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Effects of Nω-Nitro-L-Arginine Methyl Ester on Benzodiazepine Binding in Some Limbic Areas of Hyperlipidaemic Rats

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FACCIOLO, R. M., R. TAVOLARO, A. CHINELLATO, E. RAGAZZI, M. CANONACO AND G. FASSINA. *Effects of* Nω*-nitro-*L*-arginine methyl ester on benzodiazepine binding in some limbic areas of hyperlipidaemic rats.* PHARMACOL BIOCHEM BEHAV **54**(2) 431–437, 1996.—Quantitative autoradiography techniques were used to evaluate the chronic effects of the potent nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester, on the binding pattern of [^{3}H]flunitrazepam (benzodiazepine agonist) in some behaviorally key limbic areas of the genetic hyperlipidaemic Pittsburg Yoshida rat. Administration of this potent synthase inhibitor was capable of supplying higher and moderately higher binding levels in the basolateral amygdala nucleus (+52%) and in the oriens-pyramidalis CA1 hippocampus layer (+38%), respectively. When we tested for the binding changes in the presence of GABA (principal benzodiazepine modulator) we noticed that a physiological concentration (20 μM) of this inhibitory neurotransmitter was sufficient to induce notable changes in other limbic areas. In fact, lower binding values (−65%) were reported for the bed nucleus of stria terminalis whereas moderately higher values (+38%) were obtained for the radiatum-lacunosum molecular CA1 hippocampus layer. From the saturation studies, it was possible to observe that the major receptor variations provoked by the potent synthase inhibitor were not only due to changes in the total number of binding sites because there were variations, as in the case of the basolateral amygdala nucleus, that were instead due to differences in the affinity binding state. These results provide evidences of a GABAergic–nitric oxide synthase inhibitor interaction that might also be involved in the regulation of convulsive, anxiolytic, and aggressive behaviors that are modulated at the benzodiazepine site.

Pittsburg Yoshida rat Nitric oxide [^{3}H]Flunitrazepam binding L-NAME Limbic areas

THE highly reactive endothelium-derived relaxing factor nitric oxide (NO), synthesized from L-arginine and noted for its involvement in the control of vascular functions (24), has only recently been recognized as a neurotransmitter in the vertebrate CNS (10). Pharmacological studies have established that NO is capable of regulating, at the brain level, neuroendocrine secretory functions (25,34) as well as promoting convulsant type of behaviors (19). In this latter case, however, contradictory effects have been attributed to NO because proconvulsive features were obtained when the highly specific NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME), which accounts for the suppression of this endothelium-derived relaxing factor, was administered (26). These behavioral variations could very likely be a consequence of NO interacting in a regional-specific manner with the different neurotransmitter propagating systems and/or of the neuronal receptor complexes being structurally and functionally localized in a heterogeneous fashion (13).

It is now well established that GABA, a major CNS inhibitory neurotransmitter diffusely located within the mammalian brain, is prevalently involved in anticonvulsant, anxiolytic, and antiaggressive activities (4,20). Such activities are achieved via a postsynaptic modulatory response of benzodiazepine. The central benzodiazepine recognition site is part of

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a multimeric $GABA_A$ membrane protein that includes a binding site for, among others, GABA and an associated chloride ion channel [for review see Olsen and Tobin (21)]. To date, a NO-dependent activity linked to benzodiazepine receptor changes, aside from that dealing with the peripheral type of benzodiazepine sites (3), has not been fully elucidated. On the basis of epileptic seizures being correlated to the modifications of benzodiazepine receptor binding activities (16,22) and on the effects exerted by L-NAME, it would be of particular interest to determine whether the influences of this NOS inhibitor on convulsive behaviors are linked to the variations of benzodiazepine binding activities in limbic areas. These areas are, apart from their involvement in memory and spatial tasks, strongly linked with the control of sociosexual behaviors, especially of the aggressive-defensive type (31). For this purpose, binding changes of the preferentially specific benzodiazepine receptor radioligand [^{3}H]flunitrazepam (FLU) were assessed in some limbic areas of the hyperlipidaemic Pittsburg Yoshida (YOS) rat following prolonged treatment with L-NAME. Binding levels were also tested in the presence of GABA to establish whether the effects of this inhibitor require the participation of other GABAergic sites from the moment that benzodiazepine modulatory functions, which are tightly coupled to $GABA_A$-evoked chloride conductance mechanisms (27), rely on the allosteric binding of GABA to its supramolecular complex site.

The utilization of animals with elevated endothelial NOS levels, as a consequence of high cholesterol concentrations (30), has already demonstrated their usefulness in the evaluation of the endothelium-derived relaxing factor on cardiovascular functions. If elevated cholesterolaemic conditions tend to enhance the production of NO, then the selection of the genetic hyperlipidaemic YOS rat (9) could prove to be an advantageous feature in the neurobiological field, especially because high NO levels may be supplied without the adaptation of a severe cholesterol-enriched diet (17), which does not always assure either homogeneous or suitable experimental conditions.

METHOD

Animals

Male YOS rats (approximately 5 months old) were allowed a brief habituation session before treatment. This age was preferred over others because 6-month-old animals displayed diminished endothelial functions (about 30%; data from our laboratory) as well as considerably high ematic cholesterol levels (approximately 250 mg/dl) whereas lower levels (approximately 70 mg/dl) were obtained in 2-month-old rats. The animals were assigned to different treatment groups and received daily via IP 30 mg/kg of L-NAME (Sigma) dissolved in saline solution for the first month and afterwards they were given daily a 70-mg/kg IP dose for another month to gradually assure a greater inhibiting effect on NOS activity. Controls were only treated with 0.5 ml IP saline solution. Selection of the doses and treatment schedule (23) was based on preliminary experiments, on the necessity to suppress the atherosclerosis-dependent high NO levels (30) plus on the efficiency of a chronic L-NAME treatment in evoking behavioral changes at the cerebral level (1).

To another group ($n = 4$) of YOS rats, 100 mg/kg of L-arginine dissolved in saline solution was administered for 2 months. The effect of this treatment on benzodiazepine receptor levels was checked to establish whether the hyperlipidaemic condition alone was sufficient to alter receptor levels or whether L-arginine was required, especially because a high and a somewhat notable endothelial NOS activity has been reported in the lung of hypercholesterolaemic animals (15). Recently, such a endothelial isoform type has also been identified in rodent brain tissue (8).

During the entire experimental session, all animals were maintained at a 14 L : 10 D light cycle and received standard diet (4 RF18 Mucedola, Settimo Milanese-Italy) and water ad lib. Twenty-four hours after the last treatment, all animals were killed by decapitation; their brains were rapidly dissected out of the skull and immediately frozen ($-40°C$) until autoradiographic analysis.

Quantitative Receptor Autoradiography

The influence of L-NAME on benzodiazepine binding in some limbic areas of YOS rats was investigated using in vitro quantitative autoradiography. Coronal sections (16 μm) of the rat brain were cut on a cryostat, thaw-mounted onto gelatin-coated slides, thoroughly dried in a vacuum desiccator at 5°C, and stored at $-40°C$ until binding assay.

Benzodiazepine receptors were labelled in vitro using the specific ligand [^{3}H]FLU according to the method applied in previous studies (5), plus some modifications. Briefly, brain sections from rats treated with L-NAME ($n = 6$), L-arginine ($n = 4$), and saline solution ($n = 4$) were thawed and preincubated for 3 × 10 min in 50 mM cold Tris-HCl buffer (pH 7.4) to remove as much endogenous ligand of the $GABA_A$ supramolecular complex as possible. Following the washes, brain slices were incubated in the same buffer containing 2.5 nM [^{3}H]FLU (85.3 mmol/Ci, NEN) for 30 min on ice. Nonspecific binding was determined on brain sections adjacent to those used for the total binding evaluation by addition of 10 μM diazepam (Prodotti Roche, Milan). After incubation, the slides were washed in ice-cold Tris-HCl buffer for 80 s, rapidly dipped in ice-cold double distilled water, and blown dry with cold air. Dried brain sections were transferred to cardboard film cassettes along with plastic standards [^{3}H]microscales (Amersham) and an Ultrofilm (LKB) sheet was apposed at room temperature. After an exposure period of 12 days, the film was developed in Kodak D19 developer according to previously described methods (4).

The autoradiograms were evaluated at a Zeiss "VIDAS" image analyzer system that converts relative optical densities to fmol/mg wet tissue, based on the standard curve derived from the coexposed tritium standards. The values were subsequently transformed to fmol/mg protein by dividing by O.O94 (equivalent milligram of protein found in a milligram of wet tissue).

On the basis of the allosteric coupling of benzodiazepine to the $GABA_A$ complex, it is possible that L-NAME-induced [^{3}H]FLU binding variations might be mediated in a GABA-dependent manner. Because this well-known inhibitory neurotransmitter is removed during preincubation washes, a final physiological GABA concentration of 20 μM was included in parallel brain sections. The choice of [^{3}H]FLU and GABA concentrations was based on prior values plus on a preliminary K_d value of 2.5 nM evaluated from a three-point saturation binding study using wipe assays. The method for these assays is similar to that conducted for autoradiography study except that the brain slices were wiped into vials containing 3 ml Insta-gel (Packard) and the amount of radioactivity bound for each brain slice was determined by scintillation counting.

To determine the type of [^{3}H]FLU binding activity that might be responsible for the L-NAME-dependent changes ob-

tained in the autoradiographic evaluation, we employed analysis by the method of Scatchard to compare the receptor levels in the two treatment groups. For this part, corresponding brain sections to those of L-NAME (n = 6)- and saline (n = 4)-treated animals of the distribution study were handled in a similar manner as above except that the brain slices were incubated with 50 mM cold Tris-HCl buffer (pH 7.4) + 20 μM GABA containing varying concentrations of [^{3}H]FLU (0.75–20 nM). From the Scatchard plots of the ratio of bound to free ligand against the amount of bound ligand, it was possible to calculate, using nonlinear regression analysis, the negative slope, which provided us the mean dissociation constant (K_d) and the intercept of the curve at the abscissa provided the maximal number of binding sites (B_{max}).

Statistical Analysis

The binding differences in some of the limbic areas of YOS rats treated with either L-NAME or L-arginine were compared to those that received saline solution by using a Student's t-test ($p < 0.05$ considered significant, two-tailed test). Although the application of this statistical test increases the chances of a type I error, from a biological perspective of a system it proves to be a sufficient method because it permits us to compare neuroanatomically discrete receptor binding differences.

RESULTS

The application of quantitative autoradiography techniques enabled us to establish a heterogenous neuroanatomic binding pattern of [^{3}H]FLU in the YOS rat as displayed by the different grey densities in a representative autoradiogram of a posterior brain section (Fig. 1). From the saturation results (Fig. 2) it was possible to observe a stable and specific binding pattern (Table 1). As far as the GABAergic potentiation effect on [^{3}H]FLU binding activity in saline-treated and, for that matter, in L-arginine-treated animals is concerned, altered binding levels (although the data were not statistically tested) were obtained in all areas except BL. Additionally, when L-

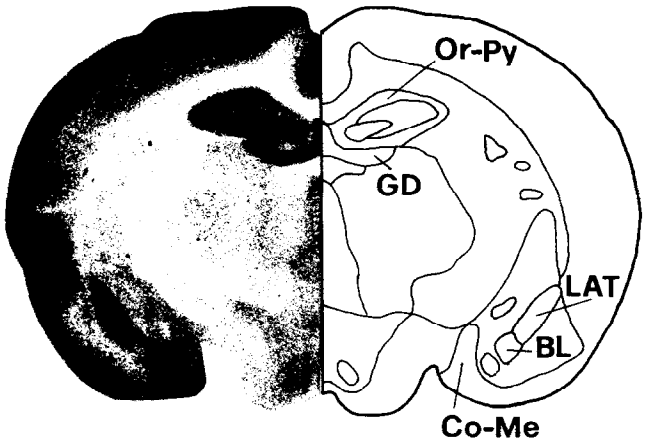

FIG. 1. A representative autoradiogram, along with its scheme, of a posterior brain area in the YOS rat treated with saline solution. Abbreviations: BL = basolateral amygdala nucleus; Co-Me = cortico-medial amygdala nucleus; GD = gyrus dentate; LAT = lateral amygdala nucleus; Or-Py = oriens and pyramidalis cell layer of CA1 hippocampal region.

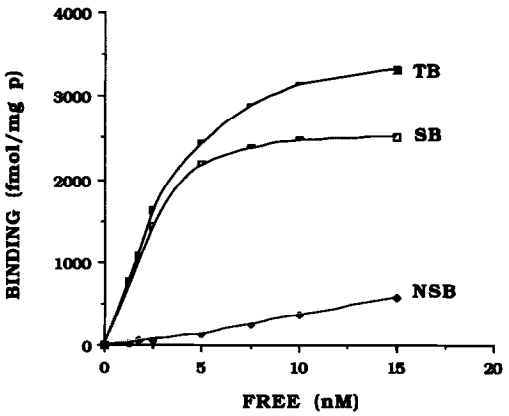

FIG. 2. Saturation curve of total (TB), specific SB), and nonspecific (NSB) binding showing the highly specific binding activity of [^{3}H]FLU in the basolateral amygdala nucleus of YOS rats treated with saline solution.

TABLE 1

AUTORADIOGRAPHIC [^{3}H]FLU BINDING LEVELS (fmol/mg p; $\bar{X}$ ± SEM) IN SOME LIMBIC AREAS OF Yos RATS TREATED WITH L-ARGININE AND SALINE SOLUTION

	Treatment Groups	
Brain Areas	Saline	L-Arginine
BNST	1509 ± 112	1603 ± 135
	2226 ± 93	2063 ± 107
LAT	2000 ± 119	1885 ± 118
	2473 ± 210	2729 ± 60
BL	1668 ± 106	1580 ± 97
	1735 ± 101	1850 ± 149
BM	2103 ± 50	1838 ± 98
	2950 ± 148	2790 ± 159
Ce	1119 ± 120	1028 ± 48
	1487 ± 132	1359 ± 135
Co-Me	1938 ± 35	2023 ± 87
	2503 ± 174	2573 ± 79
GD	1863 ± 68	2045 ± 120
	2708 ± 155	3093 ± 175
Or-Py	1354 ± 111	1260 ± 90
	1714 ± 168	1685 ± 84
RAD	1015 ± 58	963 ± 77
	1291 ± 160	1394 ± 43

Coronal brain slices were incubated in 50 mM Tris-HCl buffer containing 2.5 nM [^{3}H]FLU in the absence or presence of 20 μM GABA according to the procedure described under the Method section. Binding levels for each brain area of L-arginine-treated group in the absence and presence of GABA were compared to the corresponding saline group using Student's t-test whereas a statistical evaluation of the GABAergic influence on [^{3}H]FLU binding activity (which is already known) within the two treatment groups was not considered necessary. For abbreviations see Fig. 3.

arginine was administered, we noticed that [^{3}H]FLU bound to benzodiazepine receptors in a comparable fashion to the saline-treated group, suggesting that perhaps this amino acid is not able, as a consequence of similar NOS activity, to induce further binding changes in our hyperlipidaemic animal model.

Of all the limbic regions examined, including hypothalamic areas that are known to be key brain sites involved in the control of sociosexual behaviors, only those belonging to bed nucleus of the stria terminalis–amygdala–hippocampus circuit seemed to respond to the blocking effects of the NOS inhibitor. The administration of L-NAME was responsible for a 52% higher ($p < 0.01$) [^{3}H]FLU binding level in BL whereas only a moderately higher value (38%, $p < 0.05$) was detected in the oriens-pyramidalis cell layer of the CA1 hippocampal region (Or-Py) (Fig. 3a). In other limbic areas an even greater

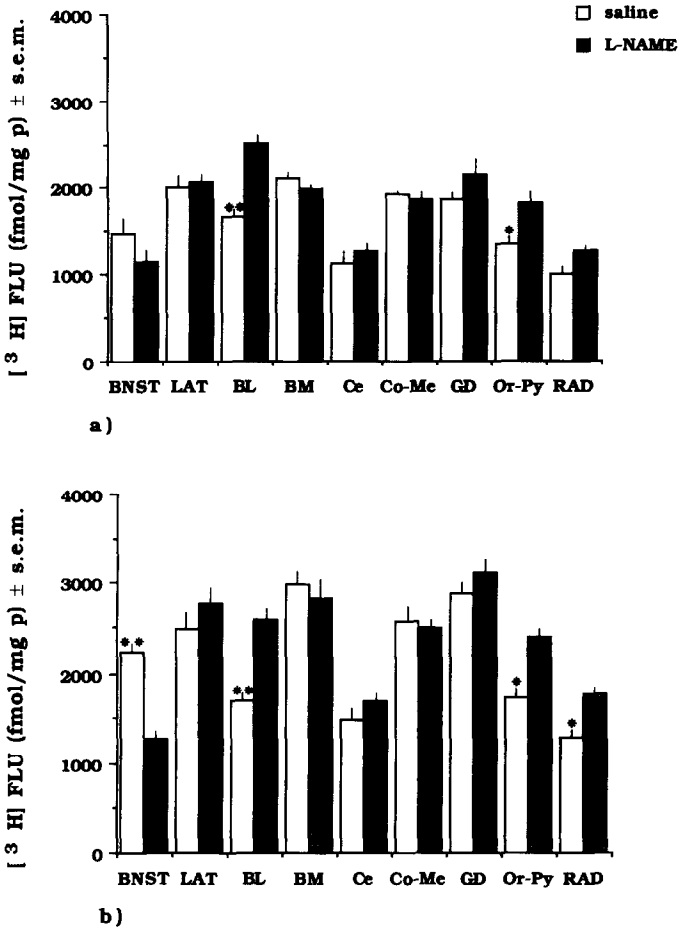

FIG. 3. [^{3}H]FLU binding levels (mean ± SEM) in the absence (a) or presence (b) of 20 μM GABA in some limbic areas of YOS rats treated with either Nω-nitro-L-arginine methyl ester (L-NAME) or saline solution as described in the Method section. The mean values of each brain area were compared using Student's t-test. *$p < 0.05$; **$p < 0.01$. Abbreviations: BL = basolateral amygdala nucleus; BM = basomedial amygdala nucleus; BNST = bed nucleus of the stria terminalis; Ce = central amygdala nucleus; Co-Me = cortico-medial amygdala nucleus; GD = gyrus dentate; LAT = lateral amygdala nucleus; Or-Py = oriens and pyramidalis cell layer of the CA1 hippocampal nucleus; RAD = radiatum lacunosum molecular cell layer of the CA1 hippocampal region.

inhibiting activity of L-NAME was observed when a physiological GABA concentration (20 μM) was added to the [^{3}H]FLU binding incubation medium, although the effect in some cases occurred in an opposite direction. In fact, a 65% lower ($p < 0.01$) binding level was obtained in the bed nucleus of the stria terminalis (BNST) whereas a moderately higher level (38%) was reported for the radiatum-lacunosum molecular cell layer of the CA1 hippocampal region (RAD) (Fig. 3b). It is worthy to note that addition of GABA did not provide any further potentiating effect on [^{3}H]FLU binding levels in either BL or Or-Py.

A saturation binding study was also conducted in the above brain sites to determine the nature of L-NAME-induced effects on [^{3}H]FLU binding variations. From the Scatchard plots (regression coefficient values between 0.85 and 0.95) it appeared that the inhibiting effect of L-NAME, independent of the GABAergic influence, was mainly responsible for changes in the number of binding sites (check B_{max} and K_d differences in Or-Py) (Fig. 4a). However, L-NAME treatment was not only involved in the variations of B_{max}, because the differences of [^{3}H]FLU binding activity, in both a GABA-dependent and -nondependent manner, for the other limbic areas such as BL also seemed to be associated to affinity state changes (Fig. 4b).

DISCUSSION

The results of the present study demonstrate that [^{3}H]FLU binds to benzodiazepine receptors in a heterogeneous manner in some of the limbic areas of the genetic hyperlipidaemic YOS rat. Overall, despite a similar distribution pattern to that of Sprague-Dawley rats in these areas, the values in some cases, such as the lateral amygdala nucleus and gyrus dentate, were 25% and 65%, respectively, lower (5), a difference that is also maintained in the presence of 20 μM GABA. It is possible that such reduced binding levels might be a consequence of the altered plasma lipid concentrations. Whether the genetic-induced lipidaemic condition represents the main cause for binding differences obtained in our experimental model, with respect to that of normal laboratory rodents, is unclear at present. Recently, studies have revealed a high sex steroid plasma content in hyperlipidaemic animals (data not published) as well as an elevated cholesterol synthetic activity being correlated to high testosterone levels in these animals (7). In this case, the high circulating sex steroid levels may be considered an important factor in the regulation of [^{3}H]FLU binding activity in the different limbic areas of the YOS rat, especially because elevated activities of the enzymes 5α-reductase and 3α-oxidoreductase, which catalyze the transformation of cerebral sex steroids, have already been linked to changes of benzodiazepine binding pattern in the different brain areas of the Sprague-Dawley rat (6).

The effects of L-NAME on [^{3}H]FLU binding activity, which proved to be of an enhancing type, occurred principally in amygdalar and hippocampal regions of the limbic system. The greater binding changes were found in the former region as registered by the remarkably high levels in BL and somewhat high levels (although in a nonsignificant manner) in the lateral and central amygdala nuclei. From the increased benzodiazepine receptor levels, it would seem reasonable to assume that BL also exerts, via a modulatory action of this $GABA_A$ supramolecular site, a prime role in the regulation of anticonvulsive activities (1) in YOS rat. It should not be so surprising to find this key cognitive amygdalar nucleus participating in such binding variations because it has already been

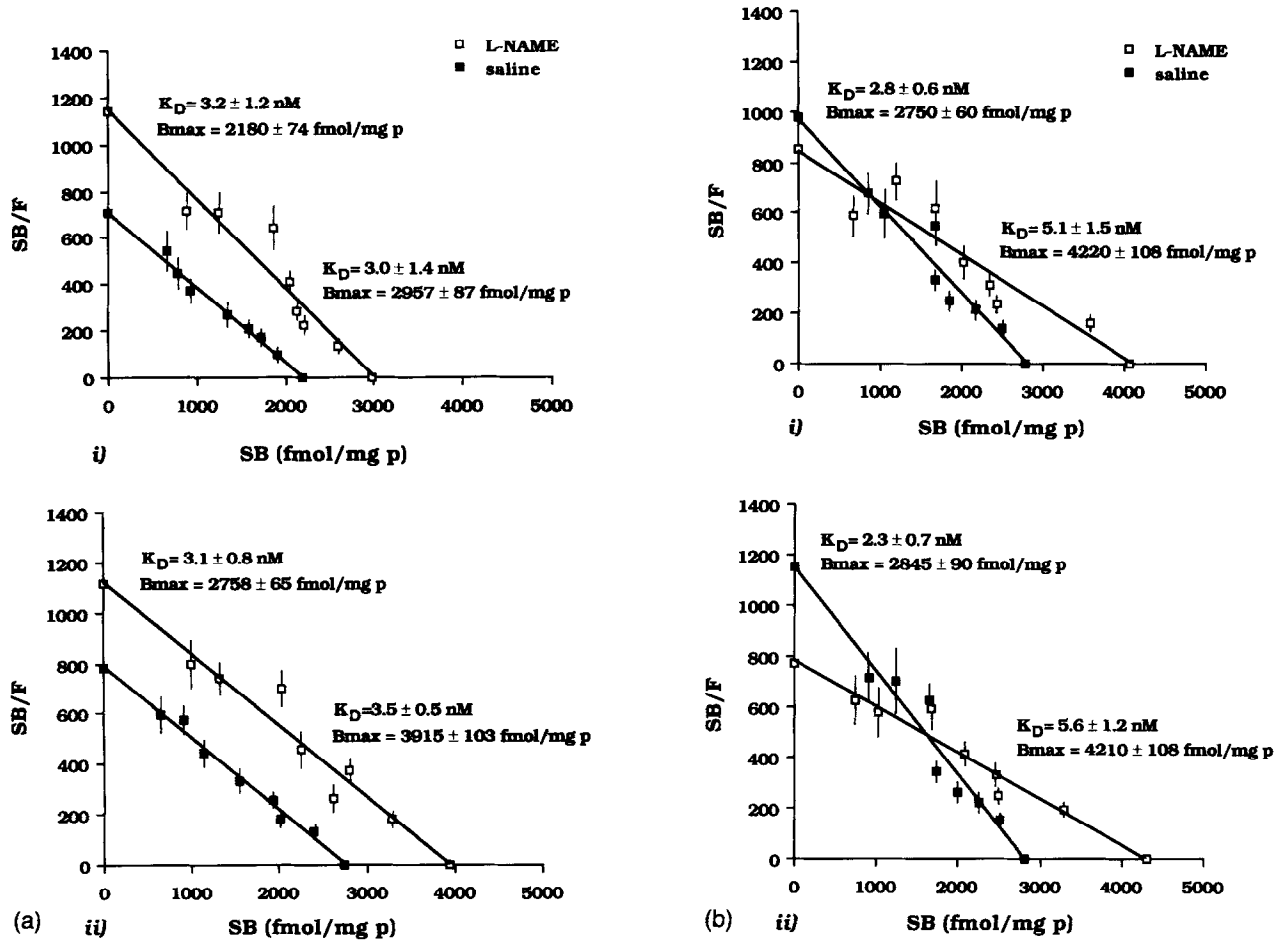

FIG. 4. (a,b) The analysis of Scatchard plots of the ratio of bound/free (B/F) concentrations (nM) against bound (B) concentrations (fmol/mg protein) was handled for the binding of [^{3}H]FLU at the benzodiazepine receptor in the (i) absence or (ii) presence of 20 μM GABA in the (a) basolateral amygdala nucleus and (b) oriens-pyramidalis CA1 layer of YOS rats treated with either $N\omega$-nitro-L-arginine methyl ester (L-NAME) or saline solution. Data are from experiments performed in triplicate with SEMs indicated by the vertical bars extending beyond the symbols.

shown to be involved in GABAergic-induced anticonflictual (28) and contextual expression of anxiety-related responses (12). Rather, it is particularly interesting to note that although L-NAME-dependent changes of the benzodiazepine receptor affinity state might be mediating a suppression of NO-dependent convulsive behaviors (19), alternatively, differences in B_{max} values seem to be linked to anticonflict activities (4). These specific types of receptor variations and consequent behavioral relationships in the same brain site could turn out to be an important and useful indicator for the determination of the differential role of this $GABA_A$ supramolecular site in the regulation of anxiolytic and anticonflictual actions.

Similar to the L-NAME effects obtained in BL, enhancing type of binding activities were also demonstrated in Or-Py and RAD of the hippocampus, even though binding differences in the latter site occurred only in a GABA-dependent manner. The fact that GABA was required to provoke greater effects of L-NAME on benzodiazepine binding seems to be in line with the GABAergic activity being recognized as a necessary factor in the promotion of pharmacological (28) and behavioral (4) functions. It is worthy to observe that the NOS inhibitor is not only responsible for elevated binding activities in the hippocampus and amygdala, limbic regions that after the cerebellum display both notable [^{3}H]L-N^G-nitro-arginine binding densities (14) and NOS-associated functions (10), because it also seems to be involved in the depression of other neuronal systems such as *N*-methyl-D-aspartate (NMDA) with consequent further worsen of limbic seizures (26). In this context, the interaction of NOS inhibitor-NMDA system seems to represent a key element in the control of certain behaviors such as spatial learning tasks (10) and epileptic crisis (26). A NOS inhibitor-benzodiazepine type of interaction might be, in a similar fashion to that observed in BL, operating towards the achievement of anticonvulsive and anxiolytic effects.

Interestingly enough, the GABA-dependent effect on L-NAME-induced binding changes did not always provoke increasing [^{3}H]FLU binding levels. Indeed, lower values were reported in other limbic sites such as BNST, suggesting that perhaps more than one type of neuronal mechanism is influencing the effects of this inhibitor on [^{3}H]FLU binding values. It is possible that the reduced benzodiazepine receptor levels in BNST, a critical olfactory site containing a remarkable quantity

of [^{3}H]L-N^G-nitro-arginine binding (14), could represent an important element in the GABAergic blocking influence of odour-derived responses in aggressive male rodents (18).

However, there were other limbic areas such as BL and Or-Py that did not show any further GABAergic potentiating effect on L-NAME-dependent binding changes. The failure of such influences leads us to suspect that perhaps a GABAergic-NOS inhibitory interaction does not occur in all brain areas. Conversely, because α, β, and γ subunits [functional domains of the $GABA_A$/benzodiazepine supramolecular complex; see (21,29)] are neither functionally nor structurally coupled in all brain regions, they might be accounting for the regional differences in the response of this complex. Although the lack of GABA influence on benzodiazepine receptor levels, which are in agreement with the results of other works (6,32), might seem to favour the latter assumption, the GABA enhancing effect reported in both the above and other brain areas under different pharmacological conditions (5) tends to instead support the former suggestion (i.e., GABA does not induce any further potentiation effect on L-NAME-dependent binding differences in all cerebral sites).

Taken together, the quantitative autoradiography data dealing with benzodiazepine binding changes under the influence of the NOS inhibitor tend to display an evident benzodiazepine receptor-L-NAME type of interaction that, in some limbic sites of YOS rat, may not necessarily be mediated via the NO pathway. On the other hand, the hyperlipidaemic conditions that are related in our animal model to an elevated endothelial NOS activity (8) seem to indicate that perhaps L-NAME effects are operating via this pathway from the moment that varying amounts of this endothelial isoform have been, in a species-specific fashion, detected in the rodent brain (2). GABAergic terminals containing NOS (33) and the modulation of $GABA_A$ receptor activity by NO (35) further strengthen the proposal of this endothelium-derived relaxing factor as an important putative neurotransmitter (11) in the promotion of GABAergic functions. On the basis of the above information plus on our results, it is tempting to speculate that the interaction between the NOS inhibitor and the $GABA_A$ supramolecular complex may exert an important neuroprotective role in the control of convulsive and anxiolytic behaviors in hyperlipidaemic rats.

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