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Changes in Tonic Immobility and the GABA–Benzodiazepine System in Response to Handling in the Chick

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FLUCK, E., S. HOGG, B. R. JONES, R. BOURNE AND S. E. FILE. *Changes in tonic immobility and the GABA–benzodiazepine system in response to handling in the chick.* PHARMACOL BIOCHEM BEHAV **58**(1) 269–274, 1997.— Changes in the GABA–benzodiazepine system were investigated following regular handling of male chicks. Compared with handling-naive chicks, those exposed to 10 days of gentle handling required a larger number of inductions and had a lower duration of tonic immobility. Corresponding biochemical changes occurred, with handling-habituated chicks having a significantly lower basal [^{14}C]GABA release from archistriatal slices and a reduction in the B_{max} of [^{3}H]muscimol binding in the forebrain. Benzodiazepine binding in the archistriatum was investigated using in vitro quantitative receptor autoradiography. Binding was localised in the anterior, mediale, dorsalis, and ventralis intermedium nuclei of the archistriatum, and there was significantly more binding in the anterior and ventralis intermedium/mediale archistriatum nuclei than in the dorsalis intermedium archistriatum nuclei. Benzodiazepine binding was not altered after handling in any of the investigated nuclei of the archistriatum. The results suggest that whereas several days of gentle handling in chicks leads to a decrease in forebrain $GABA_A$ receptors and a decrease in GABA release from the archistriatum, there are no accompanying changes in benzodiazepine receptors. Regular handling exerts a specific effect on chicks: it reduces their fear of human beings but not that of novel places or objects. It is possible that the pattern of biochemical changes observed in the present study may be specifically associated with this particular behavioural modification rather than with a change in general fearfulness. © 1997 Elsevier Science Inc.

Handling Tonic immobility $GABA_A$ Benzodiazepines Binding Neurotransmitter release Poultry

THE STRESS associated with acute handling or manual restraint is sufficient to produce profound behavioural and neurochemical changes. When comparisons are made between rats that have been habituated to handling and those which, at the time of the experiment, are subjected to acute handling stress, it is clear that extensive neurochemical changes occur in the GABA–benzodiazepine system. Thus, rats habituated to handling are less anxious when tested in the elevated plus-maze (1), have increased cortical benzodiazepine and $GABA_A$ receptor binding (2,3,21), and increased GABA and chloride uptake (4,6) compared with animals naive to handling. Regular handling also modifies the behaviour of domestic chicks. More specifically, it represents a particularly powerful method of reducing chickens' fear of human beings. For example, handled birds show lower duration of manually induced tonic immobility (TI) and less avoidance of and increased approach towards humans than do their nonhandled counterparts (8–10,13,14,16–18). Interestingly though, unlike the relatively global effects reported in rats (22), handling exerts no detectable effects on nonspecific underlying fearfulness in the domestic fowl; and fails to affect chicks' responses to unfamiliar places and objects (16,17).

The present experiment reexamined the effects of regular handling on the chicks' TI responses. TI is an unlearned anti-

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predator response to brief manual restraint that is characterised by a catatonic-like state of reduced responsiveness and its duration is thought to be positively related to the antecedent fear state (7,9,15). We also explored possible differences in GABA release from the archistriatum and in forebrain $GABA_A$ receptor binding. In a previous study, we confirmed the effects of handling on TI in chicks, but were unable to find any differences in forebrain benzodiazepine binding between handled and unhandled chicks (11). This was somewhat surprising, since we have found differences in forebrain benzodiazepine binding in quail selectively bred to exhibit long or short durations of TI (12). In the present study, we therefore investigated benzodiazepine binding in specific regions of the archistriatum, which is an area analogous to the limbic system in mammals (24), by using quantitative receptor autoradiography.

METHODS

Animals and Housing

Three batches (A, B, and C) of newly hatched Isa Brown chicks (a commercial medium-hybrid line originally derived from a Rhode Island Red × Rhode Island White cross) were obtained from ISA (Peterborough, UK). They were housed in groups of four in wooden boxes measuring 70 × 40 × 30 cm. The floors of the boxes were covered with wood litter beneath a 2 cm high wire mesh grid (1 cm mesh), which allowed the passage of excreta but denied the chicks access to the litter. Food (chick starter mash) and water were supplied ad lib in semi-circular plastic hoppers attached to grids suspended from the tops of the walls. The photoperiod was 0700 to 1900 h, warmth was provided by dull-emitter heaters fitted with 250-W bulbs, and wire-mesh lids prevented the chicks from jumping out.

Experimental Procedure

Handling. In each batch of chicks, each home-cage group of four chicks was randomly allocated to one of two treatments: handled or unhandled (Table 1; Figs. 2, 3 for group sizes). The cages containing unhandled chicks were screened to avoid visual contact between the chick and the experimenter. Chicks in the handled group were individually handled twice daily for 10 successive days from the day of arrival. Handling consisted of gently removing the chick from its home cage, stroking it for approximately 30 s, placing it in the cage that would be used for transport on the experimental day, and then placing it back in the home cage. During handling the chick could see the experimenter's whole body, with the hands, arms, and face being the predominant features. The experimenter wore similar clothing (white laboratory coat) during handling and behavioural testing.

Behavioural testing. Tonic immobility tests were performed at 11 days of age, between 0900 and 1130 h. Each chick was individually removed from its home cage and placed on a bench covered with several layers of cloth. Testing occurred in the same room as the home cage. We recorded the number of inductions required to attain TI, lasting at least 5 s, and the duration of TI. If TI was not maintained for more than 5 s, another induction of TI was attempted and if, after a total of five attempts, TI was unsuccessful, the chick was deemed not to be susceptible and it was placed back in its home cage. Scoring in this instance was 5 for number of inductions of TI and 0 s for duration. The chick was placed on its back and was gently restrained by the experimenter, by placing one hand over the body of the bird and the other over its head for 10 s. The hands were then withdrawn and a stopwatch started. Cagemates were tested in succession, and cages designated for handled and unhandled chicks were tested alternately. All chicks used in the succeeding neurochemical experiments had been previously tested for TI.

GABA release. Chicks from batch A were used for measurements of GABA release. They were removed from their home cages and carried in individual cages to a separate room for immediate sacrifice. After decapitation, the forebrain was removed and the left archistriatum dissected and sliced (0.2 mm) using a McIlwain tissue chopper. After a preliminary incubation for 10 min in normal Krebs bicarbonate medium, [^{14}C]GABA was added to the medium to give a final concentration of 0.23 μM. After a further 30 min incubation, five slices (approximately 10 mg tissue wet weight) were placed in perfusion chambers (0.3 ml) between Whatman GF/B filters, where they were superfused with Krebs buffer at a rate of 1 ml/min. The superfusate obtained during the first 15 min was discarded, before 20 fractions (2 ml each) were collected. A stimulated release was achieved by perfusing the slices for 2 min during fraction 7 (in a total of 20 fractions) with perfusing buffer containing 30 mM KCl; this was replaced with normal perfusing buffer for the remainder of the collection period.

At the end of the collection period, the brain slices were removed from the perfusion chambers and placed into vials along with 1 ml of Soluene and left for at least 4 h. After this time, 200 μl of glacial acetic acid and 3 ml of scintillation fluid were added, the vials were shaken, and the radioactivity in the tissue and in each fraction was measured using a LKB Rackbeta 1214 liquid scintillation counter. The efflux of radioactivity was expressed as the fractional rate coefficient (FRC)-radioactivity released during a particular fraction expressed as a percentage of the total amount of radioactivity present in the slices at that time. The total amount of K^+-evoked release was expressed as the sum of the FRCs after stimulation that were greater than the baseline FRC (fraction 6). An estimate of the uptake of the neurotransmitters into the slices was made by adding the total number of counts (dpm) collected during the collection period to the number of counts present in the tissue slices at the end of the collection period.

$GABA_A$ binding. Chicks from batch B were used for $GABA_A$ binding determination. They were killed by decapitation as described previously, the brains were removed, frozen, and stored at −20°C until required for assay. The tissue was thawed and homogenised in distilled water (tissue concentration 50 mg/ml) and centrifuged at 26,000 × *g* for 20 min. The pellet was resuspended in 50 mM Tris-HCl buffer and centrifuged at 48,000 × *g* for 20 min; this process was repeated three times, and the resultant homogenate was frozen at −20°C overnight. Before binding, the homogenate was thawed, centrifuged (48,000 × *g* for 20 min), and resuspended in 50 mM Tris HCl (50 mg/ml).

The $GABA_A$ receptors were labelled with [^{3}H]muscimol (0.3–25 nM), and nonspecific binding was determined for each concentration of tritiated ligand with 10 μM GABA. In the binding assays, 100 μl of membrane homogenate was made up to a final volume of 1 ml by addition of labelled and unlabelled ligand or Tris-HCl buffer. Samples were incubated for 30 min on ice.

Assays were terminated by vacuum filtration through Whatman GF/B filters (presoaked for 1 h in 0.1% polyethylenimine) followed by two 5-ml washes with ice cold 50 mM Tris-HCl buffer. Radioactivity was determined by liquid scintillation spectroscopy and, following protein concentration determination using the method of Lowry et al. (20), the concentration of ligand specifically bound was calculated. Data

from saturation studies were individually converted for Scatchard analysis, checked for one-site fits using Enzfitter (Elsevier-Biosoft), and fitted using linear regression analysis. For each individual animal, the K_d (receptor affinity^{-1}) and B_{max} (number of receptors) values were calculated from the reciprocal of the slope and the intercept on the abscissa, respectively.

Autoradiography Experiment

Preparation of sections. The chicks from batch C were used for autoradiography. They were killed by decapitation and the brain was rapidly removed, divided into two coronally, frozen in an isopentane/CO_2 mixture, wrapped in foil, and stored at −70°C until required. Coronal sections (10 μm) were collected on a Reichert cryostat at −18°C and thaw mounted onto poly-L-lysine coated coverslips. The sections were taken from two levels within the chick forebrain. Reference points used were from the Kuenzel and Masson chick atlas (19). The first level included the anterior archistriatum, and at least 30 sections were collected starting 8.8 mm anterior of the zero reference plate (Fig. 1a). The sections taken from the second level included dorsalis and ventralis intermedium archistriatum and medial archistriatum, and at least 30 sections were collected starting 7.6–7.4 mm anterior to the zero reference plate (Fig. 1b). The sections were then air-dried and stored air-tight at −20°C until the day of assay.

Benzodiazepine binding. Sections were brought to room temperature, placed in racks, lightly fixed in 0.5% paraformaldehyde for 5 min, and then washed twice (for 10 and 15 min, respectively) in assay buffer to remove fixative and endogenous ligands. Benzodiazepine binding was measured using [^{3}H]Ro-15-4513 (2 nM), which labels both diazepam-sensitive and insensitive benzodiazepine receptors. Two displacer ligands used were diazepam (100 μM), which labels only diazepam-sensitive benzodiazepine receptors, and flumazenil (100 μM), which labels both diazepam-sensitive and insensitive benzodiazepine receptors. Consecutive sections from each level for each chick brain were assayed in the order of a) total binding, i.e., 2 nM [^{3}H]Ro-15-4513 only b) 2 nM [^{3}H]Ro-15-4513 plus 100 μM cold flumazenil c) 2 nM [^{3}H]Ro-15-4513 plus 100 μM cold diazepam; and so on. Therefore at least 10 sections from each chick at each brain level were used to measure suppressed and unsuppressed binding. The whole assay was carried out on ice, with the assay buffer of 50 mM Tris HCl, pH 7.4, at 4°C. The sections were incubated in 2 nM [^{3}H]Ro-15-4513, with or without the appropriate displacer ligand, for 90 min. The incubations were terminated by washing three times (10 s for each wash) in ice cold buffer, and then briefly dipped in ice cold distilled water before rapid air-drying.

Following the binding assay, the sections were glued to a card, apposed to ^{3}H-Hyperfilm, secured between aluminium sheets, and left in the dark at 4°C for 55 days. The films were developed in Agfa G150 developer for 4 min at 20°C, fixed in Ilford Hypam for 3 min, and washed in distilled water for at least 1 h. Densitometry of the film autoradiograms were performed on a Joyce-Loebl Magiscan MD image analysis system.

A series of brain paste standards were prepared according to the method of Davenport and Hall (5) containing [^{3}H]leucine. A set of standards was apposed to each sheet of film containing sections, and a standard curve was generated of optical density vs. radioactivity from which the levels of benzodiazepine binding could be calculated.

In order to reduce variability in measurements between chicks, a standardising technique was applied and caried out in each nuclei of the archistriatum investigated. The medial hyperstriatum ventrale (MHV) in the anterior archistriatum (Fig. 1a) and the intermediate and medial hyperstriatum ventrale (IMHV) in the more posterior sections (Fig. 1b) were used as the standardising areas. The left and right hemispheres were adjusted separately, as follows. The binding for each section of the archistriatum in each chick was divided by the corresponding binding in the hyperstriatum ventrale (HV); this factor was then multiplied by the mean binding value in the HV for all chicks and both the unadjusted and the adjusted binding values are expressed as fmol/mg/protein.

Owing to the lack of distinction between the ventralis intermedium archistriatum and the mediale archistriatum nuclei, the binding analysis was carried out combining the two nuclei, and therefore, the results presented are for an unqualitative ventralis intermedium/mediale archistriatum mixture.

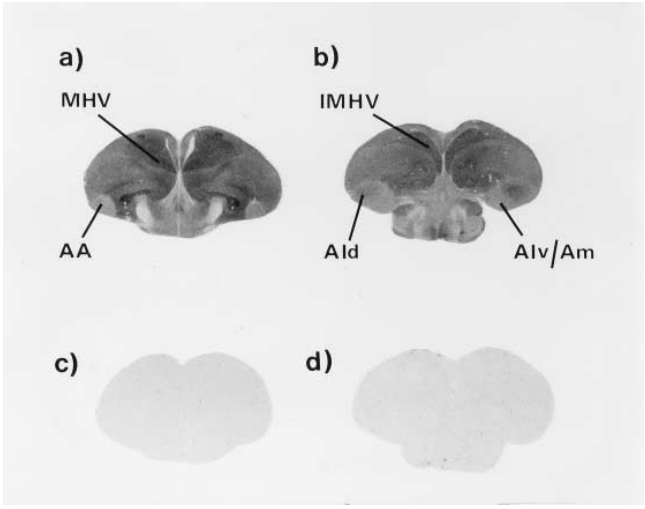

FIG. 1. Example sections of total benzodiazepine binding in the anterior archistriatum (AA; section a) and in the dorsalis and ventralis intermedium/mediale archistriatum (AId and AIv/Am, respectively; section b). The mediale hyperstriatum ventrale (the area used for standardising the binding) is present in both sections a (MHV) and b (IMHV), with mean (±SEM) binding (fmol/mg protein) in the left and right hemispheres, respectively, of 27.07 ± 1.74 and 27.3 ± 1.7 in handled chicks and 25.9 ± 1.8 and 26.0 ± 1.8 in unhandled chicks (effect of handling: $F < 1.0$). Section c is an example of nonspecific binding in sections including anterior archistriatum, and section d is an example of nonspecific binding in sections including dorsalis and ventralis intermedium/mediale archistriatum, using diazepam as the displacer.

Chemicals

[^{14}C]GABA (228 mCi/mmol), [^{3}H]muscimol (10.0 Ci/mmol), [^{3}H]leucine (52.0 Ci/mmol), and [^{3}H]Ro-15-4513 (20.8 Ci/mmol) were purchased from DuPont, NEN (Stevenage, UK). Diazepam and flumazenil were a gift from Roche Products Ltd (Welwyn Garden City, UK). GABA was purchased from Research Biochemicals Incorporated (St. Albans, UK), and the constituents of all buffers used were obtained from Sigma (Poole, UK). Soluene and Ultima Gold scintillation fluid were purchased from Canberra Packard (Pangbourne, UK), ^{3}H-Hyperfilm from Amersham International (Little Chalfont, Amersham), and Agfa G150 film developer and Hypam fixative from Ilford (Cheshire).

Krebs bicarbonate buffer of the following composition was prepared for neurotransmitter release studies: 118 mM NaCl, 4.8 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 9.5 mM glucose, 50 μM aminooxyacetic acid, 50 μM pargyline, 100 μM ascorbic acid, and 30 μM EDTA. The medium was gassed continuously with 5% CO_2 in O_2. Tris-HCl and Tris base buffers were used in the autoradiography technique and for $GABA_A$ binding of forebrain homogenate. Additional constituents to the buffer used for $GABA_A$ binding were 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$.

Statistical Analysis

Data from the GABA release and $GABA_A$ forebrain binding studies were analysed with a single-factor analysis of variance (ANOVA), the factor being handling state. Data from the autoradiographs were analysed using a between–within ANOVA, with handling state as the between-groups factor and hemisphere and brain area as the within-group factors. Comparisons of binding between individual nuclei were then made with Duncan's post hoc tests.

RESULTS

Behaviour

In all three subsets of chicks used for the different neurochemical assays, handling significantly decreased the duration of TI (see Table 1) and increased the number of inductions needed to elicit TI (Figs. 2, 3).

GABA Release

The unhandled birds had a significantly higher basal GABA release than the handled group [$F(1, 13) = 9.8$, $p < 0.05$] (see Fig. 2). There were no significant differences in K^+-evoked release or in uptake [mean ± SEM; release (sum of FRCs above baseline): unhandled = 10.0 ± 2.1, handled = 15.5 ± 4.7, $F(1, 13) = 1.3$, NS; uptake (dpm × 10^{-3}): unhandled = 22.4 ± 2.2, handled = 22.2 ± 2.9, $F(1, 13) = 0.003$, NS].

$GABA_A$ Receptor Binding

Saturation binding of [^{3}H]muscimol at the $GABA_A$ receptor and subsequent conversion for Scatchard analysis satisfied a single site fit for all animals studied. Handling did not significantly affect K_d [mean ± SEM (nM); handled = 27.6 ± 5.3, unhandled = 27.7 ± 3.3, $F(1, 10) = 0$, NS], but there was a significant difference in the B_{max} values, with a greater number of binding sites in the unhandled birds [$F(1, 10) = 4.7$, $p = 0.05$] (see Fig. 2).

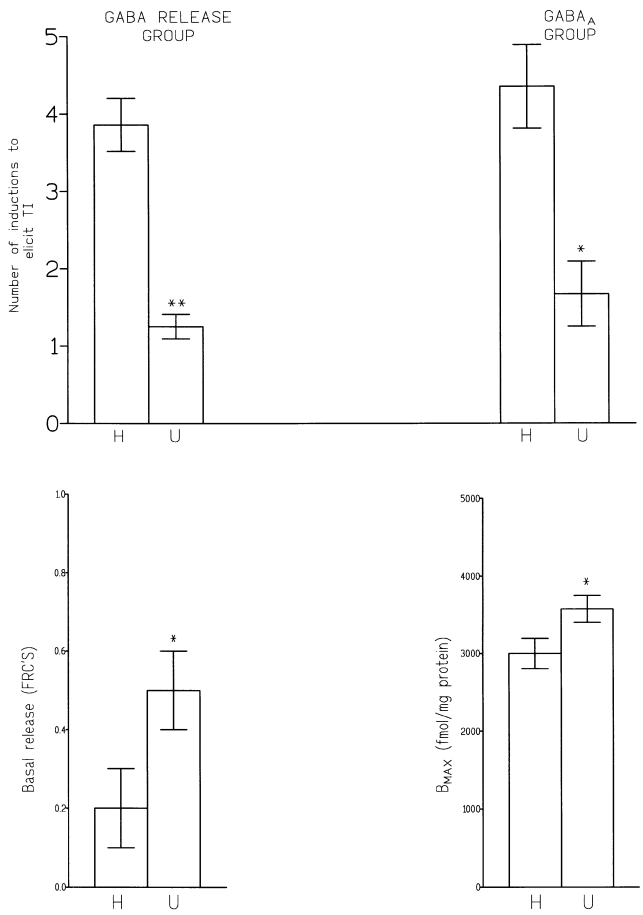

FIG. 2. Top panel: Mean (±SEM) number of inductions needed to elicit TI in handled (H) and unhandled (U) chicks used for GABA release (top left: $n = 7$ for handled and $n = 8$ for unhandled chicks) and $GABA_A$ binding (top right: $n = 6$/treatment). The corresponding basal GABA release (FRCs) and $GABA_A$ binding (fmol/mg protein) are shown in the bottom left and right panels, respectively. *$p < 0.05$ and **$p < 0.001$ compared with the handled group.

TABLE 1

DURATION OF TONIC IMMOBILITY IN HANDLED AND UNHANDLED CHICKS SUBSEQUENTLY USED FOR GABA RELEASE, $GABA_A$ BINDING, AND BENZODIAZEPINE BINDING STUDIES

Chick Batch	Handled	Unhandled
A	4.4 ± 1.7	55.6 ± 9.5**
B	15.5 ± 10.1	126.3 ± 39.2*
C	35.0 ± 13.1	225.1 ± 30.3**

Values are mean ± SEM duration (s) of TI in handled and unhandled chicks subsequently used for GABA release (batch A, $n = 7$) for handled and $n = 8$ for unhandled groups), $GABA_A$ binding (batch B, $n = 6$/treatment), and benzodiazepine binding in the archistriatum (batch C, $n = 10$/treatment). *$p < 0.05$ and **$p < 0.001$ compared with the handled group.

Benzodiazepine Binding

Total [^{3}H]Ro-15-4513 binding was heterogeneously distributed in the chick brain at the sites under investigation (Fig. 1a, b). Nonspecific binding using flumazenil as the displacer ligand was below levels of detection. Nonspecific binding using diazepam was very low (8 fmol/mg protein) and homogeneous in distribution across the brain sections (Fig. 1c, d). This indicates an even but low level of diazepam-insensitive receptors, and therefore the data presented are for the two sites combined. There were differences in binding among the various archistriatum nuclei [$F(2, 32) = 30.9$, $p < 0.005$], with significantly ($p < 0.05$) less binding in the dorsalis intermedium than in the anterior and intermedium/mediale nuclei. There were no differences in binding between the hemispheres of any of the archistriatal nuclei ($F < 1.0$ for all areas)

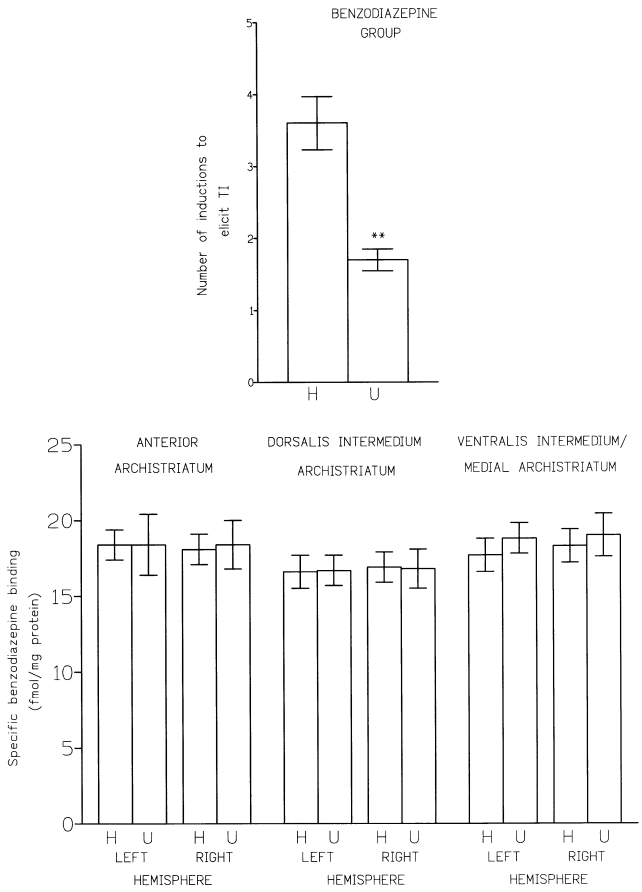

FIG. 3. Top panel: Mean (±SEM) number of inductions needed to elicit TI in handled (H) and unhandled (U) chicks used for benzodiazepine binding (n = 10/treatment). Lower panel: Benzodiazepine binding in both the left and right hemispheres of each investigated archistriatal nuclei. $^{**}p < 0.001$ compared with handled chicks.

and no changes following handling stress ($F < 1.0$ for all areas), whether analysis was conducted on unadjusted data or on data standardised with reference to the HV. The unadjusted data are presented in Fig. 3.

DISCUSSION

Regular gentle handling of chicks resulted in changes in the GABA system. Handled chicks had lower basal GABA release from archistriatal slices and fewer forebrain $GABA_A$ binding sites than unhandled chicks. These changes are in contrast to the increase in $GABA_A$ receptors found in the cortex of well-handled rats (3). Handling-habituated rats are less anxious than rats naive to handling when tested in the elevated plus-maze (1); thus, in rats, the increase in forebrain $GABA_A$ receptors could mediate the decrease in anxiety. However, the decrease in GABA release from cortical slices in rats would seem more likely to be a presynaptic compensatory mechanism acting to reduce stress (6). In handling-habituated chicks, the decrease in TI is compatible with a reduction in fear of humans, but it is difficult to see how the decrease in $GABA_A$ binding would be mediating this. However, decreased $GABA_A$ binding would be compatible with less motor inhibition, which in turn would lead to a reduced duration of TI. Thus, the change in GABA binding could be mediated in the changed motor expression of fear.

Benzodiazepine binding in the archistriatum of the chick did not change after handling, and we have previously reported that forebrain benzodiazepine binding was not affected (11). If the attenuation of the duration of TI induced by handling were associated with reduced general anxiety, an increase in benzodiazepine receptors might have been expected, because benzodiazepines decrease measures of anxiety in pigeons (23). Similarly, Andrews et al. (2) reported that rats habituated to handling were less anxious in the elevated plus-maze and had significantly more benzodiazepine receptors than rats naive to handling; the receptor number was increased to that of well-handled rats when the handling-naive rats were given an acute dose of diazepam. Furthermore, Japanese quail selectively bred for short rather than long TI showed a higher affinity for the benzodiazepine receptors in the forebrain (12).

It is therefore possible that in the genetic selection of Japanese quail it is a general responsivity to predators that is selected, and hence the trait is closer to general anxiety. However, in the case of modification of TI after human handling, the response may be situation specific and hence does not reflect a general change in anxiety. Indeed, this is just what has been reported (16,17).

From the results of the present study, the situation-specific reduction in TI would not seem to involve a change in benzodiazepine receptors. However, possible changes in $GABA_A$ binding in other brain areas and other measures of the functional state of the GABA–benzodiazepine receptor complex would have to be investigated before a role for this system in the reduction of the specific fear of humans can be dismissed. It is possible that the reduced $GABA_A$ function was mediating the modification of the motor response of immobility independent of any change in fearfulness. However, there is evidence that handling does not produce motor inhibition in all situations, and although active escape from humans is reduced, approach is increased (16–18). A change in specific fearfulness might occur along with a change in motor responses and may be mediated by other changes in GABA–benzodiazepine function or by other neurotransmitters.

In conclusion, although in some cases (e.g., genetically selected lines of quail) the duration of TI may reflect general anxiety or fearfulness, this is not necessarily always the case. Handling in the chick produces a change in TI not associated with a general change in anxiety, but one perhaps associated with a specific decrease in fear of humans and reduced GABA inhibition that may contribute to the purely motor aspects of the response.

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