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Correlation of [^{3}H]Diazepam Binding Density With Anxiolytic Locus in the Amygdaloid Complex of the Rat

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Using [^{3}H]diazepam binding, high concentrations of receptors were found in the frontal cortex and lateral amygdala. Infusions of chlordiazepoxide into the lateral amygdala induced a release of responding measured during the component of a conditioned emotional response task previously associated with an aversive stimulus. The lateral amygdala appears to be an important component of the forebrain circuitry involved in the expression of anxiety and sensitive to benzodiazepine drugs.

INTRODUCTION

The amygdaloid complex has long been identified as an important centre for the regulation of emotional behaviour[4]. Weiskrantz originally suggested that the effect of amygdalectomy in monkeys tested on avoidance tasks was to make it difficult for animals to identify reinforcing stimuli[32]. Later recording[22] and lesion studies in monkeys[7,14] and rats[23] have supported the idea of an amygdaloid mechanism for evaluating the motivational significance of sensory stimuli. Lesions and disruptive electrical stimulation of the amygdaloid complex in rats selectively release spontaneous and conditioned avoidance behaviour, indicating that the amygdaloid complex in this species is perhaps most critically involved in the evaluation of aversive stimuli[5,6,27]. One of the most striking and characteristic behavioural effects of benzodiazepine drugs is also to release behaviour suppressed by aversive stimuli such as electric shock[3]. The present experiments test the hypothesis that activation of benzodiazepine receptors concentrated within the amygdaloid complex would produce anxiolytic effects. The distribution of benzodiazepine receptors in the amygdaloid complex and other major components of the limbic system was estimated primarily to provide a guide for complementary microinfusion experiments, and also as an additional comparison with mapping studies from other laboratories[33]. The behavioural effects of microinfusions of benzodiazepines into subregions of the amygdaloid complex rich in receptors were measured using a 'conflict' test[26] modified for intracerebral microinfusion studies[27]. The modified test is sensitive to benzodiazepines administered parenterally and to blockade of these effects by the benzodiazepine antagonist Ro 15-1788, allows repeated drug treatments on a stable baseline, yet requires only 8 sessions of training[27,28].

The effects of the intra-amygdaloid infusions on pain threshold in the tail-flick test were also measured to evaluate the possible analgesic effects[21] of BZ at doses showing an anxiolytic action. [^{3}H]Diazepam was used for the binding studies, but as the ethanol/propylene glycol/saline solvent vehicle necessary for i.v. administration of diazepam was unsuitable for direct intracerebral infusion, the water-soluble compound chlordiazepoxide was used for the infusion experiments.

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MATERIALS AND METHODS

[^{3}H]Diazepam binding

Animals were killed by decapitation. The brain was exposed by a single median sagittal scissor cut through the skull and retraction of the cranial bones. The brain was removed on a spatula and positioned ventral surface uppermost on an ice-cooled stainless steel plate. Transections and finer cuts were made with mounted razor blades, and the tissue manipulated with fine forceps. The dissection typically took 4–5 min to generate samples of frontal cortex, striatum, septum, hypothalamus, hippocampus, amygdaloid complex, entorhinal cortex, cerebellum, brainstem and spinal cord. Samples were frozen immediately on aluminium foil overlying solid CO_2 and then stored in polythene vials. Subregions of the amygdaloid complex were dissected from frozen sections on a freezing-stage microtome (Frigistor). Frozen brains were transferred from liquid nitrogen, truncated posteriorly and placed anterior surface uppermost on the freezing-stage. Four successive sections of 500 μm thickness were then cut through successive levels of the amygdaloid complex, beginning at the level of the anterior commissure (0.0 in the atlas of Pellegrino and Cushman[19]).

As each section was exposed, 4 subregions of the amygdaloid complex (lateral, basolateral, centralmedial and cortical) were dissected out under a binocular microscope with fine scalpel blades (Swann-Morton No. 11), using the anterior commissure, optic tract and lateral ventricle as landmarks.

Frozen tissue was homogenized to 10 mg·ml^{-1} in 50 mM Tris/HCl (Trizma) buffer (pH 7.4) at 4 °C. Pre-cooled assay reagents were then added to assay tubes in an ice bath at 4 °C. Final concentrations of the reagents in the assay (200 μl total volume) were, in order of addition: tissue homogenate 10 mg·ml^{-1}, 5 micromolar 'cold' diazepam (Roche) or de-ionized water, and 10 nM [^{3}H]diazepam (3.22 TBq/mmol, or 87 Ci/mmol, Amersham Radiochemicals). Assay tubes, replicated in triplicate, were incubated for 45 min at 4 °C. Tube contents were transferred to a 12-port binding manifold (Millepore) in two 5 ml washes of 50 mM Tris, and vacuum filtered (minimum vacuum 20 mm Hg: Whatman GF/C filters). Filters were removed and dried in a jet of warm air for a minimum of 60 min, then immersed in scintillation fluid composed of 4.2 g PPO (2.5-diphenyloxazole) and 50 mg PDPOP (1,4-bis-2-5-phenyl-oxazolyl-benzene) per litre of sulphur-free toluene. Samples were then counted to constant period (10 min) on an Isocap 300 liquid scintillation counter (Nuclear Chicago). The ESP-variable quench mode of a more sophisticated counter (Searle Model 6880 MK. 3) was used to check quench. Quench was sufficiently low and constant for the direct use of untransformed counts per minute (cpm) data to be used in subsequent calculations. The range of tissue counts was 1000–22,000 cpm, with filter blanks typically below 100 cpm. Bound radioactivity in cpm was converted to binding density in fmol per mg tissue (w. wt.); the reference standard was always 2 pmol of ligand spotted directly onto the filter. Each data point was the mean of the 3 assay replicates, the samples for which were all based on a minimum of 6 pooled samples.

Two methods were employed to estimate maximal specific receptor binding in the brain regions assayed: the estimates of choice were based on extrapolation from Scatchard plots, but where this was impractical, as in the case of very small intra-amygdaloid samples, relative estimates of binding density were made at fixed ligand concentration.

Conflict test

Rats (n = 11) were first trained to press a lever on an FR1 schedule in a standard conditioning chamber. All animals then received a 20-min conditioning session each day for 8 days. Each session was partitioned into alternating 5-min light (L) and 2.5-min dark (D) periods in a fixed LDLDL sequence. During both periods (L,D), every lever-press delivered a food pellet; in the dark periods only, a random 50% of lever-presses also elicited a footshock (0.6 mA, 0.5 s). All conditioning contingencies were controlled by BASIC programs running on a low-cost laboratory microcomputer (Acorn). All drugs were administered i.p. in 0.9% saline. Animals were tested in extinction sessions (i.e. shock off) separated by 1 or 2 sessions of re-training to maintain baseline responding. Results are expressed as mean lever-pressing rates over the 5-min periods. The Wilcoxon test was used throughout for statistical comparisons.

Analgesia test

Rats (n = 11) were lightly restrained and their tails

positioned in a groove over a 1 cm hole shielded from a 1 kW heat lamp positioned 6 in. below it. The shield plate was then removed to expose the 1 cm hole and the latency for the rat to flick its tail away from the heat source measured with a stopwatch.

RESULTS

Binding

Specific binding was saturable and linear around the range of tissue concentration used for estimates of binding density. Binding estimated by extrapolation from Scatchard plots was high in frontal cortex, intermediate in hippocampus, entorhinal cortex, amygdaloid complex and hypothalamus, and low in striatum, septum and brainstem (Fig. 1A). Estimates at fixed ligand concentration yielded a very similar pattern of binding density except for a slightly lower value for the hippocampus (Fig. 1B). Within the amygdaloid complex, binding was highest in the lateral sample (Fig. 1B).

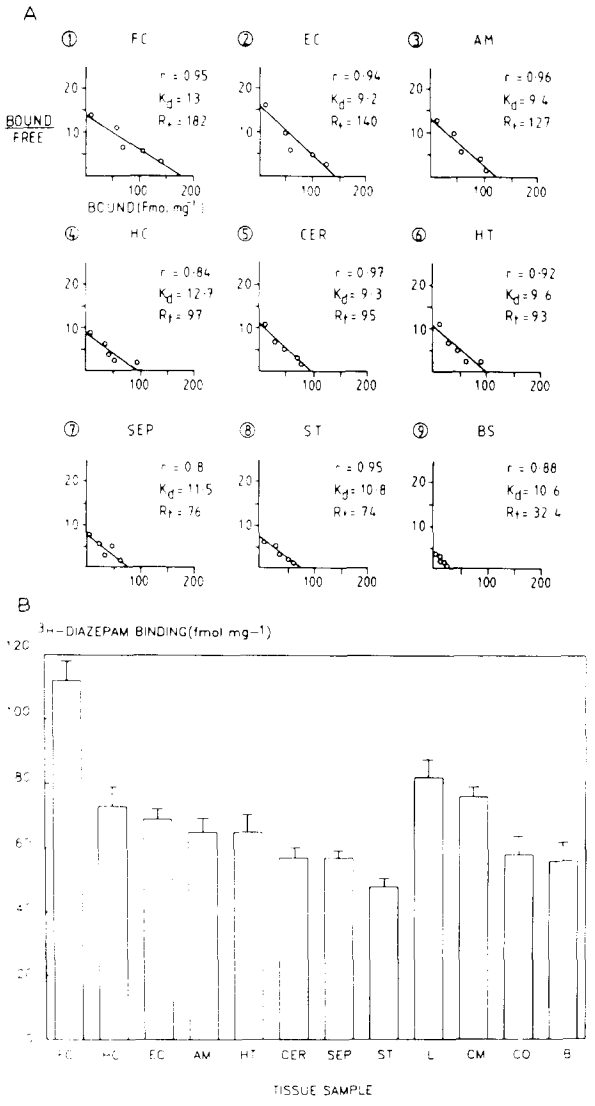

Fig. 1. Estimates of [^{3}H]diazepam binding density in various regions of rat brain and within the amygdaloid complex. A: estimates from Scatchard plots. B: estimates taken at 10 nM ligand concentration (means I s.c.s., n = 6). FC, frontal cortex; HC, hippocampus; EC, entorhinal cortex; AM, amygdaloid complex; HT, hypothalamus; CER, cerebellum; SEP, septum; ST, striatum; L, lateral amygdaloid area; CM, central-medial amygdaloid; CO, cortical amygdaloid area; B, basal amygdaloid area.

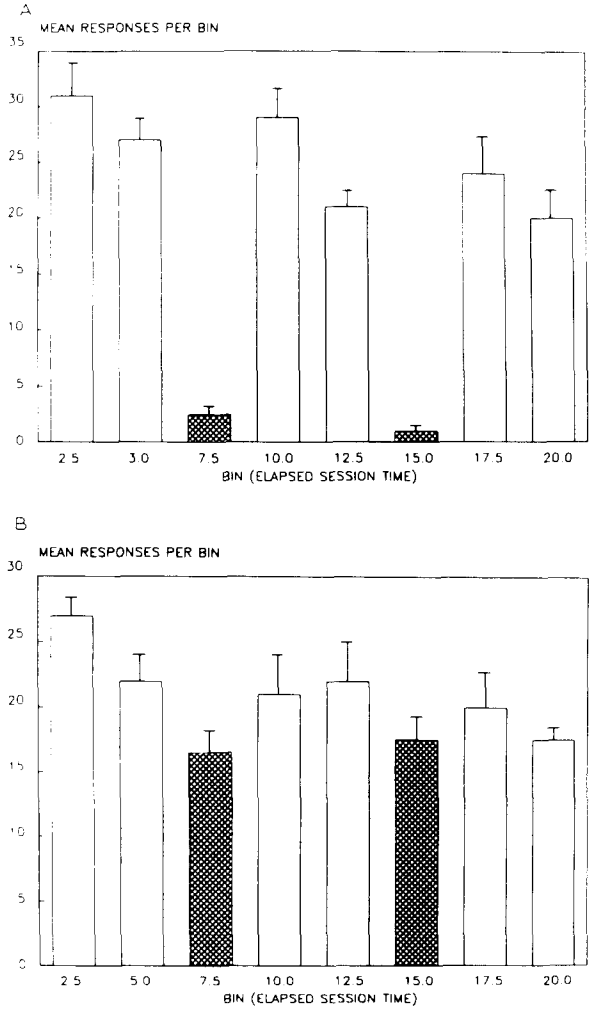

Fig. 2. Effect of infusion of chlordiazepoxide into the lateral amygdaloid complex on the rat conflict test (means ± S.E.M., n = 11). Dark periods shaded, light periods unshaded. A: saline control infusion (1 μl at 0.5 μl per min). B: chlordiazepoxide infusion (10 μg in 1 μl at 0.5 μl per min); release effect significant with respect to saline control ($P < 0.001$, Wilcoxon test).

Conflict test

Acquisition was very rapid. Subjects showed suppression confined to the dark periods during the first training session, which after 8 sessions reached a stable asymptotic level. Microinfusions of chlordiazepoxide (10 μg in 1 μl saline at a rate of 0.5 μl· min^{-1}) into the lateral amygdaloid complex produced highly significant release of responding in the dark periods with no effect on responding in the light (Fig. 2). Control infusions of saline into the lateral amygdaloid complex, and of the same dose of chlordiazepoxide to a site 1 mm dorsal to the amygdaloid complex produced no release (Fig. 3). The releasing effect of CDP was severely attenuated when the shocks normally omitted on test days were instead included ($P < 0.001$, Wilcoxon; Fig. 3). The inclusion of shock did not however quite reduce responding to saline control levels ($P < 0.01$, Wilcoxon; Fig. 3). According to Myers[16], the small, low-rate infusions used would be expected to produce a radial spread of about 0.5 mm from the site of infusion. The spread of an infusion of 1 μl of [^{3}H]diazepam stock (10 nM) into the lateral amygdaloid area was measured directly. Samples dissected and frozen 5 min after a standard infusion (1 μl over 2 min) revealed approximately 17,000 cpm of activity in the amygdaloid complex, compared to 300 cpm in the next highest region, the ventral hippocampus.

Analgesia test

An identical infusion of chlordiazepoxide into the lateral amygdaloid complex in the same group of 11 rats had no effect on tail-flick latency when compared with saline vehicle (5.7 ± 0.5 S.E.M. and 4.8 ± 0.7 s, respectively; n.s., Wilcoxon test).

DISCUSSION

The binding results from this study are in striking agreement with the most recent findings based on

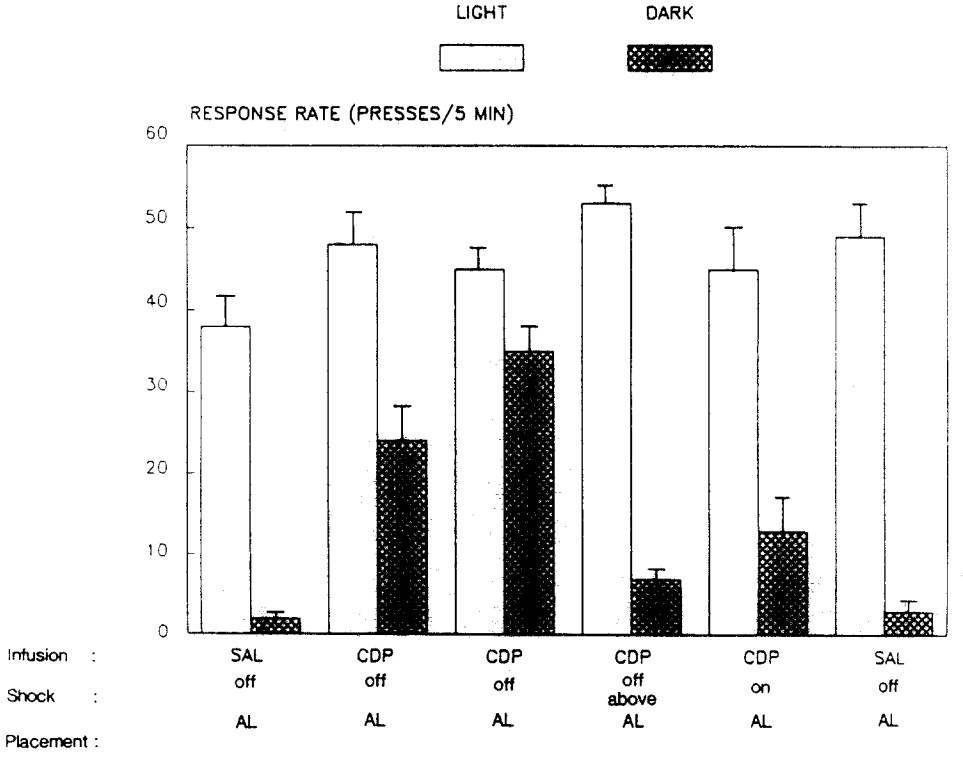

Fig. 3. Summary (means ± S.E.M., n = 11) of the effects of intra-amygdaloid chlordiazepoxide on the rat conflict test for all combinations of infusions, shock and placement tested. Compared with saline controls (SAL), chlordiazepoxide (CDP) infusions into the lateral amygdaloid complex (AL) significantly increased responding in the dark (shaded) periods in the absence ($P < 0.001$, Wilcoxon test) and presence ($P < 0.005$, Wilcoxon test) of shock. The central placement had no significant effect, and response values in the light (unshaded) periods were also unaffected (all n.s., Wilcoxon test).

autoradiographic mapping[17] revealing the lateral area to have the highest density of [^{3}H]diazepam binding within the amygdaloid complex. Microinfusions of a small dose of chlordiazepoxide into the lateral area of the amygdaloid complex produced a selective release of suppressed responding with no non-specific effects. This effect was not produced by extra-amygdaloid infusions of chlordiazepoxide or of saline, and the effective intra-amygdaloid dose of chlordiazepoxide did not show any analgesic effect on the tail-flick test. Similar infusions using [^{3}H]diazepam solution suggested that spread after 5 min was minimal. Such a time-course and Myers' studies[16] render it likely the infusion had its anxiolytic effect before it had spread significantly beyond the site of injection.

Peripheral administration of chlordiazepoxide has an identical releasing effect, recently shown to be blocked by the benzodiazepine antagonist Ro 15-1788 (ref. 28). These findings strongly suggest that the lateral amygdaloid complex is a key site for the anxiolytic action of the benzodiazepines.

The presence of shock greatly attenuated the releasing effect of CDP, indicating the release effect was specific to the conditioned emotional response (CER) and did not extend to behaviour suppressed by immediate punishment. Other actions of chlordiazepoxide cannot account simply for the confinement of the release phenomenon to the dark (shock signal) period. Muscle relaxant or sedative actions would have produced further suppression of responding and neither of these actions would be predicted to be specific to the dark periods.

The wider neural mechanisms by which activation of benzodiazepine receptors in the amygdaloid complex produces a specific anti-anxiety effect are unclear[24]. Recent anatomical studies[1,12,25,31] have supported earlier suggestions that the amygdaloid complex may be an important cortico-hippocampal relay[2,7]. The lateral area of the amygdaloid area contains the lateral nucleus which receives projections from the neocortex and projects to the hippocampal formation[11]. The lateral amygdaloid projection to the hippocampal system is known to have a strong modulatory electrophysiological influence on the entorhinal input to the dentate gyrus[29,30]. The population of benzodiazepine receptors in the lateral amygdaloid area is therefore in a position to modulate cortico-hippocampal neurotransmission, and this may be an important facet of the anxiolytic action of benzodiazepines.

In addition to its reciprocal links with the hippocampal formation, the basolateral area of the amygdaloid complex has prominent links with frontal cortex both directly and via the nucleus medialis dorsalis of the thalamus[10,20]. Recent cytoarchitectural studies have also supported the notion that the lateral and basolateral areas of the amygdaloid complex play a critical role in coordinating limbic forebrain areas that direct behavioural output[13]. Both the hippocampal formation and the amygdaloid complex send output to the nucleus accumbens septi[8,9].

Thus, different sectors of the amygdaloid complex project to hippocampus and to association cortex, a dissociation supported by the cytoarchitectural data. In turn, both the hippocampus and basolateral amygdaloid complex project to the nucleus accumbens, which receives a rich dopaminergic innervation[12] and serves as an interface between limbic structures and motor output mechanisms of the striatum[18]. Both in terms of its interactions with circuits in the limbic system and its access to motor mechanisms, the amygdaloid complex is viewed as a critical structure coordinating limbic forebrain areas that direct behavioural output. Lesion studies suggest that whereas the amygdaloid complex is critical for the coding of meaningful events, the hippocampus is involved in the memory of those events[15]. In the absence of these limbic structures, present experience fails to guide future behaviour.

One might speculate then that the amygdalo-hippocampal and amygdalo-frontal circuits, respectively, subserve the mnemonic and affective aspects of emotional experience. It is particularly striking that all of the key sites in this speculative scheme exhibit high local concentrations of benzodiazepine receptors. The strong agreement of biochemical, anatomical, physiological and behavioural data from this and other work suggests a prevalent role of benzodiazepine receptors in the amygdaloid and related limbic sites in anxiety.

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