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Opioidergic, GABAergic and serotonergic neurotransmission in the dorsal raphe nucleus modulates tonic immobility in guinea pigs

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ABSTRACT

Tonic immobility (TI) is an innate defensive behavior that can be elicited by physical restriction and postural inversion and is characterized by a profound and temporary state of akinesis. Our previous studies demonstrated that the stimulation of serotonin receptors in the dorsal raphe nucleus (DRN) appears to be biphasic during TI responses in guinea pigs (*Cavia porcellus*). Serotonin released by the DRN modulates behavioral responses and its release can occur through the action of different neurotransmitter systems, including the opioidergic and GABAergic systems. This study examines the role of opioidergic, GABAergic and serotonergic signaling in the DRN in TI defensive behavioral responses in guinea pigs. Microinjection of morphine (1.1 nmol) or bicuculline (0.5 nmol) into the DRN increased the duration of TI. The effect of morphine (1.1 nmol) was antagonized by pretreatment with naloxone (0.7 nmol), suggesting that the activation of μ opioid receptors in the DRN facilitates the TI response. By contrast, microinjection of muscimol (0.5 nmol) into the DRN decreased the duration of TI. However, a dose of muscimol (0.26 nmol) that alone did not affect TI, was sufficient to inhibit the effect of morphine (1.1 nmol) on TI, indicating that GABAergic and enkephalinergic neurons interact in the DRN. Microinjection of alpha-methyl-5-HT (1.6 nmol), a 5-HT_2 agonist, into the DRN also increased TI. This effect was inhibited by the prior administration of naloxone (0.7 nmol). Microinjection of 8-OH-DPAT (1.3 nmol) also blocked the increase of TI promoted by morphine (1.1 nmol). Our results indicate that the opioidergic, GABAergic and serotonergic systems in the DRN are important for modulation of defensive behavioral responses of TI. Therefore, we suggest that opioid inhibition of GABAergic neurons results in disinhibition of serotonergic neurons and this is the mechanism by which opioids could enhance TI. Conversely, a decrease in TI could occur through the activation of GABAergic interneurons.

1. Introduction

Tonic immobility (TI), also known as “animal hypnosis” or “death feigning”, is an innate defensive behavioral response that is characterized by profound physical inactivity and a relative lack of responsiveness to environmental stimuli. Tonic immobility may be experimentally induced by postural inversion in conjunction with slightly restricting the animal's movement. The duration of TI can vary across species and depends on environmental conditions [1]. The end of a TI episode is characterized by an abrupt restoration of the animal's typical posture [2]. This behavioral response is the last line of defense used by prey to survive during a predatory attack [3]. Confirming the efficacy of this tactic, Sargeant and Eberhardt [4] showed that 58% of wild ducks that exhibited TI when attacked by foxes (*Vulpes fulva*) survived the initial attack. Another indication of the adaptive value of TI was demonstrated by Thompson and colleagues [3], who demonstrated that TI reduced the time that cats attacked quails.

Studies from our laboratory have investigated the involvement of neural structures in the TI defensive response. We have shown the involvement of different structures, including the parabrachial area, the medial hypothalamus, periaqueductal gray matter (PAG), the amygdala, the nucleus raphe magnus (NRM), the anterior cingulate cortex, the dorsal hippocampal formation (DHP, unpublished results) and the dorsal raphe nucleus (DRN) [5–13].

Recently published data show that the activation of different serotonin (5-HT) receptors in the DRN may modulate distinct aspects of the TI defensive response. Microinjection of the 5-HT_{1A} agonist 8-OH-DPAT into the DRN promotes a reduction in TI duration, while 5-HT_2 receptor stimulation by alpha-methyl-5-HT increases the duration of TI [13]. The DRN is located in the ventromedial PAG [14,15], and is one of major sources of serotonergic afferents in the forebrain and midbrain [16]. The neurotransmitter 5-HT has an important role in modulating cognitive function and affective and neuroendocrine responses [17]. The heterogeneous nature of the DRN is reflected in the variety of behaviors influenced by the 5-HT that is released from the DRN. These behaviors include sleep, sexual behavior, feeding,

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motor activity, pain, circadian rhythm, aggression and anxiety [15,17]. Defensive behaviors can also be modulated by 5-HT, based on its pattern of release to different brain structures [18], and TI seems to be highly influenced by the level of 5-HT in the brain [19]. In the DRN, serotonergic neurons are the most abundant type of neuron [14,15]. However, neurons that release other neurotransmitters and neuromodulators are also present, including enkephalin and gamma-aminobutyric acid (GABA) releasing neurons [15,20–22].

It is known that opioid peptides can alter extracellular levels of 5-HT. According to Grauer and colleagues [23] and Tao and Auerbach [21], morphine microinjection into the DRN increased the extracellular concentration of 5-HT in neural areas that are innervated by this nucleus. Some studies have reported the presence of opioid receptors in the DRN [24–26], and μ-opioids reduce GABA-mediated postsynaptic currents in 5-HT neurons [22]. GABA is the main inhibitory neurotransmitter in the brain and can be found in 40% of vertebrate neurons [27]. Tao and colleagues [28] demonstrated that $GABA_A$ and $GABA_B$ receptors are present in the DRN and could be involved in reducing 5-HT release. GABAergic afferents make inhibitory synapses directly on 5-HT neurons in the DRN [29,30].

Tao and Auerbach [31] showed, using microdialysis, that opioid receptor activation is responsible for 5-HT release through the inhibition of GABAergic afferents in the DRN. Another study shows that approximately 80% of DRN GABAergic cells are μ-opioid receptor immunoreactive [25]. In accordance with these results, GABAergic stimulation in the DRN of rats attenuated the increase in 5-HT release produced by morphine [32]. Neurochemical methods utilized by Tao and Auerbach [33] also showed that morphine administration into the DRN in anesthetized rats caused an increase in 5-HT metabolism and release [32,34] and a concomitant decrease in GABA release in the DRN [35]. Therefore, GABA inhibition by morphine could facilitate serotonergic neurotransmission.

Opioid, GABA and serotonin circuits in the DRN that regulate 5-HT release in other brain areas [15–17,32,34,36] could modulate defensive responses, including TI. Thus, the purpose of this study was to investigate the role of opioidergic, GABAergic and serotonergic neurons and their interactions with each other in the DRN in the modulation of tonic immobility behavioral responses in guinea pigs.

2. Materials and methods

2.1. Animals

In this study, we used 82 male guinea pigs (*Cavia porcellus*), weighing 430–480 g, from the animal care facility of Ribeirão Preto Medical School (FMRP). Throughout the experimental period, the animals were housed in plexiglass cages (56×37×39 cm, five animals per cage) lined with wood shavings, with controlled illumination (12/12 h) and temperature (24±1 °C). The guinea pigs had free access to food (guinea pig specific food and fresh grass) and water ad libitum. All experiments were performed in compliance with the recommendations of the College of Animal Experimentation (COBEA) and with the approval (Proc.no.089/2007) of the Ethical Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto of the University of São Paulo. All efforts were made to minimize animal suffering.

2.2. Drugs

The substances morphine sulfate (opioid agonist), naloxone hydrochloride (opioid antagonist), muscimol ($GABA_A$ receptor agonist), bicuculline methiodide ($GABA_A$ receptor antagonist), 8-OH-DPAT ($5\text{-}HT_{1A}$ agonist) and alpha-methyl-5-hydroxytriptamine ($5\text{-}HT_2$ agonist) were diluted in saline (0.9% NaCl) on the day of the experiment, according to previous studies [37–40]. All substances were obtained from Sigma, St. Louis-USA.

2.3. Surgical procedures

Animals were anesthetized by intramuscular injection of both ketamine (40 mg/kg) and xylazine (5 mg/kg). Following anesthesia, we performed the dorsal head trichotomy in a stereotaxic apparatus (David-Kopf Instruments, USA) for small animals. A guide cannula was implanted in the DRN, which was located using the Rössner atlas [41] for guinea pigs: AP: +10.8 mm caudal to bregma, L: +3.0 mm lateral to the midline and P: −8.0 mm from the skull, with a 30° angle. The cannula was placed 1.0 mm dorsal to the DRN. The guide cannula was prepared from hypodermic needles segments that had a 0.6 mm outer diameter and were 14 mm long.

2.4. Microinjection procedure

We used a Hamilton microsyringe (10 μl) that was connected through a segment of polyethylene (PE 10) to a thin dental needle (Mizzi, USA) that was 15 mm long and had a 0.3 mm inner diameter. The 15 mm needle injection length extended 1 mm beyond the cannula guide and reached the DRN. The microinjection was performed in 60 s, and the needle was left in place for an additional 60 s to prevent reflux of the injected solutions. All solutions were microinjected in a total volume of 0.2 μl.

2.5. Tonic immobility recordings

The tonic immobility procedure was performed during the same period of the day, (from 13:00 to 18:00 h) to reduce variability in the results. The animals were maintained for 20 min in the experimental room prior to the start of the experiment to habituate the animal and reduce environmental stress. The animals were placed in V-shaped boxes (25 cm long×15 cm high) that were lined with nylon foam. TI induction was performed by quickly posturally inverting the animals and then manually restraining them until the animal did not resist this procedure. At this moment, the experimenter slowly withdrew his hands and a chronometer was activated to measure (in seconds) the duration of the TI episode. The episode was determined to be over when the guinea pig returned to a habitual posture on four legs. Each naive animal was subjected to five consecutive TI inductions, with a randomized interval of between 40 and 90 s between each episode (control condition). Animals that had an average TI episode length of greater than 45 s under control conditions had a guide cannula implanted in the DRN. Five to seven days after stereotaxic surgery, the animals were submitted to a TI session in the absence of drug injection to control for postoperative effects, (sham condition). The following day, the animals were microinjected with drugs and, after each treatment, five TI episodes was again measured. A fourth day of experiments was performed when we microinjected an antagonist ten minutes before the agonist (as was the case in experiments 4, 9 and 10) prior to inducing TI. The behavioral observations started 1 min after the drugs had been microinjected.

2.6. Experimental procedures

To evaluate the effect of opioidergic stimulation in the DRN, animals were divided into four experimental groups: experiment 1 (n=7), the animals received a microinjection of saline (NaCl 0.9%) as a vehicle control; experiment 2 (n=10), the animals were microinjected with morphine (1.1 nmol); experiment 3, the guinea pigs were administered with naloxone (0.7 nmol) (n=6); and experiment 4 (n=11), the animals received a microinjection of naloxone (0.7 nmol) followed ten minutes later by a microinjection of morphine (1.1 nmol).

Three other groups of animals were tested for the role of GABA in guinea pig DRN stimulation. In experiments 5 (n=8) and 6 (n=11), the animals were microinjected with 0.26 and 0.5 nmol of muscimol,