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BRAIN RESEARCH

Research Report

Extracellular serotonin level in the basolateral nucleus of the amygdala and dorsal periaqueductal gray under unconditioned and conditioned fear states: An *in vivo* microdialysis study

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ARTICLE INFO

Article history:
Accepted 22 July 2009
Available online 30 July 2009

Keywords:
Basolateral nucleus of the amygdala
Unconditioned fear
Conditioned fear
Dorsal periaqueductal gray
Serotonin
Microdialysis

ABSTRACT

Serotonin (5-HT) plays a key role in the neural circuitry mediating unconditioned and conditioned fear responses related to panic and generalized anxiety disorders. The basolateral nucleus of the amygdala (BLA) and the dorsal periaqueductal gray (dPAG) appear to be mainly involved in these conditions. The aim of this study was to measure the extracellular level of 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) in the BLA and dPAG during unconditioned and conditioned fear states using *in vivo* microdialysis procedure. Thus, for the unconditioned fear test, animals were chemically stimulated in the dPAG with semicarbazide, an inhibitor of the γ-aminobutyric acid-synthesizing enzyme glutamic acid decarboxylase. For the conditioned fear test, animals were subjected to a contextual conditioned fear paradigm using electrical footshock as the unconditioned stimulus. The results show that the 5-HT and 5-HIAA level in the BLA and dPAG did not change during unconditioned fear, whereas 5-HT concentration, but not 5-HIAA concentration, increased in these brain areas during conditioned fear. The present study showed that the 5-HT system was activated during conditioned fear, whereas it remained unchanged during unconditioned fear, supporting the hypothesis that 5-HT has distinct roles in conditioned and unconditioned fear (dual role of 5-HT in anxiety disorders).

1. Introduction

A defensive system comprised of the frontal cortex, the amygdala, and the ventral periaqueductal gray (vPAG) is involved in the organization of aversive associative learning (De Oca et al., 1998; Fanselow, 1994; Walker and Carrive, 2003, but Molchanov and Guimarães, 2002, reported neural substrates for unconditioned fear in the vPAG). Fear-conditioning paradigms have been considered useful animal models of generalized anxiety disorder (GAD). On the other hand, the medial hypothalamus, the amygdala, and the dorsal periaqueductal gray (dPAG) have long been recognized to integrate innate defensive responses to environmental threats (Brandão et al., 1994, 2003; Graeff, 1990). The aversiveness of the

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doi:10.1016/j.brainres.2009.07.074

stimulation of these brain areas is attested by the fact that rats engage in different kinds of behavior to interrupt or switch off this stimulation (Graeff et al., 1993; Olds and Olds, 1962). In human patients, electrical stimulation of the dPAG evokes strong feelings of fear, impending death and marked autonomic changes, as in a full-blown panic attack (Amano et al., 1978; Nashold et al., 1969). Given the striking similarities between the autonomic and behavioral effects of the dPAG stimulation and the symptoms of panic attacks, it has been suggested that the dPAG is involved in the genesis of panic disorder in humans and that stimulation of this midbrain area in animals can model panic attacks (Jenck et al., 1995; Lovick, 2000; Schenberg et al., 2001). It is also well-known that this neural substrate is under tonic inhibitory control exerted by GABA mechanisms (Di Scala et al., 1984; Brandão et al., 1986).

It is well-known that the BLA participates in the mediation of conditioned fear (Graeff et al., 1997; Li et al., 2006; Martinez et al., 2007; Macedo et al., 2007; Yokoyama et al., 2005) as well as in unconditioned fear responses (Campbell and Merchant, 2003; Cornélio and Nunes-de-Souza, 2007; Costall et al., 1989, 1990; Duxon et al., 1997; Gonzalez et al., 1996; Higgins et al., 1991; Macedo et al., 2007; Schreiber and De Vry, 1993a). Microdialysis studies have shown an increase of extracellular level of glutamate and dopamine in the amygdala during different conditioned fear states, while the gamma-aminobutyric acid is reduced (Stork et al., 2002; Venton et al., 2006; Yokoyama et al., 2005). The serotonergic system in the amygdala as well as in the dPAG also plays an important role in the mediation of different fear states induced experimentally (Davis et al., 1994; Deakin and Graeff, 1991; Graeff, 2004; Zangrossi et al., 2001). Deakin and Graeff (1991) proposed that distinct serotonergic pathways modulate conditioned and unconditioned fear behaviors related, respectively, to generalized anxiety disorder and panic disorder, suggesting a dual role for serotonin (5-HT) in these anxiety disorders. In the first, the ascending 5-HT pathway arises in the dorsal raphe nucleus (DRN) and innervates the amygdala and frontal cortex and facilitates defensive behaviors that occur in response to potential or distal threats. In the second, the DRN-periventricular pathway innervates the medial hypothalamus and dPAG and inhibits innate fight-or-flight reactions to proximal danger (Deakin and Graeff, 1991; Deakin et al., 1992; Graeff, 1990). This hypothesis of a dual role for 5-HT has been supported by evidence showing that 5-HT acting in the amygdala facilitates the conditioned fear response, whereas 5-HT mechanisms in the dPAG impair the unconditioned fear response (Davis et al., 1994; Deakin and Graeff, 1991; Graeff et al., 1997; Zangrossi et al., 2001). Indeed, intra-dPAG injections of 5-HT receptor agonists (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}) inhibit unconditioned fear responses induced by dPAG electrical stimulation or escape behavior assessed in the elevated T-maze (Jacob et al., 2002; Nogueira and Graeff, 1995; Zanoveli et al., 2003). Additionally, behavioral studies using microdialysis to assess 5-HT concentrations in the basolateral nucleus of the amygdala (BLA) showed that 5-HT release is increased during stress and anxiety states (Kawahara et al., 1993; Rueter and Jacobs, 1996). Recently, it has been found that intra-BLA injections of the selective serotonin reuptake inhibitor fluoxetine were shown to increase the contextual conditioned fear response (Martinez et al., 2007).

As reported earlier, taking into account that 5-HT system in the dPAG is also involved in the mediation of unconditioned fear, but few studies have investigated its role in this region during conditioned fear states, the present study evaluated 5-HT neurotransmission in the dPAG and BLA during unconditioned and conditioned fear states using the *in vivo* microdialysis procedure. Thus, although there are several studies in the literature involving the direct manipulation of the 5-HT system in the dPAG and that several studies used measurements of 5-HT concentrations in the amygdala and prefrontal cortex of rats submitted to different types of fear (Holmes, 2008 for a review; Kawahara et al., 1993; Miyata et al., 2007) to our knowledge this is the first study to measure endogenous levels of 5-HT in the dPAG under different fear states. The chemical stimulation of the dPAG with semicarbazide and the contextual fear conditioning procedures were used as the unconditioned and conditioned tests, respectively. The use of chemicals that stimulate only postsynaptic receptors is preferable to electrical stimulation, which has the disadvantage of exciting not only perikarya but also axons and fibers of passage. The present study takes advantage of the fact that injections of appropriate doses of semicarbazide—an inhibitor of the γ-aminobutyric acid (GABA)-synthesizing enzyme glutamic acid decarboxylase—gradually reduces the pool of GABA in the synaptic cleft to slowly promote defensive reactions (Hoshino et al., 1979). Thus, with increasing doses of semicarbazide injections into the dPAG these reactions are initially characterized by freezing followed by fleeing locomotion and escape leaps (Borelli et al., 2005; Brandão et al., 1986; Vianna et al., 2001).

2. Results

After histological screening for the correct placements of the dialysis and/or injection cannulae, only animals with correct placements were included in the statistical analysis. The representative sites of cannula placements are shown in Fig. 1.

2.1. *Experiment 1: extracellular levels of 5-HT and 5-HIAA in the dPAG and BLA during unconditioned fear states*

As shown in Figs. 2A and B, intra-dPAG injections of semicarbazide induced the freezing response compared with saline-injected animals ($p<0.05$). This result was observed in animals submitted to 5-HT level analysis of the BLA [$F(1,13)=27.82$, $p<0.05$] and dPAG [$F(1,13)=126.06$, $p<0.05$].

Figs. 2C and D illustrate the effects of intra-dPAG injections of semicarbazide or saline on extracellular 5-HT level in the BLA and dPAG, respectively. Two-way ANOVA revealed that chemical stimulation of the dPAG did not alter extracellular 5-HT levels in the BLA [$F(1,13)=0.83$, $p>0.05$] or dPAG [$F(1,13)=3.31$, $p>0.05$].

Table 1 (supplementary material) shows that intra-dPAG injection of semicarbazide did not affect extracellular 5-HIAA levels in the BLA [$F(1,13)=1.56$, $p>0.05$]. ANOVA also showed that although treatments caused significant differences in extracellular 5-HIAA levels in the dPAG [$F(1,13)=6.68$, $p<0.05$], *post hoc* comparisons showed that this difference was due to reduction in the levels of this metabolite in the control group.