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DIFFERENTIAL EFFECTS OF TESTOSTERONE ON PROTEIN SYNTHESIS ACTIVITY IN MALE AND FEMALE QUAIL BRAIN

C. R. DERMON,[a] A. STAMATAKIS,[a] S. GIAKOUMAKI[a] AND J. BALTHAZART[b]*

[a]*Department of Biology, University of Crete, Heraklion 714 09, Crete, Greece*

[b]*Center for Cellular and Molecular Neurobiology, Research Group in Behavioral Neuroendocrinology, University of Liège, 17 place Delcour (Bat. L1), B-4020 Liège, Belgium*

Abstract—In Japanese quail, testosterone (T) increases the Nissl staining density in the medial preoptic nucleus (POM) in relation to the differential activation by T of copulatory behavior. The effect of T on protein synthesis was quantified here in 97 discrete brain regions by the *in vivo* autoradiographic ^{14}C-leucine (Leu) incorporation method in adult gonadectomized male and female quail that had been treated for 4 weeks with T or left without hormone. T activated male sexual behaviors in males but not females. Overall Leu incorporation was increased by T in five brain regions, many of which contain sex steroid receptors such as the POM, archistriatum and lateral hypothalamus. T decreased Leu incorporation in the medial septum. Leu incorporation was higher in males than females in two nuclei but higher in females in three nuclei including the hypothalamic ventromedial nucleus. Significant interactions between effects of T and sex were seen in 13 nuclei: in most nuclei (n=12), T increased Leu incorporation in males but decreased it in females. The POM boundaries were defined by a denser Leu incorporation than the surrounding area and incorporation was increased by T more in males (25%) than in females (6%). These results confirm that protein synthesis in brain areas relevant to the control of sexual behavior can be affected by the sex of the subjects or their endocrine condition and that T can have differential effects in the two sexes. These anabolic changes should reflect the sexually differentiated neurochemical mechanisms mediating behavioral activation.

*Corresponding author. Tel: +32-4-366-5970; fax: +32-4-366-5971.
E-mail address: jbalthazart@ulg.ac.be (J. Balthazart).

Abbreviations: Aid, archistriatum pars dorsalis; AIv, archistriatum pars ventralis; AL, ansa lenticularis; ANOVA, analysis of variance; AR, androgen receptor; CCM, cloacal contact movements; CDL, area corticoidea lateralis; Cer Mol, cerebellum molecular layer; cGnRH, chicken gonadotrophin-releasing hormone; DLA, nucleus dorsolateralis anterior; DMA, nucleus dorsomedialis anterior; ER, estrogen receptor; F, female(s); FL, Field L; GnX, gonadectomized; GnX+T, gonadectomized and testosterone treated; GP, globus pallidus; LH, luteinizing hormone; LHy, lateral hypothalamic; LPO, lobus parolfactorius; M, male(s); MA, mount attempt; MLd, nucleus mesencephalicus lateralis, pars dorsalis; MM, mamillaris medialis; NG, neck-grab; OMv, oculomotor complex; OV, nucleus ovoidalis; PHN, nucleus periventricularis hypothalami; PLSD, protected least significant difference; PMI, nucleus paramedianus internus; POM, medial preoptic nucleus; PP, paleostriatum primitivum; SAC, stratum album centrale; SM, medial septum; SP, nucleus subpretectalis; SRt, nucleus subrotundus; T, testosterone; TT, tractus tectothalamicus; VIP, vasoactive intestinal polypeptide; VMN, nucleus ventromedialis hypothalami.

Key words: sexual dimorphism, medial preoptic nucleus, leucine incorporation, sex behavior, sex difference.

In most species of birds and mammals, testosterone (T) activates male-typical copulatory behavior by acting on specific brain regions. The medial preoptic area represents one critical site for T action on behavior but other brain regions such as the amygdala, bed nucleus of the stria terminalis or mesencephalic central gray are also involved (Ball and Balthazart, 2002). In many species, this activation is sexually differentiated: T normally activates the full range of reproductive behaviors in males but not in females that either do not react at all or only show a partial activation of behavior when exposed to the steroid. Although many morphological, neuroendocrine and neurochemical sex differences have been identified in the vertebrate brain (e.g. Goy and McEwen, 1980; Breedlove, 1992; de Vries, 1995), the specific mechanisms that are responsible for the sexually differentiated behavioral responses to steroids are not fully understood (Balthazart et al., 1996).

The Japanese quail (*Coturnix japonica*) displays an extreme form of sexually differentiated behavioral response to T. While this steroid regularly activates the full copulatory sequence in castrated males, this response is never observed in ovariectomized females even after treatment with doses of T that are several times larger than the behaviorally active doses in males (Adkins, 1975; Balthazart et al., 1983). The origins of this sexually differentiated response to T have been traced to the endocrine environment of the quail embryo. Ovarian estrogens secreted before the 12th day of incubation suppress in genetic females the capacity of responding to T by showing male typical behavior: they are demasculinized (Adkins, 1975; Balthazart and Adkins-Regan, 2002). This process can be blocked by injecting female embryos with an aromatase inhibitor that will prevent synthesis of endogenous estrogens (Balthazart et al., 1992) and it can be mimicked in males by injecting the embryos with exogenous estrogens before day 12 of incubation (Adkins, 1979). The neuroendocrine/neurochemical mechanisms underlying this behavioral demasculinization are however only partly understood (Balthazart et al., 1996).

A combination of studies based on electrolytic lesions and stereotaxic implantation of T clearly demonstrate that T action in the medial preoptic nucleus (POM) of the preoptic area is necessary and sufficient for the activation of male copulatory behavior in castrated males provided, of course, that adequate sexual stimuli (a sexually receptive female) are present (Panzica et al., 1996a). Interestingly

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the volume of the POM is sexually differentiated and controlled by the circulating T levels: it is larger in males than in females, reduced by castration and increased back to normal levels (i.e. those seen in sexually mature reproductively active males) following treatment of castrates with exogenous T. However, in gonadectomized female quail treated with exogenous T, the POM volume increases to the level normally seen in sexually mature males whereas these females still do not display male typical copulatory behavior (Panzica et al., 1996a). The sex difference in POM volume observed in gonadally intact sexually mature birds thus reflects the differential levels of circulating T (the nucleus is differentially activated by circulating hormones in adulthood) rather than a permanent differentiating effect of embryonic estrogens. In contrast the behavioral dimorphism affecting male-typical copulatory behavior is the result of the differential exposure to estrogens during the embryonic life. The POM volume is therefore a good signature of T action in the brain but cannot be used to predict the behavioral phenotype of a given subject. Other aspects of the POM anatomy (neuronal size in the lateral part of the nucleus) and neurochemical organization (aromatase activity, number of aromatase-immunoreactive neurons, dopamine turnover...) are also sexually dimorphic in adult quail (Balthazart et al., 1996) but available data do not permit to determine whether these sex differences result from a differential activation by steroids in adulthood or from a differential organization by estrogens during embryonic life (see Balthazart et al., 1996; Balthazart and Adkins-Regan, 2002 for review).

The POM can be identified from the surrounding preoptic area by a denser staining with dyes such as Toluidine Blue, Thionine Blue or Cresyl Violet that more or less specifically label the Nissl substance (Balthazart et al., 1991; Panzica et al., 1991). This presumably reflects a higher protein synthesis activity in the nucleus as compared with its surroundings. T treatment of castrated male quail also increases the Nissl staining density in the POM in parallel with the differential activation of copulatory behavior, suggesting an increased protein synthesis following exposure to the steroid (Balthazart et al., 1991). This suggests that the effects of T on behavior could be mediated, at least in part by changes in the protein synthesis activity in the POM. Nissl stains do not however represent a very specific technique to assess protein synthesis. Therefore in the present study, we used the *in vivo* L-[1-^{14}C]-leucine autoradiographic method to map protein synthesis activity in the brain of adult castrated male quail that had been submitted or not to a replacement therapy with T. This experiment specifically tested whether T increases protein synthesis activity in the POM but also in other steroid-sensitive areas and in brain regions that are known to be implicated in the control of male sexual behavior based on lesion studies (Balthazart et al., 1998a; Absil et al., 2002) and on the analysis of the induction of immediate early genes (measured by immunocytochemistry; Ball et al., 1997; Meddle et al., 1997, 1999) or of changes in oxidative metabolism (determined by the quantification of 2-deoxyglucose uptake; Dermon et al., 1999) following expression of these behaviors. Ovariectomized females treated or not with T were studied in parallel by the same technique to assess the possible existence of sex differences in protein synthesis that could be related to the sex differences in the behavioral effects of T.

EXPERIMENTAL PROCEDURES

Subjects and *in vivo* procedures

Experiments were carried out on eight male and eight female Japanese quail (*C. japonica*) that were purchased at the age of 3 weeks from a local breeder in Belgium (Elevage Paulus, Vinalmont-Wanze). Five days after their arrival in the laboratory, all birds were gonadectomized under total anesthesia (Hypnodil; Janssen Pharmaceutica, Beerse, Belgium; 15 mg/kg) through a unilateral incision behind the last rib on the left side using procedures that were previously described (Schumacher and Balthazart, 1984). Three weeks later, all subjects received two 20 mm long subcutaneous Silastic implants (Dow Corning, Midland, MI, USA; nbr 602–252; 1.57 mm internal diameter; 2.41 mm outside diameter) filled with crystalline T (n=4 males and four females) or left empty as control (n=4 males and four females). This generated four groups of subjects: gonadectomized males (M) and females (F) that were treated with T (GnX+T) or with control implants (GnX). Birds were left undisturbed for 4 weeks and then were submitted on 3 consecutive days to three behavior tests in order to quantify their copulatory behavior when introduced to a sexually mature female.

Presentations lasted 5 min each and took place in a small arena (60×40 cm) following a standard procedure that has been previously described (Schumacher and Balthazart, 1984; Balthazart et al., 1998a). Briefly, the stimulus female was introduced in the arena containing the experimental subject and the frequencies for the following behavior patterns were recorded: neck-grab (NG), mount attempt (MA; counted only when a bird showing a NG raised one leg and put it over the back of the test female), mount (Mo) and cloacal contact movements (CCM; see (Adkins and Adler, 1972; Hutchison, 1978) for a detailed description). Because the different sexual behaviors that are directly related to copulation occur in sequence (NG-MA-Mo-CCM), their frequencies are highly correlated. The following analyses will be limited to the behaviors MA and CCM. The analysis of NG and Mo leads to similar results and their presentation would be redundant.

The cloacal gland of each subject was measured with calipers (greatest length×greatest width=cloacal gland area) just before the implantation of Silastic capsules (beginning of the endocrine treatments) and at the end of the experiment in order to confirm the effectiveness of the treatment with T. This gland is an androgen-dependent structure (Sachs, 1967) and its size is highly correlated with plasma T levels (Delville et al., 1984). The body weight of all birds was also recorded on these occasions. Throughout their life at the breeding colony and in the laboratory, birds were maintained under a photoperiod simulating summer long days (16L:8D). During the behavioral experiment, all birds were isolated in individual cages. All experimental procedures were in agreement with the Belgian laws on “Protection and Welfare of Animals” and on the “Protection of Experimental Animals” and the International Guiding Principles for Biomedical Research involving Animals published by the Council for International Organizations of Medical Sciences. The protocols were approved by the relevant authorities of the University of Liège.

Protein synthesis assay

One week after the last behavior test, the brain protein synthesis activity of all subjects was studied with radiolabeled L-leucine, an

essential amino acid present in most proteins. Local L-[1-^{14}C]-leucine incorporation into proteins was determined by means of an i*n vivo* autoradiographic method. Labeled leucine was chosen as tracer because the only pathway for the metabolic degradation of this amino acid transfers the label to α-ketoisocaproic acid and ultimately to $^{14}CO_2$. The ^{14}C that is released during metabolism of labeled leucine is thus not (or only negligibly) re-incorporated into proteins due to its dilution by large amounts of unlabeled CO_2 produced by the cerebral metabolism (Banker and Coltman, 1971). The radioactivity retained in the brain thus only represents the product of the reaction, the labeled proteins and the residual non incorporated amino acid. The concentration of free radiolabeled amino acids in the tissue can be minimized by allowing a sufficiently long survival time (more than 45 min) between the pulse injection and the killing of the animal, allowing its clearance from plasma and tissue (Sokoloff and Smith, 1983) and by extensively washing the sections in running water before autoradiography (see below).

Food and water were removed for 1–2 h and each subject then received an i.p. injection of L-[1-^{14}C] leucine (sp. Act. 56 mCi/mmol; Amersham Corporation, Arlington Heights, IL, USA) at a dose of 100 μCi/kg in sterile saline. Immediately after the injection, quail were returned to their home cage. Sixty minutes later, the animals were killed by decapitation. Their brain was immediately dissected out of the skull, frozen on dry ice, and stored at −75 °C until sectioned for autoradiographic experiments.

Autoradiography and data analysis

All brains were cut in the coronal plane at 20 μm thickness with a Leica cryostat at −20 °C. Throughout the brain, sets of three adjacent sections were collected and thaw-mounted on gelatin-coated slides while the next 140 μm were skipped. Three sections were thus available for study every 200 μm. Two series of these sections were fixed overnight in 37% formalin, washed under running water for 2 h, dipped in double distilled H_2O, dried and exposed to Amersham ^{14}C-sensitive autoradiographic Hyperfilm β max, along with a set of ^{14}C-methylmethacrylate standards (Amersham), as previously described (Sokoloff et al., 1977). The last series of adjacent sections was counterstained with Cresyl Violet for cytoarchitectonic identification. After 3 weeks of exposure, the films were developed and their optical density analyzed with a MacIntosh based image analysis system (NIH Image, v. 1.61, Wayne Rasband; NIH, Bethesda, MD, USA). Leucine incorporation into proteins was determined in 97 discrete brain structures. Each structure was outlined and measured in four to six consecutive sections depending on its rostro-caudal extent, using the corresponding Nissl-stained sections as reference. Mean protein synthesis activity was transformed in nCi/g tissue based on the optical density of the radioactive standards, in each structure of each subject. These data were then normalized by a factor females, to minimize overall individual subject differences as described before (Dermon et al., 1999). The average leucine incorporation in the whole brain was estimated in each subject by measuring the incorporation in all sections of this animal and dividing the total by the number of sections. The females factor for each subject was determined as the ratio of the average ^{14}C leucine incorporation in the whole brain of all animals analyzed (n=16) divided by the individual animal's whole brain mean ^{14}C-leucine incorporation. For each brain structure considered, data were analyzed by a two-way analysis of variance (ANOVA) with the sex of the birds and their endocrine treatment (GnX and GnX+T) as factors. All tests were carried with the software SuperANOVA (Abacus Concepts, Inc, CA, USA). All data presented in the text are means±S.E.M. When significant interactions were observed between the two factors, additional post hoc comparisons were made with the Fisher protected least significant difference (PLSD) test to further identify the origin of the interaction.

It must be noted that in order to limit animal suffering, reduce the number of subjects to be killed and due to the costly and labor-intensive aspects of the present procedures, four subjects only were included in each experimental group during the present experiment. As a result, the statistical power of all analysis must be taken into account when interpreting the results. Differences or effects that are detected with such a statistical power are likely to represent very strong biological effects but conversely the failure to identify a difference may result either from the lack of a difference in the population or from the low power of the statistical analysis. Accordingly a number of effects are suggested by the average values presented in this study that did not reach statistical significance. This limitation concerns in particular effects of T that appear, in some nuclei, to be different in males and females but not to a large enough extent to induce a significant interaction in the ANOVA (see for example results concerning the nuclei SRt, PHN, VMN, PMI, POM and MM in Figs. 5 and 6). These sex differences are therefore not systematically discussed in the text although they may warrant future work to test their reliability.

Nomenclature used in this paper is based on the atlas of the chicken or quail brain (Baylé et al., 1974; Kuenzel and Masson, 1988) with minor modifications for the preoptic area and anterior hypothalamus as described in Panzica et al. (1991) and Aste et al. (1998). It must be noticed that the avian brain nomenclature is now in a process of profound revision and some of the terms used in the present paper perhaps will be changed soon. A summary of the discussion on this topic is currently at the Avian Brain Nomenclature Exchange web site (http://jarvis.neuro.duke.edu/nomen/index.html).

RESULTS

Behavioral and morphological data

During the behavior tests, all experimental subjects conformed to what could be expected based on previous studies. The four castrated males (GnX group) were sexually inactive when presented to a female and the four castrates that had been treated with T showed an active copulatory behavior in the same circumstances. All females showed absolutely no male-typical sexual behavior even when treated with exogenous T (See Fig. 1). Analysis of the total MA and CCM occurrence frequencies during the three tests by the nonparametric Kruskal-Wallis ANOVA confirmed the presence of statistically significant differences between the four groups of subjects (H corrected for ties=14,619, df=3, P=0.0022 for both behaviors). Subsequent nonparametric Mann Whitney tests indicated that for each behavior, the occurrence frequency was significantly higher in the male GnX+T group than in the three other groups (P<0.05 in each case; see Fig. 1).

Similarly, the measures of the cloacal gland areas taken before and at the end of the T treatment confirmed its effectiveness and the well-documented sex difference in responsiveness to the steroid (larger response in males than in females; Schumacher and Balthazart, 1984). A three way ANOVA of these data (sex and treatment= independent factors, successive measures=repeated factor) confirmed the overall statistical significance of the differences between sexes ($F_{1,12}$=27,97, P<0.0002), between castrates and castrates treated with T ($F_{1,12}$= 636,30, P<0.0001), and between dates (before and after treatment: $F_{1,12}$=514,55, P<0.0001). There was also a significant interaction between all these factors taken two

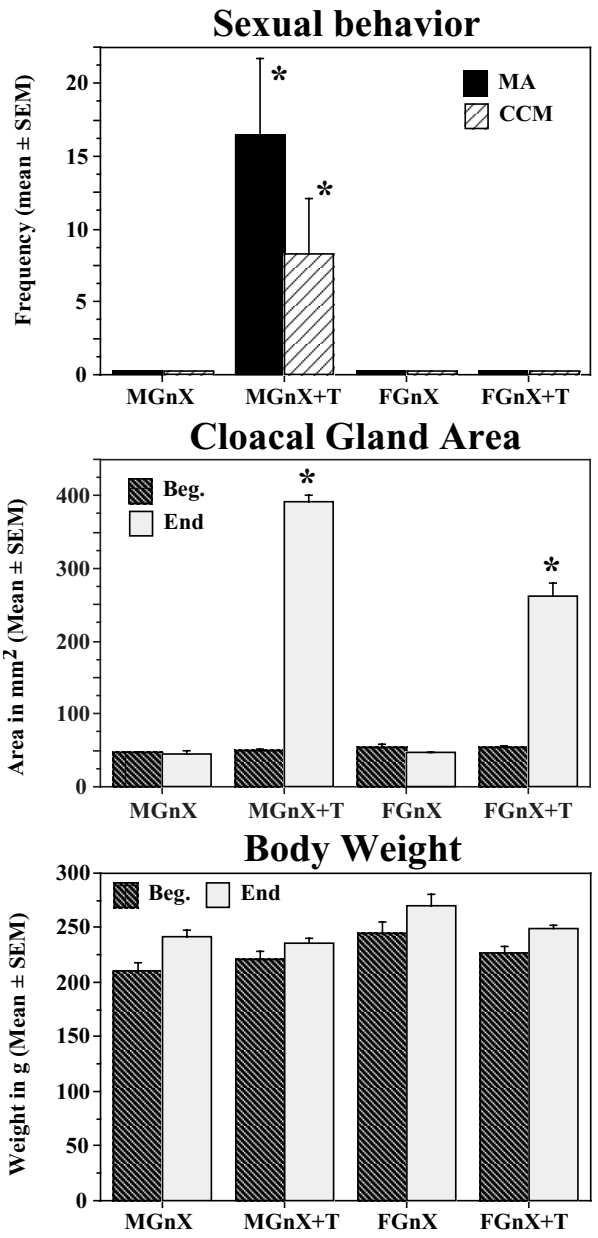

Fig. 1. Sex differences and effects of T on the expression of male sexual behavior (top), on the size of the cloacal gland (middle) and on the body weight (bottom) in GnX male and female quail. Behavioral data present the total frequencies of MA and CCM that were observed during the three 5 min tests. Cloacal gland areas and body weight were measured just before the beginning of the treatment with T (Beg.) and at the end of the experiment. See text for additional information. * $P<0.05$ Compared with all other groups.

by two ($P<0.0001$ in each case) and a significant second order interaction between all three factors ($P<0.0002$). A re-analysis by two way ANOVA (sex and treatment of the birds) of the cloacal gland areas at the end of the experiment confirmed the overall sex difference ($F_{1,12}=34.42$, $P<0.0001$), the significant effect of the treatment ($F_{1,12}=646.30$, $P<0.0001$) as well as the significant interaction between these two factors ($F_{1,12}=35.04$, $P<0.0001$). Post hoc (Fisher PLSD) tests indicated that the cloacal gland area was significantly larger in GnX+T males than in all other groups including the GnX+T females, which had in turn a larger gland than the castrated males and females not treated with T ($P<0.05$ in each case). These effects and differences were not present before the exposure to T ($0.8093>P>0.1249$).

The analysis by the same methods of the body weight of the subjects (three way ANOVA with the sex and endocrine treatments as independent factors, measures at the beginning and end of the experiment as repeated factor) confirmed the increase in body weight that is normally observed in young quail at that age (from 225.6±19.8 at the beginning to 249.5±4.5 g to at the end of the experiment) and this increase was highly significant ($F_{1,12}=95.41$, $P<0.0001$, see Fig. 1 bottom panel). This increase however affected all groups equally and there was no interaction between the repeated factor and the independent factors (sex and treatment) and no second order interaction in the ANOVA ($P>0.05$ in each case). This analysis indicated however a significant effect of the sex of the subjects ($F_{1,12}=8.16$, $P=0.0144$) but no overall effect of T ($F_{1,12}=1.26$, $P=0.2823$). A reanalysis of the two measures of body weight by two way ANOVA with the sex and treatment of the birds as independent factors showed that females were heavier than males (Beginning: $F_{1,12}=5.75$, $P=0.0337$; End: $F_{1,12}=9.85$, $P=0.0086$) but that T treatment did not affect body weight significant and there was no interaction between these two factors (all $P>0.05$).

Effect of sex and T on overall cerebral ^{14}C- leucine uptake

Because the T systemically administered to castrated quail could have affected the protein synthesis in the entire brain and this value was used to normalize data from different subjects, we first analyzed whether the steroid had any effect on the average ^{14}C-leucine accumulation throughout the brain. The average ^{14}C-leucine incorporation in the entire brain was estimated in each subject by measuring the global uptake in all sections available for that subject (two sections every 200 μm) and then calculating the average of all these values (total of the measures divided by the number of sections). Comparison of these data for the four experimental groups by two way ANOVA revealed no significant effect of the sex of the birds ($F_{1,12}=3.035$, $P=0.107$), their endocrine condition ($F_{1,12}=0.069$, $P=0.796$) and the interaction between the two factors ($F_{1,12}=1.349$, $P=0.268$).

Pattern of regional ^{14}C- leucine uptake in the brain of castrated male quail

A very heterogeneous ^{14}C-leucine uptake was detected by autoradiography in the quail brain (Table 1 and Fig. 2). Three- to five-fold differences in uptake were detected between the areas showing the lowest and highest protein synthesis activity. This heterogeneous pattern was affected in some brain areas by the sex of the subjects and/or their endocrine status (see below) but these effects

Table 1. Local cerebral leucine incorporation into protein in males and F quail that had been GnX and treated or not with T

Brain regions	MGnX	MGnX+T	FGnX	FGnX+T	SEX		Hormone		S*H	
					F-value	*P*	*F*-value	*P*	*F*-value	*P*
Dorsal ventricular ridge										
Hyperstriatum accessorium (HA)	51±2	53±2	52±1	52±3	0.002	0.964	0.307	0.589	0.281	0.605
Hyperstriatum ventrale (HV)	54±2	55±2	56±1	57±2	1.288	0.278	0.201	0.661	0.023	0.880
Hyperstriatum dorsale (HD)	56±2	57±1	55±1	55±2	0.861	0.371	0.050	0.825	0.119	0.736
Hyperstriatum intercalatum supremum (HIS)	55±2	54±1	54±1	56±2	0.015	0.904	0.304	0.591	0.134	0.720
Neostriatum (N)	55±4	52±2	54±1	57±2	0.515	0.486	0.034	0.855	1.459	0.250
Neostriatum lateral (LN)	52±4	45±3	48±2	46±2	0.244	0.629	2.789	0.120	0.948	0.349
Nucleus basalis (Bas)	57±1	61±0.3	57±3	59±3	0.088	0.770	1.756	0.209	0.069	0.796
Neostriatum intermedium (NI)	58±1	53±1	55±4	56±1	0.015	0.901	0.911	0.358	1.891	0.194
Ectostriatum (E)	64±4	61±4	59±3	63±3	0.138	0.150	0.027	0.870	1.015	0.333
Nucleus accumbens (Ac)	55±6	45±2	47±2	53±2	0.003	0.954	0.357	0.560	4.643	0.052
Nucleus commissurae pallii (nCPA)	74±6	82±3	85±7	81±2	1.088	0.317	0.124	0.730	1.332	0.270
Paleostriatal complex										
Paleostiatum augmentatum (PA)	58±2	56±1	56±1	56±2	0.863	0.371	0.165	0.691	0.346	0.567
Paleostriatum primitivum (PP)	39±1	37±1	36±2	35±1	5.542	**0.036**	1.509	0.242	0.689	0.688
Lobus paroflactorius (LPO)	42±1	48±1	48±1	43±2	0.098	0.756	0.002	0.960	14.330	**0.002**
Archistriatal complex										
Archistriatum anterior (AA)	50±2	50±2	52±2	48±1	0.014	0.905	0.998	0.337	0.611	0.449
Archistriatum ventrale (Alv)	51±2	55±2	50±1	59±1	0.657	0.433	14.990	**0.002**	2.252	0.159
Archistriatum dorsale (Ald)	52±3	56±1	53±2	58±2	0.241	0.632	4.851	**0.047**	0.100	0.756
Nucleus taeniae (Tn)*	56±1	60±2	61±4	62±3	1.474	0.248	1.034	0.329	0.212	0.653
Area corticoidea lateralis (CDL)	42±2	45±2	46±1	49±2	5.570	**0.036**	3.372	0.091	0.053	0.821
Area parahippocampalis (APH)	54±2	58±4	61±5	55±2	0.204	0.659	0.133	0.72	1.785	0.206
Hippocampus (Hp)	51±5	53±1	54±1	57±3	1.475	0.247	0.493	0.495	0.062	0.807
Field L (FL)	60±1	63±2	66±1	61±1	1.791	0.205	0.122	0.731	7.195	**0.019**
Cortex piriformis (Cpi)	44±2	42±1	42±2	39±3	1.347	0.268	0.991	0.339	**0.004**	0.946
Tuberculum olfactorium (TO)	41±3	49±2	46±1	48±4	0.346	0.566	2.603	0.132	0.791	0.391
Nucleus septalis lateralis (SL)*	42±2	39±1	41±1	39±2	0.121	0.733	3.186	0.099	0.252	0.624
Nucleus septalis medialis (SM)	44±1	39±1	48±2	40±2	2.242	0.16	16.610	**0.001**	1.888	0.194
Preoptic area										
Anterior part (POA)*	43±2	39±1	45±2	44±2	3.167	0.100	2.844	0.117	0.409	0.534
Nucleus preopticus medialis (POM)*	51±1	64±3	53±4	56±4	0.786	0.392	6.697	**0.023**	2.618	0.131
Diencephalon										
Thalamus										
Nucleus geniculatus lateralis										
Pars ventralis (GLv)	54±2	61±1	58±2	58±2	0.036	0.852	0.232	0.161	3.164	0.100
Tractus tectothalamicus (TT)	37±1	40±1	45±3	34±2	0.317	0.583	3.449	0.088	13.390	**0.003**
Nucleus triangularis (Tr)	58±1	63±0	67±5	57±3	0.102	0.754	0.784	0.393	6.040	**0.03**
Nucleus subrotundus (SRt)	70±3	78±3	83±3	79±3	4.989	**0.045**	0.484	0.499	3.734	0.077
Nucleus rotundus (ROT)	52±1	54±8	63±3	54±1	1.338	0.269	0.488	0.498	1.523	0.24
Nucleus ovoidalis (OV)	90±4	108±3	109±6	96±2	0.725	0.41	0.551	0.472	15.290	**0.002**
Nucleus dorsolateralis anterior (DLA)	50±2	61±1	63±6	57±2	1.791	0.205	0.357	0.561	5.330	**0.039**
Nucleus dorsomedialis anterior (DMA)	46±3	56±1	55±3	51±2	0.562	0.467	1.906	0.192	8.092	**0.014**
Nucleus subpretectalis (SP)	62±3	73±1	66±4	62±2	1.955	0.187	1.103	0.314	7.832	**0.016**
Nucleus spiriformis medialis (SpM)	86±5	87±5	97±5	80±4	0.200	0.662	2.548	0.136	3.179	0.099
Nucleus spiriformis lateralis (SpL)	77±4	86±4	95±9	82±4	1.676	0.219	0.177	0.680	4.203	0.062
Nucleus ruber (Ru)	57±5	64±4	65±5	63±5	0.548	0.473	0.291	0.599	1.068	0.321
Ansa lenticularis (AL)	30±1	35±2	42±5	30±2	1.239	0.287	1.132	0.308	8.164	**0.014**
Nucleus paramedianus internus thalami (PMI)	56±2	68±4	65±2	71±6	2.317	0.153	4.812	**0.048**	0.735	0.408
Nucleus habenularis medialis (HM)	57±3	59±3	55±2	57±4	0.622	0.445	0.429	0.524	0.008	0.928
Nucleus habenularis lateralis (HL)	79±5	95±7	95±6	91±2	1.282	0.279	1.115	0.311	2.862	0.116
Nucleus tractus septomesencephalicus (nTSM)	61±3	56±3	55±1	60±6	0.037	0.850	0.002	0.961	1.903	0.192
Tractus habenulointerpeduncularis (Hip)	42±1	41±5	48±4	44±3	1.465	0.249	0.317	0.583	0.163	0.693
Nucleus dorsolateralis posterior (caudalis) thalami (DLP)	58±2	62±2	61±4	61±3	0.174	0.683	0.391	0.543	0.609	0.450
Nucleus dorsomedialis posterior (caudalis) thalami (DMP)	52±3	58±2	58±2	56±2	0.704	0.417	1.409	0.258	3.395	0.090
Stratum cellulare externum (SCE)	53±3	51±1	52±3	56±4	0.676	0.426	0.179	0.679	1.342	0.269

Table 1. continued

Brain regions	MGnX	MGnX+T	FGnX	FGnX+T	SEX		Hormone		S*H	
					F-value	*P*	*F*-value	*P*	*F*-value	*P*
White matter										
Fasciculus prosencephali lateralis (FPL)	43±1	43±2	44±2	43±2	0.234	0.636	0.223	0.645	0.431	0.523
Commissura anterior (CA)	31±2	30±2	33±2	30±3	0.243	0.630	1.049	0.325	0.273	0.610
Commissura posterior (CP)	31±2	27±4	27±1	28±2	0.134	0.720	0.441	0.519	0.717	0.413
Nervous oculomotorius (NIII)	14±2	12±1	14±3	12±1	0.004	0.945	1.104	0.314	0.036	0.852
Tractus septomesencephalicus (TSM)	21±2	17±0	22±3	20±1	0.955	0.347	2.168	0.166	0.538	0.477
Hypothalamus										
Nucleus paraventricularis magnocellularis (PVN)*	54±5	54±1	62±3	54±1	1.439	0.253	1.903	0.192	1.745	0.211
Nucleus suprachiasmaticus (SCN)*	48±3	45±3	43±5	53±3	0.075	0.787	0.828	0.382	2.959	0.113
N. anterior medialis hypothalami (AM)*	42±3	44±1	47±2	48±2	4.719	0.052	0.487	0.499	0.031	0.862
N. ventromedialis hypothalami (VMN)*	44±2	47±2	52±2	48±0.4	9.487	**0.010**	0.103	0.753	4.216	0.064
Regio lateralis hypothalami (LHy)*	37±2	47±3	42±2	48±2	1.767	0.210	11.680	**0.005**	0.666	0.431
N. periventricularis hypothalami (PHN)*	43±1	50±2	56±2	50±1	18.700	**0.001**	0.139	0.715	13.732	**0.003**
N. inferioris hypothalami (IH)*	46±2	49±2	51±3	52±2	3.113	0.105	0.524	0.484	0.409	0.535
N. mammilaris medialis (MM)*	57±4	59±4	51±2	71±2	1.066	0.324	13.366	**0.003**	8.972	**0.012**
Mesencephalon										
Optic tectum										
Stratum griseum et fibrosum superficiale superficial (SGFSs)	33±2	34±1	32±1	34±1	0.326	0.578	1.563	0.235	0.224	0.644
Stratum griseum et fibrosum superficiale deep (SGFSd)	63±3	63±2	62±3	64±3	1.880	0.998	0.064	0.804	0.064	0.803
Stratum griseum centrale (SGC)	59±2	60±2	59±3	60±2	0.006	0.939	0.196	0.665	0.000	0.990
Stratum album centrale (SAC)	26±3	30±3	32±1	24±2	0.041	0.842	0.591	0.456	7.296	**0.019**
Stratum griseum periventriculare (SGP)	39±1	41±1	38±1	41±2	0.173	0.684	1.825	0.203	0.131	0.724
Isthmic complex										
Nucleus isthmi pars magnocellularis (Imc)	56±3	59±2	58±2	54±2	0.616	0.447	0.017	0.897	2.182	0.165
Nucleus isthmi pars parvocellularis (Ipc)	66±2	68±2	68±4	64±5	0.047	0.831	0.059	0.811	0.426	0.526
Nucleus isthmo-opticus (IO)	75±2	72±3	78±4	77±3	1.503	0.243	0.314	0.585	0.246	0.628
Area ventralis Tsai (AVT)*	45±3	49±2	45±4	42±2	1.170	0.300	0.025	0.875	1.751	0.210
Nucleus semilunaris (SLu)	76±4	84±5	82±6	85±8	0.354	0.565	0.911	0.362	0.268	0.615
Nucleus pretectalis (PT)	87±4	94±3	94±5	100±6	1.806	0.203	1.987	0.184	0.056	0.816
Nucleus opticus basalis (nBOR)	56±2	64±2	61±4	60±2	0.070	0.795	1.515	0.241	2.503	0.139
Nucleus tegmenti pendunculo-pontinusm pars compacta (TPc)	60±1	64±2	68±5	58±2	0.011	0.915	0.447	0.516	4.017	0.068
Nucleus mesencephalicus lateralis pars dorsalis (MLd)	75±2	78±2	75±4	78±3	0.001	0.975	1.337	0.269	0.014	0.905
Nucleus intercollicularis (ICo)*	45±1	47±3	43±3	47±3	0.393	0.542	1.133	0.307	0.081	0.780
Nucleus interpenduncularis (IP)	32±3	34±3	32±4	31±3	0.251	0.625	0.032	0.860	0.185	0.674
Nucleus nervi trochlearis (nIV)	78±3	88±3	88±8	89±6	0.803	0.387	1.036	0.328	0.612	0.449
Locus ceruleus (LoC)	48±2	45±2	43±2	44±1	4.027	0.067	0.249	0.626	1.011	0.334
Nucleus subceruleus (SC)	44±2	45±1	40±3	41±1	3.726	0.079	0.306	0.590	0.001	0.968
Nucleus lemnisci lateralis. pars intermedia (LLi)	61±5	41±14	58±6	58±7	0.542	0.476	1.324	0.274	1.204	0.295
Nucleus linearis caudalis (LC)	69±3	75±1	72±5	74±3	0.031	0.862	1.035	0.330	0.297	0.596
Nucleus tegmenti ventralis (TV)	55±3	66±12	59±10	51±4	0.500	0.495	0.061	0.809	1.652	0.227
Substantia grisea centralis (GCt)*	52±2	55±4	60±2	55±2	1.883	0.195	0.185	0.674	2.931	0.112
Nucleus nervi oculomotorii. pars ventralis (OMv)	72±1	84±3	82±5	73±2	0.130	0.724	0.200	0.662	11.586	**0.005**
Nucleus nervi oculomotorii. pars dorsalis (OMd)	79±3	88±4	88±8	79±3	0.000	0.988	0.017	0.896	3.456	0.087
Nucleus of Edinger-Westphal (EW)	85±4	88±1	97±7	85±4	0.962	0.346	1.086	0.317	3.098	0.103
Nucleus reticularis pontis (RP)	42±2	45±2	41±3	44±2	0.107	0.749	1.487	0.248	0.005	0.941
Cerebellum										
Molecular layer (Cer Mol)	23±1	19±3	17±1	16±1	8.488	**0.013**	1.678	0.219	0.662	0.431
Granular layer (Cer Gr)	85±4	91±4	85±5	83±3	0.936	0.352	0.126	0.728	1.060	0.323
White layer (Cer Wh)	28±4	21±1	30±3	31±5	3.511	0.085	1.082	0.318	1.101	0.314
Nucleus cerebellaris lateralis (CbL)	64±2	57±8	59±3	60±1	0.124	0.731	0.647	0.439	1.333	0.275
Nucleus cerebellaris intermedius (CbIM)	65±2	69±2	67±4	66±1	0.195	0.666	0.305	0.591	0.799	0.390
Nucleus cerebellaris internus (CbI)	68±3	65±2	67±6	65±1	0.023	0.881	0.561	0.467	0.022	0.882
Nucleus pontis lateralis (PL)	55±3	54±3	51±8	54±6	0.082	0.78	0.063	0.807	0.178	0.682
Nucleus pontis medialis (PM)	51±3	61±4	47±5	53±4	2.289	0.161	4.481	0.060	0.240	0.634

[a] Results are expressed in nCi/gr protein±S.E.M. and were analyzed by two way ANOVA with the sex (S) and hormonal treatments (H) as factors. Results (*F* values and associated *P*) are listed in the six right most columns.

The asterisk after the name of a structure indicates that it expresses significant levels of AR and/or ER.

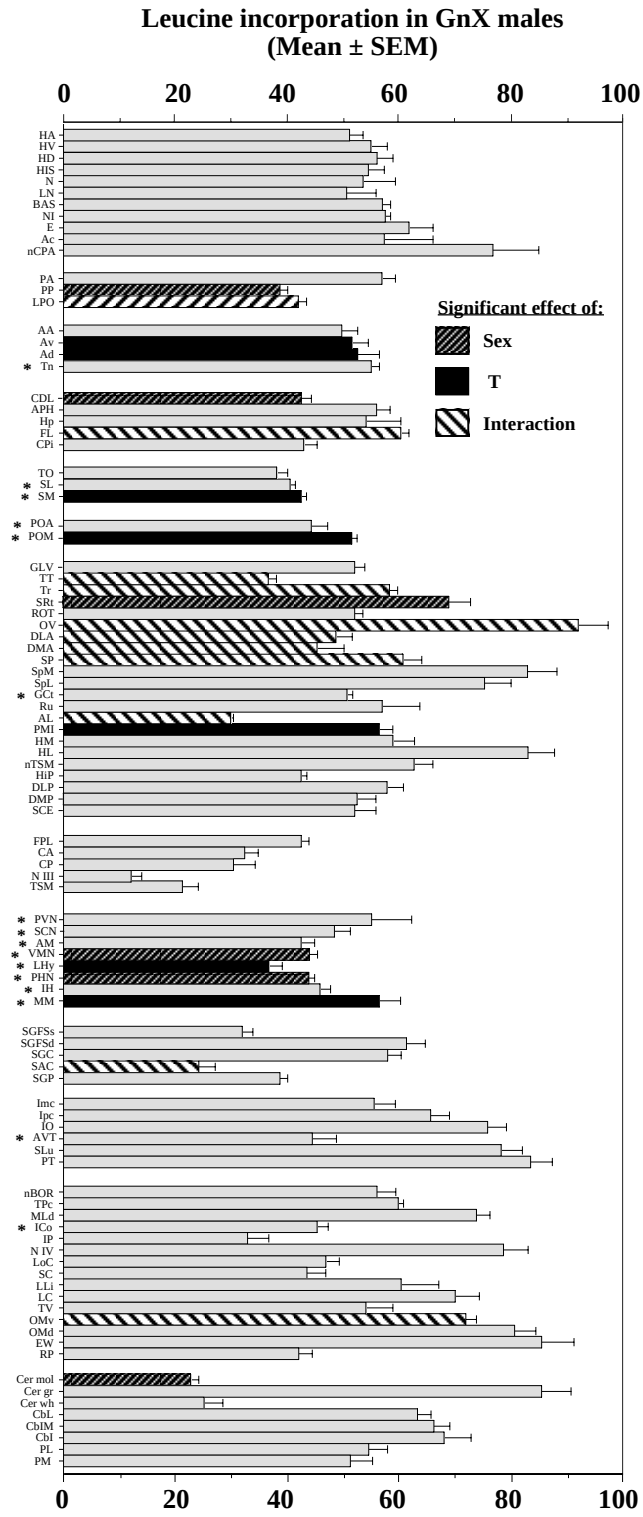

Fig. 2. Bar graph illustrating the relative incorporation of ^{14}C-leucine in 97 areas of GnX male quail. Similar data were obtained in GnX females and in birds of both sexes treated with T but although significant changes were observed as a function of the sex or T treatment, these changes were of limited absolute amplitude so that they did not substantially change the overall pattern. Data relative to each brain area were then analyzed by two way ANOVA with the sex of the birds and their endocrine condition as independent factors. The bars have been coded to indicate brain areas where significant differences in ^{14}C-leucine incorporation were detected as a function of the sex of the subjects (darkly hatched bar), their endocrine condition (black bar) or the interaction between both factors (lightly hatched bars). In addition to these significant effects encoded as described above, MM and PHN also displayed a significant interaction between sex and endocrine condition. Areas that are know to express substantial levels of androgen and/or ER are indicated by an asterisk at the left of their name.

were always of a limited magnitude (10–15% change as a maximum) so that the overall pattern of differences in activity between brain regions was not affected by these factors. This pattern is described here based on data collected in castrated males not treated with T, since previous work on sexual differentiation in quail has demonstrated that the male represents the non differentiated ("neutral") sex that develops in the absence of steroids during ontogeny (Balthazart and Adkins-Regan, 2002).

In castrated males, the highest levels of ^{14}C-leucine accumulation were observed in the nucleus ovoidalis (OV), nucleus Edinger-Westphal, granular cerebellar layer, nucleus pretectalis, nucleus habenularis lateralis, nucleus spiriformis medialis and lateralis, oculomotor complex (OMv), nucleus commissura palli, nucleus semilunaris and nucleus nervi trochlearis.

A high activity was also observed in the nucleus mesencephalicus lateralis, pars dorsalis (MLd), nucleus subrotundus (SRt), nucleus mamillaris medialis (MM) and nucleus linearis caudalis. Moderate levels of uptake were detected in the all other brain regions except white matter regions. Leucine incorporation was usually low in fiber tracts by comparison with the surrounding areas (see for example fasciculus prosencephali lateralis, commissura anterior and posterior compared with hypothalamic nuclei).

The high levels of ^{14}C-leucine uptake as compared with surrounding structures sometimes allowed one to delineate the boundaries of specific nuclei (see Figs. 3 and 4). This was for example the case for the OV, the MLd and for the POM.

Effects of sex and T on cerebral ^{14}C- leucine uptake

Analysis by two way ANOVA (sex and treatment of the birds as independent factors) of the leucine uptake in these 97 brain areas identified significant experimental effects in 24 of them (see Figs. 5–7 and Table 1 for the detail of *F* and *P* values). These ANOVAS identified sex differences in ^{14}C-leucine incorporation in both directions (males higher than females [$n=2$] or females higher than males [$n=3$]) as well as changes in uptake related to the T treatment. In most of these cases, T increased the leucine uptake ($n=5$), but in one nucleus (the nucleus septalis medialis) a decrease in protein synthesis was also observed in T-treated birds. In addition, in a large number of nuclei ($n=13$), T had differential effects in males and females which resulted in a significant interaction between the two factors in the ANOVA. In most of these cases ($n=12$), T increased leucine uptake in males but decreased it in females. All these effects are graphically presented in Figs. 5–7 and some of them are illustrated in representative autoradiograms shown in Figs. 3 and 4.

Sex differences in local cerebral protein synthesis activity

An overall sex difference in ^{14}C-leucine incorporation into protein was detected by the ANOVA in five brain areas. In two of them namely the paleostriatum primitivum (PP) and cerebellum molecular layer (Cer Mol) incorporation was higher in males than in females. In the three other regions, incorporation was higher in females. These regions are the area corticoidea lateralis (CDL), the SRt and most interestingly the nucleus ventromedialis hypothalami (VMN), a brain region that has been implicated by many studies in the control of female sexual behavior. In addition, the locus ceruleus tended to show a higher protein synthesis activity in males and a reverse trend was seen in the nucleus anterior medialis hypothalami (higher activity in females, see Table 1). These differences however fell short of significance ($P=0.067$ and $P=0.052$ respectively).

T-induced changes in local cerebral metabolic activity

Treatment with T was found to increase the ^{14}C-leucine uptake in five brain areas, the archistriatum pars ventralis (AIv) and dorsalis (AId), the POM, the nucleus paramedianus internus thalami (PMI) and the lateral hypothalamic region (LHy). The T-induced effect on leucine incorporation in the POM is clearly illustrated in the autoradiograms shown in Fig. 3.

In contrast, T induced a significant reduction in protein synthesis activity in the steroid sensitive area of medial septum (SM).

Interactions between sex and T-treatment

In 13 brain areas, T differentially affected the ^{14}C-leucine uptake in males and females. In most of these case ($n=12$), this interaction resulted from the fact that T markedly increased the leucine incorporation into proteins in males but decreased this incorporation in females. This situation was observed in the lobus parolfactorius (LPO), auditory Field L (FL), tractus tectothalamicus (TT), nucleus triangularis (Tr), OV, nucleus dorsolateralis anterior (DLA), nucleus dorsomedialis anterior thalami (DMA), nucleus subpretectalis (SP), ansa lenticularis (AL), nucleus periventricularis hypothalami (PHN), stratum album centrale (SAC), and OMv. Both effects were however not always significant as indicated by the post hoc test (See symbols on the bars in Fig. 7) but as indicated in the Experimental Procedures section this may only reflect the somewhat limited statistical power of the analyses.

In contrast in the nucleus MM, T markedly increased the leucine incorporation in females but had no or only minimal effect in males. It must be reminded that this pattern of changes also resulted in a significant overall effect of the T treatment of the leucine incorporation in this nucleus (see Table 1).

DISCUSSION

The present results demonstrate the presence of stable sex differences in protein synthesis activity in the adult quail brain. Interestingly, these differences were not associated with overall differences in leucine uptake in the entire brain suggesting that they represent a local specialization in metabolism. Furthermore, these sex

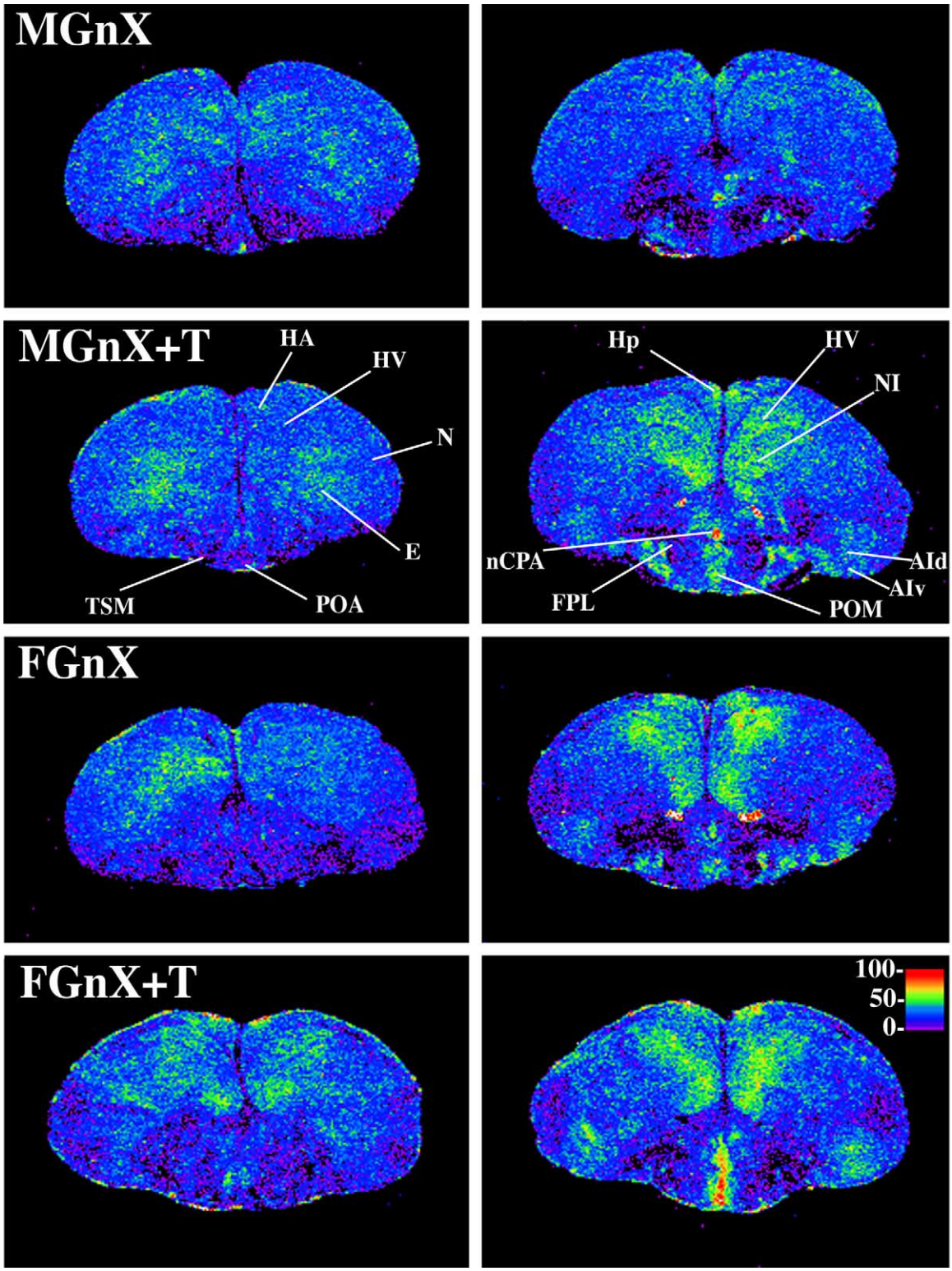

Fig. 3. Color-coded autoradiograms illustrating the neuroanatomical distribution of ^{14}C-leucine incorporation in coronal sections through the brain of the four experimental groups at the level of the preoptic area. Sections in the left columns are rostral to those presented in the right column. The color scale in the bottom right panel provides calibration of colors in nCi/gr of tissue. See Table 1 for abbreviations.

differences were observed in gonadectomized subjects that were presumably not exposed to significant levels of sex steroids. This suggests that these differences represent stable dimorphisms that do not reflect a differen-

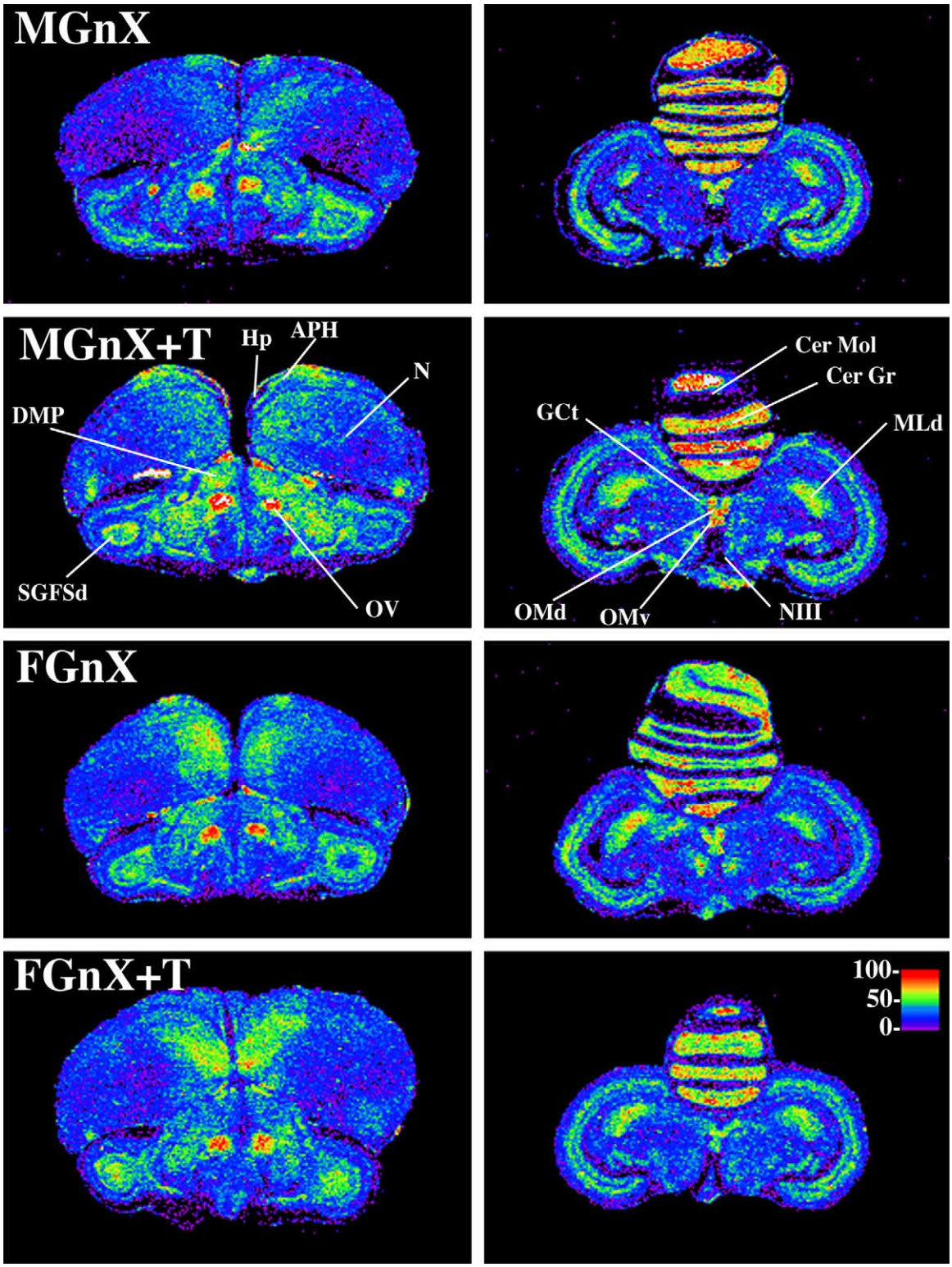

Fig. 4. Color-coded autoradiograms illustrating the neuroanatomical distribution of ^{14}C-leucine incorporation in coronal sections through the brain of the four experimental groups at the level of the caudal diencephalon (left) and mesencephalon (right). The color scale in the bottom right panel provides calibration of colors in nCi/gr of tissue. See Table 1 for abbreviations.

tial control by the endocrine milieu in the adult birds but rather the early sex differentiation during embryonic and/or early postnatal life. This notion is further supported by the fact that these sex differences were not

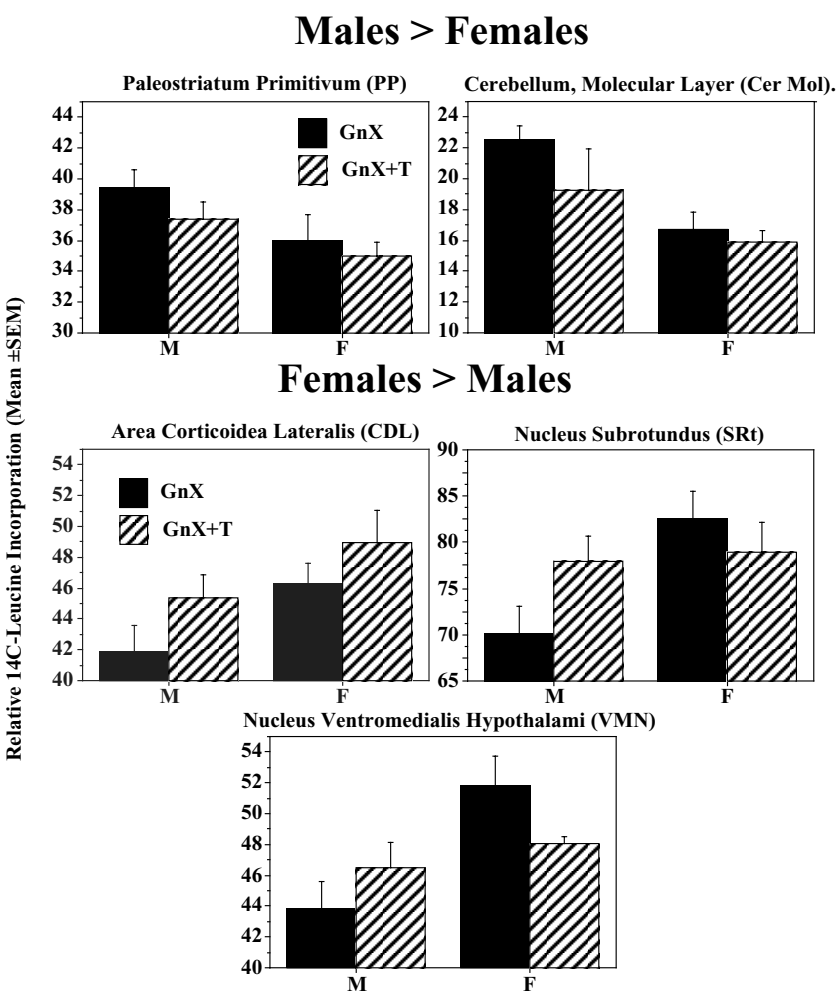

Fig. 5. Bar graphs illustrating the overall sex differences detected in the ^{14}C-leucine incorporation in the quail brain. Note that the scale used in each panel has been adjusted and truncated to permit a better illustration of the effects. Brain areas are arranged as a function of the direction of the effects observed (males higher or lower than females).

abolished when males and females were treated with identical amounts of exogenous T. However, T by itself affected the incorporation of leucine into proteins in a number of other brain areas where this aspect of the metabolism is not sexually differentiated and in yet other brain areas, this steroid differentially affected the protein synthesis in males and females. Together these data indicate that leucine incorporation into proteins is controlled both by the phenotypic sex of the birds resulting largely from their early hormonal impregnation and by their endocrine condition in adulthood. These two factors also interact in a large number of brain regions so that T exerts in these areas differential effects in males and in females. The specific location where these effects take place deserves some additional discussion with regards namely to their steroid sensitivity and their implication in sexually differentiated reproductive events. These data indeed provide new information on the metabolic activity of brain circuits that presumably underlie the sex dimorphism of sexual behavior.

Methodological issues

The *in vivo* ^{14}C-leucine autoradiographic method provides a well-established strategy for mapping protein synthesis activity in the CNS (Ingvar et al., 1985; Mies et al., 1997; Tuor et al., 1999; Smith and Kang, 2000; Hermann et al., 2001). The method of radiolabeled leucine was for example used to determine the degeneration of the auditory relay nucleus in the chick brain stem following removal of the cochlea (Stewart and Rubel, 1985) or to show that focal seizures disrupt protein synthesis in seizure pathways (Collins and Nandi, 1982). It is also known that remodeling of the nervous system in long-term events is reflected in leucine incorporation into protein in contrast to immediate function-related energy requirements that are almost exclusively met from the oxidative catabolism of glucose as can be studied by 2-deoxyglucose autoradiography (Christensen et al., 1999; Dermon et al., 1999; Balthazart et al., 2001).

Proteins are fundamental components of all structural elements in the brain tissue and as such are directly in-

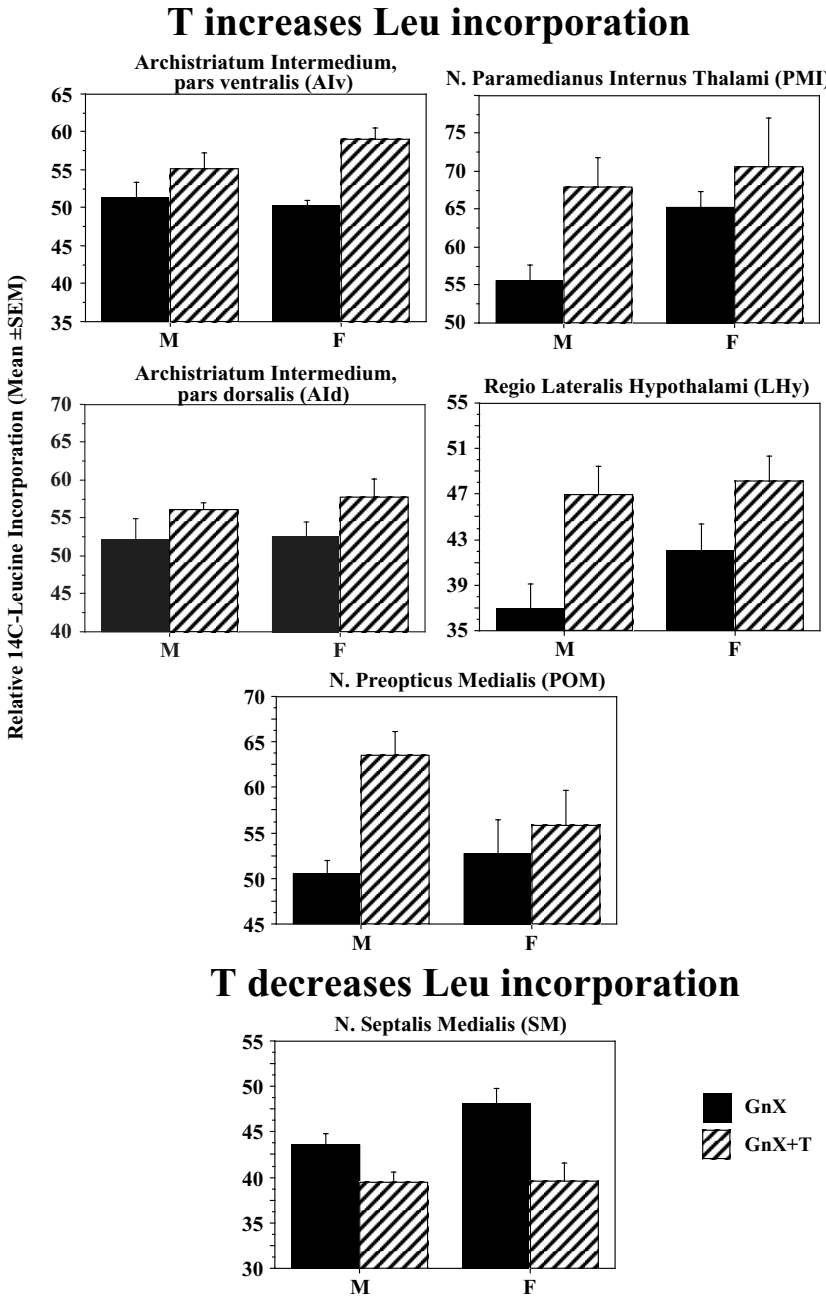

Fig. 6. Bar graphs illustrating the effects of T on the ^{14}C-leucine incorporation in the quail brain. Note that the scale used in each panel has been adjusted and truncated to permit a better illustration of the effect. Brain areas are arranged as a function of the direction of the effects observed (GnX+T higher or lower than GnX).

volved in functional events related to brain plasticity (Kennedy et al., 1981). A number of studies detected for example a stimulation of protein synthesis activity in specific areas in chicks exposed to specific learning paradigms (Schliebs et al., 1985), while degeneration events (Watanabe et al., 1998) and anesthesia (Smith et al., 1998) are associated with decreased protein synthesis rates. Persisting suppression of protein synthesis parallels apoptotic events (Hermann et al., 2001) and its recovery is essential for neuron survival after injury (Furuta et al., 1993). The rate of protein synthesis is thus a valuable cell function marker.

The present experimental procedure assessed relative changes rather than absolute values of local rates of leucine incorporation into protein. The determination of absolute values would require the knowledge of several specific kinetic parameters that are available for only a few species of mammals. In addition, the specific activity of the precursor amino acid pool (labeled plasma leucine) should be corrected for the contribution of re-

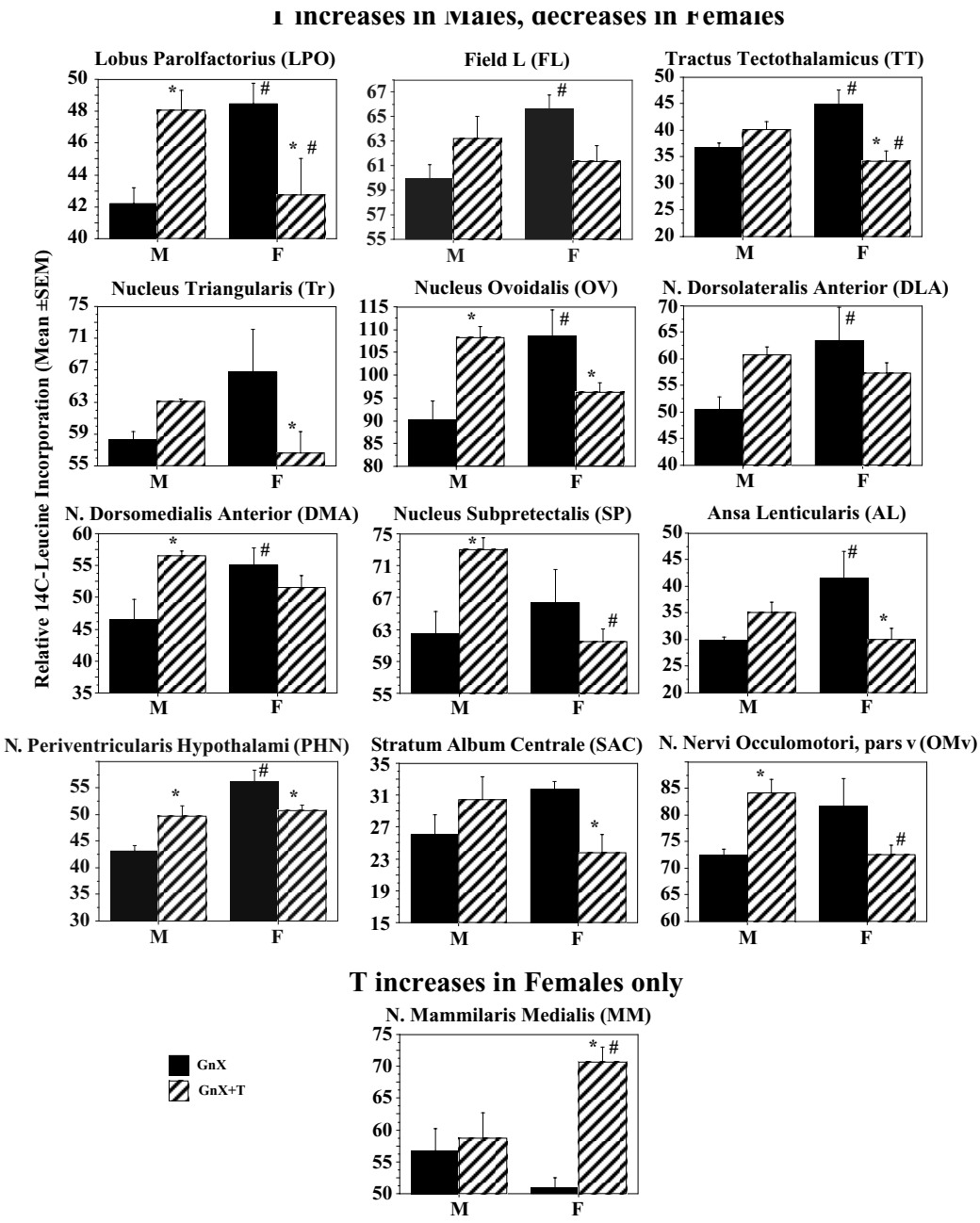

Fig. 7. Bar graphs illustrating the quail brain nuclei where significant interactions between the sex of the birds and their endocrine condition were detected in the analysis of the ^{14}C-leucine incorporation in the quail brain. Brain areas are arranged as a function of the nature of the interaction observed. Note that the scale used in each panel has been adjusted and truncated to permit a better illustration of the effects observed. The groups were compared by the Fisher PLSD post hoc tests and these results are summarized by symbols at the top of the bars as follows: * $P<0.05$ compared with GnX birds of the same sex, # $P<0.05$ compared with males submitted to the same treatment.

cycling of unlabeled leucine derived from protein degradation (Sun et al., 1992; Smith et al., 1998) which is not known for any avian species. Additional problems in the estimation of amino acid incorporation due to over- or under-estimation of the specific activity of the precursor pool must be considered when interpreting the data from tracer-dose autoradiography (Furuta et al., 1993). Conversely, decreases of leucine incorporation into protein could either reflect an increased oxidation of the precursor and/or an inhibition of protein synthesis per se. Therefore, only relative changes in incorporation could be assessed here in the absence of knowledge concerning specific kinetic parameters for the leucine uptake, distribution and catabolism in quail. These relative changes are however the only values that really matter for the purpose of the present experiment that was designed to investigate the changes in local protein synthesis activity induced by T in males and females.

The ^{14}C-leucine *in vivo* autoradiographic method provided detailed anatomical information on the brain loci where changes in anabolic activity are sexually differentiated and/or specifically associated with increases in plasma T levels. The magnitude of the significant changes in protein synthesis that were identified ranged between approximately 10 and 20% similar to changes reported in the rat brain exposed to various experimental conditions (e.g. increases due to passive avoidance learning (Schliebs et al., 1985) or to regeneration following axotomy (Sun et al., 1993; Smith and Yu, 1994); decreases due to anesthesia: (Smith et al., 1998) or in old age (Ingvar et al., 1985). The mechanisms that underlie these changes in leucine incorporation into proteins are not fully identified and must obviously be of a multiple nature. They presumably include changes in the metabolic rate in neurons and glial cells but they should also reflect the extensive plasticity that affects the avian brain even in adulthood. It is for example well established that treatment with T leads to a complete reorganization of the subcellular organization of neurons in the quail POM (Panzica et al., 1996) and major changes in their synaptic connectivity (Castagna et al., 1999). Furthermore the avian brain is well known for its capacity to incorporate news neurons in adulthood, at least in the telencephalon (Alvarez-Buylla and Kirn, 1997; Ling et al., 1997) and a similar plasticity should also affect the glial compartment. These changes in brain structure obviously require changes in protein synthesis and thus in the incorporation of labeled leucine.

Because the goals of the present study were to assess the changes in protein synthesis potentially related to the sex of the subjects and their hormone exposure in relationship to the expression of male-typical copulatory behavior, it was critical to ensure first that the subjects that would be studied were displaying the behavioral and endocrine characteristics of their sex and endocrine condition. It could actually be verified during the behavioral tests that all T-treated males displayed, as expected based on previously published studies (Adkins, 1975; Balthazart et al., 1983), an active male copulatory behavior while this behavior was completely lacking in castrated males and in females irrespective of whether they were treated or not with T. Similarly, the androgen-sensitive cloacal gland was completely regressed in all gonadectomized birds that were not treated with exogenous T. When T was administered, the size of the gland significantly increased in both sexes but, as previously reported (Schumacher and Balthazart, 1984), this response had markedly larger amplitude in males than in females. This confirmed the differential responsiveness of the male and female quail used in the present experiment to a same level of T. Together, these data indicate that the birds used here were sexually differentiated and steroid-sensitive as could be expected based on previous reports.

The present study identified complex changes in the regional incorporation of ^{14}C-leucine into specific brain nuclei as a function of the sex or endocrine treatment of the subjects. These effects and their interactions provide important information about the physiology of the brain circuitry mediating hormonal signals in quail, in particular in relationship with the activation and sexual differentiation of male reproductive behavior. Sex differences and effects of T are considered in sequence in the following sections.

Sex differences in localized cerebral ^{14}C- leucine uptake

Overall sex differences in leucine incorporation were detected here in five brain areas. Two of these areas, i.e. PP (homologous to the mammalian globus pallidus (Katren, 1968; Korzeniewska and Güntürkün, 1990) and Cer Mol, showed higher protein synthesis activity in males compared with females. A previous *in vivo* metabolic study suggested the involvement of these two areas in the performance of male sexual behavior (Dermon et al., 1999). The functional significance of the sexual differentiation of basal ganglia output circuits is discussed below.

A higher protein synthesis activity was detected here in the thalamic SRt of females as compared with males. The oxidative metabolic activity was previously shown by 2-deoxyglucose autoradiography to increase in this nucleus following POM lesions (Balthazart et al., 2001). This suggests that physiological activity in this nucleus may be indirectly controlled by steroids acting on POM circuits. Subrotundal cholinergic neurons (comparable to the mammalian posterior intralaminar nucleus) send wide ascending projections to association forebrain regions (Metzger et al., 1996; Medina et al., 1997) including CDL which interestingly also showed higher anabolic activity in females. It is therefore possible that brain functions controlled by this circuit are sexually differentiated in birds and additional studies should be carried out to investigate this possibility.

In contrast the other nucleus that displayed a higher anabolic activity in females (VMN) is obviously implicated in the control of sexually differentiated processes. The significance of the difference in VMN anabolism is intuitively easier to understand. The VMN has been identified in mammals (Barfield et al., 1983; Pleim et al., 1989; Blaustein and Erskine, 2002) as well as in birds (Gibson and Cheng, 1979; Ball and Balthazart, 2002) as a key site for the action of steroids on the activation of female sexual behavior (proceptivity and receptivity). In rodents, protein synthesis plays a critical role for the expression of female sexual behavior and the blockade of protein synthesis in the ventromedial hypothalamus prevents the expression of lordosis (Rainbow et al., 1982; Meisel and Pfaff, 1985). Exposure to steroids also produces very specific changes in protein synthesis in this nucleus (Jones et al., 1987). The significantly higher incorporation of labeled leucine in the female VMN could therefore directly reflect the neural processes that mediate the activation of female sexual behavior and represent a signature of the mechanisms that allow activation of these behaviors by steroids.

A higher leucine incorporation was also observed in females than in males in PHN but the difference was only present in GnX subjects and essentially disappeared after exposure to T, resulting in a significant interaction between sex and endocrine condition (see Fig. 7). PHN is involved in the neuroendocrine control of reproduction. It contains a

high density of chicken gonadotrophin-releasing hormone 1 (cGnRH-1)-immunoreactive cells and fibers in both quail and chicken (Van Gils et al., 1993) where they often colocalize with arginine-vasotocin (D'Hondt et al., 2000). Radioimmunoassay studies have also shown that, in turkey, the highest concentration of cGnRH-1 occurs in this nucleus and its immediate vicinity (Millam et al., 1995). cGnRH-1 is obviously the main control factor for the secretion of luteinizing hormone (LH) at the pituitary level and there is ample evidence demonstrating that the LH secretion is sexually differentiated in quail (Urbanski and Follett, 1982a,b). It is therefore not all that surprising that the level of protein synthesis is also sexually differentiated in PHN. The specific physiological link between these two types of sex differences remains however to be determined given that leucine incorporation is higher in females than in males while the latter have higher plasma level of LH than the former.

Effects of T on cerebral ^{14}C-leucine uptake

After a 4-week treatment with T, significant overall increases in leucine incorporation into protein were detected here in five brain areas (AIv, AId, LHy, POM, PMI) whereas decreases were seen in SM (see Fig. 6). A very prominent increase in incorporation was also observed following exposure to T in the MM in females but not in males (Fig. 7). Several of these brain regions are known to contain androgen receptors (AR) or estrogen receptors (ER; Watson and Adkins-Regan, 1989; Balthazart et al., 1998b). T could therefore affect the metabolic activity in these brain regions by a direct local action either as an androgen or after transformation into an estrogen by aromatization. The enzyme aromatase is indeed known to be present in the perikarya of some of these brain areas (POM, MM) or in immediately adjacent regions (AIv, AId, SM, LHy; Balthazart et al., 1990; Foidart et al., 1995). Moreover since at the subcellular level aromatase is localized in the perikarya of neurons as well as in dendrites, axons and terminals (Schlinger and Callard, 1989; Naftolin et al., 1996), it is possible that nuclei that have not been shown to contain aromatase-immunoreactive cells actually contain significant levels of active enzyme located in terminals connected to adjacent aromatase cell groups. Thus T could have acted as an androgen or as an estrogen in almost all nuclei where T treatment affected leucine incorporation. The fact that T increased or decreased local cerebral protein synthesis activity may, in this respect, be related to this differential mechanism of action (as an androgen or an estrogen) and future work should test this notion by comparing the effects on leucine incorporation of non-aromatizable androgens and of estrogens or of T in association or not with an aromatase inhibitor.

T also affected the incorporation of labeled leucine in a sexually differentiated manner in many additional brain areas that are not known as classical sex steroid targets (see Fig. 7). AR and ER have not been observed in most of these nuclei. There are three possible ways through which T could affect these nuclei (except PHN and MM which are discussed above). It is first possible that AR and/or ER are located in these regions but have escaped detection so far because there are either not present in sufficient numbers or present in a conformation that prevents their detection by methods used so far (e.g. occupied receptor not detected by *in vivo* autoradiography, receptors present in a conformation that does not allow immunocytochemical detection). Many brain regions in mammals that were not previously thought to be steroid targets have recently been shown to contain ER by method with higher sensitivity (e.g. *in situ* hybridization with probes with high specific activity).

Secondly, it must also be noted that the quail brain has recently been shown to express, like the mammalian brain (Kuiper et al., 1996, 1998), a second form of ER called ERβ (Ball et al., 1999; Foidart et al., 1999). This receptor has a broader distribution than the classical ER now renamed ERα and it is therefore possible that it is also present in some of the areas where we detected here effects of T on the ^{14}C-leucine uptake.

Thirdly and finally, it could also be argued that the effects of T on the protein synthesis in specific brain nuclei do not result only from a direct local action of the steroid or its metabolites but from the modulation of synaptic activity in projections to the nucleus under investigation that originate in classical sex-steroid sensitive neurons. Indeed it is suggested that local protein synthesis rate is not only associated with the number neuronal and glial cells, but also with the extent of terminal fields (Stewart and Banker, 1992; Crispino et al., 1997). In this context, we showed recently that some effects of T on the local oxidative metabolism, as measured by the 2-deoxyglucose accumulation, are affected in many brain areas that are not themselves steroid-sensitive by an action of T in the POM. These effects were not longer present in subjects that had received a bilateral stereotaxic lesion of the POM before they were treated with T (Balthazart et al., 2001). A similar indirect action may well take place also at the level of the control of protein synthesis activity.

Effects of T in the POM were of particular interest in the present study. It has been shown that this nucleus is a key site for the action of T and its metabolites (in particular estradiol) in the activation of male copulatory behavior (Panzica et al., 1996a; Balthazart et al., 1998a; Ball and Balthazart, 2002). In mammals, male reproductive behavior has been suggested to depend on the synthesis of proteins in the medial part of the preoptic area, based on behavioral studies after local application of protein synthesis inhibitors (cycloheximide, anisomycin; Quadagno et al., 1971; McGinnis and Kahn, 1997). In quail, we had shown previously that the treatment of castrated males with T results in a significant increase in the density of Nissl staining in the POM, which suggested that protein synthesis was augmented in this nucleus (Balthazart et al., 1991). T also causes a dramatic reorganization of the POM ultrastructure that must obviously involve an increase in protein synthesis activity (Panzica et al., 1996b; Castagna et al., 1999). The present study based on more direct and specific methods fully confirms this idea and thus indirectly supports the notion that T action in the POM through

stimulation of protein synthesis activity may be implicated in the activation of male sexual behavior. The fact that many nuclei exhibiting modified protein activity by T are mono-synaptically connected with POM (Balthazart et al., 1994; Balthazart and Absil, 1997; SM, MM, DMA, OMv) provides additional evidence in support of this argument.

It was also intriguing that, although no overall sex difference could be detected (no sex difference and no interaction in the two way ANOVA), the effect of T on leucine incorporation in the POM was numerically much larger in males than in females. Comparison of these changes in males and females (i.e. the values in males and female GnX+T expressed as percentage of the average corresponding values in the GnX groups) confirmed the numerically larger (five-fold larger) response in males but this difference failed to reach statistical significance, presumably due to the limited sample size (males: 125.7±5.3% vs females: 105.7±7.5%, $F_{1,6}=4.85$, $P=0.0698$). If future experiments demonstrate the reliability of this difference, it would provide a signature for the sexually differentiated mechanisms that control the activation by T of male copulatory behavior in quail. As explained above, the exposure of females to their ovarian steroids during embryonic life causes the irreversible loss of their sensitivity to the activating effects of T on male-typical copulatory behavior: they are demasculinized (Balthazart and Adkins-Regan, 2002). The numerically lower increase in leucine incorporation following treatment with T in females compared with males would then reflect the brain mechanisms underlying this behavioral demasculinization. Examples of such sex-dependent T effects are found in the present study in visual (DLA) and auditory (OV) relay stations. Interestingly, the nucleus DLA of birds, homologous of the mammalian dorsal lateral geniculate nucleus (Watanabe, 1987), is the main thalamic relay area of the visual thalamofugal system that is itself sexually differentiated in young chicks (lateralized projections in males but not in females; Adret and Rogers, 1989). The auditory relay nucleus OV and its cortical projection FL exhibited a similar sexually differentiated response to T. Future studies should first research whether these differential responses to T of the leucine incorporation are reliable and then try to identify the specific protein(s) whose synthesis is differentially affected by T in males and in females as well as their functional significance.

Functional considerations on neural circuits differentially affected by sex and T

The dorsal pallidum or PP and its output fibers (AL) were found here to be sexually differentiated in terms of protein synthesis activity. PP is suggested to be the counterpart of the mammalian globus pallidus (GP) and contains an intermingled population of neurons comparable to those found in the internal and external segment of GP (Gpi and Gpe, respectively; Reiner et al., 1998). The dorsal pallidum (PP) of the avian basal ganglia, as in mammals, is mainly related to somato-motor functions. It receives inputs from the striatum (PA) and projects mainly to a) the dorsal thalamic region comparable to the motor part of mammalian ventral tier (Medina et al., 1997) b) the substantia nigra pars reticulata (Medina and Reiner, 1997; Jiao et al., 2000) and c) the midbrain tegmentum via pretectal nuclei (Dubbeldam and Den Boer-Visser, 1994). Anatomical studies have shown that in addition to the major inputs coming from PA, the GABAergic PP neurons receive inputs from the AL (Brauth et al., 1978) which also contains output fibers originating in PP. This suggests the existence of a closed pallido–thalamo–pallidal loop in parallel to the pallido–thalamo–cortico–striatal loop and pallido–nigro–tectal circuit. Although the pallido–thalamo–cortical loop (similar to the mammalian pallido–ventral thalamus–motor cortex) does not show any sexual dimorphism, the thalamic output of PP via SRt (Kitt and Brauth, 1986; Medina et al., 1997) is associated with a sex differences in protein synthesis activity. This projection to SRt is comparable to the mammalian intralaminar nuclei, interconnecting motor programs of the basal ganglia to several cortical regions including the hippocampal complex and the dorsocaudal neostriatum (Miceli and Repérant, 1985; Metzger et al., 1996; Medina et al., 1997). It is therefore possible that this circuit influences in a sexually differentiated manner a number of underlying higher brain functions. In fact, the CDL was characterized here by a higher protein synthesis activity in females than in males. This area is reciprocally connected to the hippocampus and participates in the process of spatial memory in pigeons (Atoji et al., 2002). The present study also provides evidence that protein synthesis in regions involved in the pallido–pretectal–tectal and tectofugal circuits (TT, SP, T, SAC, OMv) is differentially affected by T in males and females suggesting that the control of the head, neck and eye movements may be sexually differentiated.

The basal ganglia outputs mentioned above exert a direct influence on thalamo-cortical and tectobulbar motor circuits promoting movement. This is in contrast to the recently described indirect PP output (output of the PP neurons receiving inputs from enkephalin striatal neurons via subthalamic nucleus, Ala) that exerts an indirect effect on these circuits, by suppressing unwanted movements (Jiao et al., 2000). The present study points to the existence of sexually differentiated pallidal output circuits but does not specify which subpopulations of PP neurons (those receiving inputs from substance P neurons or those receiving inputs from enkephalin containing striatal neurons, (Graybiel, 1990; Reiner et al., 1998) exhibit sex differences in protein synthesis activity and potentially influence in a sex-dependent manner specific motor circuits. It must also be noted that previous studies demonstrated an increase in oxidative metabolism (increased 2-deoxyglucose uptake) in PP following the expression of copulatory behavior in male quail (Dermon et al., 1999) while, in contrast, oxidative metabolism is inhibited in this brain region after the initiation of incubation behavior in ring dove (Georgiou et al., 1995). This provides additional evidence for an implication of PP in these two types of sexually differentiated behaviors: the increased oxidative metabolism in PP motor circuits paralleled the execution of the motor program associated with copulatory behavior, but a

decrease was observed in association with the inhibition of unwanted movements at the onset of incubation.

Parts of the limbic basal ganglia circuits that are, in birds as in mammals, related to viscero-limbic behavior were also associated here with a leucine incorporation pattern that was affected by T, sometime in a sex-dependent manner. Specifically, the protein synthesis rate was influenced by T in a sex specific pattern in the LPO (ventral striatum) that projects to ventral pallidum: T increased the synthesis activity in males but depressed it in females. Similar effects were found in several LPO projections areas such as parts of the dorsomedial thalamus (DLA and DMA). Finally T increased leucine incorporation in the LHy but decreased it in the SM. These last two effects were however present in both sexes. These limbic areas are interconnected and DMA/DLA actually represent the main thalamic input to LPO (Metzger et al., 1996) while septum is densely and bi-directionally connected to the preoptic area (Balthazart et al., 1994). It is possible that the reciprocal connections between these areas mediate, at least in part, the observed effects of T in these regions, since modified expression of protein synthesis is associated not only with differences in the number and activity of neurons and glial cells but also with the extension and activity of terminal fields (Stewart and Banker, 1992; Crispino et al., 1997).

Within the limbic system, T also increased protein synthesis activity in the dorsal and ventral intermediate archistriatum but decreased it in SM (marginal effect also in lateral septum; $P=0.099$, see Table 1). The septum, mainly in its caudal part, is characterized by high concentrations of neuropeptides such as arginine vasotocin and the vasoactive intestinal polypeptide (VIP) that are specifically affected by T. Specifically, T decreases VIP immunoreactivity in the caudal septum (Aste et al., 1997), a neurochemical change potentially related to the decrease in protein synthesis activity observed in the present study.

In male quail lesions of the posterior/medial archistriatum including nucleus taeniae have been shown to inhibit the expression of appetitive sexual behavior (Thompson et al., 1998). In contrast, a more recent study showed that more rostral lesions centered on the AIv do not affect several measures of appetitive sexual behavior (Absil et al., 2002) suggesting the existence of anatomically discrete functional specialization within the archistriatum. Even though it has been proposed, based on developmental gene expression data, that AId belongs to the lateral pallium while AIv is ventropallial (Puelles et al., 2000), both regions are steroid-sensitive and protein synthesis rate was increased by T in both regions in the present study. Anatomical, histochemical and developmental evidence suggests that AId should be homologous to the mammalian basal amygdaloid nucleus and AIv to the lateral and accessory basal nuclei of mammalian amygdala (for references and detailed discussion see Martinez-Garcia et al., 2002). To this date, homologies are however not firmly established (see internet discussion on avian nomenclature at: http://jarvis.neuro.duke.edu/nomen/index.html). In addition, the avian archistriatum can be divided in two functional regions: a somatic sensorimotor part (including AId) and a viscerolimbic part (including AIv; Zeier and Karten, 1971; Davies et al., 1997; Dubbeldam et al., 1997). The non-limbic archistriatum (AId) comprises the dorsal intermediate and medial archistriatum and essentially gives rise to specific sensory, somatosensory, and motor telencephalofugal efferents (Veenman et al., 1995). The limbic avian archistriatum (AIv) has been implicated in both fear, agonistic behavior and memory function (Phillips and Youngren, 1986; Knudsen and Kaplan, 1996; Davies et al., 1997; Kroner and Güntürkün, 1999). The present data therefore suggests that these functions are possibly modified by T. Feeding may similarly be affected by the sex of the birds and their endocrine condition as suggested by the present observation that many nuclei implicated in food intake mechanisms such as, parts of the archistriatum homologous to the mammalian amygdala, VMN, LHy, and AL (Kuenzel and VanTienhoven, 1982) were found here to exhibit sex-dependent effects of T on the leucine incorporation into proteins.

CONCLUSIONS

In conclusion, the present study demonstrates that T has widespread effects on the protein synthesis activity in the quail brain. These effects concern classical sex steroid targets that are obviously connected to the control of reproductive behavior or physiology but also brain areas that have never been related to these functions. These data thus bring further support to the idea that sex steroids have wide-ranging effects on brain function and actually control/modulate a variety of functions that are not associated with reproduction such as learning and memory, brain plasticity including neurogenesis, and a variety of pathological processes such as Alzheimer diseases or schizophrenia. This notion has gained a lot of support during recent years following the discovery of a second ERβ that has a much broader distribution in the brain than ERα and as a consequence of the phenotypical characterization of knockout mice that either do not express ER or do not produce estrogens due to the inactivation of the aromatase gene. These mice suffer from behavioral and physiological deficits that extend way beyond the realm of reproduction. The changes in leucine incorporation in a large number of brain areas identified here presumably reflect all these poorly know functions of steroids in the brain and deserve additional work.

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