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Social regulation of the brain–pituitary–gonadal axis

(gonadotropin-releasing hormone/gonads/social control/cell size change)

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ABSTRACT **Reproduction in vertebrates is regulated by the hypothalamic–pituitary–gonadal axis via neural and hormonal feedback. This axis is also subject to exogenous influences, particularly social signals. In the African cichlid fish *Haplochromis burtoni*, gonadal development in males is socially regulated. A small fraction of the males, which are brightly colored, maintain territories and aggressively dominate inconspicuously colored nonterritorial males. Here we show through manipulation of the social and endocrine environment that changes in social status and gonadal state are accompanied by soma size changes in a population of gonadotropin-releasing hormone-containing neurons in the ventral forebrain. In territorial males, these cells are significantly larger than in nonterritorial males. When an animal switches from being territorial to nonterritorial through a change in social situation, these cells shrink; in animals that change from nonterritorial to territorial status, the cells enlarge. These gonadotropin-releasing hormone-containing cells project to the pituitary and are ultimately responsible for regulating gonadal growth. This mechanism of socially induced cell size change provides the potential for relatively quick adaptive changes in the neuroendocrine system without nerve cell addition or death. Since the structure of this regulatory axis is conserved among all vertebrates, other species with socially modulated reproductive physiology may exhibit a similar form of physiological regulation.**

Social interactions regulate reproductive function in many vertebrate species. Dominance encounters, in particular, can influence access to mates (1, 2), reproductive condition (3–5), timing of maturation (6–8), and even determination of sex (9–12). Although physiological effects of social interactions are well-documented, little is known about mechanisms through which the social environment influences reproductive physiology. Any effect of changes in the social environment on gonadal function must be mediated by changes in the central nervous system, and a prime candidate is the hypothalamus. To date, however, no hypothalamic neuronal populations that respond to social signals have been identified.

In the African teleost fish *Haplochromis burtoni*, reproduction among males is monopolized by relatively few territorial (T) males (≈10%) able to defend a breeding site (13). T males are more brightly colored and have higher levels of circulating androgens (unpublished data) than nonterritorial (NT) males, which they aggressively dominate. However, when any breeding site becomes vacant, it is quickly taken by a NT male, which rapidly changes its behavior patterns and its coloration and becomes T (14). When males are reared together, maturation rate is socially regulated (8). Males reared with adults have delayed maturation relative to those reared without adults and also exhibit smaller gonadotropin-releasing hormone (GnRH)-containing neurons in the preoptic area (POA) of the ventral hypothalamus (15). We investigated whether, among cohorts of sexually mature adults, T males suppress gonadal function in NT males and, if so, whether these effects of social status are accompanied by changes in the POA neurons containing GnRH. We also asked whether this suppression of gonadal function was reversible among mature males.

MATERIALS AND METHODS

H. burtoni used in this experiment were progeny of laboratory-bred animals derived from wild-caught fish from Lake Tanganyika (13). All fish used in this study were between 12 and 18 months old, well past the age of sexual maturity (3–7 months). The fish were maintained in conditions that mimic those found in their natural environment [pH 7.8–8.2; 27°C; 12-hr light/12-hr dark cycle with full spectrum lights (Vita light)]. The fish were housed in 200-liter aquariums (10 × 20 × 40 cm), which typically contained 3–5 T males, 3–5 NT males, and 10–15 females. The fish were fed once daily with dried pellets (Wardley, Secaucus, NJ).

Social Manipulation. To understand the relationship between social status and reproductive state, males were changed from T to NT or vice versa. This was accomplished by moving T males into communities with larger T males, as a result of which they became NT (T → NT). Conversely, NT males were moved to new communities consisting of females and smaller males they could dominate, as a result of which they became T (NT → T). In each case, the subjects remained in the altered social setting for 4 weeks, after which they were sacrificed, along with control T and NT males from the original communities.

The gonads were removed and weighed and the brain was immersion-fixed in 4% paraformaldehyde [buffered with NaH_2PO_4 (pH 7.4)] for 4–8 hr. Tissue was then rinsed in phosphate buffer for 1 hr, embedded in agar, and placed in 30% sucrose for a minimum of 24 hr. The brains were then frozen and sectioned sagittally at 40 μm on a cryostat (Microm, Zeiss).

GnRH-immunoreactive (irGnRH) neurons were located in the sectioned tissue by labeling with an antibody to salmon GnRH (GF-5; kindly provided by Nancy Sherwood, University of Victoria, Canada). Immunoreactive neurons were identified by using a secondary antibody made visible with horseradish peroxidase diaminobenzidine reaction product (ABC; Vector Laboratories). We measured the cross-sectional area of labeled neurons in the terminal nerve and POA on captured video images (Macintosh; Quadra 700) with software provided by National Institutes of Health (Image 1.43; Wayne Rasband). We measured all labeled neurons, which had a completely distinguishable perimeter and a visible nucleus. The data reported here are for all individual animals for which the number of neurons meeting these criteria was 50 or more.

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Abbreviations: T, territorial; NT, nonterritorial; GnRH, gonadotropin-releasing hormone; POA, preoptic area; irGnRH, immunoreactive GnRH; GSI, gonosomatic index.

Hormonal Manipulation. To understand cause and effect among the variables of POA irGnRH neuron size and gonadal development, we manipulated androgen levels through castration and selective androgen replacement. T males were randomly assigned to one of four treatment groups: sham-operated fish, castrated males, castrated males that received testosterone replacement, and castrated males that received 11-ketotestosterone (a teleost fish-specific androgen) replacement. Prior to operations, fish were anesthetized with Tricaine methanosulfate (1:10,000; MS-222), which was also applied to the gills during the procedure. A ventral incision was made from just anterior of the anal vent to the clavicle. Males assigned to the sham treatment received the incision and a placebo pellet. Males designated castrates also had their testes removed. Fish assigned to the two androgen replacement regimes received slow-release pellets containing either testosterone or 11-ketotestosterone. These 3-mm pellets contained the selected hormone and vehicle (cholesterol, microcrystalline cellulose, lactose, di- and tricalcium phosphate, calcium and magnesium stearate, and stearic acid; Innovative Research of America). The amount of hormone to be released was based on measurements of circulating hormone levels in T males (16). Replacement pellets contained either 5 mg of testosterone or 1 mg of 11-ketotestosterone. These pellets were designed to provide constant, slow release of the androgens at levels characteristic of T males for 4 weeks. All fish remained in physical isolation following the operation and were sacrificed after 4 weeks.

RESULTS

Social Manipulation. Mean soma size of the POA irGnRH-containing neurons (Figs. 1*A* and 2) and gonosomatic index (GSI) (Fig. 1*B*) were both significantly larger in the control T males than in the control NT males. Another irGnRH cell group located in the terminal nerve in the telencephalon showed no difference in mean soma sizes between T and NT males (Fig. 1*C*), indicating that the status-linked variation in soma size is not a general property of GnRH-containing neurons, but rather is confined to the POA population (see also ref. 15).

Males converted from T to NT (T → NT) status showed a significant decrease in both POA irGnRH neuron mean soma size (Figs. 2 and 3) and GSI (Fig. 4) relative to control T males. Conversely, NT → T males experienced a significant increase in both irGnRH cell size and GSI relative to control NT males (Figs. 2–4).

There was no difference between control T males and their experimentally produced counterparts (NT → T) with respect to either POA irGnRH neurons (Figs. 2 and 3) or GSI (Fig. 4). This was also true of control NT males and their experimental (T → NT) counterparts (Figs. 2–4).

Among the total population of subjects, there was a significant positive correlation between POA mean soma size and size of the testes ($r = 0.756$; $n = 27$; $P < 0.0001$).

Castration and Hormone Replacement. Castrated males had significantly larger POA irGnRH neurons than sham-operated fish (Fig. 5). This hypertrophy was reversed by replacement of either testosterone or 11-ketotestosterone. In marked contrast to the subjects in the social manipulation experiments, in the operated animals that received no androgen replacement (shams and castrates), there is a significant negative correlation in POA mean soma size and the weight of the testes ($r = -0.721$; $n = 12$; $P < 0.005$)—the less gonadal tissue the larger the size of the POA irGnRH neurons.

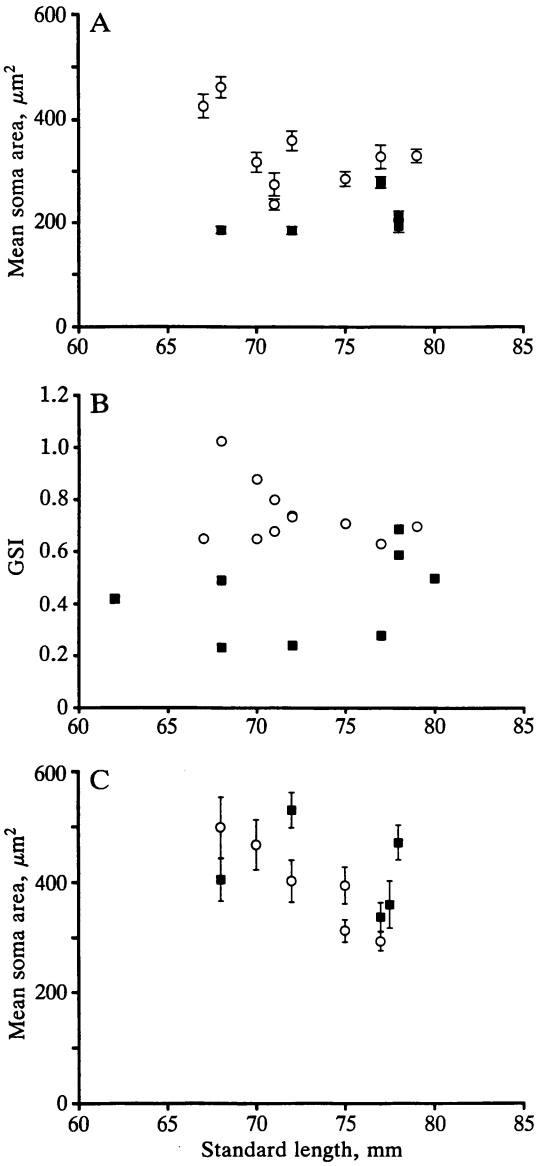

FIG. 1. Comparison of control T and NT males showing that T males have significantly larger POA neuron soma sizes, which contain irGnRH, than do NT males (*A*) ($P < 0.005$) and significantly larger GSI (*B*) ($P < 0.005$), but there is no difference in soma size of terminal nerve irGnRH neurons (*C*) ($P > 0.05$). Scatter plots show no relationship between animal size and any of the three variables in the range of animals sampled in this study. (*A*) POA irGnRH neurons in T males (○) are significantly larger than those in NT males (■) [$P < 0.005$; Mann–Whitney U test ($U = 48$; $n1 = 9$; $n2 = 5$)]. (*B*) GSI [(gonad wt/body wt) × 100] is significantly larger in T (○) than in NT (■) males [$P < 0.005$; Mann–Whitney U test ($U = 36$; $n1 = 11$; $n2 = 8$)]. Note that sample sizes are somewhat larger for the GSI data than for POA neuron mean soma size and especially terminal nerve mean soma size, because in some individuals for which we have GSI data, problems in tissue processing for the antibody label precluded us from collecting cell size data. (*C*) Terminal nerve irGnRH neurons are not significantly different between T (○) and NT (■) males [$P > 0.05$; Mann–Whitney U test ($U = 19$; $n1 = 6$; $n2 = 5$)]. Error bars represent standard error of soma size measurements.

DISCUSSION

Social status clearly determines both soma size of POA irGnRH neurons and GSI. Moreover, these effects are reversible. The relatively large testes and irGnRH neurons characteristic of T males are a consequence of their social dominance, and when this dominance is lost both neurons and testes shrink. Our social manipulations show that the

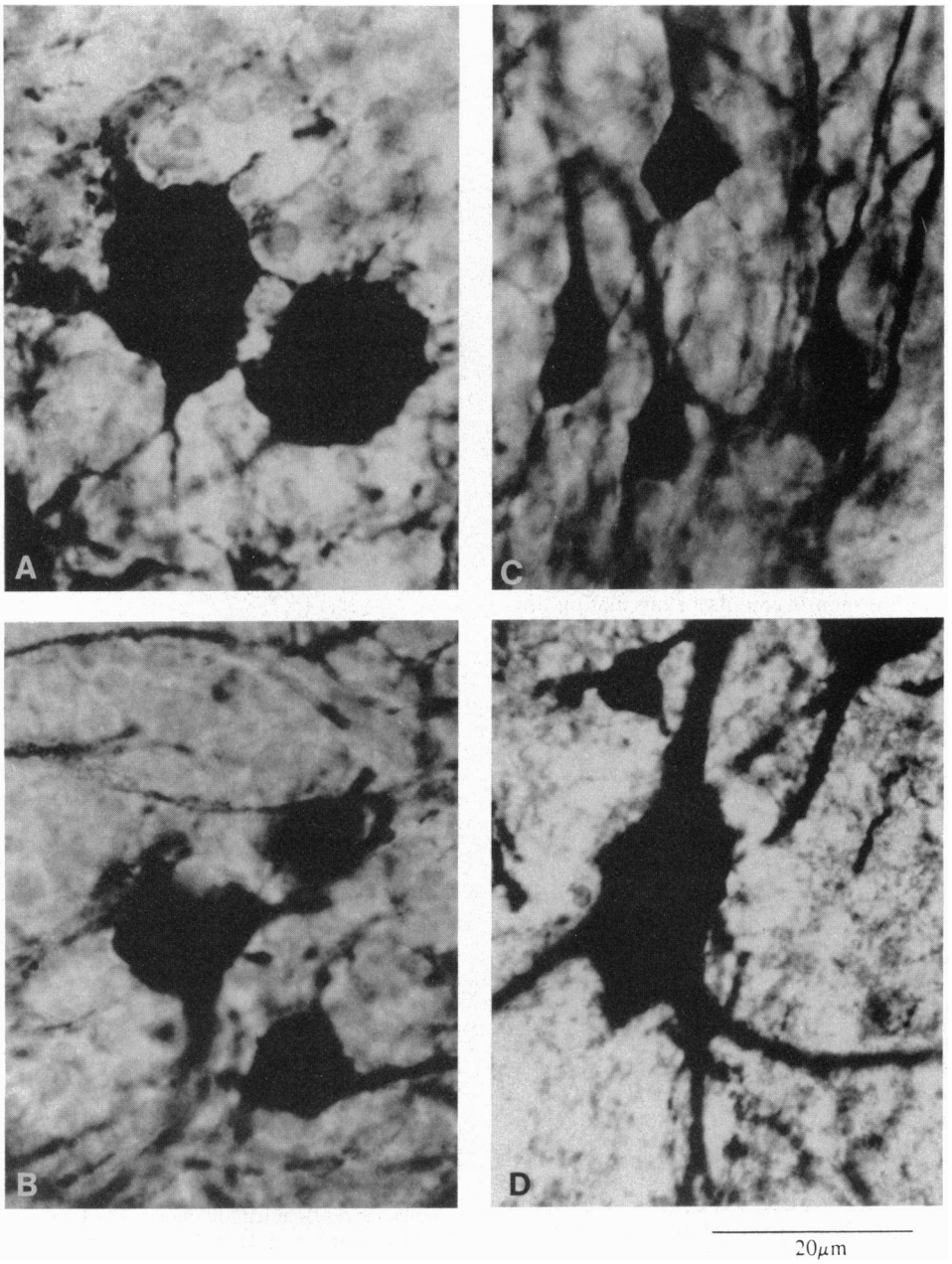

FIG. 2. Photomicrographs of representative POA irGnRH neurons for the four types of males in this study. (*A*) T male. (*B*) T → NT male. (*C*) NT male. (*D*) NT → T male. (Bar = 20 μm.)

associations between social status, testes size, and size of POA irGnRH neurons are not independently caused by a fourth factor. There are three logical ways that social status could influence POA cell size and GSI (Fig. 6): (*i*) a change in social status causes a change in gonadal size as a result of its effects on the POA neurons (Fig. 6*A*); (*ii*) a change in social status causes a change in the size of POA neurons as a result of its direct effect on the gonads (Fig. 6*B*); (*iii*) a change in social status influences the gonads and POA neurons independently and in parallel (Fig. 6*C*).

The hormonal manipulations were designed to reveal the relationship between gonadal size and POA irGnRH neuron size. The results show that gonadal steroids constrain the size of these neurons. This accords with the results of a previous experiment in which we found a significant negative regression of soma size on residual GSI in T males with various amounts of gonad removed (16). These results indicate that the association between large testes and large POA irGnRH neurons in the T males does not result from the stimulatory effects of gonadal androgens on the POA neurons. T males have larger POA irGnRH neurons than NT males despite high circulating androgen levels, not because of them. This excludes the possibility that a change in social status causes a change in the size of POA neurons as a result of its direct effect on the gonads (Fig. 6, case B).

Thus, either social status acts independently on the GnRH neurons and the gonads or the effects of social status on gonadal growth are mediated by changes in the POA GnRH neurons. We consider the latter more likely. The feedback connections between gonads and POA neurons are well-documented. Developmentally, the causal primacy of the GnRH neurons with respect to gonadal growth is consistent with the known effects of GnRH-containing neurons on gonadal development in other vertebrates (17–19). Moreover, if the correlation between gonadal size and POA neuron size results from independent but parallel effects of social status,

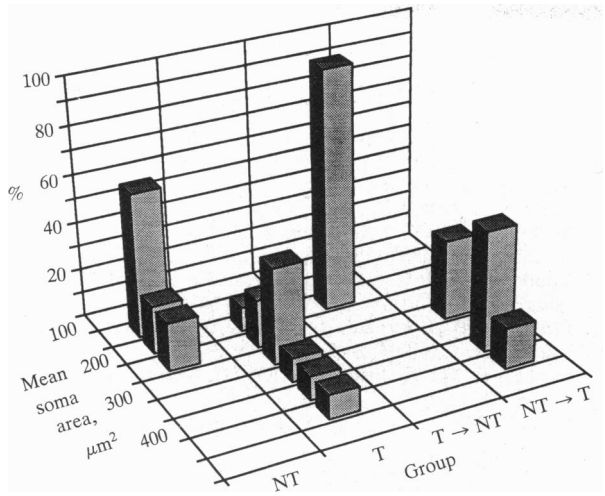

FIG. 3. Three-dimensional plot of mean soma sizes of POA irGnRH neurons, showing significant differences between T and T→ NT males and between NT and NT → T males. Percentage of individuals with mean soma size in a given size bin are plotted for each treatment condition. There are no significant differences between T males and their experimental counterparts (NT → T) or between NT males and their experimental counterparts (T → NT). Data for T and NT from Fig. 1*A* are represented in histogram form to facilitate comparisons with the two experimental groups. Values compared with the Mann–Whitney *U* test (one-tailed) are as follows: T vs. T→ NT, $P < 0.005$ ($U = 66.5$; $n1 = 9$; $n2 = 8$); NT vs. NT→ T, $P < 0.005$ ($U = 26$; $n1 = 5$; $n2 = 5$); T vs. NT→ T, $P > 0.05$ ($U = 29$; $n1 = 9$; $n2 = 5$); NT vs. T→ NT, $P > 0.05$ ($U = 9$; $n1 = 8$; $n2 = 5$).

it is difficult to understand the striking difference in their relationship when comparing intact and castrated animals. In intact animals, POA neuron size is highly positively correlated with gonad size. In contrast, within a population that includes both intact T males and those that had been largely

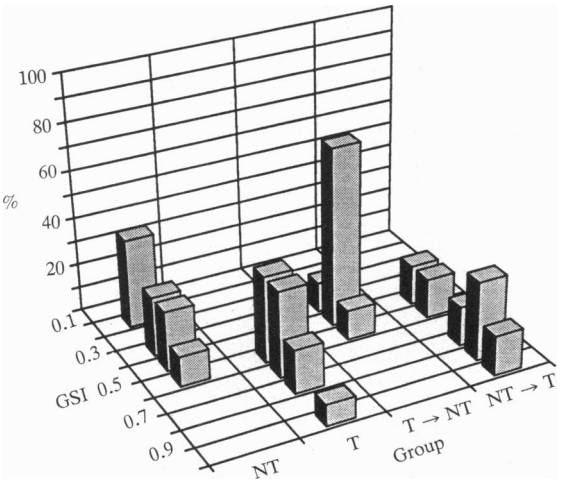

FIG. 4. Three-dimensional representation of GSI data, showing significant differences between T and T→ NT males and between NT and NT → T males. Percentage of animals within a given 0.2 magnitude range are plotted for each treatment condition. There are no significant differences between T males and their experimental counterparts (NT→ T) or between NT males and their experimental counterparts (T → NT). Data for T and NT males from Fig. 1*B* are represented again here in the first two rows to facilitate comparisons. Values compared with the Mann–Whitney *U* test (one-tailed) are as follows: T vs. T→ NT, $P < 0.001$ ($U = 54$; $n1 = 11$; $n2 = 8$); NT vs. NT→ T, $P < 0.01$ ($U = 37.5$; $n1 = 8$; $n2 = 5$); T vs. NT→ T, $P > 0.05$ ($U = 45$; $n1 = 11$; $n2 = 5$); NT vs. T→ NT, $P > 0.05$ ($U = 47.5$; $n1 = 8$; $n2 = 8$).

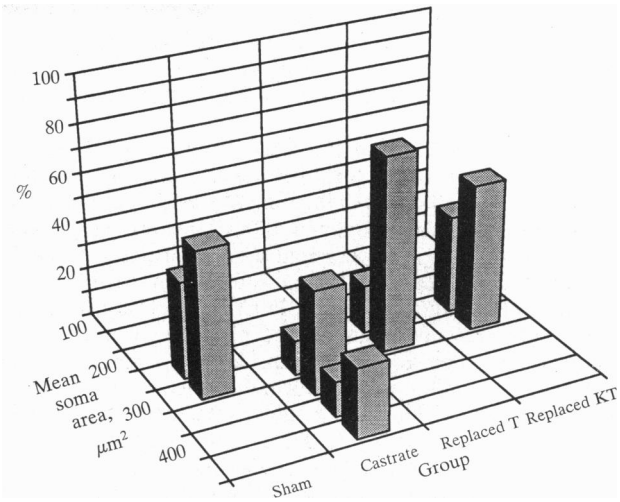

FIG. 5. Three-dimensional representation of mean soma sizes of POA irGnRH neurons showing that castrated fish (Castrate) had significantly larger mean soma sizes than did sham-operated (Sham) fish and significantly larger neurons than their counterparts to which either testosterone (Replaced T) or 11-ketotestosterone (Replaced KT) was administered. Values compared with the Mann–Whitney *U* test (one-tailed) are as follows: castrate vs. sham; $P < 0.005$ ($U = 35$; $n1 = 7$; $n2 = 5$); castrate vs. replaced T, $P < 0.01$ ($U = 33$; $n1 = 7$; $n2 = 5$); castrate vs. replaced KT, $P < 0.005$ ($U = 35$; $n1 = 7$; $n2 = 5$).

or completely castrated, POA neuron size is highly negatively correlated with gonad size. This dramatic reversal in the sign of the correlation between POA cell size and gonad size occurs without any change in social status. This would not be expected if the gonads and POA are independently regulated by social status. The most parsimonious explanation of these results is that the change in gonadal size resulting from alteration in social status is mediated by changes in the POA

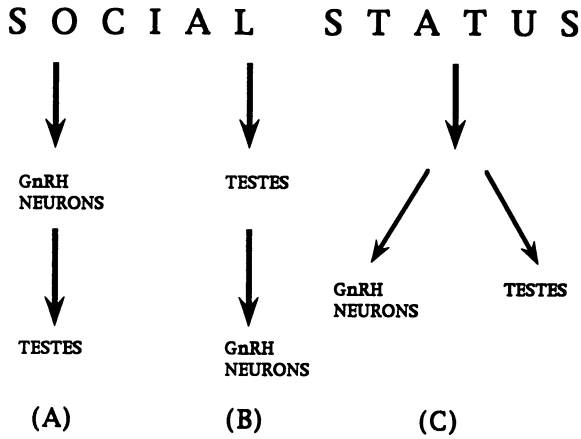

FIG. 6. Changes in social status of adult male *H. burtoni could* influence POA GnRH neuron size and gonad size in one of three ways. Case A: The influence on gonad size could be mediated by the change in GnRH neuron size directly. For reasons given in the text, we favor this interpretation of our data. Case B: The social status could influence the testes size, which, in turn, influences the GnRH neuron size. The experiments reported here eliminate this possibility. In intact animals, GnRH neuron size is positively correlated with testes size, whereas in castrated animals, GnRH neuron size is negatively correlated with testes size. Thus, androgen produced by the testes cannot be the controlling factor of GnRH neuron size. Case C: Social status influences the GnRH neuron size and the testes size via parallel pathways. While this is a logical possibility, we consider it unlikely because of the difference in the relationship between GnRH cell size and testes size when comparing intact and castrated animals. See text for further information.

neuron size. A definitive demonstration of this causal sequence, however, will require further experiments such as administration of an anti-GnRH agent or transection of connections between GnRH-containing neurons and the pituitary.

Our results suggest that the other population of GnRH-containing neurons in the terminal nerve are not involved in the socially induced changes in gonadal state. These neurons do not change size in response to social cues. Moreover, GnRH neurons of the terminal nerve project diffusely throughout the telencephalon and midbrain regions (20), whereas GnRH neurons in the POA project solely to the pituitary (21, 22) and regulate the pituitary production of gonadotropins. The gonadotropins, in turn, affect gonadal physiology, including steroid hormone release. In the transition from T to NT this axis is down-regulated, whereas in animals switched from NT to T it is up-regulated.

The POA irGnRH neurons projecting to the pituitary appear to be the final common pathway for the effects of social stimuli on gonadal function. It seems likely that similar pathways exist in other vertebrates exhibiting socially regulated reproductive physiology because key neuroendocrine components of the brain–pituitary–gonadal axis are highly conserved across vertebrate species (23–25). It will be especially interesting to discover whether a similar mechanism underlies other effects of the social environment on sexual development, such as socially regulated sex change.

The degree and form of the plasticity of POA irGnRH neurons are noteworthy. The lability of soma size in these neurons provides the potential for adaptive changes in their activity without the need for cell addition or death. Indeed, this plasticity may compensate for constraints on cell division in highly differentiated neurons. The cellular mechanisms responsible for this capacity are yet to be explored.

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