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Receptor Binding in Japanese Quail Selected for Long or Short Tonic Immobility

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HOGG, S., S. PATERSON, A. D. MILLS AND S. E. FILE. *Receptor binding in Japanese quail selected for long or short tonic immobility.* PHARMACOL BIOCHEM BEHAV **49**(3) 625–628, 1994. – Japanese quail, selectively bred for long (LTI) and short (STI) tonic immobility (TI) responses, are thought to represent high and low fear groups, respectively. To study the neurochemical mechanisms underlying the behavioral distinctions, binding parameters were determined at the benzodiazepine, 5-HT_{1A}, 5-HT_3, α_2, and opioid receptor sites in the forebrains of the two lines. No differences were found in 5-HT_{1A}, 5-HT_3, α_2, μ- or κ-opioid receptor binding between the lines. The K_D for the binding of [^{3}H]-flunitrazepam at the benzodiazepine receptor was significantly greater in the LTI than in the STI birds, indicating lower affinity for benzodiazepine ligands. The lines did not differ in benzodiazepine receptor number. Using [^{3}H]-naltrindole, the LTI line was found to have fewer δ-opioid receptors than the STI line; the birds did not vary with respect to the affinity of these receptors. Thus, the selective breeding of the two lines has resulted in differences in benzodiazepine and δ-opioid binding, and these could produce differences in activity levels, fear, and pain responses, all of which could contribute to the tonic immobility response.

Quail Tonic immobility Radioligand binding Benzodiazepine Opioid

TONIC immobility (TI) is an unlearned response, induced by temporary restraint and characterized by a catatonic-like state of reduced reactivity to external stimulation (7). It is thought to be the terminal response in a chain of antipredator behaviors and has been demonstrated in a wide range of species including amphibians, birds, fish, insects, mammals, and reptiles (12). TI has been extensively studied in domestic birds, which give a particularly robust and easily discernable reaction. It has been suggested to be related to the fear state of the animal, i.e., the more frightened a bird is when TI is induced, the longer it will remain immobile when the restraint is removed (7). This applies whether the fear results from the restraint itself or from other, nonspecific stimuli (2).

Divergent lines of Japanese quail have been selectively bred for long (LTI) and short (STI) tonic immobility reactions (9). In these birds, the behavioral distinctions are robust and thought to represent high and low fear groups, respectively. Because there is, as yet, no clear understanding of the neurochemical basis of TI, the present study investigated the involvement of different neurotransmitter systems in mediating TI by comparing radioligand binding parameters between the two lines of quail.

METHOD

Animals

The animals used were straight run Japanese quail of the F16 generation of the LTI and STI lines maintained at the Station de Recherches Avicoles, Nouzilly, France [see (9) 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 comprized 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 photo period reduced to 8 h. At 5 weeks of age, male birds from each of the lines were transferred to battery cages (20–30 birds

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per cage) and maintained at an ambient temperature of 20 ± 2°C. Food and water were freely available at all times.

Birds were killed 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]-8-OH DPAT (162.9 Ci/mmol) [^{3}H]-BRL 43694 (80.0 Ci/mmol), [^{3}H]-yohimbine (78.4 Ci/mmol), [^{3}H]-[D-Ala^2,MePhe^4,Gly ol^5]enkephalin ([^{3}H]-DAGO, 48.0 Ci/mmol) and [^{3}H]-bremazocine (25.7 Ci/mmol) were purchased from Dupont (Stevenage, UK) Ltd, [^{3}H]-CI-977 (21.0 Ci/mmol) from Amersham, and [^{3}H]-naltrindole (19.4 Ci/mmol) from Izinta (Hungary). Trizma HCl, trizma base, DAGO, and polyethylenimine were obtained from Sigma (Poole, UK), idazoxan from Research Biochemicals Incorporated (St. Albans, UK), naloxone hydrochloride from Endo Laboratories (USA), and [D-Ala^2,D-Leu^5]enkephalin (DADL) from Cambridge Research Biochemicals (Cambridge, UK). Tertatolol and ondansetron were gifts from Servier and diazepam from Roche products. Ultima gold scintillation fluid was purchased from Canberra Packard.

Dissection and Preparation of Tissue for Radioligand Binding

The birds were killed by decapitation 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, which were prepared fresh for each experiment, were then prepared as described below.

For benzodiazepine binding, brains were homogenized (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 overnight. 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.

For both 5-HT$_{1A}$ and 5-HT$_3$ binding brains were homogenized in Tris·HCl buffer (50 mM, pH 7.4 at 0°C) at a concentration of 100 mg/ml and centrifuged at 48,000 × *g* for 20 min at 4°C. Following resuspension, they were incubated for 10 min at 37°C to enhance metabolism of endogenous 5-HT and recentrifuged. The final binding homogenate was suspended in Tris·HCl buffer (50 mM, pH 7.4 at 37°C) and the assay performed immediately using a tissue concentration of 10 mg/ml.

For binding to the α_2-adrenoceptors, the tissue was homogenized at a concentration of 50 mg/ml in Tris sucrose buffer (50 mM Tris·HCl, 250 mM sucrose, 1 mM $MgCl_2$; pH 7.4 at 0°C) and centrifuged at 1,100 × *g* for 10 min at 10°C. The supernatant was recentrifuged at 40,000 × *g* for 20 min at 4°C. A binding homogenate was prepared by resuspending the pellet in 50 mM Tris·HCl with 0.1% ascorbate (pH 7.4 at 25°C) and the assay performed immediately at a tissue concentration of 5 mg/ml.

In investigation of binding parameters at opioid receptors tissue was homogenized in 0.32 M sucrose at a concentration of 10 mg/ml and centrifuged at 1000 × *g* for 10 min at 4°C. The resultant supernatent was centrifuged at 38,000 × *g* for 20 min at 4°C. The pellet was resuspended in Tris·HCl buffer (50 mM, pH 7.4 at 25°C), incubated at 37°C for 45 min, and the samples were then spun at 49,000 × *g* for 15 min. The assay was performed immediately at a tissue concentration of 10 mg/ml.

Binding Assays

For saturation assays, the benzodiazepine binding sites were labeled with [^{3}H]-flunitrazepam (0.005–16 nM), the 5-HT$_{1A}$ sites with [^{3}H]-8-OH-DPAT (0.024–6.5 nM), the 5-HT$_3$ sites with [^{3}H]-BRL43694 (0.1–10 nM), the α_2-adrenoceptor sites with [^{3}H]-yohimbine (0.008–14 nM), the μ-opioid sites with [^{3}H]-DAGO (0.1–15 nM), the δ-opioid sites with [^{3}H]-naltrindole (0.05–2.5 nM), and the κ-opioid sites with [^{3}H]-CI-977 (0.1–4 nM) or [^{3}H]-bremazocine (0.02–4 nM). When bremazocine was the labeled agent, the μ and δ binding was suppressed by the addition of a constant ratio of 100 nM unlabeled DAGO and DADL to each 0.3 nM [^{3}H]-bremazocine. Nonspecific binding was determined for each concentration of tritiated ligand with 3 μM diazepam (benzodiazepine binding), 3 μM tertatolol (5-HT$_{1A}$), 1 μM ondansetron (5-HT$_3$), 3 μM idazoxan (α_2 binding), or 1 μM naloxone (opioid). In the binding assays, 100 μl (benzodiazepine, 5-HT$_{1A}$, 5-HT$_3$, and α_2) or 900 μl (opioid) of membrane homogenate was made up to a final volume of 1 ml by addition of labeled and unlabeled ligand or Tris·HCl buffer. Samples were incubated for 60 min on ice for benzodiazepine binding, for 60 min at 25°C for opioid binding, for 30 min at 25°C for α_2 binding, for 10 min at 37°C for 5-HT$_{1A}$ binding, and for 30 min at 37°C for 5-HT$_3$. At the end of the incubation, samples were filtered over Whatman GF/B filters that had been presoaked for 1 h in 0.1% polyethylamine and washed with two or three times 5 ml ice cold Tris·HCl buffer.

Radioactivity was determined by liquid scintillation counting at an efficiency of 40–50%. Protein concentration was determined by the method of Lowry (8) using bovine serum albumin as standard, typically, 1 mg tissue yields 40–70 μg protein.

Opioid binding data were analyzed using the Ligand computer program (10) and results from benzodiazepine, 5-HT$_{1A}$, 5-HT$_3$, and α_2 receptor binding studies were checked for two site fits using Enzfitter (Elsevier-Biosoft) and were then fitted using linear regression analysis. For each individual animal the K_D (reciprocal of receptor affinity) and B_{max} (number of receptors) values were calculated from the reciprocal of the slope and the intercept on the abscissa, respectively.

Statistical Analysis

The K_D and B_{max} data were analyzed using single-factor analyses of variance (ANOVA).

RESULTS

Benzodiazepine Binding Assays

The characteristics of the binding of [^{3}H]-flunitrazepam to benzodiazepine binding sites are shown in Table 1. In both strains, [^{3}H]-flunitrazepam labeled a single class of binding sites (see Fig. 1 for a representative Scatchard from each line). Although the B_{max} values for the two strains were similar, the K_D in the LTI birds was greater than that in the STI strain, $F(1, 13) = 12.2, p < 0.01$.

5-HT$_{1A}$ Receptor Binding Assays

The characteristics of the binding of the [^{3}H]-8-OH DPAT to 5-HT$_{1A}$ binding sites are shown in Table 1. In both lines,

TABLE 1

K_D AND B_{MAX} VALUES FOR FOREBRAINS OF QUAIL FROM THE STI AND LTI LINES

	K_D (nM)		B_{MAX} (fmol/mg protein)	
Receptor	STI	LTI	STI	LTI
Benzodiazepine	1.80 ± 0.10	2.35 ± 0.17*	2079.8 ± 184	2120 ± 135
5-HT_{1A}	3.29 ± 0.15	3.02 ± 0.23	169.9 ± 14.1	150.1 ± 13.2
α_2	2.78 ± 0.43	3.62 ± 0.76	364.8 ± 32.1	487.2 ± 63.4
μ-Opioid	1.64 ± 0.37	1.18 ± 0.33	35.1 ± 3.85	43.2 ± 7.2
δ-Opioid	0.18 ± 0.07	0.25 ± 0.06	159.4 ± 5.0	118.9 ± 15.0†
κ-Opioid (CI 977)	0.67 ± 0.05	0.52 ± 0.09	54.7 ± 5.4	47.6 ± 10.2
κ-Opioid (brem)	0.24 ± 0.05	0.24 ± 0.05	112.9 ± 13.0	88.2 ± 14.2

Data are expressed as mean (±SEM) values for four to five (opioid), six (5-HT_{1A} and α_2), or eight (benzodiazepine) saturation binding experiments. K-Opioid receptor binding was determined using both the specific ligand CI 977 and the nonspecific agent bremazocine (brem) in the presence of DAGO and DADL.
*$p < 0.01$.
†$p = 0.05$.

[^{3}H]-8-OH DPAT labeled a single class of binding sites (data not shown). No significant differences were found in the binding between the two lines.

5-HT_3 Receptor Binding Assays

The levels of binding at the 5-HT_3 receptor were very low. At 15 nM [^{3}H]-BRL 43694 they were less than 2 fmol/mg protein for both lines and, as such, it was not possible to make any true comparisons between the lines.

α_2 Adrenoceptor Binding Assays

Saturation binding at the α_2-adrenoceptor was investigated using [^{3}H]-yohimbine, the K_D and B_{max} values are shown in Table 1. Two site fits were not observed in either of the two lines, and the binding parameters did not differ between the lines.

Opioid Receptor Binding Assays

The characteristics of saturation binding to the opioid sites in the quail are shown in Table 1. No significant differences were found in the affinity of binding of any opioid ligand and B_{max} was found to differ for the δ-opioid site only, $F(1, 7) = 5.3$, $p = 0.05$. See Fig. 2 for a representative example of Scatchard analysis for each strain; unfortunately lower concentrations of [^{3}H]-naltrindole could not be used because of the low specific activity of this ligand.

DISCUSSION

Although the serotonergic (4,6) and adrenergic (3,5) systems have been shown to affect the duration of TI, it would appear that the behavioral differences between the two lines of quail are not due to distinct binding properties at 5-HT_{1A} or α_2 binding sites. Binding at the 5-HT_3 receptor sites was not detectable in either of the lines, and, thus, these forebrain receptors are unlikely to be contributing significantly to the profound difference in TI.

If TI does, indeed, reflect the fear state of the bird, the differences in benzodiazepine receptor affinity binding may underlie the distinctions in predator-induced tonic immobility.

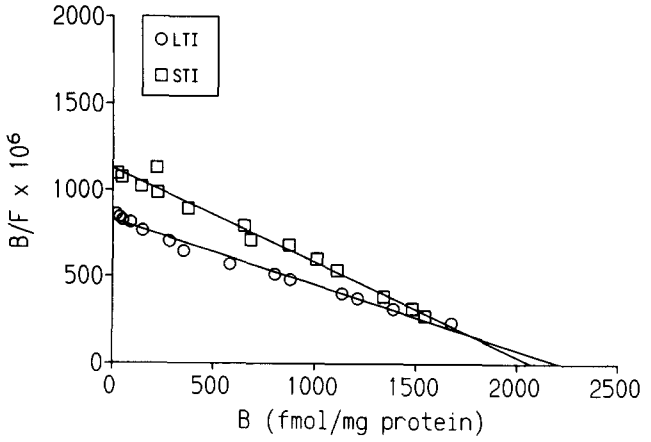

FIG. 1. Representative Scatchard analyses, of bound/free (B/F) concentrations (nM) against bound (B) concentrations, for binding of [^{3}H]-flunitrazepam at the benzodiazepine receptor site are shown for quail from the LTI and STI lines.

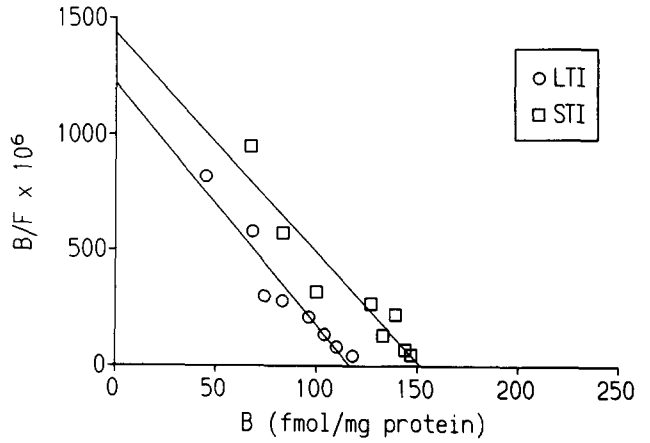

FIG. 2. Representative Scatchard analyses, of bound/free (B/F) concentrations (nM) against bound (B), for binding of [^{3}H]-naltrindole at the δ-opioid receptor site are shown for quail from the LTI and STI lines.

The benzodiazepine receptor agonist, midazolam, has been shown to increase punished responding in pigeons (13), an effect reported to be indicative of fear reduction. Therefore, a greater sensitivity, in the STI line, to a circulating benzodiazepine agonist ligand may explain the lower fear levels in this line. Low doses of benzodiazepines are stimulant and high doses are sedative; although the molecular basis of this remains unknown, the binding difference between the STI and LTI lines might mediate differential effects of a benzodiazepine ligand on activity. Without some independent measure of activity, it cannot be excluded that differences in activity could underlie the differences in TI between the lines.

Our data are not the first to implicate the opioid system in mediating the TI response, and opioid peptides have been shown to potentiate TI in both chickens (11) and rabbits (1). Aloisi et al. (1) also showed that the TI response was potentiated by pain, and that anti-β-endorphin attenuated the effects of pain on TI. Although the aforementioned studies do not provide a simple explanation for opioid control of TI, the differences in δ-opioid receptors between the lines suggests that the different TI responses may be due to the presence of endogenous endorphins in the LTI line but not the STI, line.

We hypothesized that the genetic selection of quail for long or short tonic immobility responses should be accompanied by neurochemical distinctions. In this study we have shown that the behavioral selection has, indeed, resulted in significant neurochemical differences between the lines. The precise manner in which the differences in the benzodiazepine and δ-opioid receptor binding properties contribute to the genetic differences in TI, and whether each receptor mediates a different component of the TI response, remain to be elucidated.

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