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Stress-induced decrement in the plasticity of the physical properties of chick brain membranes

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Summary

The molecular basis underlying the stress-induced increment in the density of central benzodiazepine receptor from chick forebrain, observed previously at 4°C, was studied from a biophysical perspective. The thermal dependence of [^{3}H]flunitrazepam binding to the central benzodiazepine receptor and the supramolecular organization were studied in forebrain membranes from chicks submitted to partial water immersion. The equilibrium dissociation constants increased with temperature in membrane from both control and stressed chicks. The heat capacity values in control samples ($\Delta C_{p,\,CON}$) were significantly less negative than $\Delta C_{p\,STR}$. Changes in ΔH and ΔS between 4–37°C were greater in stressed chicks compared to control; however, the binding was exothermic and driven by enthalpy in both conditions. At 4°C, the receptor density (B_{max}) was higher in stressed chicks compared to control. Such a difference was lost irreversibly upon temperature elevation, possibly due to the hysteresis between the heating and cooling behaviour of $B_{max,\,CON}$ and the constancy in $B_{max,\,STR}$. The fluorescence anisotropy of diphenylhexatriene was higher in control samples with respect to stressed chicks below 10°C. A temperature-induced increment in protein intrinsic-fluorescence was observed only in control, and was quenched by acrylamide more easily at 4°C than at 25°C. A higher microviscosity at 4°C in control favoured more external localizations of integral proteins; at higher temperatures, tryptophan residues moved to hydrophobic membrane-regions. Changes in the membrane-organization towards more fluid states favoured the accessibility of benzodiazepine to the central benzodiazepine receptor, expressed by the higher values of B_{max} found in stressed samples at low temperatures with respect to control samples.

Keywords: Stress, benzodiazepine binding, $GABA_A$ receptor, thermodynamics, membrane fluidity, DPH fluorescence, tryptophan fluorescence, dynamic quenching.

Abbreviations: ANOVA, analysis of variance; B_{max}, maximal binding; BZD, benzodiazepine; CBR, central benzodiazepine receptor; CON, membrane samples of control chickens; ΔC_p, heat capacity change; CV, variation coefficient; DBI, double bond index; DPH, diphenylhexatriene; DZ, diazepam; FNZ, flunitrazepam; GABA, gamma amino butyric acid; ΔH, enthalpy change; K, equilibrium constant of the binding reaction; K_d, equilibrium dissociation constant; PWI, partial water immersion; ΔS, entropy change; SEM, standard error of the mean; STR, membrane samples of stressed chickens.

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Introduction

Stress is an organism's complex response which involves changes in various neurochemical and endocrine systems such as the opioid (Jackson *et al.* 1979, Drugan *et al.* 1985), the dopaminergic (Fekete *et al.* 1981, Anisman and Zacharko 1990), the catecolaminergic (Tsuda and Tanaka 1985, Stone 1987, Petty *et al.* 1993), the cholinergic (Anisman *et al.* 1981), the serotoninergic (Dunn 1988, Petty *et al.* 1992), the pituitary-hipothalamic-adrenocortical axis (Jain *et al.* 1991) and the GABAergic system (Petty and Sherman 1981, Drugan and Holmes 1991, Paredes and Agmo 1992, Stephen *et al.* 1994).

Gamma-aminobutyric acid (GABA) exerts its physiological role through several GABA receptor sub-types A, B, C and X by modulating pituitary hormone secretion, thermoregulation, ingestive behaviours, motor functions, reproductive behaviour, aggressive-defensive behaviours and anxiety (for review see Drugan and Holmes 1991).

It has been demonstrated that the $GABA_A$ receptor ($GABA_A$-R) is a pentameric protein that acts as a ligand-gated chloride channel and is the site of action of a variety of pharmacologically important drugs including benzodiazepines (BZDs) (Sieghart 1992). The central BZD receptor (CBR) is an allosteric modulator site at the $GABA_A$-R in the brain. The $GABA_A$ is the only GABA receptor sub-type associated with a BZD binding site (Pritchett *et al.* 1989).

There is considerable evidence indicating that environmental stressful conditions induce rapid and reversible changes on the density and the affinity of the CBR (Drugan and Holmes 1991, Stephen *et al.* 1994). Previous reports from the laboratory demonstrated that the CBR density in chicken forebrain consistently increased without change in affinity, in several test situations. For example, due to the stress accompanying a passive training task (Martijena and Arce 1993), an imprinting task (Salvatierra *et al.* 1994), a food discrimination task (Salvatierra *et al.* 1997) and a forced-swimming test (Martijena *et al.* 1992) also named partial water immersion (PWI) when applied to chickens (Marin and Jones 1999). Interestingly, the PWI test differentially affects the CBR density in chicks previously classified on the basis of their performance either in an imprinting task (Salvatierra *et al.* 1994) or in a T-maze task (Marín and Arce 1996).

It has been suggested that the CBR increment induced by PWI may be explained by the recruitment of existing receptors rather than by higher synthesis or lower degradation rates of the receptor constituent proteins (Martijena *et al.* 1992). This recruitment might be the result of a higher accessibility of ligand (BZD) to its binding site, resulting from a change in the conformation of the receptor protein and/or its localization within the membrane (Perillo *et al.* 1994a). This would be possible taking into

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account the behaviour of proteins in membrane models. Thus, extrusion or penetration of proteins can be modulated by changes in the lateral surface pressure of monomolecular layers at the air–water interface (Santini *et al.* 1990, Sanchez and Perillo 2002), a parameter that resembles the molecular packing organization of bilayer membranes (Marsh 1996).

Subtle local changes in membrane composition might be triggered by the activation of signal transduction metabolic pathways gated by stress (Yehuda *et al.* 1999). It is difficult to predict straightforward correlations between changes in composition and organization, due to the complexity of both the membrane composition and of the biochemical events triggered by signal transduction cascades, as well as the membrane chemical and biophysical lateral asymmetry (coexistence of phases with differences in chemical composition and molecular organization within the plane of the membrane). This reflects the fact that composition and organization are variables that work in different hierarchical levels of organization (the former acts at the molecular level and the latter at the supramolecular level). However, the changes in chemical composition, even if subtle, can be transduced and amplified into significant changes in molecular organization due to the typical non-linear and dynamic behaviour of complex systems such as membranes (Maggio 1994, Aon and Cortassa 1997, Garcia *et al.* 2000). The changes in the membrane's chemical composition, observed in many experimental designs to test different physiological responses, were generally very small (10–20%). In spite of this, the concomitant changes in membrane organization as well as their physiological consequences were significant. For example, the increments in the proportions of saturated fatty acids observed in stress-induced apoptosis (Singh *et al.* 1996), the changes accompanying the habituation of rats to alcohol treatments (Miller *et al.* 2001) and those associated to changes in the temperature of the environment in fishes (Buda *et al.* 1994).

The changes induced by stress can be triggered by a mechanism that involves the activation of neuronal networks, and can be followed by multiple and complex events at the cellular, membrane and molecular levels of organization. The brains as a whole, as well as each of the cells involved in a response, function like open systems in a steady state. That means that any perturbation may either be discarded, if it is small enough and the compensation mechanisms exist, or may lead the system to a new steady state. Particularly in the former case, the initial changes will be transient. The recruitment of the BZD receptor reported in the laboratory could be observed in membranes coming from animals decapitated up to 1 h after the chicks were submitted to the stressful situation. This means that the effect of stress (the perturbation) on BZD-R recruitment could be compensated for in the living birds (systems in a steady state). Therefore, this stressful task has a transient effect. However, once the brains were dissected and the membranes obtained, the difference in BZD-R density between Control and Stressed samples was observed even in membranes maintained frozen at −20°C for several months (if they came from chicks decapitated within the 1 h period mention above). Hence, the isolated membranes behaved like a closed system and the changes were stable. These results have always been very reproducible in the laboratory and they were found to be a good model to study a biophysical aspect of a physiologically-triggered phenomenon.

The present paper investigates the hypothesis that the previously demonstrated stress-induced recruitment of CBR could be associated with changes in the membrane's supramolecular organization of the receptors' environment. Through thermodynamical analysis of FNZ binding to CBR, fluorescence anisotropy of a hydrophobic probe and dynamical quenching of membrane protein intrinsic fluorescence, the stress-induced changes in the flexibility of CBR, membrane organization and in the proteins' localization within the membrane were evaluated. Possible differences in fatty acid composition of membrane lipids were also explored.

Results

Taking into account that the binding thermodynamics of [^{3}H]FNZ to $GABA_A$-R from chicken forebrain can be described by a hyperbolic function with one binding site (Martijena and Arce 1993) it was considered that the actual binding process was not affected by the stressful conditions, at least from a qualitative point of view. Upon temperature elevation, the values of K_d increased significantly in both CON and STR samples (table 1). These K_d values were transformed into K values (corresponding to the equilibrium constant of the binding reaction) and their natural logarithms were plotted against $1/T$ (figure 1). The exothermic characteristic of the binding reaction in the CON samples was qualitatively maintained in STR samples (table 2) as shown by the analysis of the van't Hoff plots. It should be noted that the regression analysis of ln K against $1/T$ according to the van't Hoff equation (equation 3), which assumes an apparent ΔC_p=0, gave apparent temperature-independent ΔH and ΔS values with no significant difference

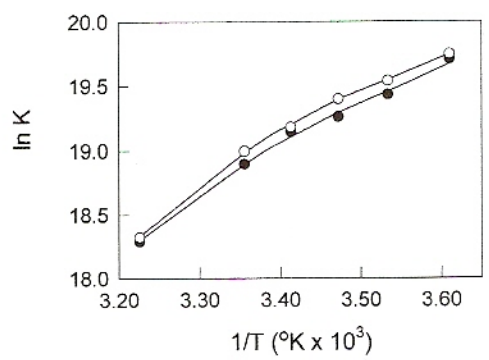

Figure 1. Van't Hoff analysis of the equilibrium constant of [^{3}H]FNZ binding to synaptosomal brain membranes from control and stressed chicks. K values were calculated as the reciprocal of the K_d values (in molar units) taken from table 1. Error bars are within the size of the symbols. The lines represent the fitting to equation (4). CON (closed circles); STR (open circles).

Table 1. Effect of acute stressor exposure on the temperature dependence of [^{3}H]FNZ binding to crude synaptosomal membranes from chicken forebrain.

Incubation temperature (°C)	B_{max} (fmol/mg protein)		K_d (nM)	
	Control	Stress	Control	Stress
4	929±30 (5)	1195±32 (5)[a]	2.76±0.24	2.66±0.28
10	1142±62 (5)[a]	1118±92 (5)[a]	3.64±0.34	3.27±0.02
15	1236±116 (5)[a]	1291±73 (5)[a]	4.32±0.86[b]	3.76±0.49
20	1099±84 (5)[a]	1358±109 (5)[a]	4.84±0.30[b]	4.67±0.36[b]
25	1190±134 (5)[a]	1285±72 (5)	6.21±0.36[b]	5.62±0.6[b]
37	1121±138 (5)[a]	1183±87 (5)[a]	11.42±1.46[b]	11.00±1.39[b]

The values of B_{max} and K_d shown represent the mean±SEM (n) of 'n' independent replicates obtained by the non-linear regression analysis of saturation curves, where the amount of [^{3}H]FNZ bound to membranes was plotted vs free [^{3}H]FNZ concentration.
[a]B_{max} values that differed from control at 4°C with $p<0.05$.
[b]K_d values that differed from the corresponding sample at 4°C with $p<0.05$.

Table 2. Effect of temperature on the values of the thermodynamic state functions calculated from van't Hoff plots.

Temperature (°C)	ΔG (KJ/mol)		ΔH (KJ/mol)		ΔS (KJ/mol/°K)		$T\Delta S$ (KJ/mol)	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
4	−44.719	−40.58	−11.28	−7.15	0.1224	0.1380	33.43	37.67
10	−45.78	−44.14	−19.92	−18.28	0.0914	0.0979	25.86	27.71
15	−46.20	−45.81	−24.24	−23.85	0.0762	0.0784	21.96	22.58
20	−46.54	−47.38	−28.57	−29.41	0.0613	0.0593	17.97	17.37
25	−46.81	−48.90	−32.89	−34.98	0.0467	0.0404	13.92	12.05
37	−47.16	−52.24	−43.26	−48.34	0.0126	−0.0035	3.90	−1.09
0–37	−45.71	−45.97	−26.5	−26.8	0.0620	0.0618	19.21 (37°C)	19.16 (37°C)

The values of ΔH were calculated by fitting data from figure 1 to linear van't Hoff equations (equation 3) (last row) or to the integrated non-linear form of it (equation 4) (other rows). The former equation considered a constant C_p value ($\Delta C_p=0$) and the latter a temperature-dependent C_p value ($\Delta C_{p\ CON}=-0.86\pm0.135$ and $\Delta C_{p\ STR}=-1.11\pm0.064$ KJ/mol/K). The calculation of other thermodynamic state functions was explained in the Experimental procedures.

between CON and STR ($\Delta H_{CON}=-26.5\pm2.8$ (CV=11%) KJ/mol and $\Delta H_{STR}=-26.8\pm3.5$ (CV=13%) KJ/mol, $\Delta G_{CON}=-45.71$ and $\Delta G_{STR}=-45.97$ KJ/mol and $\Delta S_{CON}=0.062$ and $\Delta S_{STR}=0.0618$ KJ/mol) as described by other authors (Speth *et al.* 1979, Kchman and Hirsch 1982, Quast *et al.* 1982). The inclusion of the temperature-induced variations of enthalpy in the van't Hoff equation (equation 4) gave a good fit of the experimental data (figure 1). Although the figure showed that the curve of the STR sample was slightly above that of the CON, the difference is not significant according to the statistical comparison of individual K_d values (table 1). However, a statistically significant difference ($p<0.05$) between the calculated values of $\Delta C_{p\ CON}=-0.86\pm0.135$ (CV=16%) and $C_{p\ STR}=-1.11\pm0.064$ (CV=6%) KJ/mol/°K was found. This analysis gave a 3.8 and 6.8-fold increment in the absolute values of ΔH_{CON} and ΔH_{STR}, and a 9.7 and 40.2-fold decrement in ΔS_{CON} and ΔS_{STR}, respectively, between 4–37°C in both experimental conditions (table 2). The values of ΔC_p were reasonable compared with those found in other systems (Naghibi *et al.* 1995).

A different behaviour was observed in B_{max} of CON and STR samples as a function of temperature (table 1). In CON samples, B_{max} increased with temperature from 929±30 to 1142±62 fmol/mg protein up to 10°C, and then remained constant at higher temperatures. In membranes from STR chicks, B_{max} remained constant along the whole range of assayed temperatures. Therefore, while at low temperatures (4°C) $B_{max,\ STR}$ (1195±32 fmol/mg protein) was significantly higher ($p<0.05$) than $B_{max,\ CON}$; this difference was lost with temperature elevation (table 1).

The difference between $B_{max,\ STR}$ and $B_{max,\ CON}$ observed at 4°C disappeared after a pre-incubation at 25°C because $B_{max,\ CON}$ increased significantly with respect to the samples maintained at 4°C while, under the same conditions, $B_{max,\ STR}$ remained unchanged. This phenomenon was not reversed at least until after 60 min of incubation at 4°C (note that $B_{max,\ CON,\ 25/4}=1088\pm87$ fmol/mg prot was not significantly different from $B_{max,\ CON,\ 25/25}=1179\pm44$ fmol/mg prot). The values of K_d were not affected by the pre-incubation temperature (table 3).

The percentage of fatty acid composition of CON and STR synaptosomal membranes were not significantly different. However, the existence of significant local changes, not detectable with the methodology used in this work, should be considered. A tendency towards a lower saturated/unsaturated ratio and higher double bond index (DBI) of STR compared to CON samples was observed (table 4).

The fluorescence anisotropy was significantly different between CON and STR samples (figure 2). Up to T=10°C $A_{DPH,\ CON}>A_{DPH,\ STR}$ and over 10°C $A_{DPH,\ CON}<A_{DPH,\ STR}$. As expected, anisotropy decreased with the rise of temperature from 4–37°C, with a more notable effect in CON with respect to STR. The ANOVA of the results of four experiments showed: (a) a significant effect of temperature

Table 3. Effect of the pre-incubation temperature on the temperature dependence of [^{3}H]FNZ binding to crude synaptosomal membranes from control and stressed chicks.

Pre-incubation/incubation (°C/°C)	B_{max} (fmol/mg protein)		K_d (nM)	
	Control	Stress	Control	Stress
4/4	881±20 (3)	1095±58 (3)[a,b]	2.63±0.22	2.87±0.20
25/4	1088±87 (3)[a]	1152±35 (3)[a]	2.84±0.31	2.72±0.28
25/25	1179±44 (3)[a]	1200±34 (3)[a]	5.60±0.22	5.34±0.15

The samples were pre-incubated during 25 min at the first temperature indicated and immediately incubated at the other temperature during the time indicated in the Experimental procedurs. The values of B_{max} and K_d shown represent the mean±SEM (n) of 'n' independent replicates obtained by the non-linear regression analysis of saturation curves, where the amount of [^{3}H]FNZ bound to membranes was plotted versus free [^{3}H]FNZ concentration.
[a]B_{max} values that differed from 4/4 control with $p<0.05$.
[b]Significantly different from the corresponding control sample.

Table 4. Fatty acid composition of crude synaptosomal membranes from the forebrain of control and stressed chicks.

Fatty acid	Control	Stress
14:0	0.94±0.27	0.78±0.04
16:0	30.51±0.33	30.24±0.50
16:1	2.57±0.14	2.26±0.03
18:0	28.27±1.21	27.81±0.46
18:1	19.51±0.55	20.10±0.16
18:2	1.51±0.36	1.17±0.11
20:4	10.75±0.57	11.61±0.28
22:4	5.84±0.57	6.37±0.08
Saturated/unsaturated	1.491±0.103	1.409±0.028
DBI	0.915	0.966

Lipids from crude synaptosomal brain membrane fractions were extracted, hydrolized and methylated as explained in the Experimental procedures. Methyl esters were quantified by gas–liquid chromatography. Peaks were identified either by comparisons with standards or by GC-mass spectrometry. Values are the mean±SEM from two independent experiments, of the percentage respect to total area, contributed by the area under the corresponding chromatographic peak.
DBI: double bond index calculated as the sum of the percentage of each lipid times the number of its double bonds, over 100.

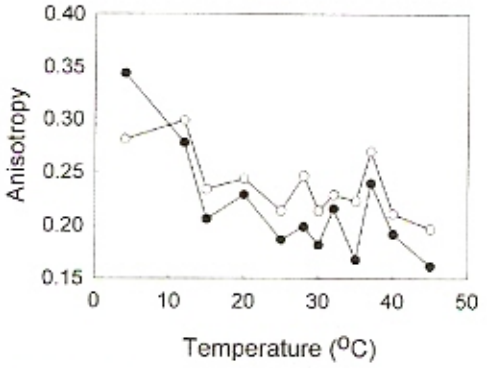

Figure 2. Effect of acute stressor exposure on the DPH fluorescence anisotropy in synaptosomal membranes from chick brain. CON and STR synaptosomal membranes were incubated for 1 h in the presence of 2 μM DPH. Fluorescence at 430 nm (excitation at 360 nm) was measured between 4–37°C with different orientations of excitation and sample polarizers (see Experimental procedures), and anisotropy was calculated according to equations (1) and (2). Values represent the mean±SEM of anisotropy values from four independent experiments. CON (closed circles); STR (open circles).

(F[11,12]=14.3, $p<0.00001$), (b) significant differences between the CON and STR samples (F[1,12]=18.06, $p<0.001$), and (c) a significant interaction between temperature and experimental condition (CON and STR) (F[11,12]=2.12, $p<0.0394$).

The effect of temperature on the partitioning of the fluorescent probe DPH ($\Delta F/F_o$) was quite similar in CON and STR, as evidenced by the positive slope of the fluorescence vs temperature plots (figure 3(*a*)). The intensity of the intrinsic protein fluorescence of membrane proteins at 340 nm increased as a function of temperature in CON and remained constant in the STR samples (figure 3(*b*)). As expected (Lakowicz 1983), the dynamic quenching of tryptophan fluorescence induced by acrylamide was dependent on the concentration of the quencher (figure 3(*c*)). In addition, the quenching efficiency in CON was higher at 4°C (ΔF=56%) than at 25°C (ΔF=44%), where $\Delta F=[100(F_{without\ acrylamide}-F_{with\ acrylamide})/F_{without\ acrylamide}]$ (figure 3(*d*)). Taken together, these results indicate that, upon temperature elevation, the exposition of membrane proteins to the aqueous environment decreased in CON but not in the STR sample.

Discussion

Acute stressor exposure affects the thermodynamics of [^{3}H]FNZ binding to synaptosomal membranes from chick forebrain

In the first communications about the existence of a BZD receptor, attention was drawn to the strong temperature dependence of the binding K_d but not of B_{max} (Baestrup and Squires 1977, Mohler and Okada 1977). Afterwards, temperature dependence of BZD binding to the CBR was widely studied (Speth *et al.* 1979, Kchman and Hirsch 1982, Quast *et al.* 1982). The present work studied and found differences in the temperature dependence of [^{3}H]FNZ binding to CBR in forebrain synaptosomal membranes from acute stressed and non-stressed (control) chicks.

According to the classical pharmacological usage, the affinity of the ligand-receptor binding process is inversely related to the value of K_d, which represents the thermodynamic equilibrium constant of the dissociation reaction of a ligand-receptor complex. However, a direct interpretation of the thermodynamics of binding is readily available from the association reaction:

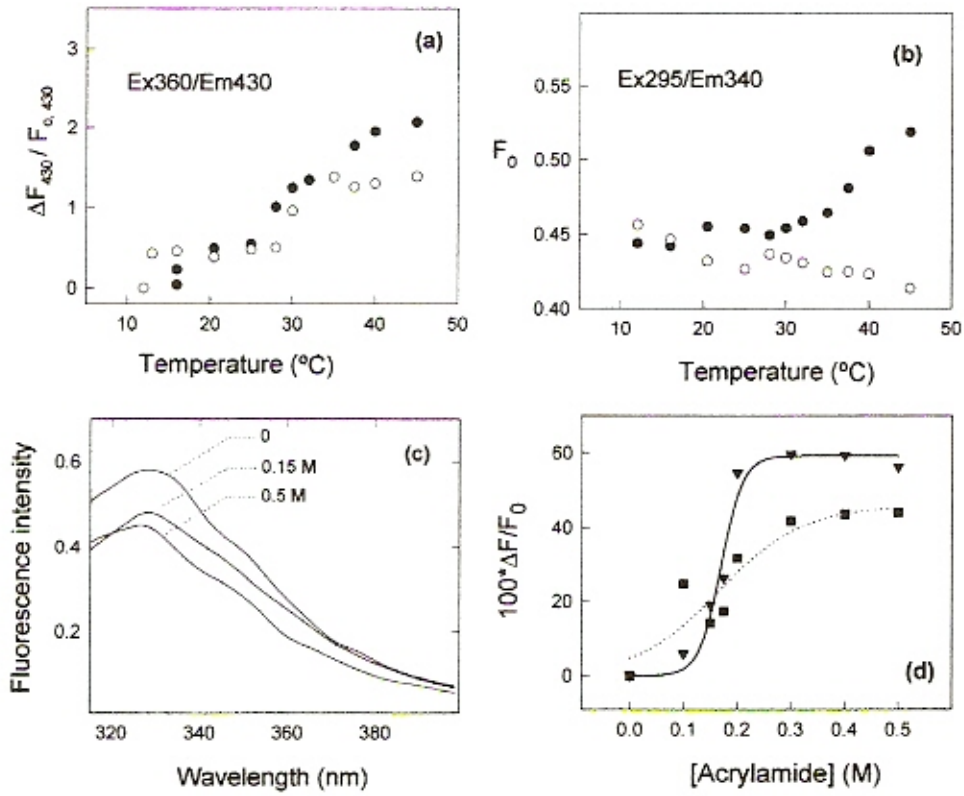

Figure 3. Effect of acute stressor exposure on the DPH and protein fluorescence in synaptosomal membranes from chick brain. (*a*) Relative fluorescence at 430 nm (excitation at 360 nm) of samples containing DPH. (*b*) Protein intrinsic fluorescence at 340 nm (excitation at 295 nm). CON (closed circles); STR (open circles). (*c*) Emission spectra (excitation at 295 nm) of intrinsic fluorescence from CON sample in the presence of the increasing acrylamide molar concentrations indicated in the figure, at room temperature. (*d*) Percentage of quenching of protein intrinsic fluorescence induced by acrylamide in CON samples at 4°C (closed triangles) and at 25°C (closed squares). Fluorescence values were obtained with both polarizers in the horizontal position.

$$\text{FNZ} + \text{CBR} \rightleftharpoons \text{CBR} - \text{FNZ}$$

Hence, the K_d values were calculated (table 1) to allow easy comparisons with published data, but association equilibrium constant (K) values were used in the thermodynamic analysis of the direct binding reaction.

As expected from classical equilibrium thermodynamics, the value of the equilibrium dissociation constant (K_d) increased with temperature (table 1), indicating a higher tendency of the ligand to dissociate from the receptor (the equilibrium constant of the binding reaction decreased one order of magnitude from 3.6×10^8 at 4°C to 8.7×10^7 at 37°C). In spite of this, ΔG of the binding became more favourable with temperature (table 2), mainly due to the contribution of the increasingly negative ΔH within the whole range of temperatures studied. ΔS also represented a favourable contribution to the binding process (except in STR at 37°C where it became negative, similar to that described by Quast *et al.* (1982) for supposedly naïve rats). Contrary to what happened with ΔH, ΔS became lower with increasing temperature. The increase in the absolute value of ΔH ($|\Delta H|$) as well as the decrease in ΔS was more marked in STR than in CON samples in accordance with the more negative values of ΔC_p in the former sample. The binding reaction, initially driven by entropy, changed towards an enthalpy-driven regime at a higher temperature, as shown by ΔH, which was less negative than the value of $-T\Delta S$ at 4°C and more negative at 37°C. Applying the linear van't Hoff plot, as $|\Delta H| > T\Delta S$ within the temperature range studied, it can be interpreted that the present reaction is driven by enthalpy, with favourable interactions occurring within the complex, which is in agreement with previous reports in rats (Quast *et al.* 1982). However, this form of the van't Hoff equation is not valid for reactions involving flexible polymers and proteins. In these systems, the energy of the bonds is of the same order of magnitude as the thermal energy, and enthalpy and entropy are indeed temperature-dependent quantities (Weber 1995, 1996). Therefore, only the results derived from the application of equation (4) should be considered (Naghibi *et al.* 1995).

Note that the values measured for ΔS were the ones intrinsic (ΔS_i) to the system studied: the binding reaction (see Weber 1996 for details). While in the system a loss in translational and rotational energy is expected due to fixation of the ligand and eventually to the immobilization of chemical groups within the receptor upon binding, the entropy of the surroundings has to suffer a high enough increase so as to overcompensate ΔS_{system} in order to follow the second

principle of thermodynamics. The major source of increment in entropy of thermodynamics is generally represented by the release, upon binding, of the water molecules tightly bound to the ligand that originally constituted its hydration sphere. This is especially true for hydrophobic binding interaction. Moreover, gains of intrinsic entropy of FNZ binding would also indicate a 'relaxation' of the $GABA_A$-R-FNZ complex to a less constrained (open) conformation. However, thermally activated motions brought about by the increased probability of bond breakage with temperature (Weber 1995) might be impaired as temperature rises (see table 2) due to a more internal protein localization within the membrane (see below). Therefore, binding may start with a hydrophobic interaction step followed by the ligand-induced conformational change whose magnitude would determine the turning towards an enthalpy-driven process upon temperature elevation.

The temperature dependence of the receptor density was significantly affected by stressor exposure (table 1). Moreover, CON membranes exhibited hysteresis between heating and cooling in the temperature dependence of B_{max}. While upon heating $B_{max,\ CON}$ increased, a subsequent cooling did not restore the B_{max} values at the starting temperature (4 °C). Conversely, $B_{max,\ STR}$ started at values equivalent to those measured in CON samples at the highest temperatures and showed a constant behaviour throughout the heating–cooling cycle (table 2).

According to the results, at 37 °C the difference in B_{max} between CON and STR is negligible or zero (statistically non-significant). Although both results might represent values quantitatively very close in a linear system, a qualitative difference may be found between both conditions in a non-linear and complex system, as previously explained. On the other hand, kinetic and thermodynamic differences in binding to the benzodiazepine sites alone have not shown correlations with the efficacies of these ligands and, thus, with ionophore function (Maksay 1996). In view of this observation, the existence of a difference in B_{max} at 37 °C, as well as its absence, might be physiologically irrelevant. However, the feasibility of a temperature-induced change in B_{max} in the CON but not in the STR samples strongly suggests that stress was able to affect membrane dynamics.

It was speculated that a difference in the environment where this process was taking place might account for the differential contribution of ΔH and ΔS (differential sensitivity to temperature) between CON and STR samples as well as for the different behaviour of receptor density change with temperature. Thus, the following experiments were directed to investigate this possibility.

Acute stressor exposure affects the dynamical organization of synaptosomal membranes from chick brain

It was reasoned that the thermal effects, induced by stress on the receptor density, might be related to changes in membrane composition associated to the activation of metabotropic receptors (Speler *et al.* 1993). The gating of signal transduction cascades leads to the activation of lipolitic enzymes (Chattergee 1999, Exton 2000, Canossa *et al.* 2001, Muzzio *et al.* 2001) and the production of second messengers and the phosphorylation of membrane proteins (Gyenes *et al.* 1994). Localized changes in the concentration of membrane components and the production of lipid derivatives with molecular areas, electric dipole moments and hydrophilic–hydrophobic relationships highly different from those of their parent compounds (Perillo 1998) should also be expected. Therefore, dramatic changes were observed in general membrane properties such as molecular packing, electrical charge densities and surface curvatures whose effects might spread, from the point of initiation, far away within the plane and the volume of the membrane. Consequently, the conformation and/or the biological activity of membrane proteins including receptors such as $GABA_A$ might be affected. In addition, enzymatic activities against organized substrates can be modulated by changes in the molecular organization of the lipid–water interface (Perillo *et al.* 1994b, Fanani and Maggio 1998). This is the case of the same lipolitic phospholipases and sphingomyelinases, previously mentioned as participating at the starting point.

The high anisotropy of CON membranes below 10 °C (figure 2) would reflect a high microviscosity and an impairment of the mobility of the membrane components that would favour a more external localization of integral proteins. The fluorescence measured at 340 nm after excitation at 295 nm is assumed to correspond mainly to the indol moiety of tryptophan amino acids (Lakowicz 1983). Therefore, its increment reflects a reduced exposition of those residues to water due to their movement to deeper localizations within more hydrophobic membrane regions (figure 3(*b*)). This was also supported by the decreased quenching ability of acrylamide on tryptophan fluorescence in CON upon temperature elevation (figure 3(*d*)). More fluid membrane organizations would favour BZD accessibility to its binding site in $GABA_A$ receptor, which in the present work is expressed by higher B_{max} values (table 1). This hypothesis is supported by a previous report (Martijena *et al.* 1992) showing that B_{max} was increased by Triton X-100 in CON but not in STR membranes (note that in the presence of the detergent, a reduction in membrane supramolecular order is expected).

Conclusions

Acute stressor exposure induced a decrease in the plasticity of the physical properties of chick crude synaptosomal membranes with respect to those from control birds. This statement was suggested by the fact that in STR samples: (a) the capability to increase B_{max} values with temperature (table 1) was lost, (b) the slope of the anisotropy of DPH fluorescence vs temperature (figure 2) was lowered, and (c) the increase in the relative fluorescence at 340 nm after excitation at 295 nm (figure 3(*b*)) disappeared and the susceptibility to be quenched by acrylamide decreased (figure 3(*d*)). The reversal of those thermal induced changes seemed to be either very slow or irreversible, as shown by the hysteresis in the variation of B_{max} between heating and cooling (tables 1 and 3).

In spite of the uncertainties about the fine mechanisms that induce them, significant differences were found in the

present work between brain membranes from CON and STR chicks. These results offer possible explanations to the similarities between the results on the thermodynamic properties of FNZ binding in STR samples and those reported by others at times when the importance of habituation to the manipulations previous to sacrifice (Rägo *et al.* 1989) had not been yet studied (Quast *et al.* 1982). It is interesting to note that after these studies, the guillotine sacrifice procedure was described as a stressor able to increase the B_{max} of the BZD receptors (Rägo *et al.* 1989); the control animals used in the present work were sacrificed by the procedure described in Experimental procedures which did not affect the BZD receptor (Martijena 1988).

On the other hand, the results point to the fact that the difference between CON and STR samples in the binding properties of FNZ has a physico-chemical basis and might be related to the stress-induced gating of signal transduction metabolic cascades. Experiments designed to investigate this last hypothesis are currently running at the laboratory. Interestingly, Benavidez and Arce (2002) demonstrated that the cytoskeleton also plays a significant role in the mechanism of receptor recruitment.

The adaptation of receptor-coupled intracellular pathways was referred to as neural plasticity. Inability to mount appropriate adaptative responses to environmental stressors was postulated to contribute to the pathophysiology of psychiatric disorders (Duman *et al.* 1999, Naisberg and Weizman 1999). The results give experimental support to that biophysical theory for neuropsychopathology.

Experimental procedures

Materials

Flunitrazepam (FNZ) and diazepam (DZ) were obtained from Hoffman La Roche (Basle, Switzerland). [^{3}H]FNZ (85 Ci/mmol) was purchased from New England Nuclear Chemistry (E.I.DuPont de Nemours & Co. Inc., Boston, MA). Diphenylhexatriene (DPH) was obtained from SIGMA Chemical Company (St. Louis, MO). Other drugs and solvents were of analytical grade.

Animals

Chicks (Cobb, of both sexes) were obtained from a commercial hatchery, INDACOR (Argentina). Birds were housed in brooders (50 × 90 cm) in groups of 10 on the evening of the day of hatch. The brooders were placed in a room (3 × 3 m) isolated from external noises, at constant temperature (32 °C) and humidity, with 12 h light–12 h dark cycle (lights on at 0700) with food and water freely available. Chicks were maintained in these conditions until they reached 15 days old.

Partial water immersion (PWI) stress

All chicks from a brooder were simultaneously removed. Half of them were immediately sacrificed by a procedure of decapitation lasting less than 5 s (control animals). This procedure did not affect the maximal binding (B_{max}) or the K_d of the BZD receptor (Martijena 1988). The other half (stressed animals) were individually placed in a cylindrical basin (22 cm diameter × 30 cm high) filled with clean water (38 °C) to a depth of ~18 cm. Thus, when the bird stood upright in the basin the water reached its neck. None of the birds exhibited signs of exhaustion during the 15 min test. At the end of this period, the chicks were removed from the basin and immediately decapitated. Then, the brains were removed and forebrains quickly dissected on ice.

Preparation of crude synaptosomal fraction

All the procedures were carried out at 4 °C. Five forebrains were pooled and homogenized in 20 volumes of ice-cold 0.32 M sucrose/g original tissue, using a potter glass Teflon homogenizer and centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 10 000 g for 30 min. Then, the pellet was resuspended again in 20 ml of 0.32 M sucrose and centrifuged at 10 000 g for 30 min to obtain a crude fraction. Finally, the pellet was resuspended in Tris-HCl buffer (50 mM, pH 7.4) to a final concentration of 0.25 mg protein/ml (Martijena *et al.* 1992).

Benzodiazepine-binding assay

The specific binding of [^{3}H]FNZ was measured by a rapid filtration technique. Binding was carried out in the presence of [^{3}H]FNZ at final concentrations ranging from 0.5–9 nM (at least eight concentration points were run). Each assay was performed in triplicate using 1-ml aliquots containing 0.25 mg proteins of crude synaptosomal fraction. Non-specific binding was measured in the presence of 10 μM DZ. The temperature and the time of incubation varied approximately according to Quast *et al.* (1982) (60 min at 4 °C, 50 min at 10 °C, 40 min at 15 °C, 30 min at 20 °C, 25 min at 25 °C and 15 min at 37 °C). In some cases, after a pre-incubation at 25 °C for 25 min, the preparation was incubated at 4 °C for 60 min. After incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24R filtering manifold. Samples were washed three times with 4 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4) and the radioactivity was measured in an LKB-1219-RackBeta Counter at an efficiency of 60% for tritium. B_{max} and K_d values were determined by computer-aided non-linear regression analysis of the experimental data from the saturation curve.

Fatty acid analysis of the synaptosomal fraction

Total lipids from chick brain membrane homogenates were extracted with 20 volumes of chloroform:methanol 2:1 and submitted to a Folch's partitioning procedure (Folch *et al.* 1975). A methanolic solution of 1% V/V sulphuric acid was added to the lower phase and boiled for 45 min in order to hydrolyse the fatty acids and obtain their ester derivatives. The fatty acid methyl esters were extracted by the addition of hexane. This last step was repeated twice and the hexane-fractions (upper phase) were collected and concentrated by solvent evaporation under vacuum. Samples were submitted to thin-layer chromatography on silica gel DG, using chloroform:diethyl ether (8:2, V/V) as the developing solvent (Ibrahim and Ghannoum 1996). The bands corresponding to fatty acids were scrapped off the plate, extracted with chloroform and the fatty acid composition determined by gas–liquid chromatography using a Shimadzu GC-R1A with a flame ionization detector and glass capillary column SUPELCOWAX 10 (30 m × 0.25 mm i.d.). Quantification was done according to the area under the peaks identified by comparison of their retention times with those of standards, and expressed as a percentage of the peaks' areas. In one experiment, samples were submitted to GC-mass spectrometry in order to confirm the chemical structure of all the molecules eluted. The GC-mass spectrometry unit consisted of a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph equipped with a SE54 fused silica column (25 m × 0.25 mm i.d.). The chromatograph was coupled to an HP5971A mass selective detector at 70 eV (Lamarque *et al.* 1994).

Steady state fluorescence

Fluorescence anisotropy. The fluorescent probe DPH (2 μM) was added to crude synaptosomal membranes (0.25 mg protein/ml).

After an incubation period of 1 h at room temperature, the fluorescence at 430 nm (excitation at 360 nm) was measured between 4–37°C. Fluorescence intensity was determined with the excitation and the sample polarizer filters oriented in parallel and perpendicularly one respect to the other, in an L format SLM 4800 spectrofluorimeter. Steady state fluorescence anisotropy was calculated as:

$$A = \frac{VV - VH.G}{VV + 2.VH.G} \quad (1)$$

$$G = \frac{HV}{HH} \quad (2)$$

where *VV*, *HH*, *VH* and *HV* are the values of the different measurements of fluorescence intensity taken with both polarizers in vertical (*VV*) and horizontal (*HH*) orientations or with the excitation polarizer vertical and the emission polarizer horizontal (*VH*) or vice-versa (*HV*). *G* is a correction factor for differences in sensitivity of the detection system for vertically and horizontally polarized light (Lakowicz 1983).

Membrane proteins intrinsic fluorescence. Crude synaptosomal membranes from CON and STR samples between 4–45°C were excited at 295 nm, and the fluorescence at 340 was recorded. As only CON membranes were responsive to temperature, they were selected to test the effect of acrylamide (between 0.1–0.5 M) at low (4°C) and at high (25°C) temperatures on tryptophan fluorescence at 340 nm (excitation at 295).

Thermodynamic calculations

The equilibrium constant for the binding reaction was: $K=1/K_d$; ΔG and ΔS were calculated as:

$$\Delta G = -RT\ln K$$

and

$$\Delta S = (-\Delta G + \Delta H)/T$$

A temperature independent value of ΔH was obtained from a plot of ln *K* vs $1/T$ (where *T*=absolute temperature) representing a straight line with a slope equal to $-\Delta H/R$ (ΔH=van't Hoff apparent enthalpy, *R*=gas constant) according to van't Hoff equation:

$$\ln K = -\Delta H(RT)^{-1} \quad (3)$$

When data was subjected to non-linear least-squares analysis, employing an integrated form of the van't Hoff equation with a non-zero but temperature-independent heat capacity change (ΔC_p) (Naghibi *et al.* 1995, Weber 1995), the curvature of the van't Hoff plot became evident:

$$\ln \frac{K}{K_o} = \frac{\Delta H_o - T_o\,\Delta C_p}{R}\left(\frac{1}{T_o} - \frac{1}{T}\right) + \frac{\Delta C_p n}{R}\,\frac{T}{T_o}$$

T_o is an arbitrarily selected reference temperature (in the present paper T_o=30°C), K_o is the equilibrium constant and ΔH_o is the van't Hoff enthalpy at that temperature. The three parameters varied to minimize the standard deviation of calculated values of ln *K* from those observed are ΔH_o, ln K_o and ΔC_o.

Statistics

Results were expressed as the mean ± SEM. Experimental data were analysed by ANOVA. In samples with non-homogeneity of variance, the non-parametric Mann-Whitney test was applied. A *p*-value ≤ 0.05 was considered to represent a significant difference between groups (Sokal and Rohlf 1980).

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References

Anisman, H. and Zacharko, R. M., 1990, Multiple neurochemical and behavioural consequences of stressors: implications for depression. *Pharmacology and Therapeutics*, **46**, 119–136.

Anisman, H., Glazier, S. J. and Sklar, L. S., 1981, Cholinergic influences on escape deficits produced by uncontrollable stress. *Psychopharmacology*, **74**, 81–87.

Aon, M. A. and Cortassa, S., 1997, *Dynamic Biological Organization. Fundamentals as applied to cellular systems* (London: Chapman & Hall).

Baestrup, C. and Squires, R. F., 1977, Specific benzodiazepine receptors in rat brain characterized by high-affinity 3H-diazepam binding. *Proceedings of National Academy of Sciences (USA)*, **74**, 3805–3809.

Benavidez, E. H and Arce, A., 2002, Effects of phosphorylation and cytoskeleton affecting reagents on $GABA_A$ receptor recruitment into synaptosomes following acute stress. *Pharmacology Biochemistry and Behavior*, **72**, 1–10.

Buda, C., Dey, I., Balogh, N., Horvath, L., Maderspach, K., Juhasz, M., Yeo, Y. K. and Farkas, T., 1994. Structural order of membranes and composition of phospholipids in fish brain cells during thermal acclimatization. *Proceedings of the National Academy of Sciences (USA)*, **91**, 8234–8238.

Canossa, M., Gartner, A., Campana, G., Inagaki, N. and Thoenen, H., 2001, Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluRI) and Trk receptor activation is mediated via phospholipase C signalling pathways. *EMBO Journal*, **20**, 1640–1650.

Chattergee, S., 1999, Neutral sphingomyelinase: past, present and future. *Chemistry and Physics of Lipids*, **102**, 79–96.

Drugan, R. C. and Holmes, P. V., 1991, Central and peripheral benzodiazepine receptors: involvement in an organism's response to physical and psychological stress. *Neuroscience and Biobehavioural Reviews*, **15**, 277–298.

Drugan, R. C., Ader, D. N. and Maier, S. F., 1985, Shock controllability and the nature of stress induced analgesia. *Behavioral Neuroscience*, **99**, 791–801.

Duman, R. S., Malberg, J. and Thome, J., 1999, Neural plasticity to stress and antidepressant treatment. *Biological Psychiatry*, **46**, 1181–1191.

Dunn, A. J., 1988, Changes in plasma and brain tryptophan and brain serotonin and 5-hydroxyindoleacetic acid after footshock stress. *Life Sciences*, **42**, 1847–1853.

Exton, J. H., 2000, Phospholipase D. *Annals of the New York Academy of Sciences*, **905**, 61–68.

Fanani, M. L. and Maggio, B., 1998, Surface pressure-dependent cross-modulation of sphingomyelinase and phospholipase A2. *Lipids*, **33**, 21079–21087.

Fekete, M. I., Szentendrei, T., Kanyicska, B. and Palkovits, M., 1981, Effects of anxiolytic drugs on the catecholamine and DOPAC (3,4-dihydroxyphenylacetic acid) levels in brain cortical areas and on corticosterone and prolactin secretion in rats subjected to stress. *Psychoneuroendocrinology*, **6**, 113–120.

Folch, J., Lees, M. and Sloane-Stanley, G., 1975, A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry*, **226**, 497–509.

Garcia D. A., Quiroga S. and Perillo, M. A., 2000, Flunitrazepam partitioning into natural membranes increases the surface curvature and alters the cellular morphology. *Chemico-Biological Interactions*, **129**, 263–277.

Gyenes, M., Wang, Q., Gibbs, T. T. and Farb, D. H., 1994, Phosphorylation factors control neurotransmitter and neuromodulator actions at the gamma-aminobutyric acid type A receptor. *Molecular Pharmacology*, **46**, 542–549.

Ibrahim, A. S. and Ghannoum, M. A., 1996, Chromatographic analysis of lipids. In: *Manual on membrane lipids*, R. Prasad, ed. (Cambridge, MA: Birkhauser Boston), pp. 52–79.

Jackson, R. L., Maier, S. F. and Coon, D. J., 1979, Long-term analgesic effects of inescapable shock and learned helplessness. *Science*, **206**, 91–94.

Jain, R., Zwickler, D., Hollander, C. S., Brand, H., Saperstein, A., Hutchinson, B., Brown, C. and Audhya, T., 1991, Corticotropin-releasing factor modulates the immune response to stress in the rat. *Endocrinology*, **128**, 1329–1336.

Kchman, R. L. and Hirsch, J. D., 1982, Thermodynamic changes associated with benzodiazepine and alkyl β-carboline-3-carboxylate binding to rat brain homogenates. *Molecular Pharmacology*, **22**, 335–341.

Lakowicz, J., 1983, *Principles of fluorescence spectroscopy* (New York: Plenum Press).

Lamarque, A. L., Modesti, D. M., Grosso, N. R. and Guzmán, C. A., 1994, Proximate composition and seed lipid components of some Prosopis (leguminosae) from Argentina. *Journal of the Science of Food and Agriculture*, **66**, 323–326.

Maggio, B., 1994, The surface behavior of glycosphingolipids in biomembranes: a new frontier of molecular ecology. *Progress in Biophysics and Molecular Biology*, **62**, 55–117.

Maksay, G., 1996, From kinetics and thermodynamics of GABA receptor binding to ionophore function. *Neurochemistry International*, **29**, 361–370.

Marin, R. H. and Arce, A., 1996, Benzodiazepine receptors increase induced by stress and maze learning performance in chick forebrain. *Pharmacology Biochemistry and Behavior*, **53**, 581–584.

Marin, R. H. and Jones, R. B., 1999, Latency to traverse a T-maze at 2 days of age and later adrenocortical responses to an acute stressor in domestic chicks. *Physiology &Behavior*, **66**, 809–813.

Marsh, D., 1996, Lateral pressure in membranes. *Biochimica et Biophysica Acta*, **1286**, 183–223.

Martijena, I. D., 1988, Cambio transitorio del receptor de benzodiazepinas-GABA como correlato bioquimico del aprendizaje en cerebro de pollo. Doctoral Thesis. F.C.E.F.y N., Universidad Nacional de Cordoba, Cordoba, Argentina.

Martijena, I. D. and Arce, A., 1993, Transient benzodiazepine-$GABA_A$ receptor increase after a passive avoidance learning in synaptosomal membranes from chick forebrain. *Canadian Journal of Physiology and Pharmacology*, **72**, 233–237.

Martijena, I. D., Salvatierra, N. A. and Arce, A., 1992, Benzodiazepine receptor recruitment after acute stress in synaptosomal membranes from forebrain of young chicks: action of Triton X-100. *Journal of Neural Transmission*, **87**, 97–104.

Miller, R. R., Heckel, C. D., Koss, W. J., Montague, S. L. and Greenman, A. L., 2001, Ethanol- and nicotine-induced membrane changes in embryonic and neonatal chick brains. *Comparative Biochemistry and Physiology*, **130**, 163–178.

Mohler, H. and Okada, T., 1977, Properties of 3H-diazepam binding to benzodiazepine receptors in rat cerebral cortex. *Life Sciences*, **20**, 2101–2110.

Muzzio, I. A., Gandhi, C. C., Manyam, U., Pesuell, A. and Matzel, L. D., 2001, Receptor-stimulated phospholipase A(2) liberates arachidonic acid an regulates neuronal excitability through protein kinase C. *Journal of Neuropharmacology*, **85**, 1639–1647.

Naghibi, H., Tamura, A. and Sturtevant, J. M., 1995, Significant discrepancies between van't Hoff and calorimetric enthalpies. *Proceedings of the National Academy of Sciences (USA)*, **92**, 5597–5599.

Naisberg, Y. and Weizman, A., 1999, Biophysical shunt theory for neuropsychopathology: pentaphasic lipid induced model for schizophrenia. *Medical Hypotheses*, **52**, 183–186.

Paredes, R. G. and Agmo, A., 1992, GABA and behavior: the role of receptor subtypes. *Neuroscience and Biobehavioral Reviews*, **16**, 145–170.

Perillo, M. A., 1998, A biophysical approach to the excitotoxic action mechanisms of some sphingolipid derivatives. *Recent Research Development in Lipid Research*, **2**, 275–298.

Perillo, M. A. and Arce, A., 1991, Determination of the membrane-buffer partition coefficient of flunitrazepam, a lipophilic drug. *Journal of Neuroscience Methods*, **31**, 203–208.

Perillo, M. A., Guidotti, A., Costa, E., Yu, R. K. and Maggio, B., 1994b, Modulation of phospholipases A_2 and C activities against dilauroyl phosphorylcholine in mixed monolayers with semisynthetic derivatives of ganglioside and sphingosine. *Molecular Membrane Biology*, **11**, 119–126.

Perillo, M. A., Yu, R. K. and Maggio, B., 1994a, Modulation of the activity of C.perfringens neuraminidase by the molecular organization of gangliosides in monolayers. *Biochimica et Biophysica Acta*, **1193**, 155–164.

Petty, F. and Sherman, A. D., 1981, GABAergic modulation of learned helplessness. *Pharmacology Biochemistry and Behavior*, **15**, 453–457.

Petty, F., Kramer, G. and Wilson, L., 1992, Prevention of learned helplessness: *in vivo* correlation with cortical serotonin. *Pharmacology Biochemistry and Behavior*, **43**, 361–367.

Petty, F., Kramer, G., Wilson, L. and Chae, Y. L., 1993, Learned helplessness and *in vivo* norepinephrine release. *Pharmacology Biochemistry and Behavior*, **45**, 231–235.

Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R. and Seeburg, P. H., 1989, Importance of a novel GABA A receptor subunit for benzodiazepine pharmacology. *Nature*, **338**, 582–585.

Quast, U., Mahlmann, H. and Vollmer, K. O., 1982, Temperature dependence of the benzodiazepine-receptor interaction. *Molecular Pharmacology*, **22**, 20–25.

Rägo, L., Kiivet, R. A., Harro, J. and Pold, M., 1989, Central and peripheral-type benzdiazepine receptors: similar regulation by stress and GABA receptor agonists. *Pharmacology Biochemistry and Behavior*, **32**, 879–883.

Salvatierra, N. A., Marin, R. H., Arce, A. and Martijena, I. D., 1994, Chick imprinting performance and susceptibility to acute stress associated to flunitrazepam receptor increase. *Brain Research*, **648**, 39–45.

Salvatierra, N. A., Torre, R. B. and Arce, A., 1997, Learning and novelty induced increase of central benzodiazepine receptors from chick forebrain, in a food discrimination task. *Brain Research*, **757**, 79–84.

Sanchez, J. M. and Perillo, M. A., 2002, Membrane adsorption or penetration differentially modulates β-galactosidase activity against soluble substrates. *Colloids and surfaces B*, **24**, 21–31.

Santini, M. T., Indovina, P. L., Simmons, J. R. and Peterson, S. W., 1990, Human erythrocyte insulin receptor down-regulation is accompanied by a transient decrease in membrane order. *Biochimica et Biophysica Acta*, **1054**, 333–336.

Sieghart, W., 1992, $GABA_A$ receptors: ligand-gated Cl- ion channel modulated by multiple drug-binding sites. *Trends in Pharmacological Sciences*, **13**, 446–450.

Singh, J. K., Dasgupta, A., Adayev, T., Shahmehdi, S. A., Hammond, D. and Banerjee, P., 1996, Apoptosis is associated with an increase in saturated fatty acid containing phospholipids in the neuronal cell line, HN2-5. *Biochimica et Biophysica Acta*, **1304**, 205–206.

Sokal, R. and Rohlf, J., 1980, *Introducción a la bioestadistica* (Barcelona: Reverté).

Speler, K., Schoch, P., Martin, J. R. and Haefely, W., 1993, Environmental stimulation promotes changes in the distribution of phorbol ester receptors. *Pharmacology Biochemistry and Behavior*, **46**, 553–560.

Speth R. C., Wastek, G. J. and Yamamura, H. I., 1979, Benzodiazepine receptors: temperature dependence of ^{3}H-flunitrazepam binding. *Life Sciences*, **24**, 351–358.

Stephen, I. D., Rosso, R. B. and Mastropaolo, J., 1994, Environmental stress-induced functional modification of the central benzodiazepine binding site. *Clinical Neuropharmacology*, **17**, 205–228.

Stone, E. A., 1987, Central cyclic-AMP-linked noradrenergic receptors: new finding on properties as related to the actions of stress. *Neuroscience and Biobehavioral Reviews*, **11**, 391–398.

Tsuda, A. and Tanaka, M., 1985, Differential changes in noradrenaline turnover in specific regions of rat brain produced by controllable and uncontrollable shocks. *Behavioral Neuroscience*, **99**, 802–817.

Weber, G., 1995, Van't Hoff revisited: enthalpy of association of protein subunits. *The Journal of Physical Chemistry*, **99**, 1052–1059.

Weber, G., 1996, Persistent confusion of total entropy and chemical system entropy in chemical thermodynamics. *Proceedings of the National Academy of Sciences (USA)*, **93**, 7452–7453.

Yehuda, S, Rabinovitz, S. and Mostofsky, D. I., 1999, Essential fatty acids are mediators of brain biochemistry and cognitive functions. *Journal of Neuroscience Research*, **56**, 565–70.

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