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Recruitment of $GABA_A$ receptors and fearfulness in chicks: Modulation by systemic insulin and/or epinephrine

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ABSTRACT

One-day-old chicks were individually assessed on their latency to peck pebbles, and categorized as low latency (LL) or high latency (HL) according to fear. Interactions between acute stress and systemic insulin and epinephrine on $GABA_A$ receptor density in the forebrain were studied. At 10 days of life, LL and HL chicks were intraperitoneally injected with insulin, epinephrine or saline, and immediately after stressed by partial water immersion for 15 min and killed by decapitation. Forebrains were dissected and the $GABA_A$ receptor density was measured ex vivo by the ^{3}[H]-flunitrazepam binding assay in synaptosomes. In non-stressed chicks, insulin (non-hypoglycemic dose) at 2.50 IU/kg of body weight incremented the Bmax by 40.53% in the HL chicks compared to saline group whereas no significant differences were observed between individuals in the LL subpopulation. Additionally, insulin increased the Bmax (23.48%) in the HL group with respect to the LL ones, indicating that the insulin responses were different according to the anxiety of each category. Epinephrine administration (0.25 and 0.50 mg/kg) incremented the Bmax in non-stressed chicks, in the LL group by about 37% and 33%, respectively, compared to ones injected with saline. In the stressed chicks, 0.25 mg/kg bw epinephrine increased the Bmax significantly in the HL group by about 24% compared to saline, suggesting that the effect of epinephrine was only observed in the HL group under acute stress conditions. Similarly, the same epinephrine doses co-administered with insulin increased the receptor density in both subpopulations and also showed that the highest dose of epinephrine did not further increase the maximum density of $GABA_A$R in HL chicks. These results suggest that systemic epinephrine, perhaps by evoking central norepinephrine release, modulated the increase in the forebrain $GABA_A$ receptor recruitment induced by both insulin and stress in different ways depending on the subpopulation fearfulness.

1. Introduction

GABA is the most important inhibitory neurotransmitter in the CNS. $GABA_A$ receptors ($GABA_A$R) are heteropentamers constituted from 19 known subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3), with an integral channel that is permeable to Cl^- ions (Lüscher et al., 2011). Many $GABA_A$R contain two α subunits, two β subunits and one γ subunit, with two GABA binding sites being formed by α and β subunits. GABAergic synapses are critical for the development and coordination of the neuronal activity underlying the majority of physiological and behavioral processes in the brain (Jacob et al., 2008; Lüscher et al., 2011). The $GABA_A$R are localized in the neuronal postsynaptic membrane. Central flunitrazepam binding expresses $GABA_A$R with the density measured ex vivo in synaptosomes from chick forebrain. Related to this, in chicks, there is evidence that neonatal environmental conditions can induce transient increases in the flunitrazepam sensitive-$GABA_A$R density, due to stress accompanying a food discrimination task (Salvatierra et al., 1997), a T maze task (Marín and Arce, 1996) or imprinting (Salvatierra et al., 1994).

The development of behavioral and endocrine responses to acute stress is greatly influenced by the early postnatal rearing environment in human infants (Denenberg, 1964), in rats (Meaney et al., 1996) and in chicks (Salvatierra et al., 2009). These environmental effects persist throughout life, resulting in stable individual differences in stress reactivity. Early stimulation, such as neonatal novelty exposure, decreases behavioral reactivity, in rats, in the Open Field (OF) (Tang, 2001) and induces reduced fearfulness to be able to cope better with later stressful events (Salvatierra et al., 2009; Cid et al., 2011). Categorization is an easy and fast method based on different emotional reactivities, which at early age can discriminate individual differences in response to a stressor agent among individuals of the same population (Salvatierra and Arce, 2001). The classification of one-day-old chicks of both sexes resulted in categories with different degrees of fear and/or anxiety in agreement with effects of anxiolytic doses of diazepam. These different pharmacological susceptibilities were also observed in the maximum density of flunitrazepam-sensitive-$GABA_A$R, and may depend on the

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underlying differences in anxiety and/or fear, indicating that the GABAergic system might be involved in this variability within the chick population (Salvatierra and Arce, 2001).

The brain noradrenergic (NEergic) system is thought to be involved in the provocation of anxiety (Tanaka et al., 2000). Several types of stress, including immobilization, psychological and conditioned fear, increase norepinephrine (NE) release in the brain (Stanford, 1995; Galvez et al., 1996; Tanaka et al., 2000) or impair the facilitating influence of NE on GABAergic inhibition in the rat amygdala (Braga et al., 2004). NE is released in various regions of the forebrain from neurones with cell bodies located in the locus coeruleus. Its release facilitates the processing of relevant or salient information, as well as the modulation of sensory, intentional, and memory functions (Gibbs and Summers, 2002). Although, the brain had long been considered an insulin-insensitive organ, this view has been challenged by the observation than insulin receptors are widely distributed in rat brain, with marked regional variations in receptor density (Biessels et al., 2004). Several lines of evidence have indicated that brain insulin is partly transported rapidly from peripheral tissues via the cerebrospinal fluid and partly synthesized by neurons in the brain (Woods et al., 1985; Born et al., 2002). Previous studies have implicated a clear role for insulin, a metabolic hormone, in the regulation of the NE transporter function by inhibiting NE uptake in whole-brain neuronal cultures, dissociated brain cells, and whole-brain synaptosomes (Boyd et al., 1986; Masters et al., 1987). Intraperitoneal administration of different doses of insulin was shown to be a neuroprotective phenomenon in the brain of birds exposed to a stressful event, which increased the strength of neuroinhibition as evidenced by an increase in $GABA_AR$ (Cid et al., 2008). Moreover, intraperitoneal injections of various doses of epinephrine in chickens of ten days of age induced an increase in the $GABA_AR$ density in a dose-dependent manner but only under stress conditions. Therefore, it is possible that the expression of forebrain $GABA_AR$ in subpopulations with different patterns of fear and/or anxiety is differentially modulated by these hormones (insulin and epinephrine) in response to an acute stressor or under normal physiological conditions. In this study, we examined the effects of the systemic administration of insulin and epinephrine on the recruitment of $GABA_AR$ in 10-day-old stressed and non-stressed chicks of two subpopulations of high latency (HL) and low latency (LL), as previously categorized on the basis of their latency to peck pebbles in a new environment.

2. Materials and methods

2.1. Animals

Chicks (*Gallus gallus domesticus*) of both sexes were obtained immediately after hatching from the commercial hatchery INDACOR (Argentina) when they were only a few hours old. A total of 260 birds were individually housed in 24 cm×20 cm cages (of white wood) on the morning of the hatching day (Day 0) and kept in quiet conditions under dim red light in a small room (3 m×3 m) with constant temperature (31–32 °C) and humidity, without food but with water freely available. Each housing cage was kept isolated from environmental noise.

2.2. Categorization of one-day-old chicks on the basis of their latency to peck pebbles

Twenty-four hours after hatching (Day 1), each bird was cupped gently and without restraint in the palm of the hand and individually transferred to a testing cage which was identical to the housing cage except for a scattering of small pebbles and placed in an adjacent room. The pebbles, which had been glued to the floor, were 2–4 mm in diameter and of varying colors and shapes. These pebbles were inedible, being similar to those previously described for a food–pebble discrimination task (Salvatierra et al., 1997).

Each testing cage was illuminated by a lamp (60 W) suspended immediately above. The values of the latency to peck at the pebbles were scored according to Salvatierra and Arce (2001). Chicks with latency values below 30 s were termed low-latency chicks and those with values of over 90 s were termed high-latency chicks. All chicks with values between 30 and 90 s were discarded.

Immediately after, being categorized all birds of the same age from the LL and HL subpopulations were banded with different colors and socially reared in white wooden cages (10 chicks/cage) until they reached 10 days of age. The cages were of dimensions 90×40×60 cm (length×width×height) and were kept in a small room (3×3 m) at a controlled temperature of 31–32 °C with a 12:12 h light:darkness schedule (lights on at 07:00 h). Feed (Cargill, broiler BB, 23% CP, 2950 kcal/kg) and water were freely available. At 10 days, all experiments were carried out. First, daily food replenishment and maintenance chores were done at 09:00 h. Then, the experiments were performed 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 the number of animals used.

2.3. 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; Cid et al., 2008) and injected ip at a volume of 0.12 ml. LL and HL chicks were injected with saline or one of two different E doses, and immediately returned to their rearing boxes (non-stressed chicks). After 15 min, these chicks were killed. Other chicks were injected as described above for insulin, and then exposed as indicated below to Partial Water Immersion (PWI) stress. Both, non-stressed and stressed chicks were decapitated as indicated below and the crude forebrain synaptosomal fractions were obtained.

2.4. Insulin administration

Ultra-rapid human insulin was obtained from Beta Laboratories (Argentina) and prepared in 0.9% saline before being injected intraperitoneally (ip) with a dose of 2.50 IU/kg bw at a volume of 0.12 ml (Cid et al., 2008). Ten-day-old chicks, individually categorized as HL or LL, group were injected with saline or insulin, and immediately returned to their rearing boxes (non-stressed chicks). After 15 min, these chicks were killed by decapitation. Other chicks from both groups of the same box were injected in the same way and immediately exposed to PWI stress as described below. Then, the stressed chicks were decapitated and crude forebrain synaptosomal fractions were obtained.

2.5. Co-administration of insulin plus epinephrine

Chicks categorized from the LL and HL groups 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) at a volume of 0.12 ml before being immediately returned to their rearing boxes (non-stressed chicks). After 15 min, they were decapitated as indicated below and the crude forebrain synaptosomal fractions were obtained.

2.6. Partial water immersion stress

Chicks from each subpopulation were stressed as described by Martijena et al. (1992). Briefly, at 10 days of age, a chick selected at

random was 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 the water was changed after each trial. None of the birds exhibited signs of exhaustion during the testing.

2.7. Terminal procedure

At the end of each trial, the test chick was removed from the basin, and immediately killed by decapitation with scissors, within 1 s after the experimental period in order 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, pallial amygdala and other pallial areas such as the hippocampus (Reiner et al., 2004).

2.8. Preparation of crude synaptosomal fraction

The crude synaptosomal fraction was obtained as described previously (De Robertis et al., 1961). 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 these were immediately used for the binding assay. All the procedures were carried out at 4 °C. Synaptosomes isolated from brain constitute a useful in vitro model to study several neuronal functions, because they are metabolically active and retain many properties of nerve endings (Nicholls, 1989).

2.9. [^{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 at 4 °C in the presence of radioligand at final concentrations of 0.5, 1, 2, 4, 6, 8, 10 and 12 nM. 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 mM diazepam. After 60 min of 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 using an LKB-1214-RackBeta counter at a 60% efficiency. The Bmax and Kd values were obtained by non-linear regression using the following equation for a hyperbola (one binding site): $Y = Bmax * X/(Kd + X)$, where Bmax is the maximal binding and Kd is the ligand concentration required to reach half the maximal binding. The Bmax of [^{3}H]-flunitrazepam binding is representative of the $GABA_AR$ density.

2.10. Statistical analysis

The results were expressed as subpopulation mean ± S.E.M. Bmax, and the Kd values for the $GABA_AR$ density were analyzed using a three-way ANOVA with a 2 × 2 × 3 factorial arrangement of treatments (categorization, LL vs HL, stressor, PWI vs control, hormone administration, saline vs insulin or E and their interactions). The Bmax and Kd values for $GABA_AR$ density after co-administration were analyzed by a two-way analysis of variance (ANOVA) with a 3 × 2 factorial arrangement of treatments (categorization, LL vs HL, hormone administration, saline vs insulin vs E, and their interactions). Whenever ANOVA indicated significant effects ($p<0.05$), a pairwise comparison of means was carried out using the Newman–Keuls test. In all cases, the assumptions of the ANOVA (homogeneity of variance and normal distribution) were attained. For all statistical analyses, a p value <0.05 was considered to represent a significant difference between categories.

3. Results

3.1. Effects of administration of epinephrine on [^{3}H]-flunitrazepam binding in forebrain synaptosomes from stressed and non-stressed 10-day-old chicks

The three-way ANOVA of the Bmax values revealed significant acute stress ($F(1,50) = 27.77$; $p<0.0011$), and Epinephrine treatment ($F(2,50) = 7.14$; $p<0.0020$) effects, but no significant effect of categorization ($F(1,50) = 1.57$; $p = 0.2100$). However, an interaction between the three variables ($F(2,50) = 4.69$; $p<0.0136$) (Fig. 1) was observed. The Newman–Keuls test revealed that acute stress significantly increased Bmax by 40% ($p<0.0004$) in the saline-treated groups of stressed compared to non-stressed ones in the LL subpopulation, and by 27% ($p<0.0210$) in HL ones. Consequently, PWI stress increased the $GABA_AR$ density in both subpopulations. There were no significant differences between different categories of unstressed chicks injected with saline, indicating similar basal $GABA_AR$ levels between the two subpopulations.

In contrast, the Newman–Keuls test revealed that epinephrine administration (0.25 and 0.50 mg/kg) results in an increase in Bmax, in non-stressed chicks only in the LL group, by 37% ($p<0.0014$) and 33% ($p<0.0031$) compared to ones injected with saline, respectively. However, we did not observe significant differences between individuals in the HL subpopulation (1.48%, $p = 0.8911$). This difference indicates that the epinephrine responses were different according to the anxiety of each category.

In the stressed chicks we observed significant differences in Bmax only in the HL group with, 0.25 mg/kg bw epinephrine producing an increase of 24% compared to ones injected with saline ($p<0.0088$), suggesting that the effect of epinephrine was only observed under acute stress conditions. However, the other dose used (0.50 mg/kg bw) did not significantly increase Bmax (14%, $p = 0.1135$).

The three-way ANOVA for the Kd values did not reveal significant differences for categorization ($F(1,50) = 2.23$; $p = 0.1410$), acute stress ($F(1,50) = 0.1718$; $p = 0.6802$), E treatment ($F(2,50) = 0.275$;

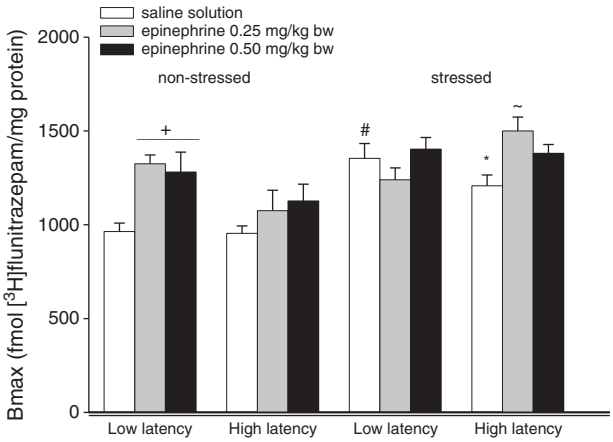

Fig. 1. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes from non-stressed and stressed categorized chicks following epinephrine administration. Epinephrine (0.25 or 0.50 mg/kg bw) or saline was administered ip 15 min before chicks were killed. Bars represent the means ± SEM, n = 5–6 chicks per group. + $p<0.0031$ compared to saline condition in LL group of non stressed chicks, # $p<0.0004$ compared to saline condition in LL group of non stressed chicks, * $p<0.0004$ compared to saline condition in HL group of non stressed chicks, ~ $p<0.0088$ compared to saline condition in HL group of stressed chicks (NK).

$p = 0.2781$) or a significant interaction between them ($F(2,48) = 0.02$; $p = 0.9981$) (Table 1).

3.2. Effects of injection of insulin on [^{3}H]-flunitrazepam binding in forebrain synaptosomes from non-stressed and stressed 10-day-old chicks

The three-way ANOVA of the Bmax values revealed significant acute stress ($F(1,27) = 24.18$; $p<0.0001$) and insulin treatment ($F(1,27) = 15.16$; $p<0.0020$) effects, but no significant effect of categorization ($F(2,45) = 1.659$; $p = 0.2220$). Furthermore, an interaction between the three variables ($F(2,45) = 11.073$; $p<0.006$) (Fig. 2) was observed. The Newman–Keuls test revealed that Bmax increased following acute stress in the LL and HL groups (40.26% ($p<0.0490$) and 57.40% ($p<0.0040$) respectively) compared to controls (unstressed) administered with saline. Therefore, the PWI stress increased the $GABA_AR$ density in the two subpopulations. However, there were no significant differences between the different categories of unstressed chicks injected with saline, indicating similar basal $GABA_AR$ levels in both subpopulations.

The Newman–Keuls test revealed that insulin administration (2.5 IU/kg) incremented the Bmax in non-stressed chicks by 40.53% ($p<0.0010$) in the HL group compared to ones injected with saline, whereas no significant differences were observed between individuals in the LL subpopulation (1.48%, $p = 0.891$). Furthermore, the Bmax rose by 23.48% ($p<0.0200$) in the HL group after insulin administration compared to the LL group, indicating that the insulin responses were different according to the anxiety of each category. However, in the stressed chicks no differences in the Bmax were observed between subpopulations, suggesting that the effect of insulin occurred through a similar mechanism to that of acute stress.

The three-way ANOVA for the Kd values did not show any significant differences for categorization ($F(1,27) = 0.55$; $p = 0.5770$), acute stress ($F(1,27) = 3.72$; $p = 0.0600$), insulin treatment ($F(1,27) = 0.27$; $p = 0.6030$) or a significant interaction between them ($F(1,27) = 0.38$; $p = 0.6830$) (Table 2).

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

The two-way ANOVA of the Bmax values revealed a significant effect of insulin and epinephrine treatment ($F(3,27) = 29.61$; $p<0.0001$), but it did not reveal an effect of categorization ($F(1,27) = 0.527$; $p = 0.4650$). However, an interaction between the two ($F(3,27) = 2.90$; $p<0.0500$) was observed (Fig. 3). Co-administration of insulin plus the lower dose of epinephrine (0.25 mg/kg bw) increased the $GABA_AR$ density in both categories by 40.84% ($p<0.0491$) and 41.19% ($p<0.0001$), in the LL and HL groups, respectively, compared to chicks of the same group injected with saline. Furthermore, insulin plus epinephrine (0.25 mg/kg bw) significantly increased the Bmax by 38.78% ($p<0.0361$) and 33.48% ($p<0.0210$), compared with the LL and HL subpopulations injected with insulin alone (Newman–Keuls test).

The co-administration of insulin (2.5 IU/kg) and the higher epinephrine dose (0.5 mg/kg) revealed a significant increase in the Bmax in both chick categories of 84.48% in the LL ($p<0.0015$) and 31.56% in the HL ($p<0.0107$) subpopulations compared to those injected with insulin alone. Furthermore, it was observed that the higher dose of epinephrine increased the Bmax (33.91%, $p<0.0312$) respect to the same group treated with the lower dose of epinephrine (0.25 mg/kg), but only in the LL subpopulation.

The two-way ANOVA for the Kd values did not show any significant differences in categorization ($F(1,27) = 1.03$; $p = 0.3661$), treatment ($F(3,27) = 0.10$; $p = 0.9600$) or a significant interaction between them ($F(3,27) = 0.76$; $p = 0.604$) (Table 3).

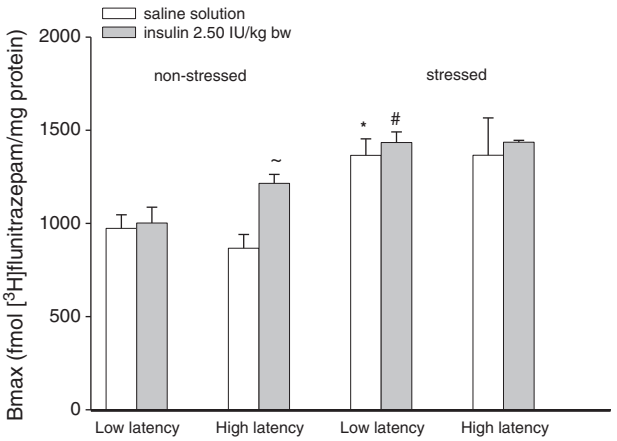

Fig. 2. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes from non-stressed and stressed categorized chicks following insulin administration. Bars represent the means ± SEM (n = 4–7 chicks per group). ~$p<0.0010$ compared to saline condition in HL group of non-stressed chicks. *$p<0.0490$ compared to saline condition in LL group of non-stressed chicks. #$p<0.0040$ compared to saline condition in HL group of non-stressed chicks (NK).

4. Discussion

This present report shows that chicks categorized into LL and HL groups according to their latency to peck pebbles in a new environment on Day 1 of life, exhibited different reactivities in the $GABA_AR$

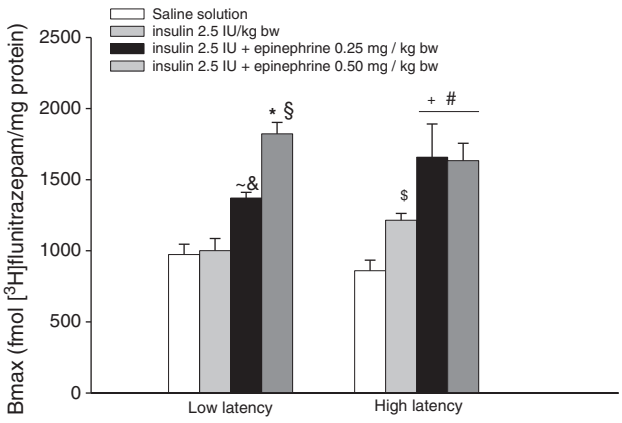

Fig. 3. Binding maximum of [^{3}H]-flunitrazepam in forebrain synaptosomes from non-stressed categorized chicks following insulin alone or insulin plus epineprhrine administration. Bars represent the means ± SEM (n = 4–7 chicks per group). ~$p<0.0491$ compared to saline condition in LL group. &$p<0.0361$ compared to insulin condition in LL group. *$p<0.0015$ compared to insulin condition in LL group. §$p<0.0312$ compared to insulin plus epinephrine 0.25 mg/Kg condition. $p<0.0010$ compared to saline condition in HL group. +$p<0.0001$ compared to saline condition in HL group. #$p<0.0210$ compared to insulin condition in HL group (NK).

Table 1
Effects of different concentrations of insulin on Kd values of $GABA_A$ R in forebrain synaptosomes from non-stressed and stressed categorized chicks, on the basis of their latency to peck pebbles.

		Kd (nM)	
Categories	Treatment	Non-stressed	Stressed
Low latency	Saline	2.98 ± 0.10	2.91 ± 0.17
	Epinephrine 0.25 mg/kg	2.59 ± 0.28	2.25 ± 0.26
	Epinephrine 0.50 mg/kg	3.01 ± 0.05	2.74 ± 0.12
High latency	Saline	2.26 ± 0.29	2.48 ± 0.30
	Epinephrine 0.25 mg/kg	2.50 ± 0.29	2.47 ± 0.27
	Epinephrine 0.50 mg/kg	2.63 ± 0.33	2.70 ± 0.53

Each Kd value represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. n = 5–6 chicks/group.

Table 2
Effects of different concentrations of insulin on Kd values of $GABA_A$ R in forebrain synaptosomes from non-stressed and stressed categorized chicks, on the basis of their latency to peck pebbles.

		Kd (nM)	
Categories	Treatment	Non-stressed	Stressed
Low latency	Saline	2.68 ± 0.29	2.01 ± 0.19
	Insulin 2.5 IU/kg	2.36 ± 0.26	2.06 ± 0.58
High latency	Saline	2.63 ± 0.33	1.12 ± 0.06
	Insulin 2.5 IU/kg	2.44 ± 0.39	2.12 ± 0.36

Each value of Kd represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. n = 4–7 chicks/group.

recruitment on Day 10, in synaptosomes from the forebrain, after acute stress and after systemic insulin and/or epinephrine administration.

Salvatierra et al. (1997) proposed that the latency to peck for the first time in a food discrimination learning in one-day-old chicks was correlated with the degree of fear/anxiety, observed as an inhibition of natural pecking behavior. As this pecking inhibition was induced by neophobia during categorization according to differences in individuals with different degrees of fear and/or anxiety; these parameters were adopted as a selection criterion for the present study. One-day-old-chicks of both sexes were individually housed and then individually categorized to avoid social-isolation stress as previously described in one-day-old chicks (Johnston and Rose, 1998; Salvatierra et al., 2009). Each category corresponded to approximately one third of the total chicks (data not shown) as found in another study (Salvatierra and Arce, 2001). It has been described that novelty is a potent fear elicitor (Boissy, 1995). In addition, it has been classified as a collative variable since the recognition of any new stimulus requires a comparison with events experienced in the past (Gray, 1979). In our present study, the pecking behavior represented a conflict between the natural tendency to peck and fear induced by novelty. Moreover, anxiolytic doses of diazepam decreased the latency to peck in a new environment only in the HL chicks but not in LL ones, suggesting that HL represented the most anxious groups (Salvatierra and Arce, 2001). These results are in agreement with Marín et al. (1997), where similar doses revealed a decrease in the locomotor activity in the OF test in two-day-old chicks. Furthermore, 15-day-old chicks individually housed for the first hours of life and individually submitted to novelty on Day 1, showed a shorter latency to ambulate in the OF test, indicating that they were less fearful and that early novelty induced a lower fearfulness at this age (Salvatierra et al., 2009).

Experimental evidence indicates that synaptically released neurotransmitters saturate their receptors (Clements, 1996), and hence the functional strength of GABAergic synapses changes in proportion to the postsynaptic $GABA_AR$ density (Nusser et al., 1997). Consistent with this idea, even modest reductions in the postsynaptic $GABA_AR$ (5%–35%) of $GABA_AR$ mutant mice had significant behavioral consequences (Shen et al., 2010). It has been reported that exposure to various types of acute stressors induced changes in the $GABA_AR$ expressed on the surface of synaptosomes (Medina et al., 1983; Martijena et al., 1992; Salvatierra et al., 1994). Martijena et al. (1992) reported, in synaptosomes from the chick forebrain, an increase in the postsynaptic density of $GABA_A$ R-flunitrazepam-sensitive induced by acute stress.

Our results (Figs. 1 and 2) showed an increased Bmax acute stress response in both categories without differences in the basal emotional reactivity. Salvatierra and Arce (2001) also observed differences in the number of $GABA_AR$ in categorized chick subpopulations exposed to Tonic Immobility and OF tests. Nevertheless, a greater increase of receptors for these subpopulations subjected to an OF was described compared to Tonic Immobility. Thus, birds suffered isolation and novelty stress in the OF test, while during PWI they experienced higher stresses through an unnatural environment such as water (Salvatierra and Arce, 2001). Related to this, Marín and Martijena (1999) observed that the locomotor response in an OF test between subpopulations with high and low performances in a T-maze was different.

The NEergic network has the potential to alter the operation of other neural circuits and modulate various physiological and behavioral responses, such as mood and anxiety (Sullivan and Gratton, 1999). Since, epinephrine is hindered by the blood–brain barrier, such effects are indirectly mediated by activation of vagal afferent projections, which release NE in the brain (Williams et al., 1998). In chicks, it was described that the systemic administration of epinephrine immediately before being exposed to PWI induced a $GABA_AR$ density increase, suggesting that both effects may occur by different mechanisms (Cid et al., 2008). In the present report, a systemic injection of epinephrine increased $GABA_AR$ by about 30%, but only in the non-stressed LL group, evidencing sensitivity to epinephrine administration. However, as no changes in Bmax in HL chicks were observed (Fig. 1). The differential modulation of epinephrine in the two subpopulations may be explained by differences in the stress responses of the endogenous NEergic system with some authors having observed that rats neonatally handled as adults showed increased levels of $GABA_AR$ in the cell NEergic body regions (Owens and Nemeroff, 1991) and reduced stress NEergic responses (Gray, 1987).

Systemic insulin administration (2.5 IU/kg) in non-stressed chicks induced an increase in $GABA_AR$ density only in the HL group (Fig. 2), which was not additional to that induced by insulin plus stress in these subpopulations, suggesting that both effects might occur by the same mechanism. Cid et al. (2008) reported that non-hypoglycaemic doses of systemic insulin significantly increased the $GABA_AR$ density in synaptosomes from non-stressed chick forebrains, but not from stressed ones. This insulin action in unstressed chicks may facilitate neuronal inhibition in the brain (Sakaguchi and Bray, 1987) by manipulation of the functional profile of the $GABA_AR$ by increased surface expression (Wan et al., 1997; Mielke and Wang, 2005). However, in the present study, insulin did not change the Bmax values in the LL subpopulation of unstressed chicks, indicating a differential response between individuals, thus making any effect on subpopulations less susceptible to novelty. The recruitment of $GABA_AR$ induced by insulin administration is possibly due to $GABA_AR$ previous phosphorylation and/or associated proteins, as was previously described for acute stress (Cid et al., 2008). Accordingly, Benavidez and Arce (2002) reported that in synaptosomal membranes from stressed chicks, the incorporation of alkaline phosphatase or ATP into the lumen abolished or increased, respectively, the receptor unmasking after incubation at 4 °C, suggesting that phosphorylation plays a role in the recruitment mechanism. Moreover, Vetiska et al. (2007) reported a possible mechanism being involved in the GABAergic potentation induced by brain insulin, which is mediated by activation of phosphatidylinositol 3-kinase facilitating insertion in the plasma membrane.

In our investigation, the co-administration of epinephrine (0.25 mg/kg) plus insulin (2.5 IU/kg) in non-stressed chicks elicited an increase in the $GABA_AR$ density in LL subpopulations compared to the group injected with insulin alone, suggesting that this increase was synergistic. However, a greater epinephrine dose (0.50 mg/kg)

Table 3
Effects of co-administration of insulin and different doses of epinephrine on Kd values of $GABA_AR$ in forebrain synaptosomes from non-stressed categorized chicks.

Categories	Treatment	Kd (nM)
Low latency	Saline	2.68 ± 0.29
	Insulin 2.5 IU/kg	2.36 ± 0.26
	Insulin 2.5 IU/kg + epinephrine 0.25 mg/kg	2.12 ± 0.57
	Insulin 2.5 IU/kg + epinephrine 0.50 mg/kg	3.18 ± 0.42
High latency	Saline	2.63 ± 0.33
	Insulin 2.5 IU/kg	2.49 ± 0.45
	Insulin 2.5 IU/kg + epinephrine 0.25 mg/kg	1.93 ± 0.11
	Insulin 2.5 IU/kg + epinephrine 0.50 mg/kg	1.85 ± 0.31

Each Kd value represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. n = 4–7 chicks/group.

plus insulin (2.5 IU/kg) showed an additional increase in the $GABA_AR$ density only in the LL group (Fig. 3). Taken together, these results suggest that LL birds were more sensitive to epinephrine modulation in the unstressed condition. Furthermore, a greater epinephrine concentration did not have a greater effect on the maximum density, in the HL subpopulation, indicating that a higher velocity of receptor trafficking was induced by the lower dose of epinephrine in the presence of insulin administration. Recently, it was described that insulin decreased high-affinity NE transporter (Robertson et al., 2010), as the high-affinity NE transporter is the primary mechanism by which NEergic synaptic transmission is terminated (Dipace et al., 2007), it is possible that insulin decreases high-affinity NE transporter, which would increase the NEergic tone principally after epinephrine administration. Insulin and stress may act by the same or by different mechanisms, which converge in an increase in the adrenergic activity. As insulin increases the NEergic strength through NE transporter down-regulation, this would lead to later NE being more time at the synaptic junction, thus regulating the $GABA_AR$. It is well documented that stress activates the sympathetic pathways, with a subsequent release of epinephrine from the adrenal glands, so it is likely that the LL group was more susceptible to epinephrine administered. Related to this, some authors have found markedly reduced levels of NE or alpha-1 adrenoceptors of selectively-bred lines after exposure to stress (Sontag et al., 2003; Weiss et al., 2008). Furthermore, it is possible that released NE in the brain by action of systemically injected epinephrine is a necessary and limiting factor for $GABA_AR$ insertion in the postsynaptic membrane (Cid et al., 2008). Therefore, an increased flux of $GABA_AR$ by insulin stimulation or induced by stress at any previous step of trafficking or docking may be then limited by the noradrenergic system at the final step of $GABA_AR$ insertion.

5. Conclusion

Taken together, these results suggest that systemic epinephrine modulated the increase in the forebrain $GABA_A$ receptor recruitment induced by both insulin and stress in different ways depending on the subpopulation fearfulness. However, this remains to be investigated through further studies.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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