Stress and putative endogenous ligands for benzodiazepine receptors: The importance of characteristics of the aversive situation and of differential emotionality in experimental animals

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Abstract. The relationships between anxiety/stress, possible endogenous ligands for benzodiazepine receptors and the behavioral modification by drugs are discussed in this short review, including the specific characteristics of elements involved in those interactions, e.g. ones concerning the aversiveness of the stressful situation and the nature of the organism under investigation. These are important factors when considering aversive tasks, insofar as they may involve stressful conditions which differ in intensity and in the degree of control afforded the subject. These characteristics may well lead to differing functional effects on GABA-gated chloride channels or, in other words, to an incongruous balance between endogenous benzodiazepine receptor agonist and inverse agonist activity. This is not surprising, as it is well known that different forms of stressors often actually produce divergent behavioral, physiological and biochemical effects. This review also illustrates the necessity of taking into account the variable effects of stressors and/or drugs on animals differing in reactivity or emotionality, even in the case of 'non-selected' stocks. The implication is made that, by genetic and/or environmental manipulation of the emotional state of the animals used, it will be possible to obtain more clearly definable results in neuropharmacological and psychopharmacological studies.

Key words. Anxiety; stress; endogenous benzodiazepine agonists and inverse agonists; aversive learning; flumazenil; Ro 15-4513; FG 7142; shuttle box avoidance; Roman high- and low-avoidance; rats; individual differences; handling effects.

There has been considerable speculation regarding the possible involvement of some endogenous benzodiazepine agonist or inverse agonist in the response to stressful situations and in the processes of aversive learning[29, 36], and this picture has been complicated further yet by much of the existing neuropharmacological and psychopharmacological data. Results from shuttle box avoidance experiments presented here, along with other studies, suggest that both the characteristics of the stressful tasks and of the animals used can determine behavioral effects obtained with benzodiazepine receptor ligands (either anxiolytic or anxiogenic substances, or even compounds without intrinsic pharmacological connotation, i.e. flumazenil). Also to be considered are the different functional roles which the GABA/chloride channels may play, depending upon the perceived aversiveness of the situation and/or the emotional state, or traits, of the subjects. As a powerful example of the role of the emotional baseline characteristics of the animals, results will be presented which were obtained in experiments performed with psychogenetically selected and bred rats.

Neuropharmacological studies using different aversive situations

Results reported in the literature dealing with the effects of stressors on benzodiazepine receptors present many inconsistencies, ranging from a decrease[40, 44] to an increase[49–51] of benzodiazepine receptor density in the brain after the application of similar stressors. This issue has been recently addressed by Havoundjian et al.[34, 35], who have found that '10 min ambient-temperature swim stress' or the sequential removal of animals from a common cage ('cohort removal') lead to an increase in the potency and efficacy of chloride ions to enhance ^{3}H-flunitrazepam binding in rat brain[34, 51], in addition to finding that this effect is confined to the chloride ionophore since, in the absence of chloride ions, neither benzodiazepine receptors nor GABA receptors (as measured by GABA-enhanced ^{3}H-flunitrazepam binding) were affected by the stressor. Consistent with these results, either '10 min swim stress' or 'cohort removal stress' resulted in an increase in the number and apparent affinity of ^{35}S-TBPS binding sites[34, 50] (which presumably represent the number of chloride channels in an 'open' conformation[52]). Furthermore, the demonstration that the in vitro addition of benzodiazepine receptor agonists (e.g. flunitrazepam) mimicked, in a non-additive manner, the stressor-induced changes in ^{35}S-TBPS binding lends biochemical support to the hypothesis that a putative endogenous modulator released during the stress response (at least from such mild stressors as swimming or cohort removal) should have benzodiazepine-like properties[50, 53]. This evidence is consistent with recent psychopharmacological findings reported by Izquierdo and coworkers[36, 37] showing that the effects of low, non-anxiogenic doses of flumazenil (as this drug has had an anxiogenic profile in certain tests[29]) enhance the acquisition or retention of some mildly stressful learning tasks. Evidence was presented to suggest that the release of a benzodiazepine agonist could be provoked by the stressor or by the anxiety accompanying some forms of learning under mildly stressful conditions[36].

Additional pharmacological and behavioral studies seem to provide further support for that suggestion. Thus, in agreement with previous results[49], Trullas et al.[53] reported that swim stress protected against DMCM- and picrotoxin-induced convulsions in mice, and flumazenil partially antagonized the effect of stress on picrotoxin-induced seizures. In addition, swim stress increased the lethality of pentobarbital in mice (from 30% in controls to 70% in swim-stressed animals)[53]. Taken together, the forementioned results from Trullas et al. suggested an increase in the function of the GABA/benzodiazepine/chloride complex following mild stressors, which would be consistent with the hypothesis that a benzodiazepine-like ligand could be released under such circumstances. Other results have, however, indicated the importance of taking into account the specific characteristics of the stressors applied (such as intensity, controllability and predictability) before any definitive conclusions may be drawn. Thus, unpredictable foot shock (which makes learning during the experimental situation and adaptation to the stressor more difficult) induces an increase in the convulsant potency of DMCM[53] suggesting, in this case, that if a putative endogenous modulator exists and is released during unpredictable foot shock stress, it should have anxiogenic rather than anxiolytic properties. In support of this proposal, Corda and coworkers have found that foot shock stress induced a decrease in the number of low-affinity GABA sites in handling-habituated (i.e. less emotional) rats, and that such a decrease was also antagonized by flumazenil at doses without per se activity in that assay[15]. As the consequences of foot shock in the last procedure were mimicked by the administration of an anxiogenic β-carboline[6], it was suggested that the ability of flumazenil to antagonize the effects of foot shock supported the contention that an anxiogenic endogenous ligand might be involved in the down-regulation of low-affinity $GABA_A$ sites following the administration of the stressor. These data, together with other findings[1,43,54], imply that variables such as stressor predictability, controllability and intensity have a critical influence on the functional consequences of stress. Therefore, predictable (or controllable) stressors, to which the organism can adapt, might have an opposed functional effect on GABA-gated chloride channels to those exerted by unpredictable (or uncontrollable) stressors.

In addition, another factor must also be taken into account when studying the effects of stressors, that being the fact that different stocks of rats differ widely in regard to their susceptibility to such procedures. A case in point would be that of the induction of shock-induced 'learned helplessness', to which many stocks of rats appear to be impervious[56]. Other important evidence supporting both the necessity of controlling for individual differences in emotional reactivity and the involvement of putative anxiogenic/anxiolytic endogenous ligands in stressful situations comes from results demonstrating that the anxiety (as measured in the elevated plus maze) induced by benzodiazepine withdrawal in rats is either prevented, or not, by 4 mg/kg flumazenil, depending upon whether the baseline anxiety levels of the animals are high or low, respectively[2,3,30], and from the divergent and/or complementary effects of flumazenil and handling on young Roman high- and low-avoidance (RHA/Verh and RLA/Verh) rats exploring a labyrinth[25,28]. These psychogenetically-selected and bred lines of rats will be returned to later in this review.

The shuttle box as an aversive situation: An introduction to the consideration of individual differences in emotional reactivity

Taking into account, as an experimental variable, differences in emotional state of the animals used, is probably of just as vital importance in neuropsychopharmacological research as are the forementioned characteristics of the aversive situation. Many discrepancies among these types of studies, which have generally been conducted within narrowly-defined boundaries with non-selected animals, can probably be explained on that basis. We have consistently observed that antianxiety agents (i.e. benzodiazepines, triazolobenzodiazepines and pentobarbital) potentiate the early acquisition of two-way, active (shuttle box) avoidance in a single, 40-trial session[4,7,8,23]. These results are in agreement with many, previously reported findings[32,41,46–48], but do not support recent suggestions[36,37] (derived from studies using different tests) that acquisition during a stressed or anxious state may be impaired by the release of endogenous benzodiazepine agonist ligands. If that were so, an impairment of early two-way avoidance acquisition would be expected following benzodiazepine administration but, in fact, just the opposite (i.e. a clear improvement) has been found by us and others[32,41,42,46–48]. We have further extended these data in studies which indicate that the acute injection of the partial inverse agonists for benzodiazepine receptors, Ro 15-4513 (Hoffmann-La Roche & Co.) or FG 7142 (Schering S.A.), two drugs with known anxiogenic and proconvulsant effects[16–18], clearly reduced two-way, active avoidance acquisition at doses devoid of effects on locomotor activity (table 1). In fact, measures of activity (= crossings between compartments during the habituation period before the session: data not shown) demonstrated that none of the doses of Ro 15-4513 (2–10 mg/kg) had sedative effects ($F_{(3.25)} = 2.2372$, n.s., ANOVA). FG 7142 at 5 mg/kg also did not reduce crossings, whereas the two higher (10–15 mg/kg) doses showed a sedative action ($F_{(3.19)} = 9.8758$, $p = 0.0004$, ANOVA; $p = 0.05$, Duncan's test vs control group). As the doses of Ro 15-4513 and FG 7142 which reduced avoidance acquisition without reducing motor activity are well within the anxiogenic range used in other tests[16–18], we interpret the

Table 1. Effects of Ro 15-4513 and FG 7142 on the early acquisition of two-way active (shuttle box) avoidance in Sprague-Dawley (IFFA-Credo) rats

	% of avoidance responses (Mean ± SEM)	F Ratio	F Prob.
Vehicle	41.2 ± 9.2		
Ro 15-4513			
2 mg/kg	42.0 ± 11.2		
5 mg/kg	33.5 ± 8.5	3.6	0.028
10 mg/kg	8.0 ± 2.5*		
Vehicle	59.5 ± 7.5		
FG 7142			
5 mg/kg	16.5 ± 3.7*		
10 mg/kg	10.0 ± 3.5*	12.2	0.0001
15 mg/kg	21.7 ± 8.7*		

5–8 rats/group were submitted to a single two-way active (shuttle box) avoidance session which consisted of 40 trials, as follows: 10 s of a tone (CS) followed by 30 s of electric foot-shock (US, 0.4 mA). The CS and US were terminated by the animal crossing to the other compartment, the response being considered to be an avoidance response when it occurred in the presence of the CS. Once the shock was over or a response was made, a 50-s rest period followed. A 10-min habituation period preceded the shuttle box session (see text for results), and crossings between compartments during that period were used as a measure of spontaneous activity. Drugs were suspended in 1% carboxymethylcellulose and intraperitoneally injected in 2 ml/kg 10 (FG 7142), or 30 min, before testing. * $p < 0.05$ vs respective 'vehicle' group.

decrease in avoidance responding as likely due to increased anxiety.

The contention that an increase in anxiety could account for these effects of the two partial inverse agonists is also supported by three previous sets of data coming out of studies using non-pharmacological approaches to the same question. First of all, behavior genetic studies have demonstrated two independent, genetic influences in the determination of shuttle box avoidance acquisition, one for poor performance during early training trials (a passive avoidance tendency) and the other for good performance on later trials (the active avoidance tendency). The same authors further suggested that the first of these influences favors the development of a conditioned emotional (i.e. fear) response, leading to freezing behavior which overrides a competing tendency to actively cross between compartments (i.e. there is a conflict between active and passive avoidance)[55]. According to Gray, the passive avoidance tendency should interfere with shuttling responses, this expectation being supported by the fact that a great variety of factors have been found to affect passive avoidance and active avoidance in opposite directions[32]. For instance, benzodiazepine receptor agonists impair passive avoidance while improving two-way, active avoidance acquisition.

Secondly, Maudsley-reactive rats (bred for high fearfulness in the open field test) show better passive avoidance, but poorer active avoidance, acquisition than do Maudsley-nonreactive rats[32]. At the same time, both Roman and Syracuse low-avoidance rats (selected for poor two-way avoidance) are more anxious, generally, than are both Roman and Syracuse high-avoidance rats, respectively[11,20]. Thirdly, environmental manipulations which reduce fearfulness of rats, such as postnatal handling[38], also lead to an improvement in active avoidance.

Both the above evidence and pharmacological results[24,26] strongly indicate that the initial stages of shuttle box avoidance acquisition are mediated by fearfulness, so that this paradigm can be considered to be a valid animal analog of anxiety, as has been previously proposed[32]. It appears, therefore, that non-selected rats which perform poorly in two-way avoidance are more fearful than animals which perform better in that task, in much the same way that non-selected rats are classified as 'high anxiety' or 'low anxiety' by their behavior in the 'elevated plus maze' test[30]. The latter two subgroups actually, in fact, differ in their GABA and benzodiazepine characteristics[33,44].

Looking at table 2 and, in addition, comparing it with table 1, it can be observed that we have typically seen two types of rats, both within the same Sprague-Dawley stock, and also when two separate stocks are compared. One type performs poorly in shuttle box avoidance, scoring about 25% of avoidance responses, and the other type performs somewhat better, or about 50% of avoidances. (For comparison, the selected RHA/Verh rats perform in excess of 80% and the selected RLA/Verh rats about 0%[20]). As shown in table 2, it has been found that an acute injection of flumazenil (Ro 15-1788, 5–10 mg/kg) increases two-way, active avoidance acquisition only in the Sprague-Dawley subsample which has low performance levels, whereas no significant flumazenil effects surfaced when high-performance rats were used. This is in general agreement with the results reported by File and Hitchcott[30] using 'high anxiety' or 'low anxiety' rats, respectively, as so judged by their baseline performances in the 'elevated plus maze'. The effects of flumazenil in the plus maze and in the shuttle box both appear to depend to a great extent upon the baseline behavior (i.e. baseline anxiety or emotional reactivity) of the subjects. This is obviously an important feature to be carefully considered when dealing with studies in which low doses of flumazenil are used with the aim of elaborating hy-

Table 2. Effects of flumazenil on the early acquisition of two-way active (shuttle box) avoidance in Sprague-Dawley (Auton. Univ. Barcelona) rats

	Experiment A, high performance controls Total avoidances	(n)	Experiment B, low performance controls Total avoidances	(n)
Vehicle	19.6 ± 1.8 (49.0%)	35	10.7 ± 2.4 (26.8%)	16
Flumazenil				
5 mg/kg	22.3 ± 1.9 (55.7%)	7	19.5 ± 3.6* (48.7%)	8
10 mg/kg	23.3 ± 1.4 (58.1%)	27	21.5 ± 2.4* (53.7%)	14

* $p < 0.05$ vs respective control group (Duncan's test). Experiment A: Pooled data from four independent studies. Experiment B: Pooled data from two independent studies. See procedure in table 1. (% of avoidances in parentheses.)

potheses about the role of putative, endogenous benzodiazepine receptor ligands on stressful or anxiety-provoking situations. In any case, from the results of the two anxiogenic drugs (table 1) and flumazenil (table 2 – Experiment B), it may be reasoned that if some endogenous ligand for benzodiazepine receptors is involved in early two-way avoidance acquisition, which mediates the positive effects of flumazenil, such a compound should be anxiogenic, rather than anxiolytic. Hence, it seems possible to discard the propositions that the results obtained could be due either to intrinsic anxiogenic-like effects of flumazenil or to a displacement of a putative benzodiazepine agonist-like endogenous ligand from benzodiazepine receptors, which have been recently put forth[36,37], because both of those possibilities should lead to an increased anxiety which should, in turn, reduce shuttle box avoidance acquisition. In summary, it is important to differentiate both a) which type of aversive situation, and b) which type(s) of subject(s) (in regard to baseline emotional reactivity) are being dealt with, and not enough merely to refer to 'mild aversive situations or learning tasks' or to 'rats' (as is demonstrated by the absence of flumazenil effects in table 2 – Experiment A). Among environmental manipulations, there is probably none which reduces the reactivity/emotionality of rats (as measured, for example, by vocalizations and defecation) as much as the postnatal habituation to handling. Handling also prevents the enhancing effect of benzodiazepine receptor agonists in shuttle box avoidance acquisition[8,23], as well as their effects on serotonin measures[9]. Accordingly, Brett and Pratt[10] have demonstrated that the habituation to handling prevents the anxiolytic action of benzodiazepine receptor agonists in the plus-maze test. Such results, coupled with the changes that handling habituation induces in ^{35}S-TBPS binding[53], in low-affinity $GABA_A$ sites[5] or in benzodiazepine receptors[45], clearly suggests once again that variables concerning emotional state must be taken into account when studying behavioral changes during stressful tasks or in any other stressful situation, in order to avoid misleading conclusions. Depending upon the animals' emotional state, their behaviors in a given learning or stressful paradigm will vary[8,20,30,39,53], as will the effects of drugs on such behaviors[8,10,30] and, as well, the functional state (i.e. balance between putative, endogenous modulators) of the GABA complex[5,30,45,53].

More on the genetic control of emotionality: Studies with RHA/Verh and RLA/Verh rats

A good example of gaining the maximum advantage from use of an emotionality-related behavioral trait, such as low or high two-way avoidance performance, is the experiment presented on table 3, which was conducted using RHA/Verh and RLA/Verh rats. Here, flumazenil also produced some improvement (i.e. a significant decrease in the amount of freezing) in the more emotional RLA/Verh rats, and at the lowest dose used (5 mg/kg), with the same tendency (although not significant) being observed at 10 and 20 mg/kg. However, in contrast to the results obtained with Sprague-Dawley rats (table 2), flumazenil was not able to induce significant changes in the avoidance behavior per se of the RLA/Verh line.

The decrease in the number of freezing responses observed in RLA/Verh rats following the administration of 5 mg/kg flumazenil is consistent with the hypothesis that a putative, endogenous anxiogenic ligand for benzodiazepine receptors could be partially responsible for the inability to escape or avoid the shock usually observed in those rats. (As previously mentioned, RLA/Verh rats are selectively bred for their failure to acquire avoidance/escape behavior in the shuttle box)[20]. Low doses of flumazenil could decrease freezing by displacing such a putative ligand from benzodiazepine receptors. As flumazenil did not actually affect avoidance behavior as such, however, it would appear that the role of the forementioned endogenous ligand in such behavior would be, at most, marginal in comparison to the role of other possible neurochemical processes[22], which are probably more directly related to the genetic profile of the RLA/Verh line.

It thus becomes clear that shuttle box avoidance, a behavior which seems to be to a large extent anxiety-mediated, can be represented by very different results depend-

Table 3. Effects of flumazenil on the early acquisition of two-way active (shuttle box) avoidance and freezing behavior in Roman high- and low-avoidance (RHA/Verh and RLA/Verh) rats

	RHA/Verh		RLA/Verh	
	Total freezing	Total avoidances	Total freezing	Total avoidances
Vehicle	0.0 ± 0.0 (0.0%)	18.0 ± 2.3 (60.0%)	16.7 ± 2.7 (55.6%)	0.5 ± 0.3 (1.7%)
Flumazenil				
5 mg/kg	0.0 ± 0.0 (0.0%)	20.2 ± 1.5 (67.2%)	7.5 ± 3.0* (25.0%)	1.0 ± 0.5 (3.3%)
10 mg/kg	0.3 ± 0.3 (1.1%)	14.8 ± 3.3 (49.4%)	10.2 ± 3.7 (33.9%)	1.3 ± 0.9 (4.4%)
20 mg/kg	0.0 ± 0.0 (0.0%)	15.8 ± 3.0 (52.7%)	10.8 ± 4.4 (36.1%)	0.5 ± 0.3 (1.7%)
40 mg/kg	0.0 ± 0.0 (0.0%)	14.3 ± 4.1 (47.7%)	21.2 ± 4.9 (76.7%)	0.4 ± 0.4 (1.3%)

* $p < 0.05$ vs respective 'vehicle' group (Duncan's test). Shuttle box sessions consisted of 30 trials. Freezing: Failures to escape or avoid the shock.

ing upon the stocks of rats being used (in this case the various subsamples of Sprague-Dawley rats, in addition to the RHA/Verh and RLA/Verh rats), and their respective interactions with the same drug at the same doses (in this case flumazenil). One point to keep in mind is that, if we are concerned with working reference models for certain kinds of human psychopathology (some forms of anxiety disorders, for example, or other behavioral disorders with a clearcut genetic component), the utilization of animal models based on psychogenetic selection for individual differences, especially combined with supplementary, selective breeding, is very likely going to prove to be a powerful and useful strategy, as many psychopharmacological studies with the selectively bred strains and lines mentioned in this review have already shown [11–14, 19–22, 27, 31, 39].

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