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The H_3 receptor protean agonist proxyfan enhances the expression of fear memory in the rat

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Abstract

Consolidation of fear memory requires neural changes to occur in the basolateral amygdala (BLA), including modulation of histaminergic neurotransmission. We previously demonstrated that local blockade or activation of histamine H_3 receptors in the BLA impaired or ameliorated, respectively, retention of fear memory. The histamine H_3 receptor is a G-protein-coupled receptor (GPCR) displaying high constitutive activity that regulates histamine neurons in the brain. Proxyfan is a high-affinity histamine H_3 receptor protean agonist exhibiting the full spectrum of pharmacological activities, from full agonist to full inverse agonist depending on the competition between constitutively active and quiescent H_3 receptors in a given tissue or brain region. Therefore, protean agonists are powerful tools to investigate receptor conformation and may be useful in designing specific compounds selective for the various receptor conformations. In the present study we examined the effect of post-training, systemic or intra-BLA injections of proxyfan on contextual fear memory. Rats receiving intra-BLA, bilateral injections of 1.66 ng proxyfan immediately after fear conditioning showed stronger memory for the context-footshock association, as demonstrated by longer freezing assessed at retention performed 72 hr later compared to controls. Comparable results were obtained when doses as low as 0.04 mg/kg of proxyfan were injected systemically. Hence, our results suggest that proxyfan behaves as an H_3 receptor agonist with a low level of constitutive activity of the H_3 receptor in the rat BLA.
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Keywords: Basolateral amygdala; Protean agonism; Contextual fear conditioning; Local administration; Systemic administration; Constitutive activity

1. Introduction

The therapeutic potential of H_3 receptor antagonist/inverse agonists for correcting cognitive deficits is raising great interest. There is convincing evidence that systemic administration of such compounds in experimental animals have procognitive effects in learning paradigms such as the five-choice, serial reaction time task (Ligneau et al., 1998), the object recognition test (Giovannini et al., 1999), the two-choice place recognition test (Orsetti et al., 2001), the five-trial inhibitory avoidance test (Fox et al., 2002a, 2003), the elevated-plus maze task (Onodera et al., 1998) and in social memory (Fox et al., 2003). However, when administered locally, into restricted brain regions H_3 ligands may have other effects on the expression of some forms of memory. For instance, post-training administrations of

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the H_3 receptor antagonists/inverse agonists thioperamide or clobenpropit into the basolateral amygdala impair memory for aversive events (Passani et al., 2001), whereas agonists such as R-α-methylhistamine improve the expression of fear memory after contextual fear conditioning (Cangioli et al., 2002). In the same learning paradigm, post-training injections into the dorsal hippocampus of R-α-methylhistamine improve memory consolidation (Giovannini et al., 2003). Almost invariably, the behavioural effects of histaminergic compounds have been studied following systemic or local administration into small brain regions, which are known to be crucial for the behavioural response under study. Both approaches have advantages and caveats: systemic treatments are clinically relevant, but histaminergic compounds are known to affect arousal, anxiety, perception (Brown et al., 2001), and stress (Westerink et al., 2002), therefore the mechanisms of action remain unexplored. On the other hand, local administrations of these compounds may reveal how the histaminergic system affects cognitive processing by modulating local neuronal circuitry, intracellular pathways and may explain potential unwanted, or side effects of the molecules under investigation. In the present study, we correlated the behavioural effects of systemic and intra-basolateral amygdala (BLA), post-training injections of proxyfan in the rat, on contextual fear memory. Aversive training tests such as contextual fear conditioning are used to assess emotional memory. They implicate the association between a neutral cue (the context, in this case) and a footshock. Re-exposure to the same environment will induce a measurable stereotyped behaviour. Furthermore, there is extensive evidence that crucial neural changes mediating emotional memory occur in the BLA (LeDoux, 2000). The interest in proxyfan stems from the recent discovery that the H_3 receptor shows a high degree of agonist-independent activity (Morisset et al., 2000), a phenomenon also referred to as constitutive activity, as demonstrated both in vivo (Morisset et al., 2000) and in vitro, in recombinant and native H_3 receptors (Rouleau et al., 2002). Several of the classical H_3 antagonists, such as thioperamide and ciproxifan, behave as potent inverse agonists, as they block the intracellular pathways associated with active H_3 receptors in transfected cells (Morisset et al., 2000; Rouleau et al., 2002). The high-affinity H_3-receptor ligand proxyfan (Ligneau et al., 2000; Morisset et al., 2001), on the other hand, is a "protean" agonist, which displays the full spectrum of pharmacological activities from full agonism to full inverse agonism (Gbahou et al., 2003). Little is known of the behavioural effects of proxyfan. In a dipsogenia test in mice proxyfan behaves as an antagonist/partial agonist (Fox et al., 2002b). Furthermore, proxyfan modulates the sleep-wake cycle in a different manner depending on the animal species (Gbahou et al., 2003), hence depending on the conformation of the receptors and their constitutive activity. The H_3 receptor is apparently one of the most highly constitutively active receptors so far detected, hence we used proxyfan to study its functional significance in a memory test that requires the activation of the amygdala, one of the brain regions with the highest density of H_3 receptor mRNA and binding sites in the rat (Pillot et al., 2002).

2. Materials and Methods

2.1. Animals

Male albino Wistar rats (average body weight 290 g) were used in all experiments. Rats were individually housed in a room with a natural light–dark cycle and constant temperature (20 ± 1 °C), and had free access to food and water throughout the experiments. Animals used in this study were cared for in accordance with the guidelines of the European Community recommendations (86/606/CEE) and were approved by the Animal Care Committee of the Università di Firenze.

2.2. Behavioural experiments

2.2.1. Apparatus

Contextual fear conditioning was induced in a basic Skinner box module (Modular Operant Cage, Coulbourn Instruments Inc., PA, USA) as in previous experiments (Cangioli et al., 2002; Passani et al., 2001). Box dimensions were 29 × 31 × 26 cm. The top and the two opposite sides were made of aluminium panels, the other two sides of transparent plastic and the floor of stainless steel rods connected to a shock delivery apparatus (Grid Floor Shocker, model E13-08, Coulbourn Instruments Inc. PA, USA). The number of the electric shocks and the inter-shock interval duration was predetermined by a stimulus programming device (Scatola di comando Arco 2340, Ugo Basile, Italy). The apparatus was placed in an acoustically insulated room kept at a constant temperature of 20 ± 1 °C. Illumination inside the room was 60 lux.

2.2.2. Contextual fear conditioning procedure

The rat was gently taken manually from the home cage, placed in a bucket and carried from the housing to the soundproof room, and then placed in the conditioning apparatus (contextual cue). The rat was left undisturbed for 3 min. and subsequently seven 1-s, 1-mA electric footshocks were administered at 30-s intervals. The rat was removed 2 min after the end of the stimulation, therefore spending a total time of 8 min inside the conditioning apparatus. The same conditioning

parameters were used for animals that received either i.p. or intra-BLA injections of proxyfan.

2.2.3. Conditioned freezing measurements

Freezing is one of the behavioural manifestations of conditional fear in rats, and consists of the complete absence of voluntary movements. Freezing duration was measured 72 hr after conditioning. To measure freezing, the animals were again placed inside the conditioning apparatus in the soundproof room and left for 6 min, during which they did not receive electrical stimulation. After that time, they were brought back to the home cage. Rat's behaviour was recorded by means of a closed circuit television system by an experimenter unaware of the animal's treatment. Freezing time was measured with a stop watch. All behavioural tests were performed between 10:00 and 12:00 hr to avoid a Kamin effect (Kamin, 1957).

2.2.4. Systemic administrations of proxyfan

Rats were injected intraperitoneally immediately post-acquisition with a constant volume (0.4 ml) of physiological solution in which proxyfan had been dissolved at the appropriate concentrations. Doses were extrapolated from those used in mice (Morisset et al., 2000). Control groups received 0.4-ml injections of physiological solution. After the injection the rats were returned to their home cages. During the following 3 days before testing, rats displayed a normal behaviour that did not differ from that of animals that received intra BLA injections of saline.

2.2.5. Surgery and intra-amygdala drug administration

Rats were anaesthetized immediately after the training session with ketamine (100 mg/kg i.p.), and bilateral injections took place within the following 10 min. Animals were restrained in the stereotaxic apparatus and secured by means of two ear pins and a maxillary bar. After incision of the scalp, 2-mm holes were drilled in the skull at the appropriate sites, and care was taken not to open the dura mater. Insertion of the injection needle was performed according to the coordinates: AP = −2.8 mm, L = −4.9, DV = 3.8 from bregma (Paxinos and Watson, 1998). Different concentrations of proxyfan were dissolved in physiological saline and injected bilaterally into the BLA. The tip of the needle was placed 8.7 mm ventral to bregma. The injection needle had an outside diameter of 0.3 mm, and was connected with a short piece of polyethylene tubing to a Hamilton syringe fixed to an electrode holder. Solutions (0.5 μl per side) were injected over a 1–2 min period and the needle was left in place for another min before withdrawing it. Control groups received bilateral injections of saline. The surgical incision was then closed with metal stitches. After surgery, the rats were returned to their home cages. During the following 3 days before testing, the animals displayed a normal behaviour that did not differ from that of controls.

2.2.6. Exploratory and locomotor activity

The intraperitoneal injections of proxyfan did not cause any modification in animals' behaviour during the 3 days between training and testing. Intra-BLA injected animals recovered from surgery and general anaesthesia within 24 hr, and in the following 48 hr did not show any abnormal behaviour. Furthermore, immediately after the retention test, all animals were placed on a round table of 1.5 m diameter and their behaviour was observed for 3 min. Data from rats showing abnormal locomotor and exploratory activities, grooming, yawning and rearing were not included.

2.3. Histology

At the end of the experiments, rats were deeply anaesthetized with ketamine, the brains were removed and stored in 10% formalin for 10 days. Brain sections were cut with a freezing microtome and Nissl-stained to verify injection sites. Only animals with correct placement of the injection needles within the boundaries of the BLA were used for data analysis.

2.4. Data analysis

For each session, data were expressed as seconds spent freezing within the 6 min of testing. One-way ANOVA and the Neuman–Keuls post hoc test were used. For all statistical tests, $P < 0.05$ was considered significant.

3. Results

Fig. 1 shows the effect of increasing doses of proxyfan on the expression of fear memory. Proxyfan was administered i.p. immediately after training, and the animals were tested for memory retention 72 hr after training. Analysis of variance and Neuman–Keuls post hoc analysis for freezing behaviour showed a significant treatment effect ($F_{4,34} = 4.614$, $P < 0.01$). Rats that received either 0.04 mg/kg ($n = 8$) or 0.2 mg/kg ($n = 9$) proxyfan spent significantly more time freezing than saline-injected controls ($n = 10$) when tested for contextual fear retention. There was no significant difference between animals that received 0.02 mg/kg proxyfan ($n = 6$), 2 mg/kg ($n = 6$) and controls ($n = 10$). As the amygdala participates in the elaboration of highly emotional, aversive memories (see Maren, 2001 for a review), we examined the effect of post-training bilateral injections of proxyfan into the BLA on contextual fear memory retention. Proxyfan was diluted in saline to permit the bilateral injection of constant

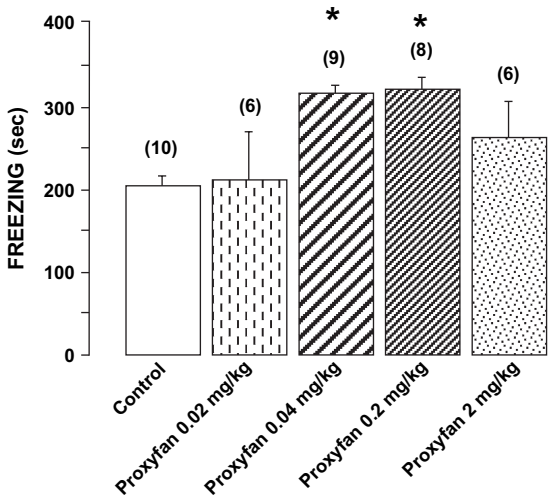

Fig. 1. Effect of post-training systemic administrations of proxyfan on contextual fear conditioning. Proxyfan was freshly prepared and diluted in sterile saline. Control rats received i.p. injections of saline. Freezing was measured 72 hr after training over a 6 min period during re-testing. Each bar represents the mean value ± S.E.M. of (n) rats. Doses are expressed as mg/kg. *$P < 0.05$ vs. controls (Neuman–Keuls test).

volumes of 0.5 μl to each rat. Analysis of variance revealed a significant treatment effect ($F_{2,27} = 3.831$, $P < 0.05$), and Neuman–Keuls post hoc analysis showed that rats receiving bilateral, intra BLA injections of 1.66 ng proxyfan (which corresponds to 10 μM; $n = 9$) spent more time freezing than saline injected controls ($n = 11$, $P < 0.05$), because these rats showed a stronger response for the footshock-context association (Fig. 2). There was no significant difference, however, between animals treated with 0.166 ng (1 μM) proxyfan and controls.

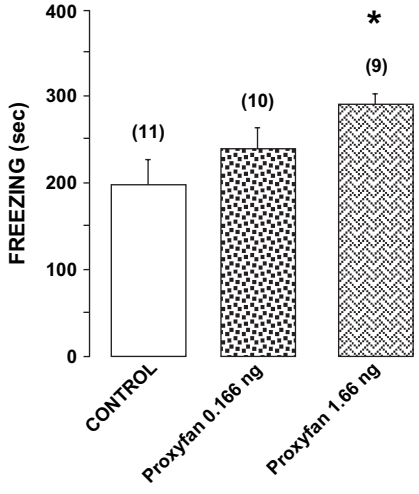

Fig. 2. Effects of post-training bilateral injection of proxyfan into the BLA on contextual fear conditioning. Immediately after training rats were injected with proxyfan under general anaesthesia. Seventy-two hours after training, freezing was measured during the 6-min period of testing. Each bar represents the mean value ± S.E.M. of (n) rats. **$P < 0.05$ vs. control (Neuman–Keuls test).

4. Discussion

The present study provides the first evidence of the enhancing effects of the "protean" H_3 receptor agonist proxyfan, on consolidation of fear memories. The H_3 receptor is one of the few GPCRs to have been described as having a physiological role in its constitutively active form controlling histaminergic neurons activity in vivo (Morisset et al., 2000). Recently, evidence was provided that the 5-HT_{2C} receptor as well is constitutively active in vivo, where it exerts an inhibitory control on nigrostriatal and mesolimbic DA neuronal pathways (Deurwaerdere et al., 2004). GPCRs are allosteric proteins that adopt inactive (R) and active (R*) conformations in equilibrium. R* is promoted by agonists or occurs spontaneously, leading to constitutive activity of the receptor. Conversely, inverse agonists promote R and decrease constitutive activity. Classical antagonists of the H_3 receptors such as thioperamide and ciproxifan block constitutive activity, and therefore were demonstrated to act as inverse agonists. The existence of another pharmacological entity, referred to as "protean" agonists, was assumed on theoretical grounds (Kenakin, 1995). It was predicted from the existence of constitutive activity that the same ligand could act either as an agonist or an inverse agonist at the same GPCR. Proxyfan behaves as a protean agonist, as it shows a spectrum of activities that range from full agonist to full inverse agonist at distinct functional responses within different animal species. In the learning paradigm used in the present study, contextual fear conditioning, proxyfan mimicked the memory-enhancing effect of H_3 receptor agonists administered locally either in the amygdala (Cangioli et al., 2002), or in the hippocampus (Giovannini et al., 2003) in contextual fear conditioning. Accordingly, rats that received systemic administrations of proxyfan showed a stronger response to the footshock-context association, as they spent more time freezing in the retention test. Full agonism of proxyfan was observed also in the cat, where it significantly increased deep slow-wave sleep, without affecting wakefulness, whereas in the mouse proxyfan behaved as a full inverse agonist (Gbahou et al., 2003), mimicking the arousal effect of the antagonist/inverse agonist ciproxifan (Parmentier et al., 2002), and as a partial agonist in the dipsogenia model (Fox et al., 2002b). Presumably, the agonist effect of proxyfan in our model implies that in the rat amygdala H_3 receptors are predominantly in a quiescent state and constitutive activity has a low efficacy. In rats, though, enzyme immunoassay experiments have shown that proxyfan decreased the levels of the histamine metabolite

t-MeHA, in the cortex, hence behaving as an inverse agonist, which presumes a high efficacy of the constitutively active H_3 receptor (Gbahou et al., 2003). This is in agreement with the hypothesis that competition between the active and inactive forms of the receptor may be responsible for the heterogeneity of pharmacological responses in different brain regions (Gbahou et al., 2003). But the pharmacology of H_3 receptors is very complex, and when evaluating behavioural responses, other factors such as polymorphism (both in humans and rodents) with a differential distribution of splice variants in the CNS, and potential coupling to different g-protein signaling pathways (see Passani and Blandina, 2004 for a review) should be kept in mind. This heterogeneity may contribute to the various effects of histaminergic ligands when tested in disparate brain regions and across animal species to alter the entire range of behavioural responses. Nonetheless, in our study proxyfan affected the expression of fear memory in a comparable manner when administered either systemically or into the BLA, a result that corroborates the theory that amygdala constitutes one of the core elements of a circuit that regulates emotional responses. As for other compounds that affect learning and memory (McGaugh, 1989), systemic administration of proxyfan induced an inverted U-shaped, dose–response curve. An analogous phenomenon could not be shown with localized injections into the amygdala, because higher doses than the ones we used do not guarantee that proxyfan diffusion remains confined to the BLA. It is worth mentioning that both systemic and local injections of proxyfan were carried out post-training, which is a protocol that excludes any influence of the treatment on acquisition and on other processes that indirectly may affect learning (McGaugh and Izquierdo, 2000). The pharmacological treatments interfered with memory consolidation as they were performed immediately after training, whereas the retention test was carried out 72 hr later; therefore, the observed memory enhancement is to be considered the expression of improved long term, contextual fear memory. Comparison between freezing time of rats that received intra-BLA injections of saline and those that received systemic saline injections confirm the hypothesis that post-trial ketamine anaesthesia and surgical procedures that were performed to inject proxyfan in the BLA do not influence negatively avoidance responses by interfering with some late stages of memory consolidation. These findings validate previously reported observations that unoperated and ketamine-anaesthetized rats that received intra-amygdala saline injections exhibited similar freezing responses to context and acoustic conditioned stimuli (Sacchetti et al., 1999). In conclusion, the complexity of the pharmacological responses induced by protean agonists is such that their therapeutic applicability may be disputable at this moment, as their efficacy may vary among brain regions and pathology addressed. However, protean agonists may help discriminate between different receptor conformations in various neuroanatomical structures and contribute to the understanding of the functional significance of constitutive activity.

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