Up-regulation of glutamate receptors is associated with LTP defects in the early stages of diabetes mellitus

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Abstract

Aims/hypothesis. Recent studies involving electrophysiology and immunolabelling indicate that short-term insulin treatment of hippocampal neurons in culture induces changes in glutamate receptor function, suggesting that this receptor system can be altered on a relatively rapid time scale during diabetic conditions. To investigate this hypothesis, we examined whether brain glutamate receptors and long-term potentiation are altered in the early stages of diabetes mellitus in non-obese diabetic mice, a genetic model of Type I (insulin-dependent) diabetes mellitus.

Methods. In vitro receptor autoradiography and immunoblotting were used to study the impact of diabetes on brain glutamate receptors. From an electrophysiological point of view, field potential recordings were also examined in area CA1 of hippocampal slices to determine the influence of diabetes on long-term potentiation.

Results. Quantitative autoradiographic analysis revealed enhanced ³H-glutamate binding to several

brain regions of diabetes mice, with maximal increases in the cerebral cortex and hippocampus. Saturation kinetics within the cerebral cortex disclosed that this change of ³H-glutamate was possibly due to an increase in the maximal number of N-methyl-D-aspartate binding sites, an interpretation that was corroborated by Western blot analysis of N-methyl-D-aspartate 2A subunits. Impairment in the expression of hippocampal long-term potentiation was also observed in diabetic mice, while the failure to elicit synaptic potentiation was prevented by insulin treatment.

Conclusion/interpretation. Because glutamate receptors are thought to be involved in several degenerative processes, our results suggest that up-regulation of these receptors in the early stages of diabetes could represent an important mechanism underlying neurological complications within the brain of diabetic patients. [Diabetologia (2002) 45:642–650]

Keywords NMDA receptor, AMPA receptor, hippocampus, NOD mice.

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Abbreviations: AMPA, α -Amino-3-hydroxy-5-methylisoxazole-4-propionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione; fEPSP, field excitatory postsynaptic potential; GluR, glutamate receptor subunit; HFS, high-frequency stimulation; IT, insulin treatment; K_d, receptor affinity; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; NOD, non-obese diabetic mice; NON, non-obese non-diabetic mice; NR, NMDA receptor subunit

According to recent projections, over 200 million people worldwide will be diagnosed with diabetes by the year 2010 [1]. Diabetes mellitus is an endocrine disorder of carbohydrate metabolism resulting primarily from inadequate insulin release (Type I insulin-dependent diabetes mellitus) or insulin insensitivity coupled with inadequate compensatory insulin release (Type II non-insulin-dependent diabetes mellitus). Forms of diabetes are associated with the development of complications in the peripheral as well as the central nervous system (CNS). For instance, learning and memory deficits are linked with Type I and Type II diabetes mellitus [2, 3, 4, 5, 6], and brain morphological abnor-

malities have been found in diabetic patients, mainly in the cortex area [7, 8]. Studies on experimental models of diabetes have provided similar results in that rats with streptozotocin (STZ)-induced diabetes exhibit morphological, behavioural and electrophysiological alterations [9, 10, 11]. In particular, numerous studies strongly suggest that dysfunction in hippocampal long-term potentiation (LTP), an electrophysiological model of synaptic plasticity thought to subserve learning and memory processes, is associated with diabetic conditions [10, 12].

Work over the past decades has shown that regulation of glutamate receptor properties can contribute to learning and memory as well as LTP formation (for review see [13]). Ironically, activation of this neurotransmitter system is also suspected to be involved in neurodegeneration following a wide range of neurological insults, including ischaemia, trauma and epileptic seizures [14, 15]. In rodents, N-methyl-D-aspartate (NMDA) and non-NMDA (kainate and α -amino-3-hydroxy-5-methylisoxazole-4-propionate, AMPA) receptors are two families of ionotropic receptors stimulated by glutamate that have been implicated in neurodegeneration [17]. Overactivation of these receptors can cause cell damage by increasing intracellular calcium concentration in neurons, thereby leading to the generation of free radicals and activation of proteases, phospholipases and endonucleases [18, 19, 20] as well as transcriptional activation of specific cell death programs [21]. Interestingly, evidence from functional and biochemical studies show that long-term exposure to hyperglycaemia in STZ-induced diabetic rats is associated with important glutamate receptor abnormalities [22, 23].

Recently, short-term insulin treatment was found to alter NMDA receptor activation [24] as well as to interact with AMPA receptor trafficking between the plasma membrane and the intracellular compartment in cultured hippocampal neurons [25] indicating that mechanisms underlying diabetic neuropathies could be initiated in the early stages of the disease, as a consequence of abnormal glutamate receptor properties. This is relevant to the clinical situation because excessive activation of glutamate receptors is a characteristic feature of brain damage during stroke and ischaemia [26], conditions that are exacerbated by hyperglycaemic states [1]. Using *in vitro* receptor autoradiography as well as immunoblotting, we have explored the possibility that NMDA or AMPA receptor properties are altered in the early stages of diabetes in non-obese diabetic (NOD) mice, a genetic model of Type I (insulin-dependent) diabetes. The functional integrity of the brain was also determined by comparing LTP magnitude in the hippocampus of NOD mice.

Materials and methods

Animals and tissue preparation. NOD mice and their controls (non-obese, non-diabetic (NON) mice) were purchased from

Jackson Laboratories (Bar Harbor, Me., USA) at 7–9 weeks of age. All mice were kept in individual cages under a 12:12-h light-dark cycle, in a facility that respected proper laboratory principles (NIH publication No. 86-23, revised 1985) and Canadian Council on Animal Care Guidelines. They had access to a standard chow and water *ad libitum*. In the NOD mouse model, Type I diabetes occurs spontaneously between the 12th and 30th weeks of age, with an onset characterized by polydipsia, glycosuria and hyperglycaemia. In this study, these diabetic mice were either killed by decapitation 1 week after the onset of diabetes, which was established by the presence of glycosuria (Roche Molecular Biochemicals, Laval, Quebec) or treated for 1 week with insulin. A subcutaneous, slow-release implant of stearate insulin was placed under the dorsal skin for 0.1 μ g insulin/day of treatment until the mice were killed (Lin-Bit, Linshin Canada, Scarborough, Ontario). Their brains were quickly removed and frozen in isopentane (methyl butane) at -20°C , then stored at -70°C . Horizontal 10- or 30- μ m thick sections were cut in a cryostat, thaw-mounted onto chrome-alum-gelatin-coated slides, and stored at -70°C until the day of use. Glucose concentration in serum, from blood samples collected at the time of killing, was determined with a Boehringer Mannheim kit, and found to be in the range of 6–8 mmol/l and 15–19 mmol/l for NON and NOD mice, respectively.

Quantitative glutamate receptor autoradiography. Adjacent brain sections were pre-incubated at 35°C for 60 min in 12 ml of Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA (Ethylene glycol-bis(β -aminoethyl ether) N,N,N'-tetraacetic acid). After washing in 50 ml of Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA, the sections were processed for AMPA or (6-cyano-7-nitroquinoline-2,3-(1H,4H)-dione (CNQX) receptor binding, as described previously [27].

In brief, brain sections were incubated for 45 min at $0-4^{\circ}\text{C}$ in Tris-acetate buffer (50 mmol/l, pH 7.4; 100 μ mol/l EGTA) containing 50 mmol/l potassium thiocyanate and either 30 nmol/l of ^3H -AMPA (specific activity 53 Ci mmol $^{-1}$, NEN-Dupont) or 15 nmol/l ^3H -CNQX (specific activity 26.70 mmol $^{-1}$, NEN-Dupont). Non-specific binding was defined as binding measured in the presence of 1 mmol/l quisqualate and 5 mmol/l glutamate. At the end of incubation, the sections were rinsed twice in cold incubation buffer for 10 s and once for 5 s in 50% of the same buffer, followed by three dips in distilled water. They were then dried rapidly under a stream of warm air and used for autoradiography. For ^3H -glutamate binding to NMDA receptors, sections were incubated with 100 nmol/l of ^3H -glutamate (51 Ci mmol $^{-1}$, Mandel Scientific Company, Guelph, Ontario, Canada) for 45 min at $0-4^{\circ}\text{C}$ in Tris-acetate buffer (50 mmol/l, pH 7.4; 50 μ mol/l EGTA) containing 5 μ mol/l AMPA, 1 μ mol/l kainic acid and 10 μ mol/l quisqualate to eliminate glutamate binding to non-NMDA sites, and 100 μ mol/l SITS (4-acetanido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid) to block glutamate uptake sites. Saturation experiments, using concentration from 20 nmol/l to 1 μ mol/l, were also done to determine whether the modulation of ^3H -glutamate binding was the result of changes in receptor affinity or in receptor number. Non-specific binding in both types of experiments was defined as binding measured in the presence of 5 mmol/l glutamate. The sections were rinsed twice in incubation buffer for 15 s and once for 10 s in 50% of the same buffer, followed by three dips in distilled water. They were dried as described above for AMPA binding.

Dried sections as well as tritium standards (American Radiolabeled Chemicals, St. Louis, Miss., USA) were exposed to tritium-sensitive film (Amersham Hyperfilm, AB, Sweden) for 14 days (^3H -AMPA), 12 days (^3H -CNQX) or 28 days (^3H -glu-

tamate). The films were processed in Sigma developer and fixer. The optical density (OD) of different brain regions was converted to radioactive units, using tritium standards after measurement with an image analysis system (Imaging Research, MCID, St. Catharines, Ontario).

Electrophoresis and immunoblotting. Horizontal 30- μ m sections were cut in a cryostat and thaw-mounted in chrome-alum-gelatin-coated slides. Adjacent sections were preincubated for 60 min in 50 ml of Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA at 35°C. Tissue was collected in 0.32 mol/l sucrose containing several protease inhibitors (leupeptin 5 μ mol/l, phenylmethylsulfonyl fluoride (PMSF) 200 μ mol/l, and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) 1 μ g/ml), then homogenized with a glass-Teflon homogeniser. The homogenates were centrifuged to obtain membrane fractions [28]. Briefly, the homogenates were centrifuged at 1,000 g for 10 min., and the supernatants were centrifuged at 11,500 g for 20 min. The resulting pellet, P2, was defined as the crude synaptosomal fraction. This fraction was resuspended in Tris-acetate buffer (50 mmol/l, pH 7.4, with the same concentrations of inhibitors as above), and protein concentrations in each fraction were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario).

Western blot analysis was conducted on the crude synaptosomal fractions obtained from control and diabetic sections. Aliquots of P2 fractions were subjected to sodium dodecyl sulfate 8% polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [29]. Proteins were transferred onto nitrocellulose membranes [30]. To block non-specific sites, the membranes were first incubated for 1 hr at room temperature in phosphate-buffered saline (PBS) containing 5% dry non-fat milk. They were then incubated with primary antibodies against glutamate receptor subunit 1 (GluR1, 0.3 μ g/ml) and GluR2/3 (0.35 μ g/ml) (Upstate Biotechnology, Lake Placid, N.Y., USA) as well as NMDA receptor subunit 1 (NR1, 0.1 μ g/ml) and NR2A (0.1 μ g/ml) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in PBS containing 5% dry non-fat milk. Bands corresponding to GluR1 or 2/3 were detected with an alkaline phosphatase-conjugated secondary antibody (Roche Molecular Biochemicals, Laval, Quebec). The OD of the immunoreactive bands was quantified with a computer imaging system (Imaging Research, MCID, St Catherine, Ontario).

Hippocampal slice preparation for electrophysiology. Experiments were performed on hippocampal slices [31] prepared from NON, NOD and insulin-treated NOD mice. Transverse slices were maintained at 35°C in medium containing (in mmol/l) NaCl 124, KCl 5, Na_2PO_4 1.25, MgCl_2 1.5, CaCl_2 2.5, NaHCO_3 26 and glucose 10. They were exposed to a humidified atmosphere of 95% O_2 : 5% CO_2 and perfused continuously at a flow rate of 1 ml/min. After a 1 h equilibration period, a glass recording electrode (1–5 M Ω ; filled with 2 mol/l NaCl) was positioned in the stratum radiatum of area CA₁ of the hippocampus to record field excitatory post synaptic potential (fEPSPs) evoked by a bipolar electrode (twisted 60 μ m nichrome), activating fibres of the Schaffer collateral system. Bipolar stimulating electrodes were placed in the stratum radiatum of two independent pathways converging on common post-synaptic target cells. After a 20-min baseline period during which responses were recorded every 30 s, theta-burst stimulations (TBS) (10 bursts of 4 stimulation pulses at 100 Hz) were given at 200-msec (5 Hz) intervals. In all cases, the response to stimulation was quantified by calculating the initial slope of the resulting fEPSP.

Statistical analysis. In most cases, the data are presented as means $\pm$ SEM. Between groups, differences in subunit composition were analysed by the two-tailed *t* test for independent samples. For differences in binding and LTP expression, groups were compared by analysis of variance (ANOVA) followed by Scheffe's post hoc analysis with conventional criteria of statistical significance: *p* values of less than 0.05.

Results

Glutamate receptor binding properties in brain sections of NOD mice. Brain sections of NON (glycaemia: 7.27 $\pm$ 0.9 mmol/l), NOD (glycaemia: 17.60 $\pm$ 2.0 mmol/l) and insulin-treated (IT) NOD (10.17 $\pm$ 1.0 mmol/l) mice were preincubated at 35°C and subjected to quantitative ^3H -glutamate ligand binding autoradiography for NMDA receptors. Visual examination of the autoradiographs clearly showed a marked increase of ^3H -glutamate binding in the hippocampus and cerebral cortex of NOD mice. The rise in ^3H -glutamate binding to NMDA sites observed in diabetic animals was distributed uniformly in the various regions of the hippocampus (Fig. 1A). A 18–35% increase in ^3H -glutamate binding was found in CA₁ and CA₃ strata radiatum and oriens as well as in the dentate gyrus of NOD mice, compared to the controls. A moderate increase of about 15% in ^3H -glutamate binding to NMDA receptors was seen in the external cortex, and among all the telencephalic regions analysed in diabetic mice, with the internal cortex showing the highest elevation (50%; see Fig. 1B), when compared to NON mice. Saturation experiments at equilibrium were performed to determine whether the increment in ^3H -glutamate binding in diabetic mice was the result of alterations in receptor affinity or maximal number (Fig. 2A). The saturation kinetics of binding in the internal cortex region yielded a linear Scatchard plot, suggesting the existence of a single binding site (Fig. 2B). In this brain region, heightened ^3H -glutamate binding during diabetes was not due to a change in affinity of NMDA receptors (K_d (receptor affinity)=206 $\pm$ 33 nmol/l in control vs K_d =202 $\pm$ 31 nmol/l in diabetic sections; means $\pm$ SEM of 4 experiments), but to a rise in the maximal number of binding sites (B_{max} =2.82 $\pm$ 0.16 in control vs 4.23 $\pm$ 0.23 pmol/mg of prot. in NOD sections; means $\pm$ SEM of 6 experiments, **p*<0.01, Student's *t* test).

Ligand binding autoradiography with ^3H -AMPA and ^3H -CNQX was also used to test the effects of diabetes on the binding properties of AMPA receptors in various brain regions. Adjacent brain sections were preincubated at 35°C and then subjected to ^3H -AMPA ligand binding autoradiography. Visual examination of these autoradiographs clearly indicated that the increased ^3H -AMPA binding in various areas of the hippocampus was generated by diabetes. We found a significant increase in around 30% in ^3H -AMPA binding

Fig. 1A, B. NMDA receptor binding in NOD mice. Adjacent horizontal sections at the level of the hippocampus were preincubated at 35°C for 1 h in Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA. Autoradiographs of 3 H-glutamate binding to NMDA receptor, like those shown in the upper part of this figure, were analysed with an image analysis system for the various hippocampal (or, stratum oriens; rad, stratum radiatum; DG, molecular layer of the dentate gyrus) and nonhippocampal (IntCx, inner layers of the parieto-temporal cortex; ExtCx, outer layers of the parieto-temporal cortex; Stria, striatum; Thal, thalamic nuclei) regions. The results obtained for the different regions of control (*open bars*) and NOD (*filled bars*) mice sections were expressed in pmol/mg of protein. **A, B** NMDA receptor binding to hippocampal and nonhippocampal structures, respectively. The data are means $\pm$ SEM of values obtained from 6 mice with at least 11 different measurements per mouse for each of the cerebral regions examined. * $p < 0.05$, ** $p < 0.01$ (Scheffe's test), control vs NOD sections

Fig. 2A, B. Binding properties of NMDA receptors in the cortex of NOD mice. Saturation curve (**A**) and Scatchard plots (**B**) of 3 H-glutamate binding to NMDA sites in the parieto-temporal cortex of control ($\square$) and NOD mice ($\blacksquare$) sections. NMDA receptor binding was determined using 3 H-glutamate concentrations from 20 nmol/l to 1 μ mol/l, and non specific binding was defined in the presence of 5 mmol/l of glutamate. The data are presented as Scatchard plots (bound/free) and analysed by the Inplot program from GrafPad to generate K_d and B_{max} ; bound values are means $\pm$ SEM of values obtained with eight different measurements for the cerebral region examined. The saturation binding curve and the saturation plots are representative of experiments done on four different animals

to CA₃ and CA₁ strata radiatum and oriens of the hippocampus in NOD sections. A similar increase (20–30%) was also observed in the dentate gyrus and cerebral cortex of diabetic mice (Fig. 3A, B). As illustrated in Fig. 4A, B, quantification and averaging of the data obtained from several sections indicated that no significant changes in 3 H-CNQX binding were generated by diabetes in the various brain regions analysed, compared to NON mice.

Fig. 3A, B. AMPA receptor binding in NOD mice. Adjacent horizontal sections at the level of the hippocampus were preincubated at 35°C for 1 h in Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA. Autoradiographs of 3 H-AMPA binding, like those shown in the upper part of this figure, were analysed with an image analysis system for the various hippocampal (A) and non-hippocampal (B) regions. Abbreviations are the same as in Fig. 1. The results obtained for the different regions of control (□) and NOD (■) mice sections were expressed in pmol/mg of protein. The data are means $\pm$ SEM of values obtained from 6 mice with at least 11 different measurements per mouse for each of the cerebral regions examined. * p <0.05, ** p <0.01 (Scheffe's test), control vs NOD sections

Fig. 4A, B. 3 H-CNQX binding in NOD mice. Adjacent horizontal sections at the level of the hippocampus were preincubated at 35°C for 1 h in Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μ mol/l EGTA. Autoradiographs of 3 H-CNQX binding to AMPA receptors were analysed with an image analysis system for the various hippocampal (A) and non-hippocampal (B) regions. Abbreviations are the same as in Fig. 1. The results obtained for the different regions of control (□) and NOD (■) mice sections were expressed in pmol/mg of protein. The data are means $\pm$ SEM of values obtained from 6 mice with at least 11 different measurements per mouse for each of the cerebral regions examined. * p <0.05, ** p <0.01 (Scheffe's test), control vs NOD sections

Expression of NMDA and AMPA receptor subunits in brain sections of NOD mice. To evaluate the protein concentrations of glutamate receptors, Western blot analyses were performed on crude synaptosomal fractions obtained from control, diabetic and diabetic-treated mouse sections (30 μ m thick). Membranes were immunoblotted with antibodies against the C-terminal domains of NR1, NR2A, GluR1 or GluR2/3, as reported previously [32, 33]. Figure 5B shows that NR2A immunostaining was increased in the telencephalic membranes of diabetic compared to control mice. The mean ($\pm$ SEM) OD values for NR2A (0.58 ± 0.1 in control vs $1.15^* \pm 0.03$ in diabetic mice; $n=5$, * p <0.01) increased by about 84% in NOD mouse sections, while no changes were reported in NOD mice that received insulin (0.69 ± 0.04 in control vs 0.70 ± 0.04 in IT NOD). In contrast, Western blot analysis of NR1 (0.78 ± 0.1 in control vs 0.91 ± 0.05 in diabetic mice) showed no detectable difference in immunostaining between controls and NOD mice. As for

Fig. 5A, B. Glutamate receptor subunits in NOD mice. Adjacent horizontal sections from control (□) and NOD mice (■) at the level of the dorsal hippocampus were preincubated at 35°C for 1 h in Tris-acetate buffer (100 mmol/l, pH 7.4) containing 100 μmol/l EGTA. After incubation, crude synaptosomal (P2) fractions were prepared by subcellular fractionation. Western blots were performed using antibodies against the C-terminal domain of GluR1 and GluR2/3 subunits of AMPA receptors (**A**) as well as antibodies against the NR1 and NR2A subunits of NMDA receptors (**B**). Note that NMDA expression is normalized by insulin treatment (IT). A representative Western blot showing modulation of the level of AMPA and NMDA receptor subunits in P2 fractions is presented above the graph. Blots were digitized and the bands were quantified with an image analysis system

NR1, both GluR1 (0.48 ± 0.04 in control vs 0.53 ± 0.05 in diabetic mice) and GluR2/3 (0.35 ± 0.04 in control vs 0.41 ± 0.05 in diabetic mice) concentration were similar in control and diabetic mice (Fig. 5A).

Long-term potentiation (LTP) in diabetic mice before and after insulin treatment. Because glutamate receptors contribute to synaptic plasticity in the brain [35, 36], electrophysiological experiments were conducted to examine the influence of diabetes on LTP expression. Hippocampal slices from control, diabetic and insulin-treated diabetic mice were prepared and perfused for 1 h with artificial cerebrospinal fluid (ACSF) before applying high-frequency stimulation (HFS) to the Schaffer commissural pathway in area CA1 of the hippocampus. Theta-burst stimulation

Fig. 6. LTP magnitude in hippocampal slices of NOD mice. Hippocampal slices were incubated in artificial cerebrospinal fluid (ACSF) and LTP was elicited by high-frequency stimulation (HFS) of the Schaffer collateral system. Evoked fEPSPs were recorded in the stratum radiatum of CA1 pyramidal cells in NON mice (△), NOD mice (□) and insulin-treated NOD (○) mice. The values obtained during the period preceding TBS were averaged to derive baseline value. The data are expressed as percentages of baseline values and each point represents the mean $\pm$ SEM of 7–9 different experiments in which the responses were measured before and after HFS

(TBS) was used to elicit LTP and consisted of 10 HFS bursts (four pulses at 100 Hz) repeated at 5 Hz. During recording, responses were tested at 30-sec intervals for at least 10 min before TBS and again for at least 40 min after HFS. In the present investigation, two evoked responses were averaged 1 and 30 min after TBS to determine the possible effect of diabetes on short-term potentiation (STP) and LTP, respectively. As shown in Figure 6, both STP and LTP were reduced in hippocampal slices of NOD mice. Statistical comparisons revealed that LTP magnitude was decreased in diabetic mice ($24.9 \pm 6\%$; $n=7$, $p<0.01$; Scheffe's test) compared to their controls ($87.5 \pm 6\%$; $n=8$), while the diminution in the capacity of diabetic slices to generate LTP was markedly prevented by IT ($61.52 \pm 6\%$; $n=8$).

Discussion

This study shows that up-regulation of glutamate receptors occurs in the early stages of diabetes mellitus. Binding experiments indicate that the increase in ^3H -glutamate binding to NMDA receptors is due to an increase in the maximal number of NMDA-binding sites. Supporting this, we showed that levels of NR2A (but not NR1) are enhanced in crude synaptosomal fractions from NOD mice. There were also indications of enhanced binding affinity of ^3H -AMPA for its receptor within the brain of NOD mice, especially in the hippocampal area. In CA1 hippocampal slices, these

changes in glutamate receptor properties were associated with LTP impairment and we found that both biochemical and electrophysiological abnormalities were prevented by IT of NOD mice.

The effect of diabetes on NMDA receptor binding reported here appears to be variable between brain structures, being larger in the internal layers (III-VI) of the cerebral cortex and almost absent in the striatum. The glutamate-binding site of NMDA receptors seems to be located on NR2, whereas the glycine-binding pocket is on NR1 [17]. Therefore, regional variations of NMDA receptor binding during diabetes could reflect the differential expression of NR2 in the various brain structures of NOD mice, as indicated by the increase in NR2A (but not NR1) in the telencephalic membranes of NOD mice. Of course, it remains to be seen whether the expression patterns of NR2A are subject to modification in specific cells of the brain regions. In particular, it is known that NR1-NR2A receptor channels are mainly expressed in pyramidal cells, and immunohistochemical studies of brain sections are currently examining whether the expression of NR subunits is differentially regulated by diabetes in these cells. From the functional point of view, experiments done on recombinantly expressed NMDA receptors have revealed that NR2 composition strongly influences the electrophysiological characteristics of NMDA receptor subtypes [17]. NR2A-containing receptor channels differ from other channel subtypes, showing fast deactivation and prominent calcium-dependent desensitisation [34]. Interestingly, NR2A seems to be necessary for LTP formation at several synapses, emphasising the functional significance of our study revealing up-regulation of NR2A in synaptic membranes from NOD mice [35, 36]. Our finding that NR2A concentrations are increased in synaptic membranes of NOD mice suggests that expression of abnormal NMDA receptors during diabetes could have functional as well as pathological consequences. This contention is supported by the present data indicating that LTP magnitude in area CA1 of hippocampal slices is altered in NOD mice. Of course, this reduction in the capacity of diabetic slices to generate LTP seems to be dependent on the diabetic state, because the LTP defect was prevented by IT of NOD mice. The possible target mechanisms contributing to the impairment in synaptic plasticity, however, remain to be determined but could include promotion of NMDA-induced cell damage or NMDA-mediated activation of silent synapses in hippocampal neurons [37, 38].

Our study shows that ^3H -AMPA binding is enhanced in most brain regions of NOD mice and indicates that the amounts of GluR1 and GluR2/3 are not different in synaptic membranes of NOD mice, suggesting that change in receptor binding is not due to modification of AMPA receptor expression. On the other hand, the characteristic effect, ie increased bind-

ing of an agonist, ^3H -AMPA, and no changes in binding of an antagonist, ^3H -CNQX, at AMPA receptors, indicates that diabetes could be associated with alteration in receptor affinity rather than changes in the number of receptors. Indeed, the molecular mechanism responsible for the increased AMPA receptor affinity in NOD mice are not known. Nevertheless, it is noteworthy that activation of the calcium-dependent enzyme phospholipase A₂ (PLA₂) could be part of the molecular mechanisms underlying changes in AMPA receptor conformation. In particular, it was reported previously that treatment of telencephalic membranes or tissue sections with exogenous PLA₂ or phospholipase C (PLC) heightened AMPA receptor affinity [39, 40, 41, 42]. Various studies have suggested that the effect of phospholipases could be due to modifications of the lipid environment of the receptors, as incorporation of phosphatidylserine (PS) into rat brain membranes reproduced the increased affinity of AMPA receptors elicited by calcium-dependent phospholipases [43]. Therefore, augmented affinity of AMPA receptors in the brain of NOD mice could arise from pre-existing modulation of receptors by endogenous lipases. This notion is, indeed, supported by recent data revealing that PS-induced enhancement of ^3H -AMPA binding is reduced in the hippocampus of NOD mice (data not shown). However, it is important to point out that proteolysis as well as phosphorylation of GluR subunits might also be important for regulating AMPA receptor binding [30, 32]. Thereby, it is possible that up-regulation of AMPA receptor affinity in NOD mice could reflect previous alteration of GluR by proteases or kinases.

To the best of our knowledge, no one has directly investigated the properties of glutamate receptors in the brain of spontaneously diabetic animals, but previous works have shown that they can be down-regulated in STZ-treated rats. At the neurochemical level, NMDA receptor expression and phosphorylation were reported to be down-regulated in postsynaptic densities from the brain of chronic STZ-induced diabetic rats [23], and quantitative autoradiographic experiments have revealed that AMPA receptor affinity was reduced in several brain regions [22]. This apparent discrepancy between our results and those of others [22, 23] could be related to the diabetic animal models used. The main difference between the two models (non-genetic STZ and genetic NOD mice) is that the former is chemically induced by a compound (STZ) which is known to possess toxic effects on various tissues, including the brain [44]. In this regard, regulation of glutamate receptors in STZ-treated animals could represent a biochemical response that is not necessarily related to the diabetic state. Moreover, down-regulation of glutamate receptors in STZ-treated rats has only been investigated after a diabetic duration of 3 months, which contrasts with the shorter duration (1 week) used in the course of this study. Therefore, it

could be hypothesized that glutamate receptor properties can be differentially modulated, depending on the duration of diabetes [3]. Nevertheless, our data are concordant with a recent study reporting up-regulation of both NMDA and AMPA receptors in thoracic spinal cord sections from obese-diabetic ob/ob mice, another model in which animals develop diabetes spontaneously and which closely resembles the Type II (insulin-independent) condition [45].

Together, the present findings are consistent with the possibility that brain glutamate receptor properties can be modified in diabetic subjects [3]. In particular, our data indicating that up-regulation of glutamate receptors is related to LTP defects in the early stages of diabetes, are in line with the recent observation that early changes in glutamate receptors could be causally involved in the peripheral neuropathies which can accompany diabetes mellitus [45]. Identifying the mechanisms by which these biochemical and electrophysiological changes are exerted could provide important clues about the cellular events responsible for diabetes-induced neuropathies.

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