Research

Involvement of basolateral amygdala α_2 -adrenoceptors in modulating consolidation of inhibitory avoidance memory

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These experiments investigated the role of the α_2 -adrenoceptors of the basolateral nucleus of the amygdala (BLA) in modulating the retention of inhibitory avoidance (IA). In Experiment 1, male Sprague Dawley rats implanted with bilateral cannulae in the BLA received microinfusions of a selective α_2 -adrenoceptor antagonist idazoxan 20 min either before or immediately after training. Retention was tested 48 h later. Idazoxan induced a dose-dependent enhancement of retention performance and was more effective when administered post-training. In Experiment 2, animals received pre- or post-training intra-BLA infusions of a selective α_2 -adrenoceptor agonist UK 14,304. The agonist induced a dose-dependent impairment of retention performance and, as with the antagonist treatments, post-training infusions were more effective. These results provide additional evidence that consolidation of inhibitory avoidance memory depends critically on prolonged activation of the noradrenergic system in the BLA and indicate that this modulatory influence is mediated, in part, by pre-synaptic α_2 -adrenoceptors.

Emotionally arousing experiences generally create strong, long-lasting memories (Christianson 1992; McGaugh 2004). Extensive evidence supports the hypothesis that the hormonal systems activated by emotional arousal strengthen memory by modulating the neurobiological processes underlying memory consolidation (Gold and van Buskirk 1975; McGaugh 1989, 2000; McGaugh and Roozendaal 2002).

Several kinds of evidence indicate that adrenal stress hormones affect memory storage via an interaction with noradrenergic mechanisms in the amygdala (McGaugh et al. 1996 for review). Norepinephrine (NE) is released in the amygdala by stressful or arousing stimulation of the kind used in inhibitory avoidance (IA) training (Galvez et al. 1996; Quirarte et al. 1998) and retention performance varies directly with the amount of NE released in the amygdala by the training experience (McIntyre et al. 2002). There is extensive evidence that post-training intra-amygdala administration of NE or β -adrenoceptor agonists enhances memory consolidation and that β -adrenoceptor antagonists impair memory (Gallagher et al. 1977; Liang et al. 1986, 1990; Introini-Collison et al. 1991). Further, the memory-modulating effects of stress hormones or drugs affecting adrenergic, gamma-aminobutyric acid (GABA), opioid peptidergic, and glucocorticoid systems are known to be mediated by the activation of β -adrenergic system within the amygdala (Liang et al. 1986; McGaugh et al. 1988; Introini-Collison et al. 1989, 1995; McGaugh et al. 1996; Quirarte et al. 1997). These findings, considered together with the evidence high density of β -adrenoceptor subtypes within the amygdala (Alexander et al. 1975; Bylund and Snyder 1976), provide strong evidence that noradrenergic effects on memory consolidation are mediated, at least in part, by an activation of β -adrenoceptors in the amygdala.

There is also extensive evidence that the memory-modulatory effects of the β -adrenoceptor system are mediated selectively by the basolateral nucleus of the amygdala (BLA). Post-training infusions of NE or the β -adrenoceptor agonist clen-

buterol administered selectively into the BLA enhance retention of IA and water-maze training, as well as contextual fear conditioning and extinction (Ferry and McGaugh, 1999; Hatfield and McGaugh 1999; LaLumiere et al. 2003; Berlau and McGaugh 2006). Further, infusions of β -adrenoceptor antagonists administered selectively into the BLA block the memory-enhancing effects of post-training systemic injections of glucocorticoids (Quirarte et al. 1997).

The amygdala also contains two types of α -adrenoceptors (U'Prichard et al. 1980; Unnerstal et al. 1984; Zilles et al. 1993). Whereas the α_1 -adrenoceptor subtype is located post-synaptically (for review, see Hardman et al. 1996), the α_2 -adrenoceptor subtype is predominantly located on presynaptic noradrenergic terminals and its activation inhibits NE release (Langer 1974; Starke 1979; Talley et al. 1996). The findings of studies of the effects of non-selective α -adrenoceptor agonists or antagonists on memory consolidation have provided conflicting evidence concerning whether the α_1 - and α_2 -adrenoceptor subtypes play different roles in the processes underlying memory storage. Post-training infusions of the non-selective α -adrenoceptor antagonist, phentolamine, into the amygdala were reported to induce dose-dependent enhancement of IA retention (Gallagher and Kapp 1981) whereas administration of the selective α_1 -adrenoceptor antagonist, prazosin, did not induce significant effects (McGaugh et al. 1988; Liang et al. 1995). Post-training activation of BLA α -adrenoceptors with phenylephrine induced a complex pattern of effects (Ferry et al. 1999a). Although phenylephrine has been reported to be a selective α_1 -adrenoceptor agonist (Hardman et al. 1996), phenylephrine also stimulates pre-junctional α_2 -adrenoceptors (Wikberg 1973; Flavahan and McGrath 1981; van Meel et al. 1981); thus, the combined activation of α_1 - and α_2 -adrenoceptors could have conflicting effects on memory storage.

In previous experiments investigating the involvement of BLA α_1 -adrenoceptors in memory consolidation, we found that post-training infusions of the selective α_1 -adrenoceptor antagonist prazosin administered into the BLA impaired IA retention, whereas selective activation of α_1 -adrenoceptors enhanced retention (Ferry et al. 1999a). Furthermore, the memory-modulatory

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effects of NE in the BLA appear to be mediated by an interaction between α_1 - and β -adrenoceptors as post-training infusions of the β -adrenoceptor antagonist atenolol into the BLA blocked the memory enhancement induced by selective α_1 -adrenoceptor activation (Ferry et al. 1999a).

Anatomical findings indicate that there is a higher density of α_2 -adrenoceptors than α_1 -adrenoceptors in the amygdala (Unnerstal et al. 1984; Zilles et al. 1993). Although there is extensive evidence that β - and α_1 -adrenoceptor subtypes are involved in memory modulation, studies have not, as yet, examined the involvement of α_2 -adrenoceptors in the BLA in memory consolidation. These receptors are known to be involved in regulating NE release in the central nervous system (Dennis et al. 1987) and more precisely in the amygdala (Fendt et al. 1994). Moreover, the amount of NE released in the amygdala directly varies with the intensity of a footshock like that used in IA training (Galvez et al. 1996; Quirarte et al. 1998) and significantly correlates with retention latencies (McIntyre et al. 2002). Therefore, selective activation or blocking of these receptors should be expected to modulate memory storage. To investigate this issue, the present experiments investigated the effects of post-training activation or blockade of α_2 -adrenoceptors in the BLA on retention of IA training. Experiment 1 examined the effects of pre- or post-training α_2 -adrenoceptor activation in the BLA on IA retention whereas Experiment 2 examined the effect of pre- or post-training blockade of BLA α_2 -adrenoceptors.

Results

Experiment 1: Effects of pre- or post-training BLA α_2 -adrenoceptor activation

Six animals did not survive surgery. Histological examination revealed that 83 animals had correct bilateral cannulae placements in the BLA. A representative cannula placement in the BLA is shown in Figure 1. The data of 30 animals with cannulae tips located outside the BLA were excluded from the analyses. Final groups were constituted as follows. Groups injected pre-training:

Figure 1. (Top) Schematic representation of the amygdaloid complex. The solid lines indicate the position of the photomicrograph (bottom) representing the cannula (upper arrow) and the injection tip (lower arrow) placement. BLA, basolateral nucleus of the amygdala; CN, central nucleus of the amygdala.

Figure 2. The effects of pre- and post-training infusions of various doses of idazoxan (a selective α_2 -adrenoceptor antagonist) into the basolateral amygdala on inhibitory avoidance retention latencies. Error bars represent mean $\pm$ SEM latency (in seconds) to enter the dark compartment on the retention test. Pre-training groups were infused 20 min before training whereas post-training groups were infused immediately after the footshock administration. * $P < 0.05$ compared with the corresponding group of the other condition; *** $P < 0.001$ compared with vehicle-injected group; • $P < 0.05$; ••• $P < 0.001$; •• $P < 0.01$ compared with 0.3 μ g of idazoxan-injected group in the same condition. $n = 9$ –13 per group.

Vehicle: $n = 9$; idazoxan 0.2 μ g: $n = 11$; idazoxan 0.3 μ g: $n = 9$; idazoxan 0.4 μ g: $n = 8$. Groups injected post-training: Vehicle: $n = 211$; idazoxan 0.2 μ g: $n = 13$; idazoxan 0.3 μ g: $n = 12$; idazoxan 0.4 μ g: $n = 10$.

The retention test latencies are shown in Figure 2. The mean ($\pm$ SEM) retention latencies of the pre- and post-training vehicle injected groups were 118.66 ± 17.88 and 128.21 ± 13.29 sec, indicating that the 0.4 mA footshock induced retention of the IA training, as training latencies were ~ 15 sec for both groups. A two-way ANOVA revealed a significant effect of idazoxan ($F_{(3,75)} = 8.83$; $P < 0.001$); an effect of time of injection ($F_{(1,75)} = 6.24$; $P < 0.05$) and no interaction between the two factors. In the pre-training condition, post-hoc between-group comparisons indicated that group infused with 0.3 μ g idazoxan had significantly longer latencies than those infused with 0.2 μ g idazoxan ($P < 0.05$) and approached significance when compared to the control group ($P = 0.057$). In the post-training condition, post-hoc between-groups comparisons indicated that the latencies of the group infused with 0.3 μ g of idazoxan were significantly longer than those of the controls ($P < 0.001$) as well as those infused with either the 0.2 or 0.4 μ g doses of the α_2 -adrenoceptor antagonist ($P < 0.05$ and 0.01 respectively). Post-hoc comparisons also indicated that the group infused with 0.3 μ g of idazoxan post-training had longer retention latencies than those of the corresponding group infused pre-training ($P < 0.05$).

Experiment 2: Effects of pre- and post-training BLA α_2 -adrenoceptor blockade

Three animals did not survive surgery. Histological examination revealed that 81 animals had correct bilateral cannulae placements in the BLA. Nineteen animals that had placements outside the BLA were excluded from the analyses. Final groups were constituted as follows. Groups injected pre-training: vehicle: $n = 10$; UK (UK 14,304) 0.3 ng: $n = 9$; UK 1.0 ng: $n = 10$; UK 3.0 ng: $n = 9$.

Figure 3. The effects of immediate post-training infusions of various doses of UK 14,304 (a selective α_2 -adrenoceptor agonist) into the basolateral amygdala on inhibitory avoidance retention latencies. Error bars represent mean $\pm$ SEM latency (in seconds) to enter the dark compartment on the retention test. Pre-training groups were infused 20 min before training whereas post-training groups were infused immediately after the footshock administration. ** $P < 0.01$ compared with the corresponding group of the other condition; *** $P < 0.001$ compared with vehicle-injected group; ••• $P < 0.001$ compared with 0.1 and 1.0 ng of UK 14,304-injected group; ♦♦♦ $P < 0.01$ compared with 1.0 and 3.0 ng of UK 14,304-injected group. $n = 9$ –12 per group.

Groups injected post-training: vehicle: $n = 9$; UK 0.3 ng: $n = 10$; UK 1.0 ng: $n = 12$; UK 3.0 ng: $n = 12$.

The retention test latencies are shown in Figure 3. The mean ($\pm$ SEM) retention latencies of the vehicle groups injected pre- and post-training were 273.88 ± 26 and 296.47 ± 47.48 sec, respectively, indicating that the 0.5 mA footshock induced strong retention of the IA training. A two-way ANOVA revealed a significant effect of UK 14,304 ($F_{(3,73)} = 16.37$; $P < 0.001$), no effect of time of injection ($F_{(1,73)} = 0.49$; $P = 0.48$) and a significant interaction between these two factors ($F_{(3,73)} = 3.00$; $P < 0.05$). In the pre-training condition, post-hoc between-group comparisons indicated that only the group infused with 3.0 ng UK 14,304 had significantly shorter latencies than those of the vehicle controls ($P < 0.01$), the 0.3 and 1.0 ng of UK 14,304 ($P < 0.001$). In the post-training condition, post-hoc between-group comparisons indicated that the animals infused with 1.0 and 3.0 ng of UK 14,304 had significant shorter latencies than those infused with the vehicle ($P < 0.001$) and the animals infused with the 0.3 ng of α_2 -adrenoceptor agonist. Moreover, comparisons indicated that the group infused pre-training with 1.0 ng of UK 14,304 had significant higher latencies than those of the corresponding post-training group ($P < 0.01$).

Discussion

The findings of these experiments provide evidence that pre-synaptic α_2 -adrenoceptors in the BLA are involved in modulating the consolidation of IA memory. Post-training intra-BLA infusions of the selective α_2 -adrenoceptor antagonist idazoxan induced a dose-dependent memory enhancement, whereas post-training infusions of the selective α_2 -adrenoceptor agonist UK 14,304 induced memory impairment. In experiment 1, post-training injection of 0.3 μ g of idazoxan induced an enhancement of retention that was greater than that induced by injections administered 20 min before training. In the second experiment, pre-training intra-BLA infusions of only the highest dose (3.0 ng)

of UK 14,304 impaired retention, whereas post-training infusions of both the 1.0 ng and the 3.0 ng doses impaired retention.

These results are consistent with those of studies in which systemic injection of selective α_2 -adrenergic drugs were found to disrupt and enhance consolidation of IA learning in the rat (Chopin et al. 2002). In addition, they fit with previous reports suggesting that the effects of peripheral administration of α_2 -adrenergic compounds on learning and memory performance are mediated through a direct action on central NE release (Abercrombie et al. 1988; Thomas and Holman 1991; Zarrindast et al. 2000; Chopin et al. 2002).

These findings are consistent with extensive prior evidence indicating a selective involvement of the BLA in adrenergic influences on memory storage (Roosendaal and McGaugh 1996, 1997; Quirarte et al. 1997). They are also consistent with previous results implicating amygdala α_2 -adrenoceptors in footshock based learning (Fendt et al. 1994; Schulz et al. 2002). Moreover, they clearly indicated that, in addition to involvement of α_1 - and β -adrenoceptors in the BLA (Ferry et al. 1999a,b), the α_2 -adrenoceptors participate in mediating the effects of training-induced or experimentally administered NE on memory storage. As pre-synaptic α_2 -negative feedback is known to regulate NE release (Starke 2001), including NE release in the amygdala (Langer 1974; Starke 1979; Fendt et al. 1994; Talley et al. 1996), the present findings provide additional evidence that memory consolidation is regulated by noradrenergic activation within the BLA.

Previous studies have found that IA memory, as well as memory for other kinds of training, is enhanced by post-training intra-BLA infusions of NE or the β -adrenoceptor agonist clenbuterol (Ferry and McGaugh 1999; Hatfield and McGaugh 1999). Further, the memory modulating effects of drugs affecting several modulatory influences, including those of the adrenal stress hormones epinephrine and corticosterone, GABAergic effects, and opioid peptidergic effects, are blocked by intra-amygdala infusions of the β -adrenoceptor antagonist propranolol (Liang et al. 1986; McGaugh et al. 1988; Introini-Collison et al. 1989; Introini-Collison et al. 1995; McGaugh et al. 1996; Quirarte et al. 1997). These findings are consistent with evidence from studies using *in vivo* microdialysis and high-performance liquid chromatography to assess NE release in the amygdala. Drugs that enhance memory, including epinephrine, the GABAergic antagonist picrotoxin, or the opioid antagonist naloxone enhance amygdala NE release after a footshock administration, whereas drugs that impair memory, including the GABAergic agonist muscimol and the opioid receptor agonist β -endorphin decrease the footshock-induced potentiation of NE release in the amygdala (Galvez et al. 1996; Quirarte et al. 1998; Williams et al. 1998; Hatfield et al. 1999). Additionally, footshock stimulation has been shown to increase NE levels in the amygdala and the amount released varies with the footshock intensity (Quirarte et al. 1998). Further, the level of NE release in the amygdala during IA training correlates highly with subsequent retention (McIntyre et al. 2002); i.e., better memory for IA training is displayed by animals that had higher levels of NE release in the BLA following training.

Several studies have shown that noradrenergic activation is critically important for physiological and behavioral responses to stressors (Redmond and Huang 1979; Bremner et al. 1996). The amygdala receives noradrenergic input from the locus coeruleus and the nucleus of the solitary tract (Fallon et al. 1978; Foote et al. 1983) which is activated by aversive stimuli such as footshock (Cedarbaum and Aghajanian 1978; Chiang and Aston-Jones 1993; Williams et al. 1998; Hassert et al. 2004). And, as noted above, increased amounts of NE are released in the amygdala by aversive footshock stimulation (Tanaka et al. 1991; Galvez et al.

1996; Quirarte et al. 1998; Williams et al. 1998). In addition, the α_2 -adrenoceptor antagonist yohimbine has been reported to amplify the immobilization stress-induced release of NE in the amygdala (Khoshbouei et al. 2002) with an amplitude comparable to that observed in the same brain region after a footshock administration (Quirarte et al. 1998; Hatfield et al. 1999), whereas clonidine, an α_2 -adrenoceptor agonist, has been reported to attenuate the footshock-induced NE release (Erb et al. 2000). Thus, the present findings are consistent with the hypothesis that the memory modulation induced by NE release in the amygdala result from the binding to α_2 -adrenergic autoreceptors that regulate the stress-induced release of NE in the BLA.

For example, because of the limits of the microdialysis technique cited, it is difficult to speculate when and for how long after a single infusion our adrenergic drugs induce their effects on NE release, since the minimal time interval between samples collection reported with this technique is about 15 min. However, the maximal effect of α_2 -adrenergic drugs on NE release has been observed 30 min after their infusion (Van Gaalen et al. 1997; Mateo and Meana 1999). In reference to the delay between administration of IA footshock during training and the peak of extracellular NE that is maximal after 30 min (McIntyre et al. 2002), it is likely that the effects of pre-training local injections of UK 14,304 and idazoxan into the BLA mainly resulted from the binding to α_2 -adrenergic autoreceptors that regulate the stress-induced release of NE. The fact that retention latencies were only influenced by the highest dose of UK 14,304 suggests that the first pool of footshock-induced NE release occurring seconds or minutes after shock administration is sufficient to enable the CS-US association. Additional studies using more sensitive measures to detect NE release with modern dosage techniques will probably help to confirm such a hypothesis.

Our finding that pre-training intra-BLA infusions of UK 14,304 and idazoxan induce effects on memory that were similar but of a smaller amplitude to those obtained in post-training groups suggests that the α_2 -adrenoceptor system in the BLA is more probably involved in a very fine memory-modulated tuning control of IA consolidation rather than in the encoding of CS-US association (see Schulz et al. 2002) during IA. The post-training effects are clearly consistent with the hypothesis that IA consolidation depends critically on the training-induced prolonged release of amygdala NE. As noted above, the evidence from microdialysis studies indicates that drugs infused into the BLA post-training affect the NE release at a maximal level around 30 min after their infusion (Van Gaalen et al. 1997; Mateo and Meana 1999). Moreover, this time interval has been described to be critical for the involvement of the amygdala during consolidation of IA (Bevilaqua et al. 1997; Izquierdo et al. 1997). Therefore, it is likely that the enhanced and inhibitory effects obtained by post-training injections of idazoxan and UK 14,304, respectively, on IA retention were due to the modulation of the prolonged training-induced increase in NE levels (McIntyre et al. 2002). Additionally, and importantly, our findings are consistent with the evidence of Pelletier et al. (2005) that a single footshock increases the firing of neurons in the basolateral amygdala and that the increase peaked after 30 min and subsided within 2 h.

In conclusion, our findings show that α_2 -induced modulation of NE release during post-trial consolidation significantly influences IA retention performance. Thus, they provide additional evidence of the memory-modulating role of NE release in the BLA. In addition, the findings are consistent with other evidence suggesting that α_2 -adrenoceptors in the BLA are a critical component in the modulating influence of NE on the IA memory consolidation and that the effect is probably due to prolonging the increase in training-induced levels of NE within the BLA.

Materials and Methods

Animals

Male Sprague Dawley rats ($n = 222$; body weight, 275–300 g at the time of surgery) obtained from Charles River Laboratories were used. After arrival, they were housed individually in a temperature-controlled (22°C) colony room and maintained on a standard 12-h light/dark cycle (light on at 07:00 h) with free access to food and water. All experiments were carried out during the light phase of the cycle between 10:00 and 14:00 h. The number of animals in each group is shown in the figure legends.

Surgery

One week after arrival, the animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and given atropine sulphate (0.4 mg/kg, i.p.) to suppress salivation. The skull was fixed in a flat position to a stereotaxic frame (Kopf Instruments) and stainless steel guide cannulae (23 gauge, 15 mm long) were implanted bilaterally 2 mm dorsal to the BLA (coordinates: anteroposterior, -2.8 mm from Bregma; mediolateral, $+5.0$ mm from midline; dorsoventral, -6.7 mm from the skull surface) according to the atlas of Paxinos and Watson (1998). The cannulae and two anchoring screws were affixed to the skull with dental cement. Stylets (15-mm long 00 insect dissection pins) were inserted into each cannula to maintain patency and were removed only for the infusion of drugs. The rats were allowed to recover from surgery a minimum of 7 d before training was initiated.

Inhibitory avoidance (IA) apparatus and procedures

The IA apparatus consisted of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, 6.4 cm wide at the floor) divided into two compartments separated by a sliding door that opened by retracting into the floor. The starting compartment (31 cm long) was illuminated and the shock compartment (60 cm long) was dark (McGaugh et al. 1988). The apparatus was located in a light- and sound-attenuated room.

The rat was placed in the starting compartment, with the door opened, and was allowed to enter the dark compartment. After the rat stepped completely into the dark compartment, the door was closed and a mild inescapable footshock with a duration of 1.0 sec was administered. Animals showing entrance latencies longer than 30 sec were eliminated from the study. The footshock intensity was adjusted for each experiment (0.4 mA for Experiment 1; 0.5 mA for Experiment 2). Bilateral microinfusions of adrenergic drugs were administered either 20 min before being placed in the dark alley (pre-training) or immediately after being removed from the dark alley (15 sec after termination of the footshock, post-training). On the 48-h retention test trial, the rat was placed in the starting compartment, as in the training session, and the latency to re-enter the dark compartment (maximum latency of 600 sec) was recorded and used as the measure of retention. Shock was not administered on the retention test trial.

Drugs

For Experiment 1, idazoxan hydrochloride (0.1, 0.2, 0.3, or 0.4 μ g; kindly provided by the Center de Recherche Pierre Fabre, Castres, France), a selective α_2 -adrenoceptor antagonist (MacDonald and Scheinin 1995), was dissolved in 0.9% saline solution. Control animals received saline only. The doses of idazoxan were selected on the basis of previous behavioral experiments (Liang et al. 1995; Ferry et al. 1999a).

For Experiment 2, UK 14,304 [5-bromo-N-(4,5-dihydro-1-H-imidazol-2-yl)-6-quinoxalinamine] (0.3, 1.0, or 3.0 ng; Tocris Cookson Inc.), a selective α_2 -adrenoceptor agonist (Atkinson and Minneman 1991) was dissolved in 0.9% saline solution. Control animals received saline only. The doses of UK 14,304 were selected on the basis of previous behavioral experiments (McGaugh et al. 1988; Ferry and McGaugh 1999). Solutions of all drugs were prepared freshly before each experiment.

Infusion procedures

Bilateral pre- or post-training infusions of adrenergic agonists and antagonists into the BLA were administered through 30-gauge injection needles connected to a 10- μ L Hamilton microsyringe by polyethylene tubing. The injection needles protruded 2 mm beyond the cannula tips to reach the BLA. A 0.2- μ L injection volume per side was infused for 30 sec by an automated syringe pump (Sage Instruments). To allow diffusion of the drug, the injection needles were retained within the cannulae for an additional 50 sec after drug infusion. The injection volume was selected on the basis of previous experiments showing that selective neurotoxically induced lesions of the BLA are produced with an infusion volume of 0.2 μ L (Ferry et al. 1995). Furthermore, drug infusions of this volume into either the BLA or the adjacent central amygdala induce differential effects on memory storage (Parent and McGaugh 1994).

Histology

After completion of behavioral testing, the rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 0.9% saline (w/v) solution followed by 10% formaldehyde (v/v). At least 24 h before sectioning, the brains were placed in a 15% sucrose (w/v) solution for cryoprotection. Sections of 40 μ m were made using a freezing microtome and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the BLA was made according to the standardized atlas plates of Paxinos and Watson (1998).

Statistics

Retention data were analyzed with two-way ANOVAs with idazoxan (four levels) or UK 14,304 (four levels) both as between-subject variables, and time of injection (two levels). Further analysis used Fisher's post hoc tests to determine the source of the detected significances in the ANOVAs. A probability level of <0.05 was accepted as statistically significant. The number of animals per group is indicated in the figure legends.

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