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Regulation of Galanin and Gonadotropin-Releasing Hormone Gene Expression in the Hypothalamus and Basal Forebrain of the Rat[1]

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Galanin is a cotransmitter in GnRH neurons and is thought to play a role in the control of gonadotropin secretion. The aim of our research has been to learn how galanin mRNA is regulated in GnRH neurons with the goal of understanding galanin's physiological significance. We have used double-label *in situ* hybridization and computerized image analysis to identify GnRH neurons coexpressing galanin mRNA and to estimate cellular levels of galanin message in these cells under different physiological conditions in the rat. In adult females, levels of galanin mRNA in GnRH neurons increase two- to fourfold with the onset of the proestrous and steroid-induced LH surges. Pharmacological blockade of synaptic transmission with either a general anesthetic (pentobarbital) or an α-adrenergic receptor antagonist (phenoxybenzamine) inhibits both the steroid-induced LH surge and the associated induction of galanin expression in GnRH neurons. Compared with the day of diestrus of the estrous cycle, during lactation cellular levels of galanin mRNA in GnRH neurons are profoundly reduced. In contrast to galanin mRNA in GnRH neurons, we could adduce no evidence for changes in cellular levels of GnRH mRNA under any physiological conditions or with any pharmacological manipulations. We conclude that alterations in galanin gene expression play a fundamental role in governing the functional activity of GnRH neurons, possibly by acting presynaptically to shape GnRH pulses, thereby determining the biological efficacy of GnRH action at its target cells in the pituitary.

Gonadotropin-releasing hormone (GnRH) neurons are the final common pathway through which the brain regulates reproduction in virtually all vertebrate species. The cell bodies of the approximately 1000 GnRH neurons are found scattered throughout the ventral forebrain and hypothalamus. Many of these neurons project to the median eminence where they form terminals on capillaries of the hypophyseal portal system. When

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the GnRH neurons are activated and release GnRH, it is efficiently delivered to the anterior pituitary where it stimulates the gonadotropes to synthesize and release the gonadotropins, LH and FSH. The gonadotropins are transported via the general circulation to the gonads where they stimulate the maturation of germ cells and the production of certain protein (e.g., inhibin) and steroid hormones. These gonadal hormones then provide feedback signals to the brain-pituitary axis, the interplay of which is responsible for controlling reproductive function in the adult. While the importance of the GnRH neuron in reproductive processes is widely appreciated, its molecular physiology is less well understood.

Galanin is a 29 amino acid gut–brain peptide first isolated from porcine gut. A growing body of literature indicates that galanin has a wide distribution in the central nervous system and is often colocalized with other neurotransmitters. Of particular interest is the finding that galanin is colocalized with GnRH (for a review see Merchenthaler, Lopez, and Negro-Vilar, 1993), and galanin has been implicated in the regulation of gonadotropin secretion. For example, injection of galanin into the cerebral ventricles (icv) increases LH secretion in rats (Sahu, Crowley, Tatemoto, Balasubramani, and Kalra, 1987), and galanin-induced LH secretion can be inhibited by icv administration of galantide, a galanin receptor competitive antagonist (Sahu, Xu, and Kalra, 1994). Together, these studies suggest that *in vivo,* galanin plays an important role in the regulation of gonadotropin secretion.

If galanin is a cotransmitter with GnRH and modulates gonadotropin levels, its level of expression in GnRH neurons may be a target of physiological regulation. We examined this idea by seeking evidence for alterations in cellular levels of galanin mRNA in GnRH neurons that might occur as a function of the animal's physiological state and as the result of challenges with centrally active pharmacological agents.

TECHNICAL APPROACH

To accomplish this, we used *in situ* hybridization and image analysis to estimate relative levels of galanin mRNA and GnRH mRNA in the brains of rats under different physiological conditions. Although this method detects only relative mRNA levels at a given point in time, we argue that the amount of cellular mRNA generally reflects the biosynthetic capacity of a cell to produce a certain protein.

Our studies comprised single-label *in situ* hybridization assays to measure GnRH mRNA levels and double-label *in situ* hybridization assays to measure galanin mRNA levels only in GnRH mRNA-containing neurons. In the single-label assays for GnRH mRNA, a complementary (c)RNA probe containing radioactive uridine was used to measure endogenous GnRH mRNA. The silver grains produced in the photographic emulsion layer were counted by a computerized image analysis system to provide a numerical index of GnRH mRNA levels. Likewise, in the

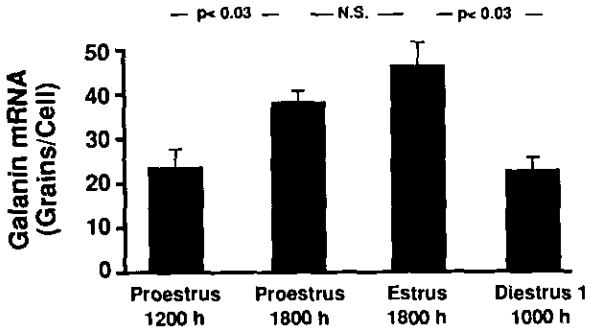

FIG. 1. Relative levels of galanin mRNA in GnRH neurons (grains/cell + SEM) in the forebrain and rostral hypothalamus of rats during the estrous cycle. (Reprinted from D. L. Marks, M. S. Smith, M. Vrontakis, D. K. Clifton, and R. A. Steiner (1993). Regulation of galanin gene expression in gonadotropin-releasing hormone neurons during the estrous cycle of the rat. *Endocrinology* **132,** 1836–1844. © The Endocrine Society.)

double-label *in situ* hybridization assays, relative levels of galanin mRNA were measured with a radioactive cRNA probe. GnRH mRNA was detected with a cRNA probe containing uridine linked to the hapten digoxigenin. An enzyme-conjugated anti-digoxigenin antibody was used to produce a product that stained GnRH mRNA-containing neurons a dark purple color. Because galanin acts as a cotransmitter in a wide variety of hypothalamic neurons, the double-label *in situ* hybridization assay is essential for determining levels of galanin mRNA in the small subset that also produces GnRH.

ESTROUS CYCLE

By double-label immunocytochemistry, it has been shown that adult male rats express galanin in about 20% of their GnRH neurons, whereas adult female rats express galanin in about 65% of their GnRH neurons (Merchenthaler, Lopez, Lennard, and Negro-Vilar, 1991). We and others have suggested that greater expression of galanin in GnRH neurons in female rats indicates that galanin is required for reproductive events unique to the female, such as the preovulatory LH surge.

To examine the possible physiological significance of galanin coexpressed in GnRH neurons, we sacrificed normal cycling female rats at different times during the estrous cycle and compared cellular levels of galanin mRNA in GnRH neurons by double-label *in situ* hybridization. Levels of galanin mRNA in GnRH neurons on the late afternoon of proestrus, during the LH surge, were significantly higher than those found on the morning of the same day (Fig. 1). No differences in galanin mRNA levels were found in non-GnRH cells in the same brain sections; moreover, there were no differences in GnRH mRNA levels observed over the estrous cycle (Marks, Smith, Vrontakis, Clifton, and Steiner, 1993a).

These results support the hypothesis that induction of galanin expression in GnRH neurons is an important event in generating the preovulatory LH surge.

SEX STEROID INDUCTION

The preovulatory surge of LH is triggered by the positive feedback action of estradiol and progesterone on the brain–pituitary axis, a process that can be simulated in ovariectomized animals by treating them with an appropriate regimen of sex steroids (estradiol and progesterone, E/P). If the induction of galanin mRNA in GnRH neurons were physiologically linked to the LH surge, we argued that ovariectomized rats that are induced to have a LH surge by the E/P treatment should likewise show an induction of galanin mRNA in their GnRH neurons. To test this hypothesis, groups of adult female rats were ovariectomized. After 3 weeks, the animals received a regimen of either estradiol benzoate and progesterone (E/P) or the oil vehicle as a control. The animals were sacrificed, and their brains and blood were collected. Serum LH was measured by radioimmunoassay (RIA) and the brains were processed in the double-label *in situ* hybridization assay for galanin mRNA and GnRH mRNA and in the single-label *in situ* hybridization assay for GnRH mRNA. We found that the steroid-primed animals exhibited LH surges and showed a dramatic induction of galanin mRNA in GnRH neurons compared to control animals, whereas GnRH message levels were unaffected by E/P treatment (Marks, Lent, Rossmanith, Clifton, and Steiner, 1994). These results confirm that sex steroids regulate galanin gene expression in GnRH neurons.

SYNAPTIC ACTIVATION

Sex steroids are capable of inducing expression of galanin mRNA in GnRH neurons, but the mechanism of this induction phenomenon is unclear. GnRH neurons themselves do not appear to express either the estrogen or progesterone receptor. Therefore, it seems plausible that there is a population of steroid receptor-positive "relay" neurons that synapse with the GnRH neurons and transduce the sex steroid signals to the GnRH neurons. These steroid-sensitive, relay neurons then act via their own neurotransmitter upon the GnRH neurons to induce the expression of galanin mRNA. We argued that if this were true, then the steroid-induced LH surge and the associated induction of galanin mRNA in GnRH neurons should be blocked by inhibition of synaptic transmission. We tested this hypothesis by examining the effect of pharmacological blockade of steroid-induced activation of GnRH neurons with a general anesthetic, pentobarbital, and with a specific alpha-adrenergic receptor antagonist, phenoxybenzamine. Three weeks after ovariectomy, adult female rats were divided into the following treatment groups: E/P injections followed

by saline, oil control injections followed by saline, E/P injections followed by pentobarbital, E/P injections followed by phenoxybenzamine. Several hours after the final treatment, all animals were sacrificed. Blood was assayed for LH by RIA and the brains were processed in double-label *in situ* hybridization assay. We found that the E/P injections induced the expression of galanin mRNA in GnRH neurons (galanin mRNA signal was fourfold higher in the E/P group compared with the oil-treated control group) and generated surge levels of LH in the blood. Animals receiving pentobarbital after E/P failed to exhibit either an LH surge or an induction of galanin mRNA in GnRH neurons. Phenoxybenzamine blocked the LH surge in the majority of E/P-treated animals. In these LH surge-blocked animals, no induction of galanin mRNA in GnRH neurons occurred. Animals treated with E/P and phenoxybenzamine that escaped the LH surge blockade (i.e., displayed an unequivocal LH surge) showed a marked induction of galanin mRNA content in GnRH neurons, statistically indistinguishable from the E/P only group (Fig. 2). Single-label *in situ* hybridization and analysis for GnRH mRNA revealed no significant differences among the treatment groups (Marks, *et al.*, 1994). These results indicate that the synaptic activation that leads to the generation of the LH surge is also required for the induction of galanin message in GnRH neurons. Furthermore, the induction of galanin mRNA in GnRH neurons is highly correlated with the activity of the GnRH neuron, whereas no evidence could be adduced for transcriptional regulation of the GnRH gene.

LACTATION

The attention of the postpartum female is, by necessity, focused on the care and nurture of her young. Estrous cycles are temporarily halted by virtue of diminished pulsatile secretion of GnRH. The proximate cause of this imposed quiescence of GnRH secretory activity involves the complex interplay of suckling, elevated plasma levels of prolactin, and altered synaptic input to GnRH neurons. We hypothesized that during lactation, blunted GnRH neuronal activity would be associated with altered levels of galanin mRNA in GnRH neurons. To test this hypothesis, we compared galanin mRNA levels in GnRH neurons of females killed at diestrus with those of lactating females. The brains were processed in the double-label *in situ* hybridization assay. We found that lactating females had levels of galanin mRNA in their GnRH neurons that were significantly lower than those of diestrous rats (Marks, Smith, Clifton, and Steiner, 1993b). In contrast, GnRH mRNA levels were similar in diestrous and lactating rats. These results are consistent with the hypothesis that the functional capacity of GnRH neurons in lactating animals is limited by their diminished expression of galanin.

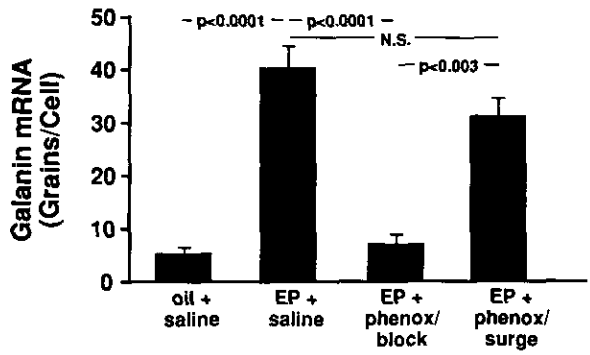

FIG. 2. Relative levels of galanin mRNA signal (grains per cell + SEM) in GnRH neurons throughout the forebrain and rostral hypothalamus in groups of adult, ovariectomized rats under different treatment regimens. The groups included control animals (oil + saline; $n = 6$), steroid-primed animals (E/P + saline; $n = 7$), steroid-primed, phenoxybenzamine-treated animals with blocked (block; $n = 3$) and unblocked (surge; $n = 4$) LH surges. (Reprinted from D. L. Marks, K. L. Lent, W. G. Rossmanith, D. K. Clifton, and R. A. Steiner (1994). Activation-dependent regulation of galanin gene expression in GnRH neurons in the female rat. *Endocrinology* **134,** 1991–1998. © The Endocrine Society.)

GALANIN'S FUNCTIONAL SIGNIFICANCE

Galanin is colocalized with a variety of neurotransmitters throughout the central nervous system, but the physiological function of this colocalization phenomenon is uncertain. The functional significance of galanin is perhaps best understood in the septo-hippocampal cholinergic system. Here, galanin is released from the same axon terminals as acetylcholine and acts on pre- and post-synaptic receptors to reduce further acetylcholine release and to antagonize the post-synaptic effects of acetylcholine in the ventral hippocampus. In this instance, galanin acts as a negative neuromodulator, but whether this mechanism can be generalized to other systems is unknown. (See review by Merchenthaler *et al.*, 1993.)

By analogy to the septo-hippocampal system, galanin in GnRH neurons may be coreleased with GnRH and have both pre- and post-synaptic effects. The putative presynaptic effects of galanin on GnRH neurons should, by some undefined mechanism, result in increased gonadotropin secretion from the pituitary. Theoretically, one could conceive of either a direct stimulatory action of galanin on GnRH neurons or a complex inhibitory presynaptic mechanism that would ultimately manifest increased gonadotropin secretion. The most obvious (but not necessarily correct) explanation is that galanin acts presynaptically to increase GnRH release, presumably via a galanin receptor subtype different from the septal presynaptic galanin receptor. Conversely, it is conceivable that galanin acts presynaptically to briefly halt GnRH secretion, which would "sharpen" GnRH pulses. This altered GnRH pulse profile could be a more efficient signal to the pituitary gonadotrope. It is also possible that galanin has post-synaptic effects at the pituitary. In support of the hypothesis that

galanin acts both pre- and postsynaptically, galanin binding sites have been found in the hypothalamus and the anterior pituitary (Merchenthaler *et al.*, 1993, and Wynick, Smith, Ghatei, Akinsanya, Bhogal, Purkiss, Byfield, Yanaihara, and Bloom, 1993).

FUTURE DIRECTIONS

Important questions regarding the function of galanin in GnRH neurons remain to be answered. What is the functional significance of enhanced galanin gene expression in GnRH neurons that is coincident with the proestrous LH surge? What is the molecular mechanism of galanin's actions on GnRH neurons (or gonadotropes)? What is the mechanism that underlies the inhibition of galanin gene expression in GnRH neurons during lactation? Perhaps, by generating lines of transgenic animals that carry alterations in the expression of galanin in GnRH neurons, greater insights into the molecular physiology of GnRH neurons will be gleaned.

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