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Nitric Oxide Synthase Inhibition and Glutamate Binding in Quinolinate-Lesioned Rat Hippocampus

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Summary

The effect of lesions induced by bilateral intracerebroventricular (ICV) injection of quinolinate (250 nmol of QUIN/ventricle), a selective N-methyl-D-aspartate (NMDA) receptor agonist, on [^{3}H]glutamate ([^{3}H]Glu) binding to the main types of both ionotropic and metabotropic glutamate receptors (iGluR and mGluR) was investigated in synaptic membrane preparations from the hippocampi of 50-day-old rats. The membranes from QUIN injured brains revealed significantly lowered binding in iGluR (by 31 %) as well as in mGluR (by 22 %) as compared to the controls. Using selected glutamate receptor agonists as displacers of [^{3}H]Glu binding we found that both the NMDA-subtype of iGluR and group I of mGluR are involved in this decrease of binding. Suppression of nitric oxide (NO) production by N^G-nitro-L-arginine (50 nmol of NARG/ventricle) or the increase of NO generation by 3-morpholinylsydnoneimine (5 nmol of SIN-1/ventricle) failed to alter [^{3}H]Glu or [^{3}H]CPP (3-((D)-2-carboxypiperazin-4-yl)-[1,2-^{3}H]-propyl-1-phosphonic acid; NMDA-antagonist) binding declines caused by QUIN-lesions. Thus, our findings indicate that both the NMDA-subtype of iGluR and group I of mGluR are susceptible to the QUIN-induced neurodegeneration in the rat hippocampus. However, the inhibition of NO synthesis did not reveal any protective action in the QUIN-evoked, NMDA-receptor mediated decrease of [^{3}H]Glu binding. Therefore, the additional mechanisms of QUIN action, different from direct NMDA receptor activation/NO production (e.g. lipid peroxidation induced by QUIN-Fe-complexes) cannot be excluded.

Key words
Quinolinic acid • NMDA receptor • Membrane binding • Nitric oxide synthase (NOS) • Neurodegeneration

Introduction

Quinolinic acid (QUIN), an endogenous metabolite of L-tryptophan, is produced by infiltrating monocytes, macrophages and activated microglia as an integral part of the inflammatory response in the central nervous system (CNS) (Heyes *et al*. 1992a, 1992b, 1996). Within the CNS, QUIN activates some heteromers of the N-methyl-D-aspartate (NMDA) receptor (Prado de Carvalho *et al*. 1996, Šťastný *et al*. 1999) that mediate its neurotoxic action. Therefore, an intracerebroventricular (ICV) infusion of low doses of this excitotoxin damages the rat hippocampal formation with some similarities to the hippocampal damage observed in chronic neurodegenerative diseases (Keilhoff *et al*. 1990, 1991). The accompanying changes in [^{3}H]glutamate ([^{3}H]Glu) receptor binding to hippocampal membranes and in the equilibrium binding constant (K_d) for Glu suggest that the

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specific binding to rat hippocampal membranes can be used as a sensitive marker of QUIN-induced hippocampal damage (Lisý *et al*. 1994). Delayed losses of rat hippocampal neurons observed after the QUIN infusion seem to suggest a possible involvement of metabotropic glutamate receptors (mGluRs) in this process. This assumption has been strengthened by studies that demonstrated strong potentiation of the NMDA-receptor mediated brain damage by co-activation of group I metabotropic glutamate receptors (mGluRs) (McDonald and Schoepp 1992, Schoepp and Sacaan 1994). However, the involvement of individual types of ionotropic (iGluR) and groups of metabotropic glutamate (mGluR) receptors in the mechanism of QUIN-induced neurodegeneration still remains to be elucidated.

The QUIN-induced neurotoxicity elicited *via* NMDA receptors appears to be mediated by nitric oxide (Dawson *et al.* 1991). However, the results of Lerner-Natoli *et al.* (1992) did not support this hypothesis for hippocampal neurons. In the case of ICV administered QUIN, nitric oxide synthase (NOS) inhibitors not only failed to suppress, but even potentiated these excitotoxin-induced neuronal losses in some hippocampal subregions (Haberny *et al.* 1992). On the other hand, the prevention of acute QUIN-induced striatal neurotoxicity by inhibition of NOS has recently been reported (Pérez-Severiano *et al.* 1998). These discrepancies can be resolved on the basis of studies demonstrating that NO may either be neurotoxic or neuroprotective depending on the dose and route of administration of the insult molecule (Globus *et al.* 1995).

In the present study, we decided (a) to delimit changes in [^{3}H]Glu receptor binding to the main types/groups of both iGluRs and mGluRs in membranes isolated from the rat hippocampal formation lesioned by ICV infusion of QUIN in relation to some previous findings (Norman *et al.* 1991, Lisý *et al.* 1994) and (b) to decide whether NO can be considered as a mediator of QUIN-induced hippocampal damage (Moncada *et al.* 1992). We therefore supplemented this study with experiments in order to assess whether the pretreatment of QUIN-injected rats with an NOS inhibitor, or NO-donor, can influence the QUIN-induced changes in receptor-type selective binding of [^{3}H]Glu to hippocampal membranes.

Methods

Fifty-day-old male Wistar rats (AnLab, Prague) of 200-280 g body weight were housed in a 12 h light/dark regime with free access to food and water. All procedures (surgical techniques and tissue sampling) followed instructions of the National Committee for the Care and Use of Laboratory Animals and were approved by the Local Animal Care Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic. Rats were anesthetized by Na-pentobarbital (Nembutal; 45 mg/kg, i.p.) and a dose of 250 nmol QUIN/lateral ventricle (Sigma, dissolved in 0.25 µl sterile isotonic saline solution) was infused bilaterally through burr-drilled holes in the calva. The head was fixed in a stereotaxic apparatus and the needle of a 1 µl Hamilton syringe was inserted into each lateral cerebral ventricle using coordinates as previously described (Lisý *et al.* 1994, Haug *et al.* 1998). A selective and irreversible inhibitor of constitutive nitric oxide synthase (cNOS) (Dwyer *et al.* 1991), N^G-nitro-L-arginine (NARG), in a dose of 50 nmol/ventricle (Sigma; dissolved in 5 µl sterile saline) and/or NO donor, 3-morpholinylsydnoneimine (SIN-1) in a dose of 5 nmol/ventricle (Sigma; dissolved in 5 µl sterile saline) were administered 15 min prior to the infusion of QUIN. The controls received an injection of isotonic saline only or a combination of saline + NARG and/or saline+SIN-1. Solutions were infused over a period of 10 min (Lerner-Natoli *et al.* 1992, Lisý *et al.* 1994, Feelisch 1998).

The rats were decapitated under deep ether anesthesia four days after the ICV injection(s). Their brains were rapidly removed and the hippocampal formations were excised. Pooled tissue samples from two to five animals were homogenized in 0.32 M sucrose using a glass homogenizer equipped with a motor driven Teflon pestle. The homogenates were centrifuged at 1000 x *g* for 10 min and the pellets were discarded. The supernatants were then centrifuged at 48000 x *g* for 20 min and the pellets, suspended in 40 vol of cold distilled water, were centrifuged at 8000 x *g* for 20 min. After homogenization, the supernatants and buffy coats were centrifuged at 48000 x *g* for 20 min and the pellets suspended in cold distilled water, were recentrifuged at 48000 x *g* for 20 min. The pellets were frozen in a mixture of dry ice and acetone and were kept at –30 °C, for no longer than one week. On the day of the binding assay, the membranes were thawed and washed two more times by suspension in cold distilled water and centrifugation at 48000 x *g* for 20 min to remove any endogenous glutamate. The final pellets were suspended in 50 mM Tris-acetate buffer (pH 7.4) (Ragsdale *et al.* 1989). In the case of membranes for the binding studies

on the mGluR, a 5 mM Tris-HCl (pH 7.4) buffer containing 2.5 m*M* $CaCl_2$ was used instead of water and the resultant pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$. After 15 min preincubation with 0.2 mM agonists of either iGluR or mGluR, the membranous suspensions were incubated for 45 min with 10 nM [^{3}H]Glu (L-G-[^{3}H]glutamic acid, 21 Ci/mmol; Amersham, England) or for 10 min with 10 nM [^{3}H]CPP (3-((D)-2-carboxypiperazin-4-yl)-[1,2-^{3}H]-propyl-1-phosphonic acid, 36 Ci/mmol; NEN, USA) in 1 ml of a relevant buffer. Binding onto the iGluR was performed in 50 mM Tris-acetate buffer (pH 7.4), whilst the assay mixture used for glutamate binding on mGluR contained 50 mM Tris-HCl buffer (pH 7.4), 2.5 mM $CaCl_2$, 0.2 mM NMDA, 0.2 mM AMPA ((±)-α-amino-3-hydroxy-5-methylisooxazole-4-propionic acid), 0.2 mM KA (kainic acid) and 0.2 mM SITS (4-acetamido-4´-isothio-cyanatostilbene-2,2´-disulfonic acid; the inhibitor of [^{3}H]Glu binding to chloride dependent uptake sites) (Ogita and Yoneda 1986, Schoepp and True 1992). Incubation was terminated by the addition of 4 ml of ice-cold buffer with subsequent filtration through Whatman GF/C glass filters under a constant vacuum. After repeated washing, the radioactivity trapped on the filters was measured in modified Triton-toluene scintillant using a Beckman 980 Scintillation Counter (Yoneda and Ogita 1986). The radioactivity found in the presence of 1 mM nonradioactive glutamate was subtracted from each experimental value as nonspecific binding to obtain the specific binding of [^{3}H]Glu (or [^{3}H]CPP). The difference between total specific binding and specific binding in the presence of any agonist was taken as the binding of [^{3}H]Glu to the receptor sensitive to a given agonist.

All the preparation procedures and incubations were performed at 2 °C. Buffers and all other solutions were sterilized before use by filtration through a nitrocellulose membrane filter with a pore size of 450 nm to avoid possible microbial contamination (Yoneda and Ogita 1989).

Protein estimation was done by the method of Lowry *et al.* (1951). The results were expressed as the mean ± S.E.M. and the statistical significance was determined using Student's t test.

All chemicals used were of commercially guaranteed grade.

Results

The specific [^{3}H]Glu binding, sensitive to AMPA, QUIS, KA and NMDA, represented 21 %, 45 %, 19 % and 27 %, respectively, of total specific binding (572 ± 29 fmol [^{3}H]Glu/mg protein) in hippocampal membranes of 50-day-old rat brains (Fig. 1A). In QUIN lesioned hippocampi the total specific binding was decreased by 31 % (to 396 ± 31 fmol [^{3}H]Glu/mg protein). As for the displacing effect of glutamate agonists in iGluRs, only the QUIS and NMDA sensitive binding was decreased (by 24 % and 32 %, respectively) as a consequence of the QUIN lesion (Fig. 1A).

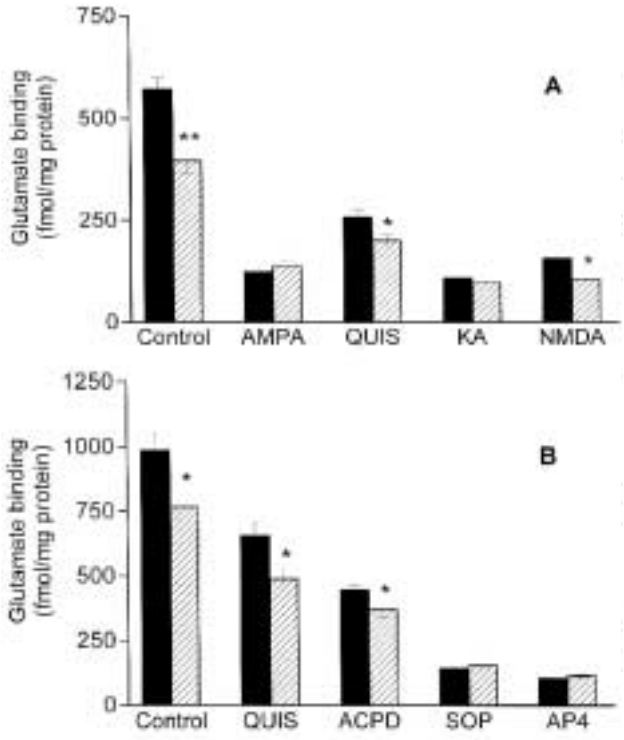

Fig. 1 A, B. *Specific binding of [^{3}H]Glu displaced by various agonists acting at different types of ionotropic (A) and metabotropic (B) glutamate receptors in membranes isolated from the rat hippocampal formation 4 days after bilateral ICV injection of 0.25 µl of sterile saline (filled columns) or QUIN in a dose of 250 nmoles / lateral ventricle (hatched columns). Each value is the mean ± S.E. of 8 - 20 estimations. *P < 0.05, **P < 0.01.*

In order to reveal the effect of QUIN-induced brain lesions on [^{3}H]Glu binding to mGluR in hippocampal membranes, we investigated the influence of mGluR agonists making it possible to distinguish between the binding to group I/II and III of this receptor (Fig. 1B). The [^{3}H]Glu binding estimated in the presence of ACPD (active at group I/II mGluRs) and QUIS (non-selective agonist at group I mGluR), which represented 45 % and 67 % of total specific binding, respectively, indicates a significant participation of mGluR (group I/II)

in hippocampal membranes. Much less binding was observed to be displaceable by mGluR group III agonists SOP (O-phospho-L-serine) and AP4 (L-2-amino-4-phosphonobutyric acid), representing 14 % and 10 %, respectively, which did not reach statistically significant level. As compared to control rats, the hippocampal membranes from QUIN lesioned brains had significantly lowered [^{3}H]Glu binding to mGluR (by 22 %). ACPD and QUIS sensitive bindings were also significantly decreased by 17 % and 26 %, respectively. The SOP or AP4 sensitive bindings remained unaffected in membranes from the QUIN-damaged hippocampi.

Table 1. Specific binding of [^{3}H]Glu on the iGluR or mGluR in the presence of QUIS, NMDA and ACPD in membranes isolated from the rat hippocampal formation 4 days after bilateral ICV injection of sterile saline (CONTROL), QUIN or NARG.

ICV injection	**Specific binding of [^{3}H] Glu (percentage of control)**					
	iGluR			**mGluR**		
	---	**QUIS**	**NMDA**	**---**	**QUIS**	**ACPD**
SALINE (CONTROL)	100 ± 2	100 ± 6	100 ± 11	100 ± 7	100 ± 8	100 ± 5
	(39)	(10)	(10)	(16)	(16)	(13)
QUIN	74 ± 2**	82 ± 5*	72 ± 6*	78 ± 6*	74 ± 7*	83 ± 6*
	(40)	(10)	(12)	(16)	(13)	(12)
QUIN + NARG	77 ± 2**	73 ± 4**	69 ± 5*	75 ± 2**	80 ± 5*	78 ± 3**
	(40)	(9)	(9)	(13)	(15)	(13)
NARG	82 ± 2**	82 ± 6*	88 ± 8$^{n.s.}$	84 ± 2*	69 ± 3**	84 ± 5*
	(29)	(9)	(9)	(6)	(6)	(6)

QUIS = quisqualic acid, NMDA = N-methyl-D-aspartate, ACPD = (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid, QUIN = quinolinic acid, NARG = N^G-nito-L-arginine

Table 2. Specific binding of [^{3}H]Glu and [^{3}H]CPP in membranes isolated from the rat hippocampal formation 4 days after bilateral ICV injection of sterile saline (CONTROL), QUIN, NARG or SIN-1.

ICV injection	**Specific binding (percentage of control)**	
	[^{3}H]Glu	**[^{3}H]CPP**
Saline (CONTROL)	100 ± 2	100 ± 6
	(39)	(20)
QUIN	74 ± 2**	43 ± 3**
	(40)	(18)
SIN-1	98 ± 5$^{n.s.}$	89 ± 4$^{n.s.}$
	(20)	(19)
QUIN + SIN-1	83 ± 5**	36 ± 2**
	(20)	(18)
NARG	82 ± 2**	63 ± 4**
	(29)	(18)
QUIN + NARG	77 ± 2**	46 ± 2**
	(40)	(20)
QUIN + NARG + SIN-1	74 ± 4**	54 ± 5**
	(20)	(18)

Each value is the mean ± S.E.M. Numbers of individual estimations are given in brackets.$^{**}P < 0.01$, $^{n.s.}$ not significant. SIN-1 = 3-morpholinylsydnoneimine, other abbreviation see Table 1.

Table 1 shows that the chronic inhibition of NO-synthase by NARG in control rats decreased the [^{3}H]Glu specific binding to a similar or lesser extent than in the case of QUIN-induced lesion. The decrease of NMDA sensitive binding was not even statistically significant in comparison with corresponding saline control. The pretreatment of the animals with NARG before the QUIN administration affected neither total nor QUIS or NMDA sensitive [^{3}H]Glu binding. Under the experimental conditions for binding to mGluR, the decrease of total (and/or QUIS- or ACPD-sensitive) [^{3}H]Glu specific binding in hippocampal membranes from QUIN-lesioned brains was very similar to that caused by treatment of the animals with NARG alone and/or with QUIN + NARG. As the inhibition of NO synthesis did not reveal any protective action in the QUIN-evoked decrease of [^{3}H]Glu binding in hippocampal membranes, we decided to test the effect of NO donor on [^{3}H]Glu binding. Table 2 shows that the ICV infusion of SIN-1, which donates NO, had no influence on the binding of [^{3}H]Glu or [^{3}H]CPP (a potent NMDA receptor antagonist). If the injection of SIN-1 preceded the QUIN administration, the binding of either of these two labelled ligands remained unchanged when compared with the hippocampi lesioned by QUIN, only. Furthermore, the binding in hippocampal membranes from the animals treated with a combination of NARG and QUIN was not affected by SIN-1 application.

Discussion

We reported previously that transiently elevated concentrations of QUIN in the cerebrospinal fluid are associated with a decline in the binding of [^{3}H]Glu to receptor sites in synaptic membranes isolated from hippocampi of young and adult rats. The changes in binding reflected alterations in the equilibrium binding constant rather than variations in the density of GluR (Lisý *et al.* 1994). Because the susceptibility to QUIN-evoked neurodegeneration can selectively affect populations of hippocampal neurons expressing different types of GluR (Pliss *et al.* 2000), we decided to study the effect of QUIN-lesion on glutamate binding into the main types of both iGluR and mGluR in the synaptic membrane preparations isolated from adult rat hippocampi. It has been shown that NO may play an important role in NMDA-mediated degeneration which has first been documented by observing the neuroprotective action of NARG, a nitric oxide synthase (NOS) antagonist, in primary cortical culture (Dawson *et al.* 1991). This was later supported by findings about the resistance of mice lacking neuronal NOS to a variety of neurotoxic insults (Huang *et al.* 1994, Schulz *et al.* 1996). QUIN interacts with high selectivity at NMDA oligomeric receptor/channel complexes of NR1/NR2B subunit composition (Curras and Dingledine 1992, Šťastný *et al.* 1999), activation of which is followed by an increase in Ca^{2+}-dependent NOS activity, with a final effect of increased NO production. Therefore, we were interested in the potential neuroprotective effect of NOS agonist NARG (Dwyer *et al.* 1991) and the toxicity potentiating action of NO donor, SIN-1 (Feelisch 1998) manifested as changes in ligand binding to synaptic membranes isolated from both normal and QUIN-lesioned hippocampi.

As it has been shown in the present results, the agonist sensitive [^{3}H]Glu specific binding was comparable in the main types of iGluR (AMPA-, KA-, NMDA-sensitive) and varied between 19 %-27 % of total specific binding. This might reflect the almost homogeneous density of all three iGluR types in the hippocampal formation in 50-day-old rats which is somewhat different from the cerebellum, for example, where the binding is significantly higher in the KA-specific type of the receptor (Rao and Murthy 1993). As for QUIS, it has a strong agonistic effect namely on mGluR (Schoepp and Conn 1993), but it also activates the AMPA receptors (Perouansky and Grantyn 1989) and we have hence used this agonist as a glutamate displacer in experiments done under conditions for [^{3}H]Glu binding in iGluR. In its presence, the binding represented 45 % of total specific binding of [^{3}H]Glu. It can be speculated that this high displacing ability of QUIS might be caused by its action on the AMPA-sensitive receptor site but a great deal of QUIS activity seems to concern mGluR.

Up to the present, the family of mGluRs is considered to consist of at least eight different subtypes, the mRNA sequences of which are known. They have been classified into three groups, according to their agonist selectivity, intracellular transduction mechanisms and sequence homology. The receptors belonging to group I (mGluR1 and mGluR5) are potently activated by QUIS and ACPD, but they are not sensitive to AP4. Receptors of group II (mGluR2 and mGluR3) are potently activated by ACPD but not by QUIS or AP4. Group III, represented by mGluR4, mGluR6, mGluR7 and mGluR8, is characterized by its sensitivity to AP4 and SOP, but not to QUIS and ACPD (Conn and Pin

1997). Thus, we used QUIS, ACPD, SOP and AP4 as agonists under the experimental conditions enabling estimation of [^{3}H]Glu binding to mGluR (Schoepp and True 1992). The capability of ACPD and QUIS to displace [^{3}H]Glu binding has been found to be much higher as compared to SOP and AP4. It means that most of the mGluRs (approx. 67 %) in the hippocampal membrane preparations are those which probably belong to groups I and II.

The binding of [^{3}H]Glu in hippocampal synaptic membranes isolated four days after i.c.v. injection of QUIN was significantly decreased against the controls in both iGluRs (by 31 %) and mGluRs (by 22 %). The use of specific agonists resulted in a decrease of QUIS and NMDA sensitive ionotropic and ACPD and QUIS sensitive metabotropic receptor binding. Thus, it can be speculated, that the QUIN-induced injury of the hippocampus insults both the NMDA subtype of iGluR and phosphoinositide-coupled group I of mGluR, which are possibly involved in neuronal degenerative disorders by enhancing hippocampal NMDA receptor coupling (Schoepp and Sacaan 1994). Moreover, excitotoxic events observed after activation of NMDA receptors by QUIN in various regions of the mammalian brain have been related to elevated cytosolic concentrations of free Ca^{2+}, ATP exhaustion and to subsequent processes (Schwarcz *et al.* 1984).

It has also been reported that QUIN is able to stimulate lipid peroxidation and to modify the activity of some endogenous antioxidant systems by an extrareceptor mechanism(s) leading to oxidative stress (Behan *et al.* 1999, Rodríguez-Martínez *et al.* 2000, Santamaría *et al.* 2001). An intrastriatal injection of QUIN produces a progressive dysfunction of neuronal mitochondria, accompanied by decreased levels of ATP which results in an inability of neurons to maintain proper ionic balances. Subsequent neurotransmitter release may further potentiate metabolic disturbances that culminate in neuronal death (Bordelon *et al.* 1997).

It is also evident that there is a close relationship between mGluRs and iGluRs in brain pathology (Schoepp and Sacaan 1994). This is documented by the fact that those mGluRs that are negatively coupled to adenylate cyclase, exert modulatory control on NMDA receptors in the membranes of murine mesencephalic neurons (Ambrosini *et al.* 1995), whereas activation of mGluR group II by ACPD limited the QUIN-induced excitotoxic damage in the rat neostriatum (Colwell *et al.* 1996). Potent neuroprotective effects of LY367385 and LY367366, which act as selective antagonists of mGluR1 and mGluR5, have also been found in NMDA receptor-mediated excitotoxicity (Kingston *et al.* 1999). We can therefore assume that neurodegenerative changes observed in the rat hippocampus after an ICV injection of QUIN involve both iGluRs (NMDA-subtype) and mGluRs group I (subtypes 1/5) which are responsible for the increases in intracellular Ca^{2+}. However, the decrease in the IP_3 receptor observed 4 days after the ICV injection of QUIN (Haug *et al.* 1998) may involve a regulatory mechanism protecting neurons against an excessive release of Ca^{2+} from intracellular stores.

As NO is thought to be a mediator of QUIN neurotoxicity elicited through the NMDA-type of iGluR (Dawson *et al.* 1991), we studied the possible effects of changed NO levels on the QUIN-evoked alterations in ligand binding onto GluRs in hippocampal synaptic membranes. We found that prolonged inhibition of NO-synthase by NARG significantly decreased the [^{3}H]Glu binding to membrane receptors into an extent comparable to the QUIN-lesions. The application of NARG which preceded QUIN infusion resulted in a similar decline of binding as in the case of the application of either of these substances. However, SIN-1, a donor of NO, had no significant effect on [^{3}H]Glu binding if administered either itself or together with QUIN and/or with NARG+QUIN. The receptor binding of [^{3}H]CPP revealed that the binding to the NMDA receptor in QUIN-lesioned hippocampi deeply declined as compared to the controls, but the inhibition of NOS or addition of the NO releaser did not influence the binding of this NMDA agonist. Using different displacers of [^{3}H]Glu binding, we found that not only NMDA receptors, but also the ACPD-sensitive subtype of metabotropic receptors, are involved in the changes caused by QUIN, NARG and SIN-1.

Thus, we cannot confirm the assumption about the protective effect of irreversible NO-synthase inhibition (Dwyer *et al.* 1991) in the case of QUIN-toxic action estimated under the conditions of our experiments. It should be mentioned in this context that the dependence of the alteration of NMDA receptor function by NO on the effective concentration of NO and on the susceptibility of specific receptor subunits expressed by particular groups of neurons to NO has already been suggested (Kashii *et al.* 1996, Kalish *et al.* 1999).

We have thus shown that the QUIN-evoked neurodegeneration in the rat hippocampus afflicts both the iGluR (NMDA-subtype) and mGluR (group I) by depressing their binding capacity. Either suppression of

NOS by NARG or increased generation of NO by SIN-1 failed to modify significantly the ligand binding alterations caused by QUIN-induced hippocampal lesion. However, the prevailing inhibition of constitutive NOS by NARG alone may exert some harmful effects on the specific binding to glutamate receptor(s) mediated by a mechanism which may involve brain hypoperfusion caused by decreased levels of brain NO. Thus, our experimental results do not confirm some previous findings about the protective effect of NOS inhibition in NMDA receptor-mediated neurodegeneration evoked by QUIN. We suggest that oxidative stress-mediated neuronal damage might be involved in this neurodegenerative process (Santamaría *et al.* 2001), the actual mechanism(s) of which would have to be studied in separate project.

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