oxidants, iron complexes, proteases and cytokines. This mechanism may also account for the fact that NOS-containing neurons and nicotinamide-adenosine dinucleotide phosphate (NADPH) diaphorase-positive neurons are less vulnerable to oxidative injury. GSNO may yield complex protein modifications. It may give rise to reactive materials which can produce both S-thiolate and S-nitrosylate reactive cysteines. This process may result from degradation of GSNO into GSSG and/or the sulfenic acid derivative of glutathione. GSNO may thus play a role in the formation of two different oxidative modifications of protein sulfhydryl groups, both glutathiolation and S-nitrosylation altering their functions (Ji et al., 1999).

5 Cysteine

L-Cysteine is an endogenous constituent of numerous proteins and peptides, including glutathione (Sagara et al., 1993; Dringen et al., 1999). It provides inorganic sulfate for detoxification reactions (Heafield et al., 1990) and may hence be involved in neuroprotection (Miyamoto et al., 1989). It also protects nerve cells by forestalling the entry of heavy metals into the brain across the blood-brain barrier (Bradbury and Deane, 1993). Since cysteine can be released from brain slices under depolarization in a Ca^{2+}- dependent manner it may act as a neuromodulator (Keller et al., 1989; Zängerle et al., 1992), exciting neurons (Olney et al., 1990). However, at high dosages cysteine evokes toxic effects similarly to glutamate. L-Cysteine has proved to be toxic in vivo in developing animals with a still immature blood-brain barrier (Karlsen et al., 1981). In vitro, at millimolar concentrations cysteine can kill neurons (Klingman and Choi, 1989). It also elicits behavioral deficits (Sharpe et al., 1975). It is thus not surprising that the excitotoxic effects of L-cysteine are involved in the pathogenesis of several neurological disorders, e.g. amyotrophic lateral sclerosis, Parkinson's, Alzheimer's (Heafield et al., 1990) and Hallerworden-Spatz's diseases (Perry et al., 1985), and hypoxic/ischemic and hypoglycemic brain damage (Olney et al., 1990; Lehmann et al., 1993; Schurr et al., 1993; Slivka et al., 1993). Even though the excitotoxic effects have been recognized for more than two decades, the exact mechanism of the toxicity of L-cysteine is not yet known (Olney et al., 1990).

The toxicity of L-cysteine manifests itself presumably only when there is an excess in the extracellular fluid in the brain, since it is an endogenous amino acid in all cells. It is released into the extracellular space mainly from glial cells upon ischemia, but rather after the release and breakdown of glutathione by γ-GT (Li et al., 1999). In this case, the enhanced release of cysteine rapidly induces neuronal necrosis in a relatively restricted brain area (Olney et al., 1990). Paradoxically, at lower doses cysteine causes a more devastating neurologic syndrome, which develops more slowly but damages a greater number of brain

regions, including the cerebral cortex, hippocampus, caudate nucleus and thalamus (Olney et al., 1990). The most vulnerable brain regions are the cerebral cortex and hippocampus (Puka-Sundwall et al., 1995). When L-cysteine was administered orally to a pregnant dam during late gestation, it caused widespread damage to the fetal rodent brain (Olney et al., 1990).

Since the toxic effect of cysteine could be prevented by D-AP5, it has been suggested that it is mediated by NMDA receptors. These receptors are thus the most obvious targets for L-cysteine both as a neuromodulator and a neurotoxin. However, in pharmacological experiments it has proved to be only a weak NMDA receptor agonist and these results are seemingly at variance with the above assumption. It has been suggested that L-cysteine is able to reduce functional cysteinyl groups in the NMDA receptor-ionophore, modifying in this manner receptor functions (Olney et al., 1990; Olney, 1993). There is a possible synergism between cysteine and glutamate. Glutamate toxicity may be potentiated by the cysteine-evoked reduction of the glutamate-activated NMDA receptor-ionophore complexes (Puka-Sundwall et al., 1995).

In addition to NMDA receptor-mediated L-cysteine toxicity, several other mechanisms are thought to be possibly involved. L-Cysteine may generate oxygen free radicals, e.g. hydrogen peroxide, by interacting with heavy metals (Plaitakis et al., 1988), which may cause lipid peroxidation and hence overexcitation of NMDA receptors. On the other hand, L-cysteine can also react with NO and produce S-nitrosocysteine (Cys-NO). Cys-NO can act neurotoxically by generating $NO^{\bullet}$ in the absence of SOD, because NO reacts readily with free oxygen radicals, producing peroxynitrite, which causes neuronal injury. In the presence of SOD, the neurotoxic effects of Cys-NO are much less marked (D'Emilia and Lipton, 1999). However, the released NO may under certain circumstances have neuroprotective effects due to its antioxidant properties. Cys-NO elevates intracellular Ca^{2+} in cultured hippocampal neurons and recovery from increased intracellular Ca^{2+} is delayed (Brorson et al., 1997). In addition to these mechanisms, L-cysteine can yield toxic oxidation products, e.g. S-sulfocysteine (Olney et al., 1975) and cysteate (Olney et al., 1971; Olney et al., 1972; Lehmann et al., 1993; Li et al., 1999). These cysteine derivatives can act at both ionotropic and metabotropic glutamate receptors (Olney et al., 1972; Patneau et al., 1990; Porter et al., 1993) and mediate toxic effects. Other excitoxic metabolites, L-cysteinesulfate and L-cysteinesulfinate, may mediate a minor part of this neurotoxicity (Olney et al., 1990). L-Cysteinesulfinate is a broad spectrum agonist at all excitatory glutamate receptors (Griffiths, 1990). It also inhibits glutamate uptake (Griffiths et al., 1989) and may exert selective effects on phospholipase D-coupled glutamate receptors (Boss et al., 1994).

AIMS OF THE STUDY

Although GSH is involved in a number of well-known physiological processes in the CNS, its role as a neurotransmitter in neurotransmission is not known.

The aim of this study was to shed light on the role of GSH as a neurotransmitter:

(1) characterizing the binding of GSH to its putative receptors,
(2) studying whether the ionotropic and metabotropic receptor ligands interact with the putative GSH receptors, and
(3) assessing how different amino-acid side-chains in the plasma membranes are involved in the binding of GSH.

In addition, GSH can scavenge free radicals and influence the release of other neurotransmitters as a neuromodulator. In order to clarify the additional role of GSH as a neuromodulator, we

(4) elucidated whether GSNO plays a role in the regulation of ionotropic glutamate receptors, and
(5) studied how glutamate receptor agonists and glutathione regulate striatal dopamine release.

Due to the breakdown of glutathione, the constituent amino acids may work independently, being even toxic in excess. Hence, the last aim was

(6) to establish how L-cysteine causes neuronal degeneration.

MATERIAL AND METHODS

1 Preparative methods

1.1 Experimental animals

The brains of domestic pigs were obtained from a local slaughterhouse and immediately cooled on ice after excision. These brains were used for preparation of synaptic plasma membranes. Seven-day-old pups of Wistar rats were the source of cerebellar granule cell cultures. NMRI mice were used in preparing striatal slices. All experimental procedures were approved by the local Committee for the Use of Experimental Animals. All efforts were made to reduce the number and the suffering of the experimental animals used.

1.2 Preparation of synaptic plasma membranes

The brains were dissected and the subfractions prepared at 0-4°C according to Cotman and Taylor (1972). The brain meninges were removed and the cerebral cortex separated from the white matter. The tissue samples were homogenized in 10 volumes of 0.32 M sucrose at 800 rpm in a glass-teflon homogenizer. The homogenisates were centrifuged at 1000 g for 10 min to remove any remaining cerebral membranes, blood vessels and white matter. The pooled supernatants were centrifuged at 20 000 g for 20 min and the pellet containing mitochondria and synaptosomes suspended in 0.32 M sucrose. The suspensions were stored at –20°C until the day of further preparations. This fraction was then thawed at room temperature, layered onto a gradient of 0-8 M sucrose and centrifuged at 8 000 g for 20 min. The pooled 0.8 M fraction which contained synaptosomes was sucked up by a syringe, mixed with 0.9% KCl and re-centrifuged at 30 000 g for 20 min. The pellet was homogenized in 40 volumes of distilled water (osmotic shock) and centrifuged at 48 000 g for 20 min. The resulting pellet was suspended in 50 mM Tris-acetate buffer (pH 7.4), layered onto a 1.2, 1.0, 0.8 M discontinuous sucrose gradient and centrifuged in a swing-out bucket rotor at 63 000 g for 45 min. The fraction between the 1.2 M and 1.0 M sucrose layers enriched in synaptic membranes was aspirated and kept frozen at –20°C until the day of experiments. The solution containing synaptic plasma membranes was then thawed in cold water bath, mixed with 0.9% KCl and centrifuged at 20 000 g for 20 min. The resulting pellet was re-suspended in distilled water, and centrifuged at 48 000 g for 20 min. The pellet from this centrifugation was homogenized in Tris-acetate buffer (pH 7.8) and incubated at 37°C for 20 min. After cooling, the suspension was again centrifuged at 48 000 g for 20 min. This last step of preparation was essential to remove endogenous ligands such as glutamate, aspartate and glycine.

1.3 Cultures of cerebellar granular cells

Cultures of cerebellar granular cells were obtained from 7-day-old Wistar rats as described by Holopainen and Kontro (1988). The cells were isolated from minced tissue by mild trypsinization followed by trituration in a deoxyribonuclease solution containing a trypsin inhibitor. They were suspended in Dulbecco's essential medium and cultured on polylysine-coated dishes. The medium contained fetal calf serum (10%), K^+ ions (25 mM), glucose (30 mM) p-aminobenzoate (7 μM), insulin (100 mU/l) and antibiotics. At 48 hours 40 μM cytosine arabinoside was added for 24 h. Ca^{2+} uptake was studied eight days later.

1.4 Chemical modification of amino acid residues in membrane proteins

1.4.1 Modification of cysteinyl and lysyl residues

5'5-Dithio-bis-2-nitrobenzoate (DTNB) (Fig. 2) and 4'4-dithiodipyridine (DDP) (Fig. 3) were used to oxidize cysteinyl residues in plasma membranes. The amount of synaptic plasma membranes in the modification mixture was 1.0-1.5 g/l. DTNB and DDP were diluted from a stock solution freshly prepared in absolute alcohol. The membranes were incubated with different concentrations of DTNB in 50 mM Tris-acetate buffer (pH 7.4) at 37°C for 15 min under shaking (Ellman, 1959). In the case of DDP, incubation temperature and time were the same but the buffer was 50 mM $KHCO_3$ (pH 7.8) (Grassetti, 1967). The control membranes were incubated in the absence of the above oxidizing agents with an equivalent amount of alcohol. The chemical reaction was stopped by addition of cold buffer and centrifugation (48 000 g, 20 min) followed by two washing steps to remove any excess of modifying agents. In order to saturate binding sites in the protection experiments, the membranes were preincubated with the ligands tested at 37°C for 15 min before chemical modification.

Different concentrations of N-ethylmaleimide (NEM) were used to alkylate cysteinyl residues (Fig. 4). The amount of synaptic plasma membranes was again 1.0-1.5 g/l. NEM was always diluted from a freshly prepared stock solution in distilled water. The incubation was carried out at 30°C for 30 min in Tris-acetate buffer (pH 7.4) under shaking (Matthews et al., 1991). It was terminated by addition of cold buffer and centrifugation (48 000 g, 20 min) and excess NEM removed by two washing steps.

The cysteinyl and lysyl residues were modified with phenylisothiocyanate (PITC) (Figs 5 and 6). PITC were diluted from a freshly prepared stock solution in absolute alcohol. The incubation with PITC was carried out in 50 mM $KHCO_3$ buffer (pH 8) at 37°C for 15 min under shaking (Edman, 1959). The modification was terminated by addition of cold buffer and centrifugation (48 000 g, 20 min). Excess PITC was removed by two washing steps.

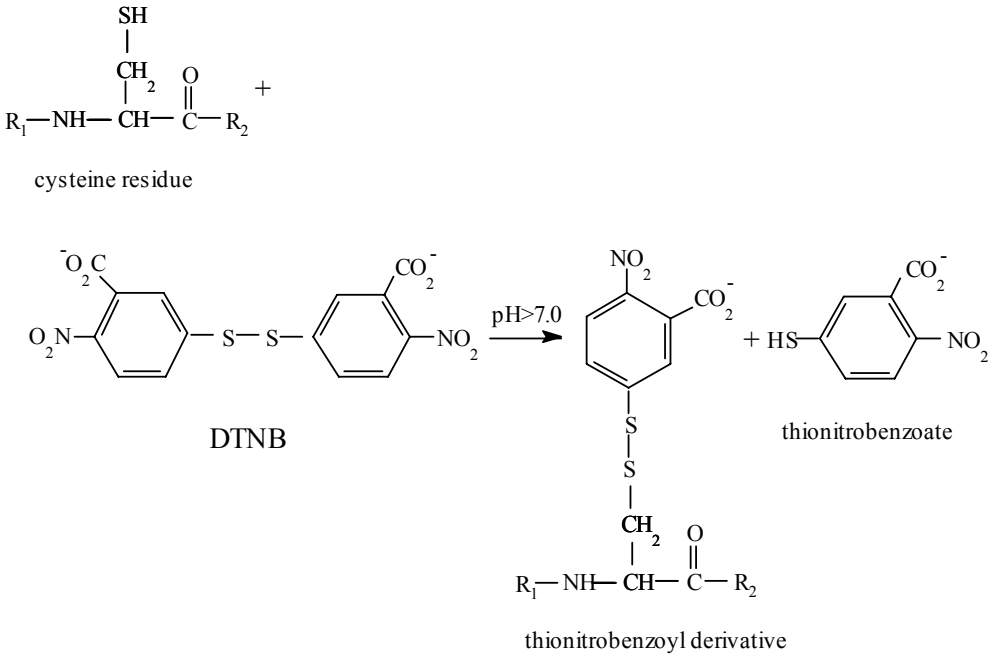

Fig. 2. The reaction of 5'5-dithio-bis-2-nitrobenzoate (DTNB) with cysteine residue.

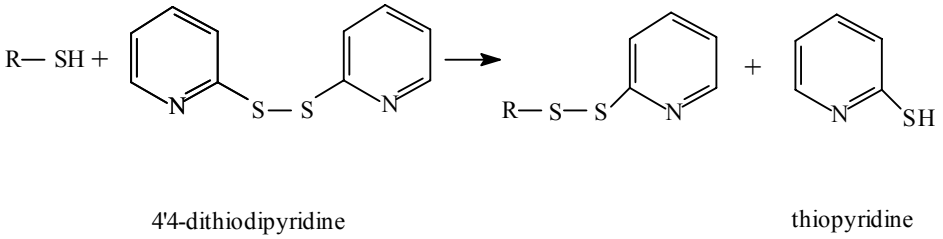

Fig. 3. The reaction of 4'4-dithiodipyridine (DDP) with cysteine residue.

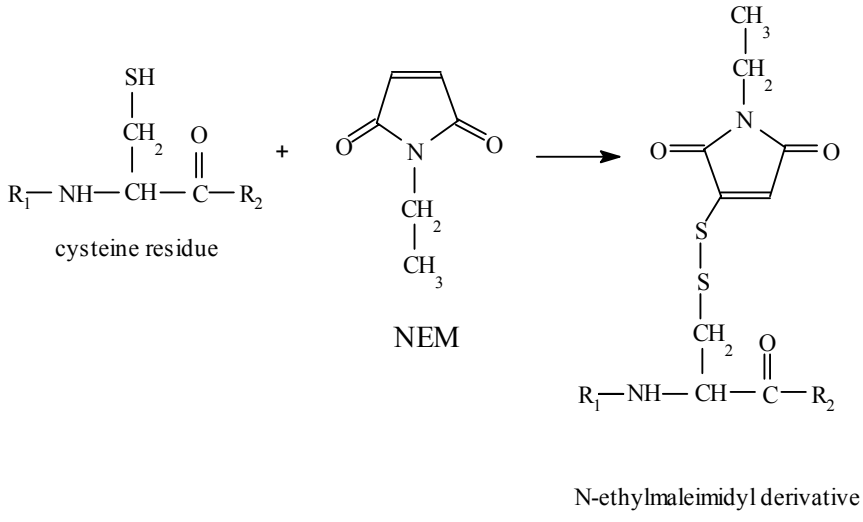

Fig.4. The reaction of N-ethylmaleimide (NEM) with cysteine residue.

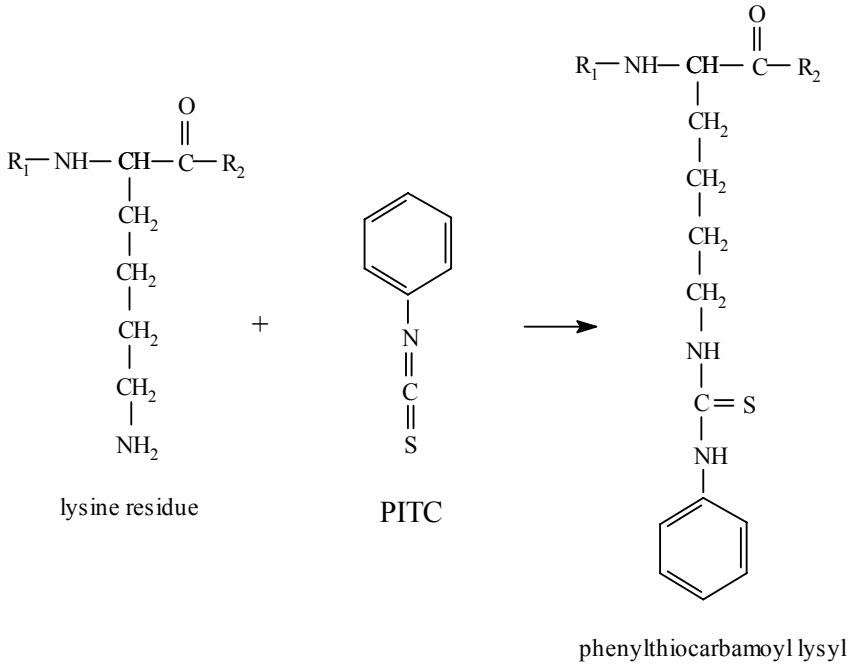

Fig.5. The reaction of phenylisothiocyanate (PITC) with lysine residue.

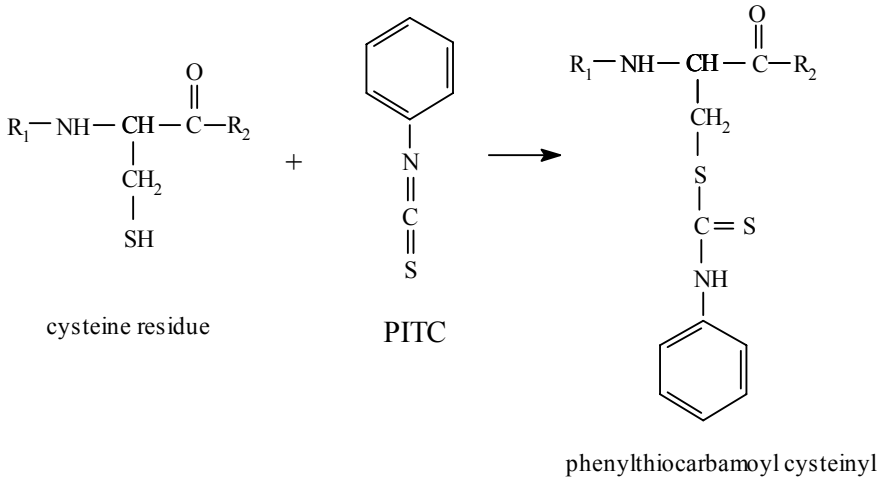

Fig. 6. The reaction of phenylisothiocyanate (PITC) with cysteine residue.

1.4.2 Disruption of disulfide bonds

The disulfide bonds in plasma membranes (1.0-1.5 g/l) were reduced with dithiothreitol (DTT) (Fig. 7). DTT was diluted from a freshly prepared stock solution in distilled water. The synaptic membranes were incubated with different concentrations of DTT in 50 mM Tris–acetate buffer at 37°C for 15 min under shaking (Cleland, 1964). The chemical reaction was terminated in the same manner as described in 1.4.1. In certain experiments the modifications with DTNB and DTT were consecutive in a different order.

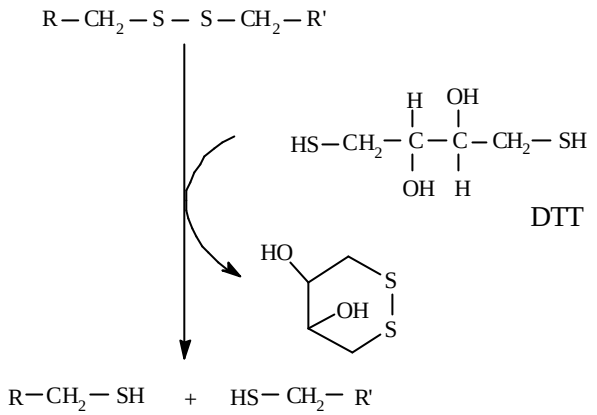

Fig. 7. Disruption of disulfide bonds by dithiothreitol (DTT).

1.4.3 Modification of arginyl residues

The arginyl residues in plasma membranes (1.0-1.5 g/l) were modified with phenylglyoxale (PGO) (Fig 8). The incubation was carried out in 50 mM $KHCO_3$ buffer (pH 8) at 25°C for 40 min in the dark under shaking (Takahashi, 1968; Jenei et al., 1997). The modifying agent was diluted from a freshly prepared stock solution in absolute alcohol. In the control experiments, the membranes were incubated in the absence of PGO with an equivalent amount of alcohol. The reactions were terminated as described in 1.4.1.

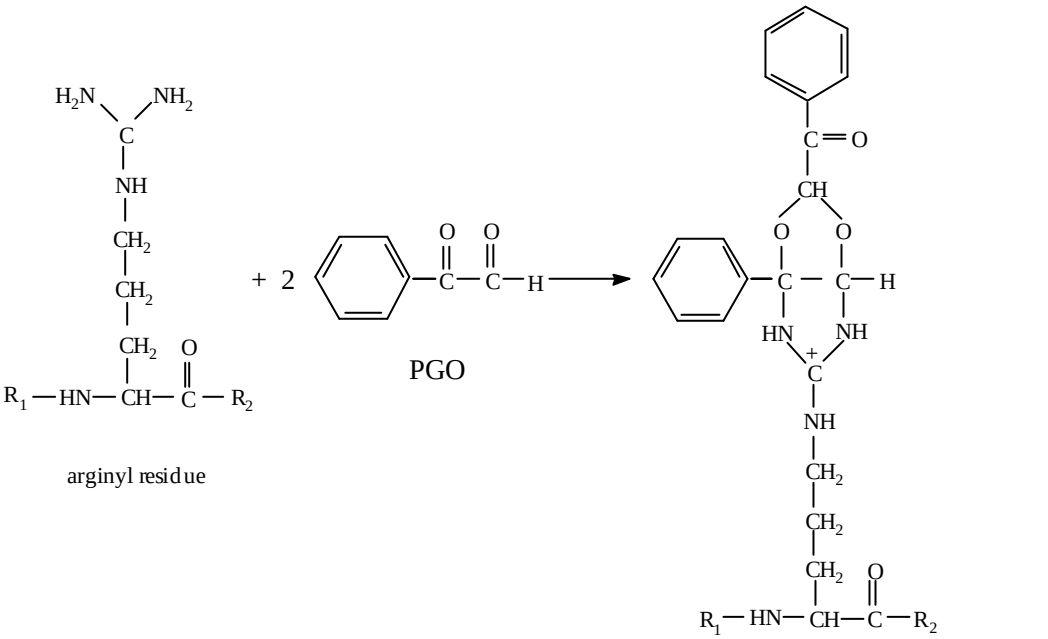

Fig. 8. The reaction of phenylglyoxal (PGO) with arginyl residue.

1.4.4 Modification of glutamyl and aspartyl residues

1-Ethyl-3-(3-dimethylaminopropyl)carbodimide (EDC) was used to modify the glutamyl and aspartyl residues in plasma membranes (1.0-1.5 g/l). Different concentrations of EDC were diluted from a freshly prepared stock solution in distilled water. The incubation was carried out in phosphate buffer (pH 6) at 37°C for 15 min under shaking (Yamada, 1981). The reaction was terminated as above and excess EDC removed by two washing steps.

2 Superfusion experiments on striatal and hippocampal slices

Slices excised from the striata of adult NMRI mice were preloaded with [^{3}H]dopamine (5 μM, 51.8 MBq/l) for 30 min in oxygenated standard Krebs-Ringer-Hepes solution (pH 7.4) containing (in mM): NaCl 126, $MgSO_4$ 1.3, NaH_2PO_4 1.3, KCl 5.0, $CaCl_2$ 0.8, Hepes 15.0, D-glucose 10.0, ascorbate 0.1 and nialamide 0.02. The slices were then transferred into continuously shaken chambers and superfused (0.25 ml/min) under O_2 at 37°C for 90 min (Korpi and Oja, 1984). The first superfusates were discarded, whereafter 0.5 ml fractions were collected at 2-min intervals. At 70 min, the superfusion medium was changed to another containing effectors, e.g. glutamate, kainate, AMPA, NMDA (stimulated release) or left unchanged (basal release). After superfusion, the slices were extracted overnight in 1 M NaOH. The superfusate samples and the extracts were then counted for radioactivity.

Slices excised from the hippocampus of Wistar rats were first preloaded with glutamate analog D-[^{3}H]aspartate (0.16 μM, 285 MBq) for 30 min at 37°C in preoxigenated standard Krebs-Ringer-Hepes solution. They were then treated in the same manner as described in the foregoing. In the first set of experiments at 70 min, the superfusion medium was changed to another containing 1 mM cysteine, while in the second set 50 mM KCl was added. After superfusion, the slices were extracted overnight in 1 M NaOH. The superfusate samples and the extracts were then counted for radioactivity.

3 Binding assays

3.1 [^{3}H]Glutamate binding

The extensively washed synaptic plasma membranes (in all cases 200-300 μg/tube) were suspended in Tris-acetate buffer (pH 7.4) in test tubes and preincubated with the studied compounds at 0°C for 10 min. After preincubation, 20 nM [^{3}H]glutamate was added and incubation continued at 0°C for 60 min under shaking. Non-specific binding was determined in the presence of 1 mM unlabeled glutamate. The experiments were terminated by centrifugation (27 000 g, 20 min). The supernatants were aspirated and the pellets washed twice with 4 ml cold buffer. The pellets were then treated with Triton X-100 and admixed to distilled water and scintillation liquid, and counted for radioactivity.

3.2 [^{3}H]Kainate binding

The plasma membranes (200-300 μg/tube) were again preincubated with the studied compounds at 0°C for 10 min in Tris-acetate buffer (pH 7.4). After preincubation, 20 nM [^{3}H]kainate was added and the incubation continued at 0°C for 60 min under shaking (London and Coyle, 1979). Non-specific binding was determined in the presence of 0.1mM kainate. The experiments were terminated by centrifugation (27 000 g, 20 min). The pellets were rinsed twice with cold buffer, treated with Triton X-100, mixed into scintillation liquid and counted.

3.3 [^{3}H]Fluorowillardiine binding

The synaptic plasma membranes (200-300 μg/tube) were also in this case preincubated with the studied compounds at 0°C for 10 min in Tris-HCl buffer (pH 7.4). After preincubation, the membranes were incubated with 20 nM (S)-5-fluoro[^{3}H]willardiine ([^{3}H]FWD) at 0°C for 40 min in the same buffer (Hawkins et al., 1995). Non-specific binding was determined in the presence of 1 mM glutamate. The experiments were again terminated by centrifugation (27 000 g, 20 min). The pellets were rinsed twice with the same cold buffer, treated with Triton X-100 and mixed into distilled water and scintillation liquid and counted.

3.4 [^{3}H]CPP binding

Here plasma membranes (200-300 μg/tube) were first preincubated with the studied compounds at 0°C for 10 min in Tris-acetate buffer (pH 7.4), then 10 nM 3-{(R)-2-carboxypiperazin-4-yl[1,2-^{3}H]}propyl-1-phosphonate ([^{3}H]CPP) was added and the incubation continued for 15 min at 25°C in Tris-acetate buffer (pH 7.4) (Compton et al., 1990). Non-specific binding was determined in the presence of 1 mM glutamate. The experiments were terminated by centrifugation (27 000 g, 20 min). The pellets were then rinsed with cold buffer, treated with Triton X-100 and counted.

3.5 [^{3}H]Dizocilpine binding

The plasma membranes (200-300 μg/tube) were preincubated at 0°C for 10 min in Hepes-Tris buffer (pH 7.6) with the studied compounds. After preincubation, 1 nM [^{3}H]dizocilpine was added and the incubation continued at 23°C for 30 min in the same buffer under shaking (Reynolds et al., 1987). Non-specific binding was determined in the presence of 30 μM unlabeled dizocilpine. The experiments were terminated by filtration. The samples were filtered onto Whatman B glass filters with suction and rinsed three times with 5 ml cold buffer using a Brandel (Gaithesburg, MD) apperatus. The filters were presoaked in 0.001% of polyethyleneamine to reduce nonspecific binding. After filtration,

distilled water and scintillation liquid were added to the filters and they were counted for radioactivity.

3.6 [^{3}H]Glutathione binding

The extensively washed plasma membranes (200-300 μg/tube) were preincubated with numerous test compounds at 0°C for 10 min in Tris-acetate buffer (pH 7.4). After preincubation, 10 nM [^{3}H]glutathione was added and the incubation continued at 0°C for 60 min in the same buffer under shaking. Non-specific binding was determined in the presence of 1 mM unlabeled GSH. The experiments were terminated by the filtration technique as in the case of [^{3}H]dizocilpine binding.

In addition to untreated, native synaptic plasma membranes, membranes were also used in which amino acid residues were chemically modified prior to the binding assays. After modification, any excess of modifying agents was removed with repeated washing steps. Finally, the samples were treated and counted as above.

The protein content in the membrane samples was determined by the method of Lowry and associates (1951).

4 Ca^{2+} uptake experiments

The Ca^{2+} uptake by cerebellar granular cells was assayed in Krebs-Ringer-HEPES solution containing (mM): NaCl 126, KCl 5.1, $CaCl_2$, 0.81, $MgSO_4$ 0.1, NaH_2PO_4 1.3, HEPES 15, D-glucose 10; pH 7.4, adjusted with NaOH. The cells were preincubated in this medium at 37 °C for 10 min and then 44 MBq/l $^{45}CaCl_2$ (0.37-1.5 PBq/kg) was added. One mM NMDA or 0.1 mM glutamate was added together with $^{45}Ca^{2+}$. When the effect of Zn^{2+} were studied, the medium was changed and 1 mM $ZnCl_2$ was added 1 min before the addition of agonists (NMDA, NMDA+cysteine) and radiolabeled Ca^{2+}**.** In other experiments, the cells were first preincubated with 1 mM $ZnCl_2$, the medium was changed and the agonists and $^{45}CaCl_2$ then added. The incubations were terminated 5 min later by aspiration of the medium and rinsing of cells with Ca^{2+}-free Krebs-Ringer-HEPES solution containing 2 mM ethylenediaminetetra-acetic acid (EDTA). The cells were then dissolved in 0.4 M NaOH, the solution neutralized with 2 M HCl and the radioactivity measured from 200 μl aliquots by scintillation counting (Holopainen et al., 1989).

5 Measurements of intracellular free Ca^{2+}

The measurements of intracellular free Ca^{2+} were carried out by the method of Holopainen and colleagues (1989) on cerebellar granular cells cultured for 7-9 days on coverslips coated with poly-L-lysine. For the fluorescence assays, the coverslips were removed from the culture dishes and carefully rinsed with a balanced salt solution; composition in mM: NaCl 137, KCl 5, $CaCl_2$ 1.0, KH_2PO4 0.44, $NaHCO_3$ 4.2, 2-[-3-hydroxyl-1-1-

bis(hydroxymethyl)ethyl]aminoethanesulfonate (TES) 20, bovine serum albumin (BSA) 0.1 (pH 7.4, 37°C). Ten µl of the acetoxymethyl ester of fura-2 (fura-2/AM) dissolved in dimethylsulfonate (DMSO) (2 g/l) was added to the dishes containing 5 ml of culture medium for the determination of intracellular free Ca^{2+}. The dishes were subsequently incubated at 37°C for 40 min in an air-ventilated incubator. The coverslips were then carefully rinsed with balanced salt solution and mounted in temperature-controlled cuvettes (37°C) containing 2.0 ml of the same medium supplemented with 0.5% bovine serum albumin. A magnetic stirrer was used to mix the sample continuously to ascertain the rapid and even distribution of the compounds added. The fluorescence at 340 nm (ex.) and 505 nm (em.) was then recorded. For calibration, the maximal fluorescence levels were obtained by addition of 1.0 µM ionomycin and the minimum levels by addition of $MnCl_2$. A K_d value of 220 nM was used for the fura-2-Ca^{2+} complex (Grynkiewicz et al., 1985).

6 Calculations

In superfusion experiments the rate constants for [^{3}H]dopamine efflux were computed as negative slopes for the linear least-square regression lines of the logarithm of the percentage of the remaining radioactivity in the slices vs. superfusion time.

In binding assays, the inhibitor concentrations for 50 per cent inhibition (IC_{50} values) with their 95% confidence intervals, maximal activation, and K_d and B_{max} constants were determined by iterative optimization using the Markwardt algorithm. Statistical significances of the differences from the corresponding controls were estimated by Student's t-test using the critical values published for multiple comparisons (Owen, 1962).

Calculation of the concentration of free Ca^{2+} was done as in Tsien and colleagues (1982).

RESULTS

1 Effects of glutamate receptor agonists and glutathione on dopamine release

Glutamate, kainate and AMPA evoked [^{3}H]dopamine release from the mouse striatum in a concentration- and time-dependent manner. Of them, kainate and AMPA (0.5 mM) were more effective than glutamate. NMDA and the metabotropic glutamate receptor agonist trans-1-aminocyclopentane-1,3-dicarboxylate (t-ACPD) failed to affect the basal release of [^{3}H]dopamine **(I, Table 1)**. Other compounds, glycine, γ-glutamylcysteine, DTNB, DTT, L-cysteine, GSH and GSSG were without any marked effect. The antagonists of ionotropic glutamate receptors, e.g. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzol[f]quinoxaline-7-sulfonamide (NBQX), DL-2-amino-5-phosphonovalerate (APV) and dizocilpine failed to affect the basal release.

GSH strongly enhanced the dopamine release evoked by 1 mM glutamate and NMDA **(I, Table 2)**. GSSG, glycine, DTT, DTNB and L-cysteine did not enhance the release, whereas γ-glutamylcysteine had a weak inhibitory effect. Interestingly, the release evoked by 1 mM kainate was enhanced by GSSG but not by GSH **(I, Table 2)**. This enhancing effect of GSSG was inhibited by CNQX and DNQX but not by NBQX. The release evoked by 0.5 mM AMPA was also enhanced by GSSG but not by GSH **(I, Table 2)**.

2 Effects of nitrosoglutathione on the binding of ligands to ionotropic glutamate receptors

GSNO, GSH and GSSG inhibited [^{3}H]glutamate, [^{3}H]CPP and [^{3}H]kainate binding to cerebral cortical membranes in a concentration-dependent manner. The estimated IC_{50} values for the displacers were in the low micromolar range **(II, Table I)**. GSNO was the most effective of them in all cases. In the case of [^{3}H]CPP, GSH and GSSG evoked only partial inhibition, about 70 and 50 per cent, respectively. The binding of [^{3}H]FWD, the agonist of AMPA receptors, was not affected by GSNO, GSH or GSSG.

GSNO inhibited [^{3}H]glutamate, [^{3}H]CPP and [^{3}H]kainate bindings more markedly at low than at high ligand concentrations **(II, Fig. 2)**. The estimated kinetic parameters for the binding of [^{3}H]CPP and [^{3}H]kainate demonstrate that GSNO inhibition was indeed competitive in nature. In the case of [^{3}H]glutamate binding, B_{max} was also altered by GSNO **(II, Table II)**.

[^{3}H]Dizocilpine binding was enhanced in a concentration-dependent manner by GSNO, GSH and GSSG. The NMDA receptor co-agonist glycine increased [^{3}H]dizocilpine binding and its effect was additive to the effects of GSNO, GSH and GSSG. NO donors, SNAP, SIN-1 or nitroglycerine, failed to displace [^{3}H]dizocilpine binding **(II, Fig. 3)**.

3 Properties of [^{3}H]glutathione binding

Marked [^{3}H]glutathione binding was discernible to synaptic plasma membranes prepared from the pig cerebral cortex. The estimated kinetic parameters revealed two binding components of high and low affinity with widely differing K_d and B_{max} values **(III, Table 1)**. The binding of [^{3}H]glutathione was increased in the presence of $CaCl_2$ and when the incubation temperature was elevated to 37°C. The estimated IC_{50} values were for unlabeled GSH in the micromolar range both in the presence of $CaCl_2$ and at a higher temperature.

4 Effects of glutamate derivatives and glutamate receptor ligands on the binding of [^{3}H]glutathione

Of the tested glutamate derivatives and mixed glutamate receptor ligands (all 1 mM), L- and D-aspartate, D-glutamate, L-glutamine and quisqualate had no effect on the binding of [^{3}H]glutathione. L-Glutamate, kynurenate and pyroglutamate slightly but significantly reduced the binding **(III, Table 2).**

Of the NMDA ligands acting at the glutamate agonist or antagonist sites, only quinolinate (1 mM) had a considerably reducive effect. NMDA, 1-aminocyclobutane-cis-1,3-dicarboxylate, and 3-[(R)-2-amino-5-phosphono-pentanoate had no effect. Glycine and the ligands of the glycine co-activatory site in the NMDA receptor, [(+)-1-hydroxy-3-amino-2-pyrrolidone, 1-aminocyclobutanecarboxylate, 7-chlorokynurenate and (RS)-(tetrazol-5-yl)glycine had no displacing effect. Of these ligands, only D-serine (0.5 mM) slightly reduced the binding **(III, Table 3)**.

Of the non-NMDA receptor ligands kainate, AMPA, CNQX, DNQX, and 5,7-dinitroquinoxaline-2,3-dione had no effects. FWD and 6-nitro-7-sulfamoylbenzol[f]quinoxaline-2,3-dione slightly displaced the binding **(III, Table 4)**.

Among the metabotropic receptor agonists and antagonists, only L-AP5 reduced the binding **(III, Table 5)** Two additional compounds were also studied which act at the glutamate uptake site. Of these, 1-aminocyclobutane-trans-1.3-dicarboxylate was inactive, while L-trans-pyrrolidine-2,4-dicarboxylate increased the binding **(III, Table 5)**.

5 Effects of glycine and GABA receptor ligands on the binding of [^{3}H]glutathione

None of the tested compounds was active in the binding of [^{3}H]glutathione **(III)**.

6 Effects of cysteine, cysteine derivatives and sulfhydryl compounds on the binding of [^{3}H]glutathione

Of the compounds tested, L-cystamine, L-cysteamine, L-cysteine, dithiothreitol, L-homocysteate and aminomethanesulfonate (all in 1 mM) proved to be the most effective displacers of [^{3}H]glutathione in this order of decreasing potency **(III, Table 6)**. However, L-cysteine was a 40 times weaker displacer than GSH itself. In spite of micromolar values of IC_{50}, these compounds had higher affinities for the GSH binding sites than glutamate receptor antagonists. L-Cysteate, taurine, homotaurine and cystathionine had no effect, but hypotaurine slightly enhanced the binding. Thiokynurenate strongly enhanced the binding of [^{3}H]GSH in a concentration-dependent manner **(III, Table 6)**.

7 Effects of some dipeptides and glutathione derivatives on the binding of [^{3}H]glutathione

γ-L-Glutamylcysteine and cysteine diminished [^{3}H]GSH binding with equal efficacy, whereas L-cysteinylglycine was less active. However, if L-cysteine in γ-L-glutamylcysteine was oxidized to cysteate (γ-L-glutamylcysteate), the binding of [^{3}H]GSH was slightly increased. γ-L-glutamyl-GABA and γ-L-glutamylleucine strongly enhanced the binding in a concentration-dependent manner. γ-D-glutamylglycine and γ-L-glutamyl-phenylalanine were weak displacers, while glycylglycine enhanced the binding **(III, Table 7)**. All GSH derivatives tested effectively displaced [^{3}H]GSH, GSH and GSNO being the most effective of them. Of the S-alkyl derivatives of glutathione, compounds possessing hydrophobic residues were more effective than those with hydrophilic residues. These compounds reduced the binding in a concentration-dependent manner with moderate efficacy **(III, Table 8)**.

8 Effects of chemical modification of cysteinyl residues and disulfide bridges on the binding of [^{3}H]glutathione

The cysteinyl residues were oxidized with 5,5-dithio-bis-2-nitrobenzoate (DTNB) and 4,4-dithiodipyridine (DDP). Treatment with DTNB considerably increased the binding of [^{3}H]GSH in a concentration-dependent manner **(IV, Fig. 2)**. Kinetic analyses revealed that treatment with 1 mM DTNB followed by washing increased the maximal binding capacity (B_{max}) 6-fold, while the affinity (K_d) was not markedly altered. The non-saturable binding also increased considerably **(IV, Table I)**. When synaptic membranes were pretreated with 1 mM GSSG for 15 min prior to addition of DTNB (protection experiment), the enhancing effect of different concentrations of DTNB was significantly inhibited, but complete inhibition could not be reached **(IV, Fig. 2)**.

Another oxidizing agent, 4,4'-dithiopyridine (DDP), also enhanced binding of [^{3}H]GSH in a concentration-dependent manner, but this enhancing effect was less pronounced than that of DTNB.

The reducing agent DTT lowered the specific binding of [^{3}H]GSH in a concentration-dependent manner. Modification of proteins with 5 mM DTT reduced [^{3}H]GSH binding by 30% **(IV, Fig. 3)**. When DTT was present only during the actual binding assays for 30 or 60 min, inhibition was greater in both

control and DTNB-treated membranes **(IV, Fig. 4)**. When membranes were pretreated with 1 mM DTT followed by washing prior to addition of 1 mM DTNB, the enhancing effect of DTNB was significantly potentiated. In contrast, when the membranes were pretreated with 1 mM DTNB followed by washing, the enhancing effect of DTNB was totally blocked by consecutive treatment with DTT **(IV, Fig. 5)**.

The alkylating agent NEM (1 mM) significantly reduced the binding of [^{3}H]GSH, but this inhibition was not concentration-dependent. The binding of [^{3}H]GSH was not altered in the protection experiments, when the membranes were preincubated with 1 mM GSSG and washed before the addition of different concentrations of NEM.

The cysteinyl and lysyl modifier agent PITC reduced the binding. Its inhibiting effect was additive with that of NEM when the membranes were treated consecutively with these compounds **(IV)**.

9 Effects of modification of arginyl residues on the binding of [^{3}H]glutathione

The specific modifier agent PGO of the guanidino groups in arginyl residues significantly reduced the binding of [^{3}H]GSH. The effect was not concentration-dependent **(IV)**.

10 Effects of modification of glutamyl and aspartyl residues on the binding of [^{3}H]glutathione

Treatment of the carboxyl groups in glutamyl and aspartyl side-chains of synaptic plasma membranes with EDC did not alter the binding of [^{3}H]GSH **(IV).**

11 Effect of cysteine on the neuronal $^{45}Ca^{2+}$ influx and the level evoked by glutamate and N-methyl-D-aspartate

L-Cysteine alone slightly enhanced $^{45}Ca^{2+}$ influx into cultured cerebellar granular cells in a concentration-dependent manner. One mM cysteine enhanced the influx only by 15%, but it also elevated the Ca^{2+} influx evoked by both 0.1 mM glutamate and 1 mM NMDA in a concentration-dependent manner. The effect of NMDA was potentiated by cysteine by as much as by 30% **(V, Fig. 1)**.

When the cerebellar granular cell cultures were pretreated with 1 mM $ZnCl_2$ or $ZnCl_2$ was applied together with cysteine, the basal influx of Ca^{2+} was not altered. The substantial potentiation of Ca^{2+} influx evoked by NMDA together with cysteine was markedly attenuated by the presence of Zn^{2+} and pretreatment with Zn^{2+} (**IV**). The effect of NMDA together with cysteine was totally blocked by the application of NMDA receptor antagonist D-AP5 and the

use-dependent blocker of the NMDA receptor-governed ion channel dizocilpine **(V, Fig. 2)**.

12 Effect of cysteine on intracellular free Ca^{2+}

Application of 0.5 mM glutamate to the incubation medium resulted in a small but significant elevation in intracellular free Ca^{2+}. Upon repetitive applications of glutamate, intracellular free Ca^{2+} increased step by step. Cysteine alone in a same concentration likewise increased free Ca^{2+}, though the effect was of lesser magnitude. Both cysteine applied after glutamate and glutamate applied after cysteine resulted in a linear increase in the content of intracellular Ca^{2+} **(V, Fig. 3)**.

13 Effect of cysteine on the release of D-aspartate from rat hippocampal slices

One mM L-cysteine evoked the release of labeled glutamate from hippocampal slices. In another experiment 50 mM KCl also evoked the release of labeled D-aspartate and this release was elevated by the addition of L-cysteine (**VI, Fig. 5**).

DISCUSSION

1 Methodological considerations

1.1 Dopamine and glutamate release

The in vitro dopamine release was analyzed using radioactive [^{3}H]dopamine. This is a common and useful approach in studying whether or not the investigated compounds influence the basal release of the preloaded labeled dopamine or the release evoked by NMDA receptor agonists from mouse striatal slices. The results from the release experiments allow us to draw inferences as to the involvement of the compounds in the modulation of neurotransmission. In the case of dopamine release, the original label was separated by thin-layer chromatography to be sure that the release of dopamine was measured and not the release of its metabolites.

In the case of glutamate release, the effect of L-cysteine on the D-[^{3}H]aspartate release was investigated to support the hypothesis that excessive release of glutamate is involved in the toxicity of L-cysteine.

1.2 Binding experiments

Well-established ligand-binding methods were used to study the effects of GSNO on the binding of specific tritiated NMDA and non-NMDA receptor ligands to the synaptic plasma membrane preparation. Pharmacological binding experiments using ligands and antagonists specific for different ionotropic glutamate receptors characterize changes at the level of receptor classes. They do not, however, differentiate between the functions of different subunits comprising the receptor. This method suffices nonetheless to reveal the involvement of GSNO in glutamatergic neurotransmission, since this compound proved to interact with glutamatergic receptors.

Binding assays are also useful in distinction of the specific binding sites for GSH to any known excitatory or inhibitory amino acid receptor. Findings here corroborate our statement that GSH possesses a receptor of its own. Opposed to the molecular biology approach, native receptors can be studied with this method. However, this method has only limited applicability, since identification of the GSH receptor protein is not possible. Molecular biological techniques are thus needed for the identification of the receptor protein, its subunits and the downstream mechanisms involved.

1.3 Chemical modification of receptor proteins

Chemical modification of proteins with specific reactions between given amino acid residues and the modifier agent results in the formation of a covalent bond (Lundblad and Noyes, 1984). The method was originally used for the identification of amino acid residues involved in substrate binding to the active center of enzyme proteins. With certain restrictions, this method is usable for the identification of amino acids involved in binding of the ligands studied. Chemical modification can be used e.g. with cell cultures, expressed receptor subunits and purified synaptic plasma membranes. The latter contain receptors in the highest density. The effect of modification can be assessed by radioligand binding studies or electrophysiological studies. The inhibition of binding induced by such modification does not necessarily indicate the direct involvement of a certain amino acid residue in ligand binding, since the inhibition can be allosteric. More reliable results can be obtained with protection experiments in which the membrane receptor proteins are modified in the presence of their specific ligands. If a given amino acid is directly involved in the binding of a ligand, then the binding site is occupied by the ligand and there are no more sites accessible to the modifier agent and it can no longer modify the residues. Hence, there is no inhibition of binding or the binding is attenuated. If inhibition is seen in the presence of the modifying agent, then it may reflect either an allosteric effect of the amino acid studied or its involvement in receptor activation. To avoid any interaction between amino acids and the modifying agent, it is necessary to keep reaction conditions stable (concentration of modifier and protein, incubation time and temperature, pH and buffers). A marked advantage of this method is that it allows investigation of the native receptor proteins.

Results from chemical modification of the native receptor proteins and molecular biology experiments can yield complementary information regarding the structure-function relations of the receptors.

2 Regulation of dopamine release by glutamate receptor ligands and glutathione

Our data indicated that striatal dopamine release is regulated by the activation of ionotropic glutamate receptors. This is in concert with the finding that dopaminergic nerve terminals possess ionotropic glutamate receptors of both NMDA and non-NMDA types, which induce the release of dopamine (Jin and Fredholm, 1994). Endogenous glutamate released from nerve terminals may act at pre- or postsynaptic ionotropic glutamate receptors and mediate fast and prolonged excitatory effects, and consequently evoke dopamine release. AMPA and kainate receptors, but to a lesser extent NMDA receptors, may thus be responsible for prolonged dopamine release. Since GSH is present in relatively high concentrations both intra- and extracellularly in the brain, it can interfere with glutamatergic neurotransmission, regulating the function of NMDA receptors by at least three mechanisms: it can bind to the agonist recognition site by means of the γ-glutamyl moiety (Janáky et al., 1993; Ogita et al., 1995), interact with the glycine coactivatory site by the glycine moiety, and regulate the redox state of functional thiol groups in the receptor proteins (Sucher and Lipton, 1991). Reduced glutathione enhances the glutamate-evoked dopamine release and this effect of GSH may be of importance in the regulation of extrapyramidal motor functions. Biochemical changes, in particular a decrease in GSH levels, occurring in the substantia nigra may be an early component in the pathomechanism of Parkinson's disease. The GSH loss appears to be global throughout the substantia nigra and not localized to the glial cells or neurons. In addition, GSH may act directly by facilitating the release of dopamine via activation of ionotropic glutamate receptors at the nigrostriatal dopaminergic nerve endings. Hence, GSH may have a beneficial effect in Parkinson’s disease (Owen et al., 1996). Glutathione may also affect the release indirectly by antagonizing the glutamate receptors at inhibitory GABAergic neurons or modulating the functions of cholinergic and somatostatinergic interneurons.

3 Interactions of nitrosoglutathione with ionotropic glutamate receptors

We showed that GSNO interacts with different ionotropic glutamate receptor binding sites and displaces glutamate and specific ligands of the glutamate receptors with high efficacy. The affinity of GSNO is highest for NMDA and kainate receptors. This interaction of GSNO with NMDA and kainate receptors is likely to be mediated by the γ-glutamyl moiety of the peptide, since a number of other γ-glutamyl peptides which do not contain cysteine or glycine displace glutamate and its analogs with similar efficacy (Varga et al., 1994). Such a conception is supported by the findings that the NO

donors SNAP, SIN-1 and nitroglycerin, which do not contain a γ-glutamyl moiety, failed to displace the specific ligands from their binding sites and did not enhance the binding of dizocilpine. However, GSH, GSSG and GSNO were all effective at NMDA and kainate receptors.

In addition, the concentration-dependent enhancing effect of GSH, GSSG and GSNO in dizocilpine binding was additive with the effect of glycine. The affinities of GSH and GSNO for the receptors are very similar. It is likely that the whole molecule of GSNO is responsible for this effect and not NO possibly liberated from the GSNO molecule.

The physiological actions of GSNO are apparently complex and do not reflect only its binding to receptors. Under physiological conditions, GSNO is broken down to GSH and NO. The binding of GSNO to plasma membranes is followed by the liberation of NO in situ and may evolve specific effects. NO inhibits NMDA receptor-mediated responses (Manzoni et al., 1992a; Butler et al, 1985; Hoyt et al., 1992; Fagni et al., 1995). It is thus likely that NO liberated from GSNO release at relatively high concentrations may react with NMDA receptors. Nitrosonium ion (NO^+) liberated from nitrosothiols interacts with the redox modulation sites of NMDA receptors and produces nitrosocysteine, which attenuates receptor activity (Lei et al., 1992; Lipton et al., 1993; Arnelle et al., 1995). This mechanism is generally referred to as S-nitrosation. GSH can also form mixed disulfides with cysteine or non-physiological thiols, which process is referred to as S-thiolation. When GSNO liberates the glutathionyl residue, mixed disulfide bridges may be formed. This process is also referred to as S-thiolation or S-glutathiolation. It is of interest that S-nitrozation of the model protein bovine serum albumin and the subsequent reaction of the formed S-nitrosothiol with GSH yields a mixed disulfide, but this reaction does not occur with the same in situ specificity as observed when S-glutathiolation is induced by GSNO (Klatt et al., 2000). NO and GSNO may thus thiolate proteins by different mechanisms. As a consequence of S-nitrozation and S-glutathiolation, a considerable conformational change ensues, followed by modulation of the receptor ionophore activity.

Protein S-glutathiolation has been implicated in the buffering of oxidative stress, stabilization of extracellular proteins, protection of critical cysteine residues in proteins against irreversible oxidation, and regulation of enzyme activity (Cotgreave and Gerdes, 1998). Disulfide bridges can also be formed under oxidative stress by means of reactive oxygen or nitrogen species, which is a kind of the defence mechanism of receptor proteins. Intracellularly, disulfides are readily broken and reduced in enzymatic reduction catalyzed by specific disulfide reductases such as thioredoxin and glutaredoxin, or non-enzymatically by alterations in the redox potentials (Prinz et al., 1997; Klatt and Lamas, 2000).

Endogenous nitrosothiols (mainly GSNO) are present at nano- to micromolar concentrations in the lung and brain (Hogg et al., 1996; Kluge et al., 1997). The concentration of GSNO is increased by the activation of NMDA receptors in the brain (Kluge et al., 1997). It has also been reported that NO and NO donors may deplete intracellular GSH pools (Balanos, 1996; Padgett and Whorton, 1998; Chatterjee et al., 2000) and the resulting decrease in the GSH/GSSG ratio is accompanied by an increase in intracellular mixed protein disulfides (Padgett and Whorton, 1998). However, a serious depletion of GSH may result in neurodegeneration. Some toxins enhance the uptake of cysteine, which stimulates the synthesis of GSH in astrocytes and simultaneously

promotes the carrier-mediated GSH release (Sagara et al., 1996). The GSH released then protects cells by capturing NO and by the above-mentioned S-thiolation. The binding of GSNO, GSH and GSSG to NMDA receptors may prevent their exaggerated activity and forestall the torrent of Ca^{2+} into the cell (Janáky et al., 1993). The Ca^{2+}-dependent stimulation of NO synthase and the further formation of NO are concomitantly intercepted. GSH and GSNO may protect cells by means of this mechanism (Werns and Lucchesi, 1990; Jain et al., 1991, Ji et al., 1999). In addition, Taguchi and associates (1995) have reported specific binding of [^{3}H]GSNO to synaptic plasma membranes. There would appear to be several different binding sites available for GSNO.

4 Binding of glutathione to synaptic plasma membranes

It was already presumed more than 50 years ago that Hydra vulgaris possesses a GSH receptor of its own, since GSH provoked a specific feeding reaction (Loomis et al., 1955). However, until recently no receptor protein had ever been identified. In rat cerebral cortical slices, GSH elicits concentration-dependent excitatory field potentials which are not blocked by any antagonist of ionotropic glutamate receptors (Shaw et al., 1996; Pasqualotto et al., 1998; Bains et al., 1998). It is hence to be presumed that GSH produces these potentials through receptors which are not any known glutamate receptors.

We now found two different binding populations (high- and low-affinity) for GSH in synaptic plasma membranes prepared from the pig cerebral cortex, which finding is in concert with the results of Ogita and Yoneda (1988). The existence of selective binding sites for GSH is also supported by other investigations. Immunocytochemically, GSH has been located in the CNS in both neurons and glia (Hjelle et al., 1998). The distribution of [^{3}H]GSH binding sites also shows regional heterogeneity within the brain (Shaw, 1998).

In order to be sure that the binding sites detected represent neurotransmitter receptors, such circumstances should be established under which it is possible to exclude the binding of GSH to other proteins. Radiolabeled GSH can namely bind to proteins other than receptors, for example, to Na^+-dependent or -independent GSH transporters (Kannan et al., 1998) and to plasma membrane ectoenzymes (glutathione transferase, γ-GT). Na^+ and Cl^- gradients supply the driving force for the transmembrane fluxes of many neurotransmitters and amino acids. The binding experiments were therefore carried out in Tris-acetate buffer, which did not contain Na^+ and Cl^-. In the absence of these ions GSH probably does not bind to transport proteins. Since the inhibitor of Na^+-dependent, high-affinity glutamate transport, 1-aminocyclobutane-trans-1,3-dicarboxylate, has been ineffective (Fletcher et al., 1991) and L-trans-pyrrolidine-2,4-dicarboxylate only slightly enhances the binding (Mitrovic and Johnston, 1994), [^{3}H]GSH is bound to proteins other than glutamate transporters. Pasqualotto and colleagues (1998) have demonstrated that [^{3}H]GSH does not bind to γ-GT. Our present experiments are in keeping with these findings. Neither acivicin, an inhibitor of γ-GT, nor any substrate of γ-GT was effective in displacing [^{3}H]GSH.

Since [^{3}H]GSH was not broken down upon incubations, the bound radioactivity represents GSH binding and not that of the labeled glycine moiety

liberated from the GSH molecule. This conception is corroborated by the findings that neither glycine nor glycinergic compounds displace [^{3}H]GSH. GSH at micromolar concentrations displaces the ligands of all ionotropic glutamate receptors and at millimolar concentrations also glycine from the co-agonist site on NMDA receptors (Oja et al., 1988, Varga et al., 1989, 1997; Ogita et al., 1995, 1998; Janáky et al., 1993, 1998; Jenei et al., 1998). [^{3}H]GSH could thus label glutamate receptors. The binding of GSH to glutamate receptors would reflect a neuromodulatory and not a neurotransmitter role. To exclude the possible overlap between the glutamate receptors and the putative GSH receptor, we tested glutamate analogs, mixed glutamate receptor ligands, NMDA receptor ligands, glycine, ligands of the glycine co-activatory site in the NMDA receptor, inhibitory amino acids, non-NMDA receptor ligands and agonists or antagonist of metabotropic glutamate group I, II and III receptors. These compounds were either ineffective or effective only at very high concentrations. Among them, the NMDA receptor subtype-specific agonist quinolinate had only a moderate effect (Monaghan and Beaton, 1991; Prado de Carvalho et al., 1996). GSH thus displaces glutamate from its binding sites but glutamate not GSH from its binding site. In addition, thiokynurenate, a cysteine derivative, evinced a strong concentration-dependent activatory effect, whereas kynurenate was ineffective. These results strongly support our assumption that GSH possesses binding sites of its own, which differ from any known glutamate receptor.

In different species and brain regions, slight pharmacological differences are discernible in GSH binding. The existence of more than one subtype of GSH binding sites is thus likely. For example, there is a difference in pharmacological profile between GSH receptors in the cerebral cortex and those in subcortical regions. The latter are sensitive to metabotropic glutamate receptor ligands, e.g. to quisqualate and L(+)-2-amino-4-butyrate, while the former are not. In addition, in the subcortical GSH receptors L-glutamate partially displaces the binding of GSH. In this respect it may be of significance that two glutamate receptor channel subunits (orphan subunits) have so far remained unassigned (Lomeli et al., 1993; Dingledine, 1999). These results and the reports cited above support the hypothesis that GSH can act as a neurotransmitter at its own population of receptors**.**

We showed here that different thiol-containing compounds effectively displace [^{3}H]GSH from its binding sites, a propensity not shared by glutamate binding. By means of tests with these compounds many structure-function relationships were revealed. The most effective compounds were cysteine and the cysteine derivatives cysteamine and cystamine. Of the dipeptides, only those compounds were effective which contain the cysteinyl moiety. Dipeptides with a similar composition have also produced GSH-like excitatory field potentials in cortical wedge preparations (Pasqualotto et al., 1998). In addition, dithiothreitol also proved to be a strong displacer. The oxidation or alkylation of the cysteinyl moiety in the GSH molecule reduces the binding of GSH to its binding sites. On the basis of these results we may assume that the cysteinyl moiety in the GSH molecule is crucial for the binding of GSH. GSH binds to the glutamate receptors by means of the γ-glutamyl moiety, while the binding of GSH to its own receptor occurs by means of the cysteinyl moiety. These findings indicate that the neuromodulatory and neurotransmitter functions of the GSH molecule stem from different constituents (Lipton and Stamler, 1994; Janáky et al., 1998; Ogita et al., 1998). The strong activation by thiokynurenate, but not by

kynurenate, indicates that the GSH receptor protein may contain a modulatory site to which co-agonists bind and allosterically activate GSH binding. On the basis of our results and the well-known properties of GSH, it can be stated that GSH has probably preserved the mediator property in addition to its antioxidant and free-radical-scavenger properties from Hydra vulgaris up to higher mammalian species. The GSH binding sites may thus be an important, largely unrecognised component in signal transduction in some neural circuits in the central nervous system.

5 Role of amino-acid side-chains in glutathione binding

In order to obtain evidence as to how different amino acids in synaptic plasma membranes are involved in the binding of GSH, the possibly functional amino-acid residues in plasma membranes were chemically modified. The results from these experiments showed that both disulfide bonds and free thiols are involved in the binding of glutathione.

The oxidation of cysteinyl side-chains with DTNB and DDP generate mixed disulfide bonds. Both compounds strongly and concentration-dependently increased the binding of [^{3}H]GSH. The reducing agent DTT, which breaks disulfide bridges, inhibited the binding of GSH, but pre-treatment of the membranes with DTT potentiated the enhancing effect of DTNB. However, when modification with DTNB was followed with DTT, the enhancement by DTNB was completely reversed. On the basis of these results we assume that reduced glutathione is also able to break disulfide bonds in synaptic plasma membranes. However, sulphur atoms in the cysteinyl side-chains and GSH molecule form new mixed disulfide bonds. In addition to this, GSH can attack disulfide bonds in membrane proteins without forming covalent bonds as a reducing agent. In this case, the cysteinyl SH in two GSH molecules donates a proton to the sulphur atoms in proteins. In this manner new sulphur atoms are reduced and new cysteine formed, while GSSG is formed from two GSH molecules. This oxidation process results in [^{3}H]GSSG molecules which can bind to free cysteinyl groups. This binding could be inhibited by irreversible alkylation of free protein thiols by NEM or by inclusion of a phenyl moiety from PITC, resulting in both cases in the formation of S-C bonds. In this case, [^{3}H]GSH binding decreases, since neither GSSG nor GSH can break these sulphur-carbon bonds. GSSG and NEM are probably competitors for the same membrane cysteinyl thiols, since treatment with both of them reduced the binding and treatment with GSSG prior to NEM failed to modify the effect of NEM.

After treatment with DTNB and DDP, [^{3}H]GSH is bound to the newly formed mixed disulfide ‘binding sites’, which are derived from the accessible SH groups in the membrane. These binding sites are artificial and not the same as physiological binding sites. They induce a marked increase in non-saturable binding. The formation of such new mixed disulfide GSH binding sites was potentiated by DTT and inhibited by GSSG added prior to DTNB or DDP. The former treatment probably yields more accessible protein thiols, while the latter masks the existing ones. The finding that the effects of NEM and PITC were almost additive renders it likely that under our experimental conditions PITC

also acted at moieties other than the cysteinyl side-chains. It is possible that GSH can bind not only to the disrupted disulfides and to free cysteine groups but can also form covalent bonds with other functional groups. Indeed, at pH 8 the lysyl and arginyl residues could also be modified by PITC (Edman, 1950) and form phenylthiocarbamoyl-lysyl and -arginyl side-chains (Ott et al., 1994). In addition, modification of arginyl residues in plasma membranes with phenylglyoxale attenuated the binding of [^{3}H]GSH. These findings suggest that the guanidino group in arginyl residues and the free amino group in lysyl residues in the plasma membranes may be involved in the stabilization of GSH binding. The flexibility of the glutathione molecule may allow simultaneous binding to the sulphur atom and the neighboring amino groups. On the other hand, both cysteinyl thiol and lysyl amino groups are involved in the formation of the tertiary structure of proteins. This simultaneous modification could therefore induce such conformational changes which would not mean direct involvement of lysyl side-chains in the binding. The glutamyl and aspartyl carboxyl groups in the membranes do not seem to have a role in the binding of GSH. The final evaluation of the character and physiological significance of the sites to which GSH binds in synaptic membranes must, however, await the purification and identification of putative receptors by molecular biology methods.

6 Effect of cysteine on Ca^{2+} influx, intracellular free Ca^{2+} and glutamate release

Activation of NMDA receptors induces Ca^{2+} influx through the associated ionophore. If cysteine interacts with the receptors it may influence the Ca^{2+} influx into neurons and give rise to a concomitant change in the levels of free intracellular Ca^{2+}. We showed that cysteine directly activates NMDA receptors in cerebellar granular cells. This finding is in concert with the results reported by Olney and associates (1990) on other preparations. Cysteine enhances the influx of Ca^{2+} evoked by glutamate and NMDA. The Ca^{2+} influx evoked by NMDA together with cysteine was attenuated by both the presence of Zn^{2+} and pretreatment with Zn^{2+}. All this indicates that cysteine reduces disulfides in the NMDA receptors and may chelate Zn^{2+} at the receptor-channel complex, relieving the physiological blockage of the channel. Cysteine also potentiates the increase in intracellular free Ca^{2+} evoked by glutamate. Cysteine can thus act through the redox modulation site of the NMDA receptor at higher concentrations. It is not precluded, however, that the Ca^{2+} flux into the intracellular space evoked by cysteine may even alter the intracellular pump mechanisms of the endoplasmatic reticulum or that Ca^{2+} releases Ca^{2+} from the reticulum (Ca^{2+}-induced Ca^{2+} release).

Cysteine can scavenge nitrosonium ion, the inhibitor of NMDA receptors, forming cysteine-NO (Cys-NO) and bringing about protective effects. On the other hand, the Ca^{2+} influx is at the same time increased. Under physiological conditions a fine-tuned balance presumably obtains between neuronal glutamate and NO and/or neuronal and glial release of L–cysteine, promoting in this manner normal activation of NMDA receptors without damage to neurons.

L-Cysteine may induce the release of glutamate or aspartate or enhance the release of glutamate evoked by K^+ depolarization. In addition, L-Cysteine has been shown to inhibit glutamate reuptake in neurons (Ferkany et al., 1986) and production of free radicals may stimulate the release (Gilman et al., 1994) or inhibit the uptake of glutamate (Volterra et al., 1994). L-Cysteine, acting together with NMDA receptor ligands, potentiates the release of transmitters evoked by these ligands. The intracellular Ca^{2+} level is thence increased this initiating toxic processes. These can be potentiated by the cysteine inhibition of the uptake of glutamate and D-aspartate into cerebellar granular cells. The elevated glutamate concentration in the extracellular space may contribute to L-cysteine toxicity. The accumulation of glutamate in the extracellular space, the concomitant enhancement of Ca^{2+} influx and the increase in the intracellular free Ca^{2+} level may thus underlie L-cysteine toxicity.

The excitotoxic effect of L-cysteine may be involved in different neurodegenerative diseases. The concentration of L-cysteine is increased in blood plasma in such diseases (Wallin et al., 1999). In brain ischemia, the level of L-cysteine in the intracellular and extracellular spaces is also elevated (Slivka and Cohen 1993). The overexcitation evoked by glutamate may thus stem partially from changes in the concentration and distribution of L-cysteine.

SUMMARY

(1) Glutamate, kainate and AMPA evoked [^{3}H]dopamine release from the mouse striatum. GSH strongly enhanced the dopamine release evoked by glutamate and NMDA, whereas the release evoked by kainate is affected by oxidized glutathione but not by GSH. GSH regulating neurotransmitter release of dopamine may act as a neuromodulator.

(2) GSNO interacts with ionotropic glutamate receptors. The interaction is likely to be mediated by the γ-glutamyl moiety of the peptide. GSNO effectively displaces specific NMDA and kainate receptor ligands and this inhibition is competitive in nature. The binding of GSNO to plasma membranes involves liberation of NO derivatives, which may induce specific effects by interacting with cysteine residues in membrane proteins. The glutathionyl residue liberated from GSNO may form disulfide bonds with membrane proteins and bring about conformational and functional alterations in the proteins. It is suggested that GSNO may act as a neuromodulator, by means of these two mechanisms, nitrozation and glutathiolation.

(3) GSH has receptors of its own in cortical synaptic plasma membranes, high- and low-affinity. The cysteinyl moiety in the molecule is essential for the binding of GSH to these receptors. According to our results, these specific binding sites for GSH differ from any known excitatory or inhibitory receptor. GSH with the specific receptor may act not only as a neuromodulator but also as a neurotransmitter in the CNS.

(4) On the basis of the results from chemical modification of amino-acid side-chains in the membrane we assume that glutathione binds to disulfides and free thiol groups in synaptic plasma membranes. The arginyl and lysyl residues are probably also involved in the binding of GSH.

(5) L-Cysteine increases the Ca^{2+} flux evoked by glutamate and NMDA into cerebellar granular cells. It relieves the blockage of NMDA receptors induced by Zn^{+}. Cysteine alone slightly elevates the levels of free intracellular Ca^{2+}. It also strongly potentiates the increase in the level intracellular of Ca^{2+} evoked by glutamate. These effects of cysteine may contribute to cysteine toxicity.

(6) Cysteine may also contribute to glutamate neurotoxicity by inducing glutamate release from nerve endings.

In this thesis additional evidence was presented for the neuromodulatory behavior of glutathione. On one hand, GSH regulates dopaminergic and glutamatergic neurotransmission, e.g., by scavenging NO and forming GSNO. On the other, cysteine derived from GSH may cause toxic effects at high concentrations by elevating intracellular Ca^{2+} and enhancing glutamate release. In addition, a neurotransmitter role of GSH is evinced by the finding that GSH possesses receptors of its own, which play a pivotal role in neurotransmission throughout in CNS.

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