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Differences in Benzodiazepine Binding in Quail Selectively Bred for Differences in Tonic Immobility

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HOGG, S., A. D. MILLS AND S. E. FILE. *Differences in benzodiazepine binding in quail selectively bred for differences in tonic immobility.* PHARMACOL BIOCHEM BEHAV **54**(1) 117–121, 1996. – We have previously found that quail selectively bred to exhibit long (LTI) or short (STI) tonic immobility responses to manual restraint differed with respect to the affinity of binding at the diazepam-sensitive benzodiazepine binding site. In the current study, binding at other components of the $GABA_A$-benzodiazepine receptor complex was investigated. Whereas the lines did not differ in either number or affinity of $GABA_A$ receptors, we found that GABA caused significantly greater enhancement of [^{3}H]flunitrazepam binding in the forebrains of LTI than in STI birds. There was also significantly higher binding to the diazepam-insensitive component (alcohol binding site) of the $GABA_A$-benzodiazepine complex in the forebrain of the LTI line. It is not known, however, whether this difference in the lines is due to differences in number or affinity of these sites. It is discussed whether these manifold differences between the lines in binding at the forebrain sites of the $GABA_A$-benzodiazepine complex could be founded on subunit differences, and whether differences in benzodiazepine binding could underlie the genetically determined behavioural differences in tonic immobility.

$GABA_A$ receptor Alcohol binding site Chloride uptake Fear Japanese quail

TONIC immobility (TI) is a reaction to a threat, such as predator approach, which can be produced experimentally. In birds, for example, a brief period of manual restraint is sufficient to induce the TI response. It has been proposed that the duration for which an animal will remain in TI and the ease with which it can be made to adopt the immobile posture are reflective of the fear state of that animal (7). Selective breeding of Japanese quail has resulted in two genetically different lines which are considered to represent low and high fear groups, because they exhibit short (STI) and long (LTI) TI responses, respectively (12).

In rats, higher levels of anxiety have been demonstrated to result in reductions in benzodiazepine binding. For example, rats which showed more distinct physical avoidance and plasma corticosterone responses on exposure to a predator odour also demonstrated lower benzodiazepine binding affinity than the less avoidant rats (5). In another situation, manual restraint in the form of handling stress resulted in a reduction in $GABA_A$ binding (2) and chloride flux (3). Handling stress, when applied to naive animals, also caused a reduction in cortical benzodiazepine-receptor number compared with binding in controls that had been habituated to handling (1,11). We have previously reported that the behavioural distinctions in selectively bred quail lines are accompanied by differences in benzodiazepine binding. The ligand [^{3}H]flunitrazepam bound with lower affinity in the more fearful LTI line than in the STI line (6).

The $GABA_A$ receptor-chloride ion channel complex consists of five subunit membrane spanning proteins with multiple allosteric binding sites; for a detailed description of its molecular morphology, see (4,15). There are distinct binding sites for GABA and other GABA agonists such as muscimol ($GABA_A$ receptor) and for the benzodiazepines. The latter can be further subdivided into diazepam-sensitive and diazepam-

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insensitive [also known as the alcohol binding site (10)] sites. The benzodiazepine-receptor antagonist flumazenil and the partial inverse agonist Ro 15-4513 bind with high affinity at both sites, and are therefore used to define the diazepam-insensitive binding site. The complex is linked to a $GABA_A$ receptor-operated chloride channel, the opening of which is modulated by benzodiazepines and other ligands (e.g., picrotoxin) acting at distinct binding sites. Benzodiazepines exert their pharmacologic effects by allosterically enhancing GABA binding and, consequently, GABA-mediated opening of the chloride channel.

The purpose of the present study was to determine whether forebrains taken from the STI and LTI lines of quail differed in binding properties at sites on the $GABA_A$-benzodiazepine complex other than the diazepam-sensitive benzodiazepine receptor. We therefore examined binding at the $GABA_A$ receptor, the effects of exogenous GABA on the binding at the diazepam-sensitive site, binding at the diazepam-insensitive binding site, and the modulatory effect of GABA on the uptake of [$^{36}Cl^-$] into synaptoneurosomes. All binding was performed in homogentes prepared from the whole forebrain of the quail; thus, homogenates contained the whole of the limbic system which is implicated in the control of fear behaviours. Although this was not ideal with respect to the study of binding in specific brain areas, it did enable the maximal use of a limited tissue supply; thus, $GABA_A$ binding, GABA enhancement of diazepam binding, and analysis of diazepam-sensitive binding could be performed in the same brains, enabling within-subject comparison of $GABA_A$-benzodiazepine complex characteristics.

METHOD

Animals

The animals used were straight-run Japanese quail (*Coturnix coturnix japonica*) of the F16 generation of the LTI and STI lines maintained at the Station de Recherches Avicoles, Nouzilly, France [see (12) for details of the selection procedure]. All eggs were incubated and hatched at the same time and in the same room, but separate trays were used for the incubation and hatching of each line. Each chick was wing-banded on the day of hatching. Immediately after hatching, the chicks were housed in a floor pen under commercial brooder lamps. At this time, the group of chicks was composed of both males and females. The lighting regimen was continuous illumination until the chicks were 3 weeks of age, at which stage they were sexed and the photoperiod was reduced to 8 h. At 5 weeks of age, male birds from each of the lines were transferred to battery cages (20 to 30 birds per cage) and maintained at an ambient temperature of 20 ± 2°C, with lights on from 0900–1700 h. Food and water were freely available at all times.

Birds were killed during the lights-on phase of the photocycle at 8 weeks of age, at which time they weighed (mean ± SEM) 200.5 ± 3.4 g and 190.1 ± 4.5 g for the LTI and STI lines, respectively.

Drugs and Chemicals

[^{3}H]Flunitrazepam (84.3 Ci/mmol), [^{3}H]Ro 15-4513 (28.8 Ci/mmol), and [$^{36}Cl^-$] (90.8 μCi/ml) were purchased from Dupont (Stevenage, UK), and [^{3}H]muscimol (10.0 Ci/mmol) from Amersham (Buckinghamshire, UK). Picrotoxin, GABA, trizma, and polyethylenimine were obtained from Sigma (Poole, UK). Ultima gold scintillation fluid was from Canberra Packard (Pangbourne, UK). Diazepam and flumazenil were gifts from Roche (Maidenhead, UK)

Procedure

The birds were decapitated and their brains removed. The forebrains were placed in vials and frozen to −70°C. Tissue was transported to London on dry ice and then stored at −70°C until use. Brains were thawed and weighed, and homogenates were then prepared as described below.

For [^{3}H]Ro 15-4513, $GABA_A$ and GABA enhancement of [^{3}H]flunitrazepam-binding brains were homogenised (polytron, 2 × 7 s at half power) in distilled water (concentration 50 mg/ml) and centrifuged at 26,000 × *g* for 20 min. The pellet was resuspended in the same volume of Tris.HCl buffer (50 mM, pH 7.4, at 0°C) and centrifuged at 48,000 × *g* for 20 min; this process was repeated three times with the resultant homogenate being frozen at −20°C until required. On the day of assay, this was thawed, centrifuged at 48,000 × *g* for 20 min, and resuspended in Tris.HCl (50 mM, pH 7.4, at 0°C); binding was performed using a tissue concentration of 5 mg/ml.

Synaptoneurosomes were prepared, according to a modified method of Schwartz et al. (14), to investigate GABA enhancement of [$^{36}Cl^-$] uptake. In brief, tissue (approximately 0.4 g) was homogenised in 2 ml of buffer containing 20 mM HEPES-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$ (pH 7.4) using a glass-Teflon homogeniser (five strokes). The homogenate was diluted to 15 ml with ice-cold buffer and then filtered by gravity through three layers of nylon mesh (160 μm). The filtered preparation was centrifuged at 1000 × *g* for 15 min. The pellet was gently resuspended in buffer and the assay was performed immediately using a tissue concentration of 30 mg/ml.

Assays

Saturation binding at the $GABA_A$ receptor was performed according to the method of Williams and Risley (16) by incubating 100 μl tissue homogenate, 100 μl [^{3}H]muscimol (5–25 nM), and 700 or 800 μl chloride Tris.HCl (50 mM Tris.HCl with 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4) for 30 min on ice. Nonspecific binding was determined for each concentration of [^{3}H]ligand with 10 μM GABA. Data were converted for Scatchard analysis, and for each individual animal, the K_d (receptor affinity^{-1}) and B_{max} (number of receptors) were calculated from the reciprocal of the slope and the intercept on the abscissa, respectively.

GABA enhancement of [^{3}H]flunitrazepam binding was studied by incubating 100 μl of the well-washed membrane preparation, 100 μl [^{3}H]flunitrazepam (1 nM), and 700 μl Tris.HCl buffer with 100 μl GABA (five concentrations between 0.01 and 100 μM, or 100 μl buffer for control) on ice for 60 min. Nonspecific binding was determined for each concentration of GABA with 3 μM diazepam. GABA enhancement of specific binding was expressed as the percentage of GABA-free control.

The diazepam-insensitive benzodiazepine binding site was investigated by incubating 100 μl of tissue homogenate with 100 μl [^{3}H]Ro 15-4513 (2 nM), 100 μl Tris.HCl buffer (pH 7.4), and either 100 μl diazepam (100 μM) or 100 μl flumazenil (100 μM) on ice for 90 min. Diazepam-insensitive binding was calculated by subtracting the nonspecific values determined using flumazenil from those with diazepam.

Binding assays, which were performed in triplicate, were terminated by rapid filtration through Whatman GF/B filters

(Maidstone, Kent, UK), presoaked in Tris.HCl with 0.1% polyethylenimine for 1 h, and two times 5-ml washes with ice-cold buffer.

To determine the levels of GABA-stimulated [$^{36}Cl^-$] uptake, aliquots of synaptoneurosomes (100 μl) were preincubated at 37°C with 800 μl of HEPES·Tris buffer (20 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$, pH 7.4) for 2 min. [$^{36}Cl^-$] uptake was initiated by the addition of 0.5 μCi of [$^{36}Cl^-$] in the presence or absence of 50 μM GABA. After 5 s, the reaction was terminated by the addition of 1 ml ice-cold buffer containing 100 μM picrotoxin. The samples were then filtered, under vacuum, through Whatman GF/B filters (presoaked for 1 h in 0.1% polyethylenimine). Measurements were made in quintuplicate, and GABA-stimulated [$^{36}Cl^-$] uptake was calculated by subtracting the mean uptake in the absence of GABA from that obtained in its presence for each individual tissue. These conditions were observed, in preliminary experiments, to produce significant uptake of [$^{36}Cl^-$] into synaptoneurosomes prepared from fresh chick brain.

Radioactivity was determined by liquid scintillation counting at an efficiency of 40–50%. Protein concentration was determined by the method of Lowry (9) using bovine serum albumin as standard; typically, 1 mg tissue yields 40–70 μg protein.

Statistical Analysis

K_d and B_{max} values for binding at the $GABA_A$ receptor were compared using one-way analysis of variance (ANOVA).

GABA enhancement of [^{3}H]flunitrazepam binding was analysed with repeated-measures ANOVA to investigate the effect of increasing GABA concentration; the differences between binding in the STI and LTI at individual concentrations were analysed with single-factor ANOVA.

Comparisons of the sensitive and insensitive components of benzodiazepine binding were made using one-way ANOVA.

RESULTS

$GABA_A$-Receptor Binding

Representative data for Scatchard analysis of $GABA_A$-receptor binding are shown in Fig. 1. Both STI and LTI lines demonstrated single-site binding; they did not differ in either K_d (STI: 18.5 ± 3.5 nM; LTI: 18.9 ± 3.4 nM) or B_{max} (STI: 5904 ± 588 fmol/mg protein; LTI: 5269 ± 410 fmol/mg protein); n = 6 per group.

GABA Enhancement of [^{3}H]Flunitrazepam Binding

Binding was enhanced to a maximum of 155.3 ± 12.8% and 107.5 ± 9.2% of control for LTI and STI, respectively. Figure 2 demonstrates the dose-dependent nature of the GABA enhancement. Repeated-measures ANOVA for the GABA enhancement of [^{3}H]flunitrazepam binding demonstrated a significant overall effect of concentration [$F(6, 54) = 2.3$, $p < 0.05$]. One-way ANOVA between STI and LTI for each individual concentration of GABA showed that the binding in the LTI birds was significantly higher than the STI at 10^{-9}M [$F(1, 4) = 12.8$, $p < 0.05$], 10^{-7}M [$F(1, 6) = 10.66$, $p = 0.01$], 10^{-6}M [$F(1, 6) = 10.7$, $p = 0.01$], and 10^{-4}M [$F(1, 6) = 7.1$, $p < 0.05$] GABA (Fig. 2).

Diazepam-Insensitive Benzodiazepine Binding

The effects of diazepam and flumazenil on the level of binding of [^{3}H]Ro 15-4513 in the STI and LTI quail were investigated (Fig. 3). In the STI line, the amounts of [^{3}H]Ro 15-4513 binding which were insensitive to both diazepam and flumazenil (67.6 ± 7.2, and 64.4 ± 1.7 fmol/mg protein, respectively) were not significantly different. In the LTI birds, however, the level of nonspecific binding defined with 100 μM flumazenil (45.8 ± 3.6 fmol/mg protein) was significantly lower than that defined using 100 μM diazepam [75.3 ± 6.3 fmol/mg protein: $F(1, 8) = 19.1$, $p < 0.01$]. This means that the LTI line has higher diazepam-insensitive binding than the STI.

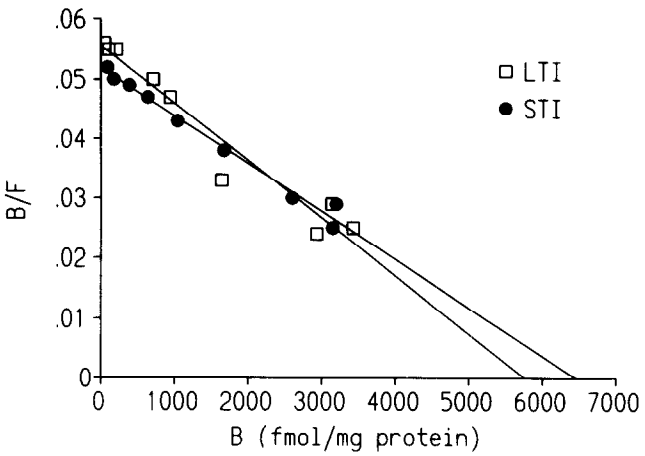

FIG. 1. Representative Scatchard analyses of bound/free (B/F) concentrations (nM) against bound (B) concentrations (fmol/mg protein), for specific binding of [^{3}H]muscimol at the $GABA_A$ receptor are shown for quail from the STI and LTI lines.

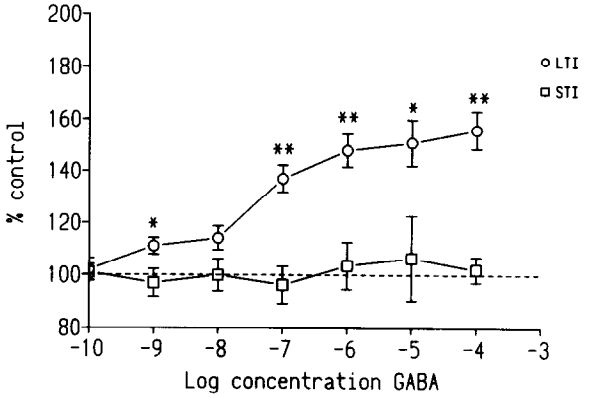

FIG. 2. GABA (M) enhancement of [^{3}H]flunitrazepam (1 nM) binding in quail from the LTI and STI lines. Data are expressed as mean (±SEM) percentage of control binding; this is the level of specific binding in well-washed membranes without the addition of exogenous GABA. *$p < 0.05$, **$p < 0.01$ for one-way ANOVA between STI and LTI at each individual concentration of GABA.

GABA-Enhanced [$^{36}Cl^-$] Uptake

Uptake of [$^{36}Cl^-$] into synaptoneurosomes prepared from previously frozen quail forebrain was not significantly affected by 50 μM GABA in either the STI or LTI line (n = 5 per line). In parallel experiments where synaptoneurosomes were prepared from fresh chick and fresh rat brains, 50 μM

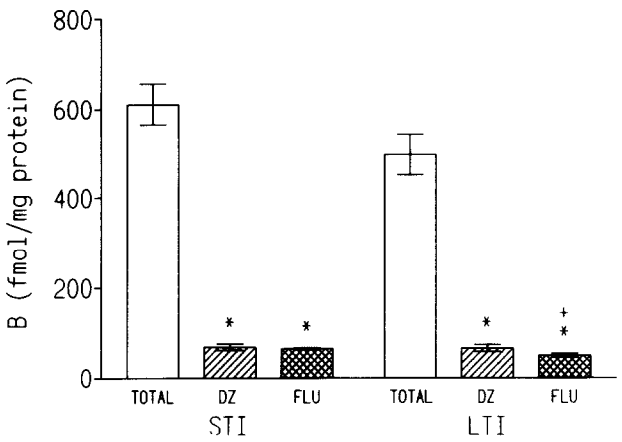

FIG. 3. Comparison of the ability of diazepam (100 μM) or flumazenil (100 μM) to displace [^{3}H]Ro 15-4513 (2 nM) from well-washed membranes prepared from the forebrains of STI and LTI quail. Data are expressed as mean (±SEM) concentration of ligand bound (B, fmol/mg protein) in nonsuppressed (TOTAL), diazepam-suppressed (DZ), and flumazenil-suppressed (FLU) samples (n = 5). *$p < 0.001$ vs. total (nonsuppressed) binding, $^+p < 0.01$ vs. diazepam-suppressed binding.

GABA resulted in 165.2 ± 11.9% (n = 3) and 408.2 ± 71.5% (n = 4) increases in [$^{36}Cl^-$] uptake, respectively. Thus, it would appear that the freezing process affects the uptake of $^{36}Cl^-$ into synaptoneurosomes.

DISCUSSION

We have demonstrated that the neurochemical distinctions between STI and LTI quail are not based solely on the diazepam-sensitive binding site of the $GABA_A$-benzodiazepine complex. However, so far, the neurochemical differences are related only to the benzodiazepine components of the complex. We cannot definitely exclude differences between the lines in chlorine uptake, as this process was affected by the freezing of the tissue.

Although the binding at the $GABA_A$ receptor itself was not different for the two lines, distinctions in the ability of exogenous GABA to enhance [^{3}H]flunitrazepam binding were observed. The LTI quail demonstrated significantly greater enhancement of binding in the presence of GABA than the STI line. It is possible that this provides a compensatory mechanism for the LTI birds which, in the absence of exogenous GABA, demonstrated lower binding affinity than the STI birds (6), or that the STI line is already maximally enhanced by a mechanism other than GABA-mediated modulation. Alternatively, it could be that GABA and ligands at the benzodiazepine-binding site are not allosteric modulators in the STI line, which questions the function of the high-affinity benzodiazepine site in these birds. What is apparent, however, is that the STI and LTI quail have different transductional mechanisms which may be the result of differing molecular subcomponents of the $GABA_A$-benzodiazepine complex in the two lines.

The differences in diazepam-insensitive or alcohol binding sites between the lines also suggest molecular distinctions between the $GABA_A$-benzodiazepine complex of the two lines. The alcohol binding sites are observed only when there is an α_6 subunit present in the structure (8,10); thus, there is likely to be a higher density of these subunits in the LTI birds. These data provide evidence for the presence of the α_6 subunit of the $GABA_A$-benzodiazepine complex in brain areas other than the cerrebellum (the unique brain region where α_6 subunits have, until now, been documented). However, without full Scatchard analyses it is not possible to conclude whether this apparent increase in density of the α_6 subunits in LTI quail manifests itself as an increase in the affinity or the number of diazepam-insensitive binding sites.

The involvement of benzodiazepines in modulating fear and anxiety is well established clinically in humans and experimentally in nonhuman primates and rodents, and behavioural studies indicate that the $GABA_A$-benzodiazepine complex has a role in the control of avian anxiety. However, the exact nature of this role is not clear, as both agonist and inverse-agonist stimulation of the diazepam-sensitive benzodiazepine receptor have been shown to increase the duration of TI in chickens (13). Stimulation of the diazepam-sensitive benzodiazepine receptor has also been demonstrated to increase punished responding in pigeons (17); in the same study, flumazenil caused an increase in the rate of unpunished responding. These data indicate a functional role for diazepam-insensitive benzodiazepine binding sites, and provide evidence for the existence of endogenous ligands for these sites in birds. Our data may suggest one of two opposing roles for the diazepam-insensitive binding site: first, increasing the receptivity of the LTI quail to the actions of potential "anxiolytics"; second, the action of "anxiogenic" endogenous ligands for this site is more pronounced in the LTI line, thus making them more fearful.

The present study showed that genetic selection of quail for a behaviour believed to reflect fear also results in differences in the neurochemical properties of several components of benzodiazepine binding. However, the manifold behavioural properties of benzodiazepines as well as the several differences in binding, which may be confounded by compensatory changes in the individual components of the binding, make it impossible to relate the two directly. Behavioural studies in the STI and LTI lines should help to elucidate the roles of the individual components of the binding differences and clarify the potential role of different receptor subunits in mediating behavioural responses to predatory stimulation.

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