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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