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RESEARCH REPORT

Acute stress or systemic insulin injection increases flunitrazepam sensitive-$GABA_A$ receptor density in synaptosomes of chick forebrain: Modulation by systemic epinephrine

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Abstract

Interactions between acute stress and systemic insulin and epinephrine on $GABA_A$ receptor density in the forebrain were studied. Here, 10 day-old chicks were intraperitoneally injected with insulin, epinephrine or vehicle and then immediately stressed by partial water immersion for 15 min and killed by decapitation. Non-stressed controls were similarly injected, then returned to their rearing boxes for 15 min and then killed. Forebrains were dissected and $GABA_A$ receptor density was measured *ex vivo* in synaptosomes by ^{3}[H]-flunitrazepam binding assay. In non-stressed chicks, insulin at 1.25, 2.50 and 5.00 IU/kg of body weight (non-hypoglycemic doses) increased B_{max} by 33, 53 and 44% compared to saline, respectively. A similar increase of 41% was observed in receptor density after stress. However, the insulin effect was not additive to the stress-induced increase suggesting that both effects occur through similar mechanisms. In contrast, epinephrine, at 0.25 and 0.5 mg/kg did not induce any changes in B_{max} in non-stressed chicks. Nevertheless, after stress these doses increased the receptor density by about 13 and 27%, respectively. Similarly, the same epinephrine doses co-administered with insulin (2.50 IU/kg), increased the receptor density by about 20% compared to insulin alone. These results suggest that systemic epinephrine, perhaps by evoking central norepinephrine release, modulates the increase in forebrain $GABA_A$ receptor binding induced by both insulin and stress.

Keywords: *Acute stress, epinephrine, $GABA_A$ R density,* Gallus gallus domesticus*, insulin, water immersion stress*

Introduction

GABA is the most important inhibitory neurotransmitter in the CNS. It is estimated that, depending on brain region, 20–50% of all central synapses use GABA as their neurotransmitter (Sieghart 1995). $GABA_A$ receptors ($GABA_A$ R) are ligand-gated chloride channels that mediate inhibitory neurotransmission. These receptors are pentameric hetero-oligomers and most native receptors are formed from multiple isoforms of the α, β and γ subunits (Moss and Smart 2001; Sieghart and Sperk 2002). Functionally, receptors containing α1–3 or α5 subunits represent the benzodiazepine-sensitive $GABA_A$ R subtype known to mediate the diverse behavioural actions of diazepam (Benson et al. 1998).

Several types of stress, including immobilization, psychological and conditioned fear increase norepinephrine release in brain (Stanford 1995; Galvez et al. 1996; Tanaka et al. 2000). Since, acute restraint stress impairs the facilitating influence of norepinephrine acting via α_1, sites on GABAergic inhibition in the rat amygdala (Braga et al. 2004), it is possible that systemic administration of epinephrine can affect the $GABA_A$ R density in synaptosomes. Since epinephrine passes the blood–brain barrier poorly, such effects are indirectly mediated by activation of vagal afferent projections that release norepinephrine in the brain

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(Williams et al. 1998). Acute stress by immobilization of rats activates the adrenomedullary system, whereas the greatest activation of the sympatho-neural system occurs during immobilization and cold (Kvetnansky et al. 1998).

Several lines of evidence indicate that brain insulin is partly rapidly transported from peripheral tissues via the cerebrospinal fluid and partly synthesized by neurons in the brain. Insulin rapidly enters the cerebrospinal fluid from the periphery in several species (Woods et al. 1985). A systemic injection of insulin under euglycemic conditions produces significant memory improvement in verbal memory and selective attention (Kern et al. 2001). Intranasal administration of insulin induces a sharp and rapid increase in cerebrospinal fluid insulin concentration without affecting blood insulin and glucose levels (Born et al. 2002). The brain had long been considered an insulin-insensitive organ, but this view has been challenged by the observation than insulin receptors are widely distributed in rat brain with marked regional variation in receptor density (Havrankova et al. 1978; Biessels et al. 2004).

Synaptosomes are fortuitous artifacts created by the pinching-off and self-sealing of the synaptic contact between two nerve cells during homogenization of brain tissue (De Robertis et al. 1961). The synaptosomes contain cytoskeleton and organelles in addition to synaptic vesicles with neurotransmitters and receptors for reuptake at the presynaptic zone (Whatley and Harris 1996). Synaptosomes isolated from mammalian brain constitute a useful *in vitro* model to study several neuronal functions, because they are metabolically active and retain many properties of nerve endings, namely neurotransmitter uptake (Weinberger and Cohen 1982; Nicholls 1989). Furthermore, $GABA_A$ Rs are localized in the postsynaptic membrane of synaptosomes.

The central flunitrazepam (FNZ) binding, reflecting $GABA_A$ R density, measured, *ex vivo*, in synaptosomes from chick forebrain, increased about 30% after an acute stressor. This increase was first reported to result from $GABA_A$ R recruitment because subsolubilizing concentrations of Triton X-100 induced it, and this was not additive to the increment induced by acute stress (Martijena et al. 1992).

In a similar system but using synaptosomal membranes in which the cytoskeleton is depolymerized, and in the absence of organelles and soluble components, it was observed that the incorporation of alkaline phosphatase into the lumen of synaptosomes abolished the unmasking of $GABA_A$ R induced by stress. Conversely, the presence of ATP in the lumen of synaptosomes increased the $GABA_A$ R unmasking in non-stressed but not in stressed chicks. Together, the results suggested that phosphorylation of the $GABA_A$ R and/or their associated proteins play a role in the stress-induced recruitment mechanisms (Benavidez and Arce 2002). The regulation of the $GABA_A$ R by phosphorylation, trafficking, channel activity, and functional plasticity of inhibitory synapses has been reviewed (Lüscher and Keller 2004).

In this study, we examined the effects of systemic administration of insulin and epinephrine on $GABA_A$ R density, in synaptosomes, from the forebrain in stressed and non-stressed chicks. Results obtained from this study may provide a greater understanding about the interaction among stress and these hormones on any step in the trafficking, docking and final insertion into synaptic membranes of $GABA_A$ Rs.

Materials and methods

Animals

Chicks (*Gallus gallus domesticus*) of both sexes were obtained immediately after hatching from a commercial hatchery INDACOR (Argentina) when they were only a few hours old. On their arrival in the laboratory, they were housed in white wooden cages (10 chicks/cage). The cages were 90 × 40 × 60 cm (length × width × height) and were kept in a small room (3 × 3 m) at a controlled temperature of 28–32°C and a 12:12 h light: darkness schedule (lights on at 07:00 h) was used. Feed (Cargill, broiler BB, 23% CP, 2950 kcal/kg) and water were freely available. In this way, chicks of the same age were socially reared until they reached 10 days of age. At this time, all experiments were carried out. Daily food replenishment and maintenance chores were performed at 09:00 h. All experiments were carried out between 10:00 and 12:00 h.

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of the Universidad Nacional de Córdoba, and efforts were made to minimize animal suffering and to reduce the number of animals used.

Insulin administration

Human Insulin ultra rapid action obtained from Beta Laboratories (Argentina) was prepared in 0.9% saline and injected intraperitoneally (ip) (at doses ranging between 0.25 and 5.00 IU/kg bw) in a volume of 0.12 ml.

Ten day-old chicks injected with saline or one of several insulin doses were immediately returned to their rearing boxes (non-stressed chicks). After 15 min, they were killed. Other chicks of the same box were injected in the same way and immediately exposed as indicated as described below to Partial Water Immersion (PWI) stress. Both non-stressed and stressed chicks were decapitated and crude forebrain synaptosomal fractions were obtained.

Epinephrine administration

Epinephrine dissolved in a sterile commercial solution (Fada Pharma), was diluted with 0.9% saline solution (Roux OCEFA) to concentrations of 0.25 and 0.50 mg/kg bw, as reported previously (Miyashita and Williams 2004) and injected ip in a volume of 0.12 ml.

Chicks were injected with saline or one of two different epinephrine doses, and immediately returned to their rearing boxes (non-stressed chicks). After 15 min, they were killed. Other chicks were injected as described above for insulin and then exposed as indicated below to PWI stress. Both, non-stressed and stressed chicks were decapitated as indicated below and the crude forebrain synaptosomal fractions were obtained.

Co-administration of insulin plus epinephrine

Chicks were injected ip with saline, 2.5 IU/kg insulin alone or insulin plus one epinephrine dose (0.25 or 0.50 mg/kg bw) in a volume of 0.12 ml and immediately returned to their rearing boxes (non-stressed chicks). After 15 min, they were killed and the crude forebrain synaptosomal fraction was obtained. Other chicks were injected as described above and immediately exposed as indicated below to PWI stress and finally, decapitated as indicated below and the crude forebrain synaptosomal fractions were obtained.

Partial water immersion (PWI) stress

Three chicks from each cage were stressed as described by Martijena et al. (1992). Briefly, at 10 days of age, a chick was randomly removed from a communal cage by an experimenter, transferred to a separate room, and placed in a cylindrical basin (22 cm in diameter × 30 cm high) containing water (38°C) approximately 18 cm deep. Thus, when the bird stood upright in the basin, the water reached only up to its neck. A test period of 15 min was used, and water was changed after each trial. None of the birds exhibited signs of exhaustion during the testing.

Terminal procedure

At the end of a trial, the test chick was removed from the basin, and immediately killed by decapitation with scissors, within 1s after the experimental period to avoid an additional stress. Then, the brains were removed and forebrains quickly dissected on ice. The forebrain hemispheres are telencephalic structures that are neurochemically and functionally comparable to the mammalian neocortex, claustrum, and pallial amygdala in addition to other pallial areas such as hippocampus (Reiner et al. 2004).

Measurement of plasma glucose concentrations

When chicks were killed by decapitation, trunk blood was collected in tubes coated with ethylenetriaminetetraacetic acid. Blood samples were centrifuged (3000 rpm for 20 min at 4°C), plasma samples were collected and plasma glucose concentrations were measured using a commercial glucose oxidase kit (Wiener Lab, Argentina) according to the manufacturer's protocol.

Preparation of crude synaptosomal fraction

The crude synaptosomal fraction was obtained essentially as described (De Robertis et al. 1961). All the procedures were carried out at 4°C. Briefly, the forebrain was homogenized in 20 volumes of ice-cold 0.32 M sucrose/g original forebrain tissue, using a Potter glass–Teflon homogenizer and centrifuged at 1000*g* for 10 min. The supernatant was then centrifuged at 10,000*g* for 20 min. Then, the pellets were resuspended in a solution containing 50 mM Tris–HCl buffer, pH 7.4, obtaining a final concentration of 0.3 mg protein/ml (Lowry et al. 1951), and were immediately used for the binding assay.

[^{3}H]-flunitrazepam binding assay

The specific binding of [^{3}H]-flunitrazepam (85 Ci/mmol) was measured by a filtration technique. Binding was carried out in the presence of radioligand at final concentrations of 0.5, 1, 2, 4, 6, 8, 10 and 12 nM, at 4°C. Each assay was performed in triplicate using 1 ml aliquots containing 0.3 mg of protein from the synaptosomal fraction. Non-specific binding was measured in the presence of 10 μM diazepam. After 60 min incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24 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 counted in an LKB-1214-RackBeta counter at 60% efficiency. B_{max} and K_d values were obtained by nonlinear regression using the equation for a hyperbola (one binding site): $Y = B_{max}$ast;$X/(K_d + X)$, where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. B_{max} of [^{3}H]-flunitrazepam binding is representative of the GABA$_A$ R density.

Statistical analysis

B_{max} and K_d values for GABA$_A$ R density and plasma glucose concentrations were analyzed by one- or two-way analysis of variance (ANOVA). Whenever ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means by Newman–Keuls test was carried out.

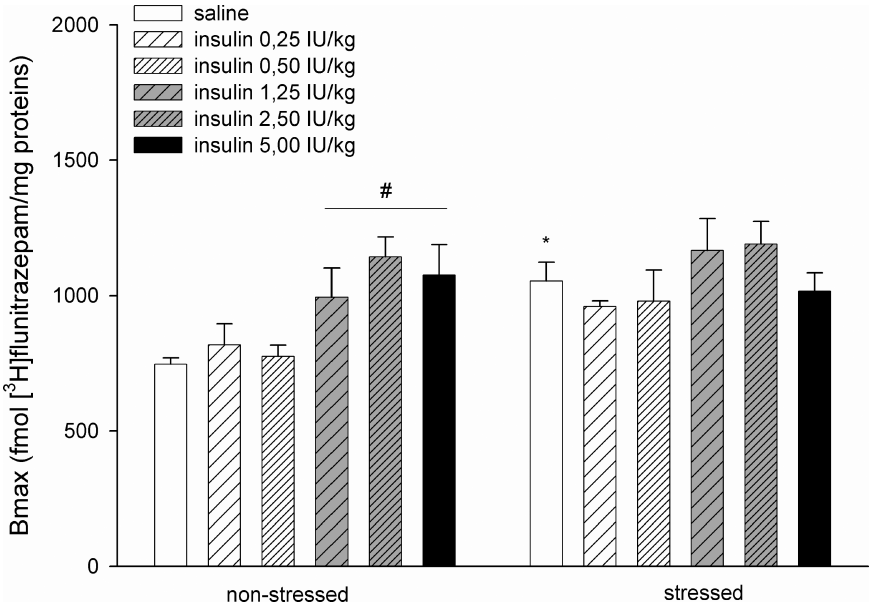

Figure 1. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes from non-stressed and stressed chicks following insulin administration. Insulin (in the range 0.25–5.00 IU/kg bw) or saline was administered ip 15 min before the chicks were killed. Bars represent the means ± SEM, $n = 6$ per group. $^{\star}p < 0.01$ compared to corresponding treatment in the non-stressed condition. $^{\#}p < 0.03$ compared to saline in the non-stressed condition (Newman–Keuls test).

Results

Effects of injection of insulin on [^{3}H]-flunitrazepam binding in forebrain synaptosomes from non-stressed and stressed chicks

Two-way ANOVA of the B_{max} values revealed significant acute stress ($F(1,52) = 5.381$, $p < 0.024$) and insulin treatment ($F(5,52) = 6.101$, $p < 0.001$) effects, with no significant interaction between the two $F(5,52) = 1.563$, $p = 0.187$ (Figure 1). The insulin doses given did not induce hypoglycemia as indicated in Table I. Two-way ANOVA for plasma glucose levels did not show significant differences for acute stress ($F(1,52) = 0.380$, $p = 0.53$) or insulin treatment ($F(5,52) = 1.532$, $p = 0.19$), or a significant interaction between the two ($F(5,52) = 1.643$, $p = 0.16$)

Acute stress increased B_{max} by 41% ($p < 0.024$) between the saline-treated groups of non-stressed and stressed chicks. Newman–Keuls tests showed, in the non-stressed condition, that insulin increased B_{max}, compared with saline, by 33% ($p < 0.01$) for 1.25 IU/kg, 53% ($p < 0.001$) for 2.50 IU/kg and 44% ($p < 0.001$) for 5.00 IU/kg. However, insulin did not increase B_{max} in the stressed chicks, suggesting that the effect of insulin occurs through a similar mechanism to acute stress. Two-way ANOVA for K_d values did not show significant differences for acute stress ($F(1,52) = 0.\ 487$, $p = 0.47$), insulin doses ($F(5,52) = 0.580$, $p = 0.71$), or a significant interaction between the two ($F(5,52) = 1.101$, $p = 0.37$) (Table II).

Table I. Plasma glucose concentrations 15 min after different doses of insulin (mg/dl) in non-stressed and stressed chicks.

Insulin doses (ip) IU/kg	Non-stressed	Stressed
0.00 (saline)	266 ± 12	264 ± 17
0.25	219 ± 16	236 ± 20
0.50	259 ± 32	208 ± 16
1.25	203 ± 23	256 ± 19
2.50	210 ± 13	219 ± 15
5.00	269 ± 28	278 ± 37

Each value represents the mean ± SEM, $n = 6$ chicks/group.

Effects of epinephrine on [^{3}H]-flunitrazepam binding in forebrain synaptosomes from non-stressed and stressed chicks

Two-way ANOVA for B_{max} values revealed significant acute stress ($F(1,82) = 183.01$, $p < 0.001$) and epinephrine treatment ($F(2,82) = 5.742$, $p < 0.004$)

Table II. Effects of different concentrations of insulin on K_d values of $GABA_A$ R in forebrain synaptosomes from non-stressed and stressed chicks.

	K_d (nM)	
Insulin (IU/mg)	Non-stressed	Stressed
Saline	1.88 ± 0.17	1.58 ± 0.09
0.25	1.78 ± 0.09	1.84 ± 0.14
0.50	1.79 ± 0.09	1.72 ± 0.09
1.25	1.74 ± 0.04	1.54 ± 0.10
2.50	1.67 ± 0.04	1.89 ± 0.18
5.00	1.81 ± 0.16	1.82 ± 0.16

Each value of K_d represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. $n = 6$ chicks/group.

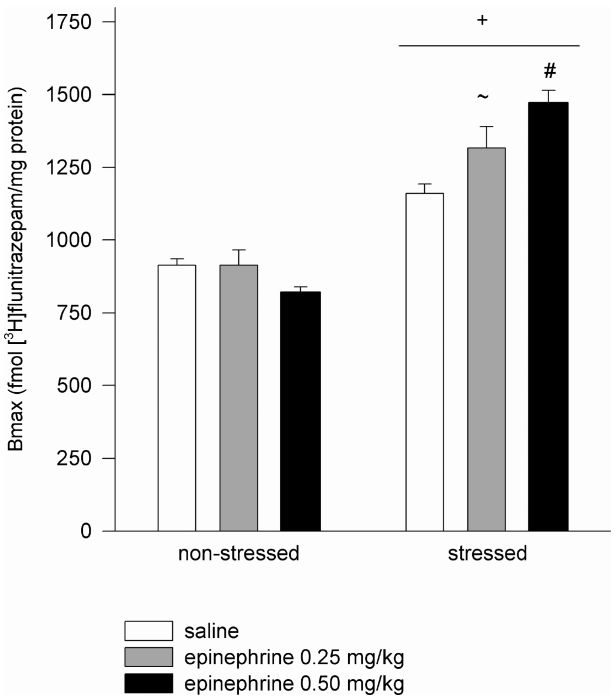

Figure 2. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes from non-stressed and stressed chicks following epinephrine administration. Epinephrine (0.25 and 0.50 mg/kg bw) or saline was administered ip 15 min before chicks were killed. Bars represent the means ± SEM, $n = 10–14$ per group. $^{+}p < 0.01$ compared to corresponding treatment in the non-stressed condition. $^{+}p < 0.01$ compared to saline in the stressed condition. $^{\#}p < 0.01$ compared to saline and epinephrine 0.25 mg/kg, in the stressed condition (Newman–Keuls test).

effects, and a significant interaction between stress and treatment ($F(2,82) = 17.709$, $p < 0.001$ (Figure 2).

Acute stress increased the B_{max} by 27% ($p < 0.01$) between the saline-treated non-stressed and stressed groups. No differences were observed in the B_{max} values between non-stressed chicks given saline or either dose of epinephrine. However, in stressed chicks, the B_{max} significantly increased after ip injections of epinephrine in a dose-dependent manner: compared with saline, by 13% ($p < 0.01$) and 27% ($p < 0.01$) at 0.25 and 0.50 mg/kg epinephrine, respectively. Therefore, the epinephrine-induced B_{max} increase was additive to the effects of the stressor, suggesting that the increase of GABA$_A$ R density after epinephrine occurs by a different mechanism than after exposure to the stressor. Two-way ANOVA for K_d values did not show significant effects of acute stress ($F(1,82) = 1.\ 887$, $p = 0.17$), epinephrine treatment ($F(2,82) = 2.403$, $p = 0.91$), or a significant interaction between stress and treatment ($F(2,82) = 1.904$, $p = 0.15$) (Table III).

Table III. Effects of different doses of epinephrine on K_d values of GABA$_A$ R in forebrain synaptosomes from non-stressed and stressed chicks.

	K_d (nM)	
	Non-stressed	Stressed
Saline	1.85 ± 0.25	1.79 ± 0.18
Epinephrine 0.25 (mg/kg)	1.88 ± 0.24	1.92 ± 0.31
Epinephrine 0.50 (mg/kg)	1.95 ± 0.20	1.74 ± 0.17

Each value of K_d represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. $n = 10–14$ chicks/group.

Effects of co-administration of insulin plus epinephrine on [^{3}H]-flunitrazepam binding in forebrain synaptosomes from non-stressed chicks

A one-way ANOVA shows that insulin (2.5 IU/kg bw) increased significantly the GABA$_A$ R density in the non-stressed chicks ($F(3,17) = 10.797$, $p < 0.001$), by 28% (Figure 3). Co-administration of insulin plus epinephrine (0.25 and 0.50 mg/kg bw) increased the GABA$_A$ R density, compared with insulin alone, by 20% ($p < 0.03$) and 20% ($p < 0.01$), respectively. Figure 3 also shows that the highest dose of epinephrine did not further increase the maximum density of GABA$_A$ R. No significant differences in K_d values were observed ($F(3,17) = 0.236$, $p = 0.89$) (Table IV).

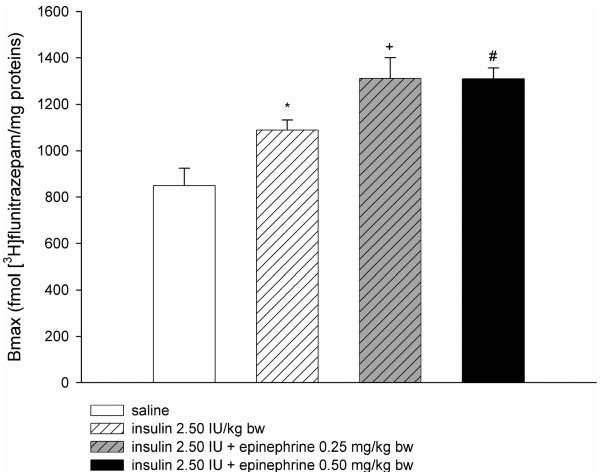

Figure 3. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes, from non-stressed chicks, following co-administration of insulin plus epinephrine. Bars represent the means ± SEM, $n = 6$ per group. $^{\star}p < 0.001$ compared to saline. $^{+}p < 0.05$ and $^{\#}p < 0.01$ compared to insulin alone condition (Newman–Keuls test).

Table IV. Effects of co-administration of insulin and different doses of epinephrine on K_d values of GABA$_A$ R in forebrain synaptosomes from non-stressed chicks.

	K_d (nM)
Saline	1.80 ± 0.46
Insulin 2.5 IU/mg	1.94 ± 0.38
Insulin 2.5 IU/mg + Epinephrine 0.25 (mg/kg)	1.52 ± 0.42
Insulin 2.5 IU/mg + Epinephrine 0.25 (mg/kg)	1.78 ± 0.20

Each value of K_d represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. $n = 6$ chicks/group.

Discussion

Since stress induces changes in hormone and neurotransmitter systems, it is possible that epinephrine and insulin are involved in the FNZ sensitive-$GABA_A$ receptor increase induced by acute stress in synaptosomes from chick forebrain. In the present report, the central benzodiazepine receptor density was used to express the $GABA_A$ R density, because the FNZ-binding site is located in the α subunit of the $GABA_A$ R (Primus and Kellog 1991). In order to study the insertion of receptors into the membrane, other authors performed experiments using cell-biology techniques in neuronal cultures. However, in our case, we asked whether or not the recruitment measured *ex vivo* of $GABA_A$ R into synaptosomes induced by acute stress, which can only be performed using live animals, is modulated by insulin and/or epinephrine systemic administration.

The results (Figure 1) show that an ip injection of insulin (1.25–5.00 IU/kg bw) increased significantly the $GABA_A$ R density, in forebrain synaptosomes, from non-stressed but not stressed chicks. Hence, the insulin-induced $GABA_A$ R increase was not additional to the increase induced by stress, suggesting that both effects might occur through similar mechanisms. It is possible that stress impairs the $GABA_A$ R increase induced by insulin or conversely that insulin prevents stress effects on the receptor density. These findings are also in accord with reported findings (Mielke and Wang 2005) indicating that insulin added to cultured neurons exerts neuroprotection by counteracting the decrease in cell surface expression of the $GABA_A$ R following oxygen–glucose deprivation, and suggesting that insulin increases the process of receptor insertion into the postsynaptic membrane surface. However, a mechanism such as phosphorylation of proteins connecting both stress and insulin effects cannot be discarded. There are two reports showing that stress and insulin effects are related, through receptor phosphorylation: (i) a previous study from our laboratory carried out on synaptosomal membranes from forebrain of stressed chicks, showed that the incorporation of alkaline phosphatase or ATP into the lumen abolished or increased, respectively, the $GABA_A$ R density. On the other hand, in non-stressed chicks ATP increased the receptor density to values similar to those in stressed chicks. These results suggested that the recruitment of receptor induced by stress depends on the degree of phosphorylation of $GABA_A$ R and/or its associated proteins (Benavidez and Arce 2002). (ii) A recent report (Vetiska et al. 2007) indicates that in cultured neurons, insulin induced a recruitment of postsynaptic $GABA_A$ Rs in a way dependent on $GABA_A$ R-associated phosphoinositide 3-kinase (PI3-K). In this report, it was hypothesized that insulin stimulation leads to tyrosine phosphorylation of one or more of the $GABA_A$ receptor subunits, promoting receptor insertion into postsynaptic domains.

It is possible that a generalized increase of synaptic strength at both pre- and post-synaptic sites can take place during an acute stress. Therefore, post-synaptic receptors are likely to be saturated by released GABA and a rapid increase or recruitment of the post-synaptic $GABA_A$ R may be a more efficient way of strengthening synaptic efficacy of GABA function (Wan et al. 1997). Previous restraint stress facilitated a fear memory by reducing GABAergic inhibition in amygdala, when rats were tested with fear conditioning 48 h later (Rodriguez-Manzanares et al. 2005). However, these authors did not measure the receptor density immediately after acute stress. From our findings, stress-induced $GABA_A$ R increase may be a phenomenon to compensate for alteration of the GABAergic system induced by stress.

IP injection of epinephrine (Figure 2) did not affect the $GABA_A$ R density in forebrain synaptosomes, in the non-stressed condition. However, after stress the $GABA_A$ R density increased in a dose-dependent way suggesting that the epinephrine effect is dependent on stress. The present findings are consistent with evidence that a single footshock increases central norepinephrine release, which affects other neuromodulatory systems, including GABAergic systems (Galvez et al. 1996; Roozendaal et al. 1996).

IP injection of insulin (2.5 IU/kg bw) plus epinephrine (0.25 and 0.50 mg/kg bw) (Figure 3) in non-stressed chicks increased $GABA_A$ R density more than insulin alone, suggesting that epinephrine action depends on, and adds to the increase induced by insulin alone. A greater epinephrine concentration did not have a greater effect on $GABA_A$ R density, indicating a maximal velocity of receptor trafficking induced by the lower dose of epinephrine in the presence of insulin administration. The velocity of trafficking and docking of receptors, within synaptic vesicles, are associated with several proteins that can be phosphorylated (Kneussel 2002; Lüscher and Keller 2004). Hence, it is possible that stress and insulin stimulate the phosphorylation of these proteins and consequently increases the velocity of vesicular transport, their fusion with membranes and final receptor insertion at the membrane surface. Alternatively, it may be that stress reduces the receptor recycling/degradation ratio and consequently could stimulate receptor biosynthesis.

Furthermore, it is possible that released norepinephrine in brain by action of systemically injected epinephrine is a necessary and limiting factor for the $GABA_A$ R insertion in the post-synaptic membrane. Therefore, an increased flux of $GABA_A$ R by insulin stimulation or induced by stress in any previous step of trafficking or docking could be limited by the noradrenergic system, at the final step of $GABA_A$ R insertion. Together, the results suggest that insulin-induced increase in $GABA_A$ R density might occur through the same mechanism that induces increased $GABA_A$ R density after acute stress. This increase might

be explained by phosphorylation of $GABA_A$ R and/or their associated proteins. Furthermore, norepinephrine release in the brain induced by epinephrine systemic injection might modulate $GABA_A$ R insertion into the post-synaptic membrane. However, this remains to be investigated through further studies.

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