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Characterization of CRF, AVT, and ACTH cDNA and pituitary-adrenal axis function in Japanese quail divergently selected for tonic immobility

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Hazard D, Couty M, Guémené D. Characterization of CRF, AVT, and ACTH cDNA and pituitary-adrenal axis function in Japanese quail divergently selected for tonic immobility. *Am J Physiol Regul Integr Comp Physiol* 293: R1421–R1429, 2007. First published July 11, 2007; doi:10.1152/ajpregu.00070.2007.—Higher corticosterone (CORT) responses to acute stress have previously been reported in quail selected for short (STI) duration of tonic immobility (TI) than for long TI (LTI), although behavioral studies indicated that LTI quail were more fearful. To investigate adrenal and pituitary function in these quail lines and their possible involvement in the differences in hypothalamic-pituitary-adrenal (HPA) axis reactivity, we measured CORT responses to adrenocorticotropin (1–24 ACTH), corticotropin-releasing factor (CRF), and arginine vasotocin (AVT) after characterizing the nucleotide acid sequences of these peptides in quail. Although maximum adrenal responses, assessed by ACTH challenge, were higher in STI quail, adrenal sensitivity was comparable for the two genotypes. It is therefore unlikely that differences in HPA axis reactivity involved the adrenal level. AVT and ACTH induced comparable CORT responses in both genotypes, whereas those induced by CRF were much lower. AVT is thus more potent than CRF in quail, but the respective maximum pituitary capacity of both genotypes to secrete ACTH was similar, and it is doubtful that the AVT pathway is involved in the difference in HPA axis reactivity between genotypes. On the other hand, the higher CORT responses induced by CRF in STI quail suggest that CRF might be involved in the differences in HPA axis reactivity between LTI and STI genotypes.

hypothalamic-pituitary-adrenal axis; corticosterone; stress

SUCCESSFUL ADAPTATION TO FRIGHTENING or stressful stimuli requires not only the ability to perceive and respond to a stimulus but also the ability to control the stress responses appropriately. One physiological characteristic of the stress response is activation of the hypothalamic-pituitary-adrenal (HPA) axis. HPA axis activation results successively in the release of corticotropin-releasing factor (CRF) and/or arginine vasotocin [AVT, the avian homolog of mammalian arginine vasopressin (AVP)] from the hypothalamus, ACTH from the pituitary gland, and glucocorticoids from the adrenal glands (5, 32, 53, 54). Nucleotide or peptide sequences of CRF, AVT, and ACTH have not yet been identified in the quail. Corticosterone (CORT) is the primary adrenal steroid secreted in bird plasma in response to stress (24, 55). Corticosteroids are required to reestablish homeostasis via feedback mechanisms (5, 51, 52). They act by facilitating behavioral adaptation via preparation of the body for the metabolic requirements of flight or fight responses and by consolidating or potentiating fear responses (5). The CORT level can therefore be used as a valuable marker of the HPA axis activity.

Differences in HPA axis reactivity following restraint (27, 32, 44) have previously been reported in two divergent genotypes of Japanese quail selected for long (LTI) or short (STI) duration of tonic immobility (TI). TI is an unlearned catatonic state, and this behavioral response has been shown to be positively correlated with other measurements of fear (31, 40). Several behavioral tests conducted in LTI and STI quail have led to the conclusion that quail that show long duration of the TI response are more fearful than quail that show a short duration of the TI response (17). Higher CORT responses to restraint previously reported in STI quail than in LTI quail were unexpected if CORT levels are considered to be indicators of the stress response. Nevertheless, similar results have been shown in humans, with anxious subjects exhibiting lower neuroendocrine activation during acute stress compared with nonanxious subjects (29). Although these quail genotypes have been widely characterized for their behavioral responses to stress, little is known about their physiological responses to stress. Thus, in view of the possible interaction (described above) between behavioral and physiological responses to stress, it seemed important to identify the origin of the genetic variations in HPA axis reactivity to stress in LTI and STI quail to explain the earlier results.

The influence of genetic factors upon variations in HPA axis activity has been suggested in humans by twin and family studies (28, 34, 37, 39, 42). Moreover, wide variations in HPA axis activity have been described between inbred strains of mice (18, 30) and rats (20, 48, 49) and between different breeds of pigs (10, 15, 25). Bird strains have been selected for divergent adrenal responses to ACTH (14), immobilization (50), cold (3), and social stress (21). Various mechanisms have been suggested to explain the genetic differences in HPA axis activity in these different models. The causes of variations in HPA axis activity may thus take place in the central structures and involve mechanisms at different organizational levels of the HPA axis (hypothalamus, pituitary, adrenal glands) or through different external and internal modulatory mechanisms such as feedback.

Several experimental findings have suggested that the functional differences in HPA axis reported between Lewis and Fisher 344 rat strains (4, 13, 57, 62) may have a central origin, with major differences in hypothalamic CRF neuron function (57), whereas others have reported changes at the pituitary (1) or adrenal cortex levels (20). Moncek et al. (41) recently proposed the hypothesis that the variations in reactivity of the HPA axis in Lewis and Fisher rats probably originates from

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pituitary and adrenal levels. On the other hand, comparing five inbred rat strains, Gomez et al. (20) showed that the lowest CORT response to immobilization stress observed in Lewis and Wistar-Kyoto rats did not result from lower ACTH secretion, which suggests that the adrenal cortex is hyporeactive in these strains. Similarly, others (10, 11, 63) have demonstrated that an adrenal origin is the cause of high cortisol responses in pigs. Furthermore, selection of Japanese quail for high serum CORT levels in response to immobilization resulted in different steroidogenic properties of adrenocortical cells in vitro (7).

The aim of the study reported here was to identify the origin of the differences in HPA axis responsiveness observed between LTI and STI genotypes. In view of the mounting scientific evidence described above, showing the involvement of adrenal and/or the pituitary levels in the genetic variations in HPA axis reactivity for several models, we hypothesized that LTI and STI genotypes of quail might differ in their adrenal and/or pituitary function. Injection of 1-24 ACTH has been reported to stimulate active CORT release from the adrenal glands in birds (2, 23, 35, 36, 43). Pharmacological challenge using various doses of 1-24 ACTH has provided a valuable approach to investigating adrenal function in LTI and STI genotypes. Various experimental findings have shown that CRF is more potent than AVP in CORT release in several mammals [rats (19, 46, 59); ewes (33)]. On the other hand, contradictory results have been reported concerning the respective effects of these two peptides in birds, with CRF being sometimes more active (8, 61) and sometimes less active (38, 58). To further investigate their respective effects on pituitary activation in LTI and STI genotypes, pharmacological studies were undertaken using CRF or AVT alone or both peptides together.

MATERIALS AND METHODS

RT-PCR

The corresponding partial regions of mRNA coding for CRF, AVT, and proopiomelanocortin hormone (POMC) were amplified by RT-PCR to characterize the nucleotide acid sequence of the active CRF, AVT, and ACTH peptide regions. Hypothalamic and pituitary total RNA were isolated using the RNeasy Mini purification kit (Qiagen). RNA concentrations were measured by absorption at 260 nm. Total RNA samples (2.5 µg) were reverse transcribed using 200 units Superscript II RNase H⁻ RT (Invitrogen) and oligo(dT) primers (Sigma Aldrich Chimie).

The sequence of the forward CRF primer (5'-TCGGAGGAGC-CGCCCATCTCC-3') was based on the consensus sequence derived from alignment of known CRF nucleotide acid sequences of the human (GenBank accession no. NM_000756), rat (GenBank accession no. X03036), pig (GenBank accession no. AF440229), sheep (GenBank accession no. J00803), and fish (GenBank accession no. AF098629). The reverse CRF primer sequence (5'-TCACTTC-CCGATGATTCCA-3') was based on the known CRF nucleotide acid sequence of the chicken (GenBank accession no. AJ621492). The sequences of the forward (5'-CTGCCCTCTCCTTCTCT-3') and reverse (5'-TCAGAGGCCAGGCTGCTT-3') AVT primers were based on the known AVT nucleotide acid sequence of the chicken (GenBank accession no. X55130). The sequence of the forward (5'-TTCCGCTGGGGCAAGCCGGT-3') and reverse [5'-TCACT(C/G)(G/A)CCCTTCTTGT(A/G)(G/C)G-3'] POMC primers were based on the consensus sequence derived from alignment of known POMC nucleotide acid sequences of the chicken (GenBank accession no. NM_001031098), human (GenBank accession no. NM_000939), bo-

vine (GenBank accession no. NM_174151), and rat (GenBank accession no. AF510391). The CRF, AVT, and POMC primers were provided by Sigma Aldrich Chimie.

The PCR cycle consisted of a denaturation step (95°C, 30 s), an annealing step (CRF 55°C, 30 s; AVT 60°C, 30 s), and an elongation step (72°C, 60 s) performed with *Taq* polymerase (Invitrogen-LifeTechnologies). After 40 cycles, the final elongation step was prolonged for 10 min. PCR fragments were analyzed by horizontal agarose electrophoresis and visualized via ethidium bromide fluorescence.

Cloning and Sequencing

The PCR fragment was ligated using a TA-cloning vector (pCR 2.1-TOPO TA Cloning kit; Invitrogen-LifeTechnologies). TOP10 chemically competent *Escherichia coli* cells were transformed with the resulting ligation mixture, and recombinant bacterial colonies were selected by blue/white screening. Recombinant plasmid DNA was purified with the NucleoSpin Plasmid DNA Purification kit (Macherey-Nagel) and sequenced on both strands using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences of the clones corresponding to partial quail CRF, AVT, and POMC cDNA were deposited in the GenBank/EMBL database.

Animals and Rearing Conditions

Japanese quail (*Coturnix japonica*) from the 36th generation of two divergent genotypes selected for STI or LTI were used in the study (40). Quail were identified by wing banding on the day of hatching. Quail were exposed to continuous light until 3 wk of age and then to a 16:8-h light-dark (16L/8D; light on at 0600) rhythm. The quail from the different genotype and age groups were reared in different collective battery cages. Food and water were provided ad libitum. The caretaker checked the quail daily in the morning (from 0830) and refilled the feeders whenever necessary. No care was provided on the day of the experiment. The birds were treated according to the European Communities Council Directive of November 24, 1986 (86/609/EEC). All procedures described here fully comply with French legislation on research involving animal subjects. All experiments were carried out with due regard to legislation governing the ethical treatment of animals, and investigators were certificated by the French governmental authority for carrying out these experiments (No. 06255).

In Vivo Experimental Procedures

Three successive experiments were performed on sexually mature quail at the age of 6 wk. Groups of quail ($n = 4-6$ /group) of the same genotype and sex were randomly constituted 1 wk before experimentation and placed in small collective battery cages. Experiments were performed between 0830 and 1730 since it has been previously reported that basal CORT levels remain stable throughout the day in LTI and STI quail (26). The experiments consisted of physical restraint in a crush cage or pharmacological challenge. An average of seven to eight quail was used for each experimental point. Blood samples were collected from each quail directly in a tube containing EDTA (2 mg/ml blood) following death by decapitation after submission to the different treatments. All samples were temporarily stored on ice. Following centrifugation at 2,000 *g* for 15 min at 4°C, plasma samples were separated and stored deep frozen at -20°C until measurement of CORT using a specific RIA (16).

A group of quail bled immediately after capture and transferred from their home cage to the test room was included in each trial to assess basal CORT concentrations in the specific conditions of each experiment.

Treatments

Restraint tests. After transfer to the test room, quail were placed in a "crush-cage" in the form of a wooden box (15 cm length × 5 cm width × 10 cm height) closed at the top by a netting cover. Quail were

restrained for a period of 10 min and then bled immediately. This restraint test differed from the selection test, which consisted of placing each quail on its back in a U-shaped cradle, hand-restraining it for 10 s to induce TI, and then releasing it.

Pharmacological challenge. After transfer to the test room, quail were weighed individually to adjust the dose injected to body weight. Quail were injected in the pectoralis major muscle with mammalian 1-24 ACTH (1 mg = 100 IU; Immediate Synacthen; Novartis), mature human/rat CRF peptide (Phoenix Pharmaceuticals), or AVT (Phoenix Pharmaceuticals) at different doses diluted in saline solution (0.9% NaCl wt/vol). Quail were placed back in their home cages immediately after the injection for various durations (ranging between 10 and 120 min) and bled thereafter. Control quail received a representative volume of the vehicle (0.9% NaCl; 400–700 μ l volume). Dose-response and time-course relationships were investigated.

In Vivo Experiments

Experiment 1: 1-24 ACTH dose-response and time-course studies.

ACTH DOSE RESPONSE. CORT dose responses to 1-24 ACTH were analyzed in two separate experiments depending on whether we were testing adrenal sensitivity or maximum CORT adrenal response capacity. Adrenal sensitivity was measured in quail injected with 1-24 ACTH at increasing doses of 0, 1.25, 2.5, 5, and 10 μ g/kg body wt and bled 10 min postinjection. The maximum CORT adrenal response capacity was measured in quail injected with 1-24 ACTH at increasing doses of 0, 10, 25, 100, and 250 μ g/kg body wt and bled 20 min after injection.

ACTH TIME COURSE. Quail were injected with doses of 2.5, 10, or 100 μ g/kg body wt of 1-24 ACTH or with a representative volume of the vehicle. Quail were then placed back in their home cages and bled 5 (only for the dose of 10 μ g/kg body wt), 10, 20, 40, 60, or 120 min after injection.

Experiment 2: CRF dose-response and time-course studies. Quail were injected with CRF at doses of 10 or 100 μ g/kg body wt. The quail were returned to their home cages immediately after the injection for 15- or 30-min periods and bled thereafter.

Experiment 3. To compare the potency of CRF and AVT peptides to activate the pituitary-adrenal axis and to investigate their respective possible involvement in the CORT response to stress, both peptides were included in a single comparative study. Quail were thus either submitted to a restraint period of 15 min or injected with 100 μ g/kg body wt of 1-24 ACTH, 100 μ g/kg body wt CRF, 10 or 50 μ g/kg body wt AVT, or injected with a combination of 100 μ g/kg body wt CRF and 50 μ g/kg body wt AVT. Quail were placed back in their home cages for a 15-min period and bled thereafter.

Statistical Analysis

CORT values were subjected to multifactorial ANOVA to assess the effects of genotype, sex, treatment, and their interactions using the Statview IV program (Abacus Concept). Normality of CORT findings and equality of the variances of the different groups were checked before performing ANOVA. Whenever specific factor and interaction effects reached significance ($P < 0.05$), post hoc tests were performed using the Fisher test (protected least significant difference). CORT values were expressed as means $\pm$ SE, and the level of significance was $P < 0.05$ unless otherwise stated.

RESULTS

Molecular Cloning of Partial cDNA Encoding CRF, AVT, and POMC

A 132-bp CRF fragment (GenBank accession no. AY786511), a 472-bp AVT fragment (GenBank accession no. AY786510), and a 342-bp POMC (GenBank accession no. AY786509) were obtained by RT-PCR and were consistent with the predicted

lengths. They corresponded to amino acids 125–167 of chicken pro-CRF, 6–161 of chicken pro-AVT, and 138–250 of chicken POMC. The degrees of similarity of the partial quail pro-CRF, pro-AVT, and POMC cDNA sequences with the corresponding chicken DNA sequences were 99% (131/132), 94% (443/472), and 93% (321/345), respectively. Comparison of the partial predicted amino acid sequences of quail CRF, AVT, and POMC with the chicken, human, rat, pig, bovine, sheep, and fish homologs are presented in Fig. 1. The quail CRF active peptide region (i.e., 41 amino acids) presented full identity with chicken, human, pig, and rat sequences, whereas it differed by 7 and 8 amino acids out of 41 from the bovine and ovine sequences, respectively (Fig. 1A). The quail AVT active peptide region (i.e., 9 amino acids) presented full identity with the chicken AVT peptide but differed by one amino acid from the human, bovine, and rat AVP active peptide region (Fig.

A - CRF

Quail	SEEPPTSLDLTFALLREVLHARAEQLAQAHNSRKLMEITGK
Chicken	VE-----REKRSEEPPTSLDLTFALLREVLHARAEQLAQAHNSRKLMEITGK
Human	LGGAQEAPE-REKRSEEPPTSLDLTFALLREVLHARAEQLAQAHNSRKLMEITGK
Pig	LSRQEAPE-REKRSEEPPTSLDLTFALLREVLHARAEQLAQAHNSRKLMEITGK
Rat	LGGAQEAPE-REKRSEEPPTSLDLTFALLREVLHARAEQLAQAHNSRKLMEITGK
Bovine	LSRQEAPEHARRKRSDEPPISLDTFALLREVLHARAEQLAQAHNSRKLMEITGK
Ovine	LSRQEAPEHARRKRSDEPPISLDTFALLREVLHARAEQLAQAHNSRKLMEITGK

propeptide → Mature peptide

B - AVT

Quail	LPLSFLCLLALSSACYQNCPRGGKRALGDALRQCLPCGPNRGRGFCGPGICCGAELGCYLGT
Chicken	MAFSPPLSFLCLLALSSACYQNCPRGGKRALGDALRQCLPCGPNRGRGFCGPGICCGAELGCYLGT
Human	MPDHLPAFLGLLAFSSACYQNCPRGGKRAHSDLELRQCLPCGPGGKGRFCGPGICCGAELGCYVGT
Bovine	MPDHLPAFLGLLAFSSACYQNCPRGGKRAHSDLELRQCLPCGPGGKGRFCGPGICCGAELGCYVGT
Rat	MLNTLLSACFLSLLALTSACYQNCPRGGKRAHSDLELRQCLPCGPGGKGRFCGPGICCGAELGCYVGT

Signal peptide → AVT → Copeptin

Quail	ETRCAEEDYPPSCQGGGKPCGSGGRCAANGYCCSADTCANDAVCLEE--GSEQEEAEKNTLYDGA
Chicken	ETRCAEEDYPPSCQGGGKPCGSGGRCAANGYCCSADTCANDAVCLEE--GSEQEEAEKNTLYDGA
Human	EALRCQENYLPSCQGGGKPCGSGGRCAANGYCCSADTCANDAVCLEE--GSEQEEAEKNTLYDGA
Bovine	EALRCQENYLPSCQGGGKPCGSGGRCAANGYCCSADTCANDAVCLEE--GSEQEEAEKNTLYDGA
Rat	EALRCQENYLPSCQGGGKPCGSGGRCAANGYCCSADTCANDAVCLEE--GSEQEEAEKNTLYDGA

Quail: AGDLLRLMALANRQ--GKQPLG
 Chicken: AGDLLRLMALANRQ--GKQPLG
 Human: AGALLRLYLQAGAPPEPAPQDGY
 Bovine: SGALLRLYLQAGAPPEPAPQDGY
 Rat: ARELLRLYLQAGTQESVDSAPRYV

→ Copeptin

C - POMC

Quail	FRNGKPYGKRRPIKYPNGVDEESAEYSYMEFRREAR-----AGDGLGLSEEEEEEE
Chicken	SYSMEHRRGKPYGKRRPIKYPNGVDEESAEYSYMEFRREAR-----PUGDPLGLSEEEEEEE
Human	SYSMEHRRGKPYGKRRPIKYPNGVDEESAEYSYMEFRREAR-----PUGDPLGLSEEEEEEE
Bovine	SYSMEHRRGKPYGKRRPIKYPNGVDEESAEYSYMEFRREAR-----PUGDPLGLSEEEEEEE
Rat	SYSMEHRRGKPYGKRRPIKYPNGVDEESAEYSYMEFRREAR-----PUGDPLGLSEEEEEEE

← α-MSH → ACTH → CLIP → β-LPH → γ-LPH

Quail	EE-----GEEKKDGGSYRHRHRAHAPLKOKRYGGFATLHESQTLPLTLFNKATIKSAYKKG
Chicken	EE-----GEEKKDGGSYRHRHRAHAPLKOKRYGGFATLHESQTLPLTLFNKATIKSAYKKG
Human	HSLLV-----AAEKKDGGSYRHRHRAHAPLKOKRYGGFATLHESQTLPLTLFNKATIKSAYKKG
Bovine	YGLVRAHEAAEKKDGGSYRHRHRAHAPLKOKRYGGFATLHESQTLPLTLFNKATIKSAYKKG
Rat	QVL-----EPDTEKADGPRYVEHFRNGNPPKOKRYGGFATLHESQTLPLTLFNKATIKSAYKKG

← β-MSH → β-end

Fig. 1. A: sequence similarity between predicted quail corticotropin-releasing factor (CRF) partial amino acid sequence and COOH-terminal part of other CRF amino acid sequences (chicken CAF18561; human NP_000747; pig AAN40888; rat CAA26838; bovine AAK83231; ovine AAA31512). B: Sequence similarity of predicted quail arginine vasotocin (AVT) partial amino acid sequence to complete sequence of other AVT [or arginine vasopressin (AVP)] amino acid sequences (chicken CAA38923; human NP_000481; bovine NP_789824; rat NP_058688). C: sequence similarity of predicted quail partial proopiomelanocortin (POMC) to COOH-terminal part of other POMC amino acid sequences (chicken NP_001026269; human CAG46625; bovine NP_776576; rat AAA41903). Positions of ACTH, β -lipotropic hormone (β -LPH), α -melanocyte-stimulating hormone (α -MSH), corticotropin-like intermediary peptide (CLIP), γ -lipotropic hormone (γ -LPH), β -endorphin (β -end), and β -melanocyte-stimulating hormone (β -MSH) resulting from POMC precursor are indicated with arrows. Differences in amino acid composition of CRF, AVT, and ACTH mature peptides are indicated with dark spots.

1B). Comparison of the partial predicted amino acid sequences of POMC in quail with homologs in other species indicated that the region encoding the 7-39 ACTH peptide was identical to the chicken region and differed by seven amino acids from the human region and by nine from bovine and rat regions (Fig. 1C).

In Vivo Experiments

Experiment 1. DOSE RESPONSE. Measurement of CORT responses 10 min after injection with doses of 1-24 ACTH ranging between 1.25 to 10 $\mu\text{g/kg}$ body wt indicated that comparable dose-response relationships between CORT concentrations and ACTH doses were found in both genotypes (dose effect, $P < 0.0001$; genotype effect, $P = 0.1$; Fig. 2A). On the other hand, a significant sex effect ($P = 0.05$) was

Fig. 2. Corticosterone (CORT) concentrations (ng/ml plasma) measured in long (LTI) and short (STI) duration tonic immobility quail 10 min after im injection of 1-24 ACTH (Immediate synacthen) at serial doses ranging from 1.25 to 10 $\mu\text{g/kg}$ body wt ($5 \leq n \leq 9$ quail/experimental point; A) and 20 min after injection of doses ranging from 10 to 250 $\mu\text{g/kg}$ body wt ($6 \leq n \leq 10$; B). Data are means $\pm$ SE. C, control noninjected quail; 0, quail injected with vehicle. x-Axis is given as a semilogarithmic scale.

observed on this dose-response relationship. Injection of the 2.5 $\mu\text{g/kg}$ body wt dose led to a significant increase ($P = 0.002$) in plasma CORT concentration (from 4.3 ± 0.8 to 13.8 ± 1.9 ng/ml in males and from 6.5 ± 1.8 to 12.7 ± 2 ng/ml in females).

Injection of 1-24 ACTH at doses of 10, 25, 100, or 250 $\mu\text{g/kg}$ body wt resulted in significant increases ($P = 0.0005$) in CORT levels in both genotypes and both sexes 20 min postinjection (Fig. 2B). CORT responses following 1-24 ACTH injection were significantly higher ($P = 0.004$) in STI quail than in LTI quail. Responses measured 20 min after injection of doses ranging between 25 and 250 $\mu\text{g/kg}$ body wt did not differ significantly ($P > 0.3$) but were significantly higher ($P < 0.0001$) than those reached after injection of the 10 $\mu\text{g/kg}$ body wt dose for both genotypes.

TIME COURSE. Injection of saline solution had no significant effect ($P = 0.9$) on CORT levels (Fig. 3A), whereas injection of 1-24 ACTH at doses of 2.5, 10, or 100 $\mu\text{g/kg}$ body wt induced significant increases ($P < 0.0001$) in CORT levels (Fig. 3, B–D) that did not differ significantly ($P > 0.23$) between genotypes. Time-course responses differed according to the dose. A dose of 2.5 $\mu\text{g/kg}$ body wt resulted in maximum CORT levels of 9.7 ± 1.2 ng/ml within 10 min postinjection. CORT concentrations measured 20 min postinjection (4.1 ± 0.7 ng/ml) were no longer significantly different ($P = 0.08$) from basal levels (2.3 ± 0.5 ng/ml) in either genotype. Maximum mean CORT levels (28.8 ± 1.8 ng/ml) for STI and LTI quail of both sexes measured in response to the injection of a 10 $\mu\text{g/kg}$ body wt dose were also reached within 10 min but remained at high levels for periods lasting between 5 to 20 min postinjection and returned to basal levels (3.4 ± 1.2 ng/ml) from 40 min (3.6 ± 0.7 ng/ml) onward. Injection of 1-24 ACTH at a dose of 100 $\mu\text{g/kg}$ body wt resulted in increases in CORT levels that remained high between 10 and 60 min postinjection in STI and LTI quail of both sexes. CORT concentrations measured 120 min after injection were similar to basal values.

Experiment 2. Injection of CRF at a dose of 10 $\mu\text{g/kg}$ body wt did not result in significant increases ($P = 0.7$) in CORT levels 15 or 30 min postinjection in either genotype or sex (Fig. 4A), whereas injection of CRF at a dose of 100 $\mu\text{g/kg}$ body wt resulted in a significant increase ($P < 0.0001$) in CORT levels. CORT levels remained similar between 15 and 30 min following injection of CRF at a dose of 100 $\mu\text{g/kg}$ body wt. CORT responses to CRF injection (100 $\mu\text{g/kg}$ body wt) were significantly higher ($P = 0.03$) in STI quail than in LTI quail, and females also showed significantly higher CORT responses than males ($P = 0.005$).

Experiment 3. Being placed in the crush cage induced significant increases ($P < 0.0001$) in CORT levels, which were significantly higher ($P = 0.0005$) in STI quail than in LTI quail. Injection of AVT at doses of 10 or 50 $\mu\text{g/kg}$ body wt resulted in significant ($P < 0.0001$) and similar increases in CORT levels 15 min postinjection in both genotypes and both sexes (Fig. 4B). CORT concentrations reached in response to injection of AVT were similar ($P = 0.7$) to those in response to injection of 1-24 ACTH at the dose of 100 $\mu\text{g/kg}$ body wt and higher than those induced by restraint ($P < 0.001$) and CRF (100 $\mu\text{g/kg}$ body wt) ($P < 0.001$) in both genotypes and sexes. In LTI quail, increases in CORT levels in response to being placed in the crush cage or injection of CRF at the dose

Fig. 3. Changes in CORT concentrations (ng/ml plasma) measured in LTI and STI quail over time following injection of a saline solution ($3 \leq n \leq 4$ quail/experimental point; A) or 1-24 ACTH (Immediate synacthen) at doses of 2.5 ($5 \leq n \leq 7$; B), 10 ($5 \leq n \leq 9$; C), or 100 $\mu\text{g/kg}$ body wt ($5 \leq n \leq 9$; D). Data are means $\pm$ SE. x-Axis is given as a semilogarithmic scale.

of 100 $\mu\text{g/kg}$ body wt were similar ($P = 0.09$). In STI quail, injection of CRF at the dose of 100 $\mu\text{g/kg}$ body wt resulted in a significant increase in CORT levels in females ($P = 0.002$) but not in males ($P = 0.4$), and CORT levels induced by CRF injection were lower ($P = 0.01$) than those induced by restraint. Injection of both CRF and AVT resulted in significantly lower levels ($P = 0.006$) than those induced in response to injection of AVT alone or 1-24 ACTH (100 $\mu\text{g/kg}$ body wt).

DISCUSSION

To make sure that the peptides used to investigate adrenal and pituitary function were appropriate for the quail model, we first characterized the respective cDNA of CRF, AVT, and ACTH and the predicted amino acid sequences. Comparison of the predicted amino acid sequences of quail CRF, AVT, and ACTH mature peptides revealed complete peptide homology with chicken peptides, which suggests that the corresponding genes are probably highly conserved, at least the coding region. These peptides are also highly homologous with the corresponding peptides of some mammalian species. Quail and chicken CRF are in fact fully homologous to human, pig, and rat CRF but differ from bovine and ovine CRF. CRF homologous to human, rat, and pig CRF should therefore be used in pharmacological investigations in quail and probably in other domestic species of birds. On the other hand, complete identity of AVT between several species of birds and mammals makes it possible to use the same AVT for pharmacological investigations in those species. The complete homology of the region encoding 7-39 ACTH in the quail and chicken suggests that the first six amino acids of the NH_2 -terminal region of quail ACTH (i.e., 1-6 ACTH), which have not been characterized, are likely to be homologous with those characterized in the chicken. On the other hand, quail and chicken ACTH differs from mammalian ACTH, particularly by two amino acids in the region encoding 1-24 ACTH, one of the two being located in the region encoding 1-18 ACTH reported to be active, as well as the complete ACTH peptide (9). Despite these differences, injection of mammalian 1-24 ACTH has been reported to stimulate very actively CORT release from the adrenal glands in birds (2, 23, 35, 36, 43). Thus the two differences in amino acids between birds and mammals in the region encoding 1-24 ACTH are likely to be mainly conservative and would probably result in the same tertiary structure of the peptide that would not impair mammalian biological 1-24 ACTH activity in the quail and chicken. Pharmacological challenge using mammalian 1-24 ACTH thus constitutes a valuable approach to investigating adrenal function in LTI and STI quail.

Investigation of adrenal function by injection of physiological doses of 1-24 ACTH ranging between 1.25 to 10 $\mu\text{g/kg}$ body wt in this study (i.e., doses inducing increases in CORT levels in the range of physical treatment or stress) suggests that adrenal sensitivity is comparable in LTI and STI genotypes of both sexes. As reported in other bird species (2, 35), the duration of CORT response following the injection of 1-24 ACTH and time intervals to reach maximum CORT levels were found to be dependent upon dose, and again the kinetics were comparable for both genotypes. Thus CORT responses were already at maximum levels within 5 min postinjection for doses up to 10 $\mu\text{g/kg}$ body wt. Maintenance of CORT concentrations at similar levels between 20 and 60 min postinjection

Fig. 4. CORT concentrations (ng/ml plasma) measured in LTI and STI quail 15 min or 30 min after im injection of CRF at doses of 10 or 100 μ g/kg body wt ($7 \leq n \leq 8$ quail per experimental point; A) and 15 min after restraint in the crush cage, with injection of 100 μ g/kg body wt 1-24 ACTH, 10 μ g/kg body wt AVT, 50 μ g/kg body wt AVT, 100 μ g/kg body wt CRF, or injection with both 100 μ g/kg body wt of CRF and 50 μ g/kg body wt of AVT ($7 \leq n \leq 10$; B). Data are means $\pm$ SE. Control quail were injected with vehicle.

of 100 μ g/kg body wt of 1-24 ACTH in three of the four groups of quail suggests that maximum CORT levels were reached in this interval or that CORT release was stable throughout this period. Longer time intervals to reach the maximum response have been reported in other studies (2, 35), but this difference in timing might be due to the exact nature of the 1-24 ACTH used. Indeed, different time-course responses have been reported in broilers using two different forms of 1-24 ACTH (immediate and delayed synacthen; see Ref. 22). On the other hand, the higher CORT levels measured in STI quail than in LTI quail in response to the injection of pharmacological doses of 1-24 ACTH (i.e., over 10 μ g/kg body wt) indicated that maximum adrenal CORT response capacity was slightly, but significantly, higher in STI than in LTI quail. We can therefore conclude that genetic selection for divergent duration of TI has somehow affected the maximum adrenal CORT response capacity. Results from previous studies have similarly indicated that the adrenal level can be a major area of genetic variability in HPA axis reactivity in various species such as broilers (7), rats (20), and pigs (10, 11, 63). However, differences in adrenal responsiveness between LTI and STI genotypes were only observed following the injection of very high doses of 1-24 ACTH, which induced greater increases in CORT levels than physical stress (i.e., restraint in a crush cage) (27) and which thus could be considered as supraphysiological

doses. On the other hand, CORT responses in the range of those measured following the restraint test were observed after injection of much lower doses of 1-24 ACTH (<10 μ g/kg body wt) and led to comparable CORT responses for the two genotypes. It is therefore unlikely that the difference in adrenal responsiveness observed can be the cause of the difference in CORT responses between LTI and STI genotypes resulting from restraint stress.

Possible involvement at the pituitary level in the genotype difference in CORT response to restraint was investigated by injecting AVT and/or CRF. It has been shown in previous studies that AVT is active in stimulating CORT release in birds, and is even more potent than CRF in ducks (8), pigeons (61), and European starlings (45). Similarly, in this study, high doses of AVT induced CORT responses of comparable amplitude to those induced in response to high doses of 1-24 ACTH in both genotypes, whereas CORT levels induced by injection of CRF were of much lower amplitude. These results indicate that AVT is very active and much more potent than CRF in stimulating the pituitary-adrenal axis in LTI and STI genotypes. On the other hand, results from other studies have indicated that central administration of CRF is more effective than AVT in inducing CORT release in the plasma of chickens (38, 58), and it has been reported that CRF is a more potent secretagogue than AVP in several mammals [rats (19, 46, 59);

ewes (33)]. However, the effects of CRF in birds were investigated in several earlier studies using the ovine CRF peptide (8, 61), but subsequent characterization of the chicken CRF sequence (60) showed that it differs from the ovine sequence by 7 out of 41 amino acids. Given these differences in peptide sequence, it could be hypothesized that the weaker potency of CRF in inducing ACTH release observed in previous studies compared with AVT was due to the fact that the natural CRF was different from the ovine CRF. Although the individual differences in amino acids are predominantly conservative, it cannot be excluded that these differences in amino acids may result in differences in the tertiary structure of the peptide, and thus impair its biological activity. This hypothesis could not be valid in the present study since we used a commercially available rat/human CRF whose structure was shown in this study to be homologous to the quail CRF. On the other hand, because it was administered peripherally, we cannot exclude the possibility that the dose of 100 µg/kg body wt of CRF was not sufficient to induce the maximum pituitary response. However, we used a very high dose compared with the doses reported in the literature. It therefore appears very likely that AVT is a greater ACTH secretagogue in LTI and STI quail.

Similar CORT levels in LTI and STI quail in response to AVT, and above stress-induced levels, suggest that the difference between genotypes might not be because of a difference in the ability of the pituitary gland to secrete ACTH. These results also suggest that there is probably no difference in receptor capacity for AVT between the genotypes. However, it remains to be explored whether the pituitary gland's sensitivity to AVT is comparable or differs between the two genotypes to demonstrate absence of involvement of the AVT pathway in the genotype differences in CORT response.

Interestingly, higher CORT responses were measured in STI quail compared with LTI quail following CRF injection at the dose of 100 µg/kg body wt. We have no ready argument to explain why lower CORT levels were induced in response to CRF injection in *experiment 3*. Moreover, the higher or similar levels induced by CRF (*experiment 2*) compared with those induced by restraint stress suggest that activation of the pituitary gland by the CRF pathway can be sufficient to induce the CORT response observed following restraint stress, with the possible exception of male STI quail. We therefore cannot exclude the possibility that a difference in pituitary responsiveness to CRF might be involved in the differential HPA axis reactivity to stress observed between LTI and STI genotypes. Pituitary gland release of ACTH through CRF activation could be the rate-limiting step regulating CORT secretion in response to acute stress because of limits in receptor capacity for CRF.

A synergic effect between AVT and CRF on CORT release has previously been reported in ducks (8). Surprisingly, although both peptides had a secretagogue effect on CORT, a synergic effect was not observed in the present study. We hypothesized that this was because of the fact that the doses of AVT used already induced the maximum responses. Interestingly, the CORT levels reached in response to combined injection of CRF and AVT peptides were comparable for the two genotypes.

Other mechanisms, not yet investigated in LTI and STI quail, might be involved in the CORT differences between the two genotypes detected after restraint stress. A regulation mechanism may occur above the pituitary gland, at the level of

the hypothalamus, and could result in differences in secretion of AVT and/or CRF (47) between the LTI and STI genotypes. This may reflect differences in cognitive processes or differences in negative CORT feedback. It would also be interesting to investigate CORT-binding globulin activity and CORT metabolism, known to be involved in regulating CORT levels (6, 12, 56), which have not been studied to date in LTI and STI quail.

In conclusion, genetic selection for short duration of TI was found to be associated with a greater maximum CORT adrenal response capacity in quail, whereas adrenal sensitivity was comparable in both genotypes. The present results strongly suggest that the differences in HPA axis reactivity to acute stress between LTI and STI genotypes do not involve functional differences at the level of the adrenal glands. We also showed that AVT was much more potent than CRF in stimulating ACTH and CORT release in LTI and STI quail and that the ability of the pituitary gland to secrete ACTH was similar between the two genotypes. The AVT pathway may not be involved in the genotype difference, but pituitary sensitivity in response to AVT remains to be investigated further. On the other hand, differences in the pituitary response to CRF might be involved in the differences in HPA axis reactivity to stress observed between LTI and STI genotypes. However, the respective involvement of CRF and AVT in the HPA axis response to acute stress requires further investigation in quail before firm conclusions can be reached.

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