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Corticosterone microinjected into nucleus pontis oralis increases tonic immobility in rats

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ABSTRACT

Tonic immobility (TI) is also known as "immobility response", "immobility reflex", "animal hypnosis", etc. It is an innate antipredatory behavior characterized by an absence of movement, varying degrees of muscular activity, and a relative unresponsiveness to external stimuli. Experimentally, TI is commonly produced by manually forcing an animal into an inverted position and restraining it in that position until the animal becomes immobile. Part of the neural mechanism(s) of TI involves the medullo-pontine reticular formation, with influence from other components of the brain, notably the limbic system. It has been observed that TI is more prolonged in stressed animals, and systemic injection of corticosterone (CORT) also potentiates this behavior. At present, the anatomical brain regions involved in the CORT modulation of TI are unknown. Thus, our study was made to determine if some pontine areas could be targets for the modulation of TI by CORT. A unilateral nucleus pontis oralis (PnO) microinjection of 1 μL of CORT (0.05 μg/1 μL) in rats resulted in clear behavioral responses. The animals had an increased duration of TI caused by clamping the neck (in this induction, besides of body inversion and restraint, there is also clamping the neck), with an enhancement in open-field motor activity, which were prevented by pretreatment injection into PnO with 1 μL of the mineralocorticoid-receptor antagonist spironolactone (0.5 μg/1 μL) or 1 μL of the glucocorticoid-receptor antagonist mifepristone (0.5 μg/1 μL). In contrast, these behavioral changes were not seen when CORT (0.05 μg/1 μL) was microinjected into medial lemniscus area or paramedian raphe. Our data support the idea that, in stressful situations, glucocorticoids released from adrenals of the prey reach the PnO to produce a hyper arousal state, which in turn can prolong the duration of TI.

Introduction

Animals show a variety of behaviors characterized by immobility, i.e. sleep, rest, freezing, maternal transport, etc.. Another kind of immobility that has been conceptualized as an evolutionarily adaptive survival strategy (Marx et al., 2008) is the tonic immobility (TI). It has also been referred to as immobility response, death feint, animal hypnosis, playing possum, fright, and immobility reflex (Gallup, 1974; Klemm, 1989). TI is a reversible state of immobility caused by physical restraint. During this state there are varying degrees of muscular activity and a relative unresponsiveness to external stimuli. Under TI animals are not asleep, instead they may be actively monitoring their environment (Gallup et al., 1980). A captured prey that becomes immobile, rather than struggling and fighting, may increase its chance of escaping if the predator temporarily loosens its grip under the assumption that its prey is indeed dead (Bracha, 2004). In our laboratory, a TI can be produced in rats by clamping the neck, followed by inversion and restraint (De La Cruz et al., 1987). The neural mechanism(s) of TI probably involve the brainstem reticular formation, with influences from other components of the brain, mostly neocortex, basal ganglia and limbic system (Klemm, 2001). Support of this mechanism comes from studies in both neonate and mesencephalic rats (De La Cruz et al., 1987, 1995), where the immaturity or absence of mid- and forebrain caused an overexpression of TI. The modulator role of the limbic system on TI has also been reported. For example, the amygdala exerts a positive modulator effect on TI (Davies et al., 2002; Leite-Panissi and Menescal-de-Oliveira, 2002; Leite-Panissi et al., 2006). In contrast, the hippocampus seems to have the opposite role (Flores et al., 2005; Woodruff et al., 1975). For this, fear on the part of the prey is considered an antecedent that facilitates TI (for review see Marx et al., 2008). Furthermore, there is broad evidence that stressful stimuli modulate TI positively (Carli et al., 1981; Faure et al., 2003; Henning, 1978; Jones et al., 1994; Miranda et al., 2006; Zamudio

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et al., 2009). Thus some stress hormones, i.e. adrenaline and glucocorticoids, must play a major role to facilitate TI under stressful situations.

To cope with stressful experiences, animals activate the hypothalamic–pituitary–adrenal (HPA) and the sympathoadrenomedullary systems (Fulford and Harbuz, 2005). Activation of HPA axis increases the release of corticotrophin-releasing hormone (CRH) from the hypothalamus, which in turn stimulates the secretion of adrenocorticotropin (ACTH) from pituitary, concluding in the secretion of glucocorticoids (corticosterone (CORT) in rodents) from adrenals into the blood (Herman and Cullinan, 1997). Additionally, sympathetic activation enhances the secretion of adrenal catecholamines, mainly the hormone adrenaline. Therefore, for an appropriate adaptation to an acute stress challenge, a coordinated action of both systems is required.

Glucocorticoids exert widespread effects that serve to coordinate the responses to cope with stressful situations. Aside from numerous metabolic and immune actions of glucocorticoids, they have been shown to exert effects on the brain to regulate some behaviors, including tonic immobility, locomotor activity, sexual behavior, memory, and learning (Coddington et al., 2007; De Quervain et al., 1998; Kent et al., 2000; Quirarte et al., 2009; Sajadi et al., 2006; Sandi et al., 1996; Tenk et al., 2006; Zamudio et al., 2009). The effects of glucocorticoids on the brain are mediated by two types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The MRs bind CORT with a high affinity and are primarily located in the limbic system and certain brainstem areas. In contrast, the GRs have a lower affinity for CORT and are widely distributed throughout the brain (De Kloet et al., 2005; Joëls and De Kloet, 1994). The actions of glucocorticoids on these intracellular steroid receptors are mediated at a genomic level (De Kloet et al., 1998), though nongenomic rapid actions of glucocorticoids have also been reported (for review see Tasker et al., 2005).

Recently, we demonstrated that systemic injection of CORT increases TI in rats (Zamudio et al., 2009), suggesting an important role of this hormone in the stress facilitation of TI. However, the site(s) of action for CORT to modulate this behavior is unknown. Klemm (2001) proposed the brainstem as one of the centers controlling TI. Thus, we hypothesized that some pontine areas could be targets for the CORT in the modulation of TI.

TI and rapid eye movement (REM) sleep share some behavioral and neurophysiological characteristics; immobility, an activated electroencephalogram (EEG), hippocampal theta rhythm, righting reflex inhibition, and some autonomic responses. However, there are also some differences. During TI, REM and muscle atonia are not seen. Moreover, it has been proposed that some brainstem structures involved in the generation of REM sleep are used as effector mechanisms in TI as well (Overeem et al., 2002). Thus, we choose two of these structures (the nucleus pontis oralis (PnO) and the paramedian raphe (PMR)) that play an important role in control of REM sleep (Sakurai et al., 2005; Xi et al., 2004) as possible sites for CORT action. The medial lemniscus area (mla) in pons was also assessed.

To test the above hypotheses, TI was measured after microinjections of CORT into the PnO, PMR, or mla. Moreover, the blockade of CORT action by pretreatment with the mineralocorticoid-receptor antagonist, spironolactone (SPIRO) or the glucocorticoid-receptor antagonist, mifepristone (MIFEP) was also tested. Finally, the effect of CORT in these three pontine areas was also evaluated on open field motor activity.

Materials and methods

Animals and housing

Eighty-two adult male Wistar rats (250 to 300 g) from our animal facilities were used. Rats were isolated in individual cages 1 week before the surgical procedures and maintained in temperature (22 ± 2 °C) and light (12 h light-dark cycles; 0007 to 1900 lights on) controlled conditions and with free access to food and water. The experimental protocol for the study was approved by the National School of Biological Sciences Ethics and Biosecurity Committee and is in accordance with guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals in 1996, and with those established by The Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999). Every effort was made to alleviate any pain or distress that might be experienced by animals during this experiment. We used minimum number of animals required to attain the goals of this study. Behavioral testing always took place between 1000 and 1400.

Surgical procedures

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) (Pfizer, México) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Rats were implanted with a guide cannula (23 gauge × 7 mm) aimed at 1) right nucleus pontis oralis (PnO) using the stereotaxic coordinates (Paxinos and Watson, 1986): AP −8.0 mm; ML − 1.4 mm; and DV − 7.0 mm, 2) right medial lemniscus area (mla): AP −8.0 mm; ML − 1.0 mm; and DV − 8.6 mm, and 3) right paramedian raphe (PMR): AP − 8.0 mm; ML − 0.5 mm; and DV − 7.0 mm. The guide cannula was held in place on skull with two screws and dental acrylic glue. A stylet was inserted into the guide cannula and rats were returned to their individual cages for 1 week before behavioral testing.

Microinjections

The drugs used were corticosterone 21-acetate (CORT); spironolactone (SPIRO), and mifepristone (MIFEP) from Sigma-Aldrich, México. All drugs were dissolved in vehicle (VEH: 45% hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, México) in 0.1 M phosphate-buffered saline, pH 7.4). Drugs were microinjected via an injection cannula (31-gauge × 10 mm) that extended 1 mm beyond the tip of guide cannula. The injection cannula was connected to a 1-μL syringe (Hamilton Co., Reno, NV, USA) with TYGON microbore tubing (ID: 0.25 mm; OD: 0.76 mm) filled with sterile water. One microlitre of CORT (0.01 and 0.05 μg/1 μL), SPIRO (0.5 μg/1 μL), MIFEP (0.5 μg/1 μL), or VEH was microinjected through the cannula in 60 s. The injection cannula was left 60-s longer to avoid backflow of drug up the cannula tract. After that, the injection cannula was removed and the stylet was returned to the guide cannula.

Histological procedures

At the end of behavioral experiments, animals were overdosed with sodium pentobarbital (150 mg/kg, i.p.) and perfused intracardially with 0.9% saline solution followed by 4% Formalin. Their brains were removed manually and stored for at least 48 h in 10% Formalin. Coronal sections 100-μm thick were obtained using a vibroslice (752 M, Cambden Instrument, Lafayette, IN, USA), stained with cresyl violet and mounted with resinous medium. Under a light microscope (SMZ-10A, Nikon Instruments Inc., Melville, NY, USA), the microinjection sites were located with reference to the stereotaxic atlas of Paxinos and Watson (1986). Only results of behavioral observations from rats in which microinjections sites were within PnO, mla, or PMR were used.

Behavioral testing

Motor activity

The animal was placed on the middle of the open field (black-painted wooden box 60 cm × 60 cm × 30 cm, w × l × h). Light