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Norepinephrine Transporter mRNA Expression After Coitus in the Rabbit Brainstem

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Abstract

In the female rabbit, coitus induces a massive release of hypothalamic gonadotropin-releasing hormone (GnRH) within 20 min. The GnRH surge is preceded by an increase in hypothalamic norepinephrine (NE) release. Presumably, coitus stimulates NE, hence GnRH, release by increasing the activity of tyrosine hydroxylase (TH, the rate-limiting enzyme for NE synthesis) and/or decreasing the activity of norepinephrine transporter (NET, the key protein for NE re-uptake). Since NE cell bodies are located primarily in the brainstem, we hypothesize that coital signals are relayed to hypothalamic GnRH-secreting neurons via brainstem NE-containing perikarya. In support of this hypothesis, we found that both c-fos and TH mRNA expressions in brainstem noradrenergic areas, particularly in the A1 and A2 cell groups, increased within 30 min and returned to precoital levels within 60 min after coitus. Here we analyzed coitally induced changes in NET mRNA expression at 0, 15, 30 and 60 min postcoitus in the brainstem by in situ hybridization, using ^{35}S-labeled rabbit NET RNA probes. In comparison with nonmated females (i.e., at 0 min), the expression of NET mRNA significantly increased ($P<0.05$) within 15 min postcoitus in the A1, but not the A2 area. By 30 min postcoitus, NET gene expression increased in the caudal portion of the A1 and in the caudal and central portion of the A2. By 60 min postcoitus, NET mRNA expression in the caudal and rostral portion of the A1 and the caudal and central portion of the A2 was still higher than NET mRNA expression in nonmated rabbits ($P<0.05$). No change in NET mRNA expression was observed in the A6. The results suggest that coitus increases NET mRNA expression in A1 and A2 noradrenergic areas within 15-30 min, and this enhanced NET mRNA expression was maintained for at least 60 min, particularly in the A2. These findings, in combination with our previous observation on increased TH gene expression within 30 min, but not 60 min, after coitus, further suggest that the coitus-induced NET transcriptional events within brainstem NE neurons may play an important role in the maintenance, and particularly in the termination, of hypothalamic NE release, hence regulating the size and duration of the coitus-induced GnRH surge.

Key Words: norepinephrine transporter, tyrosine hydroxylase, GnRH, coitus, brainstem

Introduction

Brain norepinephrine (NE) plays a key role in the initiation and maintenance of the coitus-induced release of hypothalamic gonadotropin-releasing hormone (GnRH), hence the release of the preovulatory

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luteinizing hormone (LH) surge in the rabbit (for review, see 1, 36). For example, central administration of NE stimulates (27, 34), whereas adrenergic blocking drugs inhibit (33, 43), hypothalamic GnRH secretion. Moreover, the postcoital elevation in hypothalamic GnRH secretion is preceded by a remarkable release of NE from the arcuate nucleus-median eminence (22, 41). The action of NE on hypothalamic GnRH neurons may occur either at the GnRH perikarya that are in contact with NE-containing neurites or at GnRH terminals that are in juxtaposition to NE neurites (12, 15). The effectiveness of synaptic NE on GnRH secretion depends on several neurochemical events, including the rate of NE synthesis (determined by the activity of the rate-limiting enzyme, tyrosine hydroxylase [TH]) and the rate of NE re-uptake (determined by the activity of the presynaptic NE transporter [NET; 4, 20, 31]). In mammals (10, 13, 14, 38), including rabbits (2,3), hypothalamic noradrenergic terminals are derived from cell bodies located in several areas within the brainstem, including the ventrolateral tegmentum of the medulla (A1 noradrenergic cell group), the dorsal tegmental tract (A2 noradrenergic cell group) and the locus coeruleus (A6 noradrenergic cell group). During the first 30 min of the coitus-induced NE/GnRH surge, TH gene expression increased in the A1 and A2, as detected by *in situ* hybridization (6), and in the A6, as detected by the ribonuclease protection assay (42). Moreover, NET gene expression also increased in the A6 within 30 min after coitus (42). In those studies (6, 42), the expression of NET mRNA in the A1 and A2 was not examined.

In the present study, we utilized the *in situ* hybridization technique to quantify the expression of NET mRNA in the A1, A2 and A6 noradrenergic areas at four different times (0, 15, 30 and 60 min) after coitus in female rabbits.

Materials and Methods

Animals

Adult female New Zealand White rabbits were obtained from Western Rabbitry Co. (Philomath, OR) and were housed individually under controlled light (lights on 07:00-19:00 h) and temperature (23°C). The animals were fed once daily with Purina Rabbit Chow (Ralston, NJ) and provided with water ad libitum.

Experimental Design

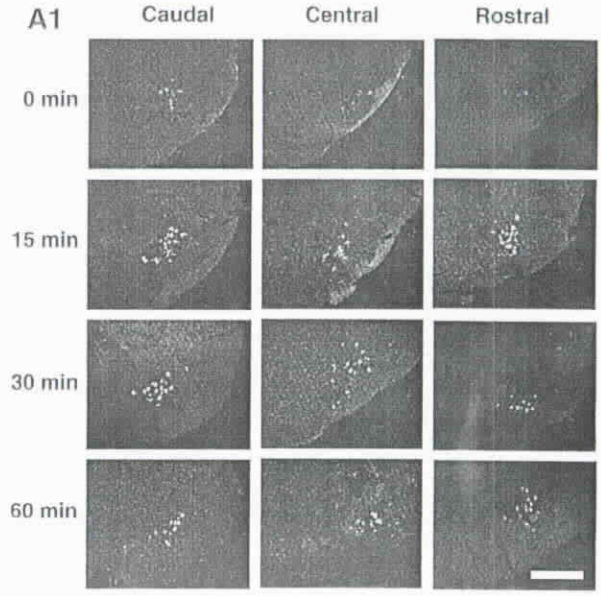

Fig. 1. Darkfield images of NET mRNA expression in the caudal, central and rostral portion of the A1 noradrenergic areas of one representative female rabbit before (0 min) and at 15, 30 and 60 min after coitus. Autoradiographic signals were captured on emulsion-dipped brainstem sections and presented in grayscale images. The magnification of each panel was the same and indicated by the bar (lower right corner panel) with bar length = 100 mm.

Rabbit mating was performed by placing a female into the cage of a vasectomized stud male for 5 min, during which time successful coitus occurred (determined by visual observation [5, 6, 7, 22, 41]). Mated female rabbits were killed under deep Nembutal anesthesia (30 mg/kg, i.v.) at either 15 (n=3), 30 (n=3) or 60 min (n=3) after coitus for *in situ* hybridization. Unmated females (0 min, n=3) were handled identically to mated females, except that they were not placed with males and were killed either immediately prior to or following the mated individuals.

Tissue Preparation

Brains were perfused through the aorta with 500 ml of saline followed by two liters of cold 4% paraformaldehyde in 0.1 M borate, pH 9.5, as described elsewhere (30). Brainstem blocks were postfixed and cryoprotected in 20% sucrose-4% paraformaldehyde-0.1 M borate, pH 9.5, for 72 h at 4°C with continuous agitation on an orbital shaker. Then the brainstems were deveined and fast frozen in dry ice/isopentane and stored at -80°C. Frozen brainstems were sectioned (20 μm thick) in a microtome, collected (1-in-6) in a cryoprotecting buffer with RNase inhibitor and mounted

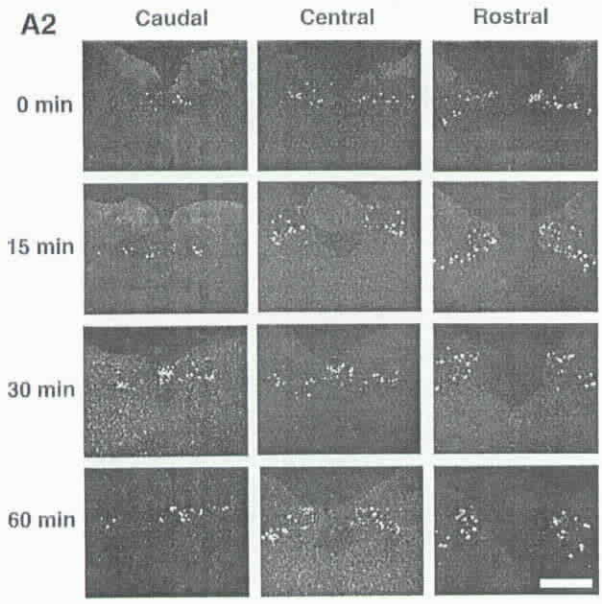

Fig. 2. Darkfield images of NET mRNA expression in the caudal, central and rostral portion of the A2 noradrenergic areas of one representative female rabbit before (0 min) and at 15, 30 and 60 min after coitus. Bar length = 100 mm. See Fig. 1 legend for details.

on coated slides (see below) for *in situ* hybridization.

In Situ Hybridization

The procedures for *in situ* hybridization were based on those by Cox et al. (9) and on those reported previously by our laboratory (30). Briefly, mounted sections on gelatin-subbed, poly-L-lysine-coated microscope slides were prepared for prehybridization with a 30-min proteinase K digestion (10 μg/ml at 37°C) and acetylation (0.0025% at room temperature), dehydrated in ascending ethanol concentrations and dried under vacuum overnight. T_7 polymerase was used to transcribe the ^{35}S-labeled antisense NET cRNA probes (503 bp, [30]). The radiolabeled cRNA probes were purified by passing the transcription reaction solution through a Sephadex Quick-Spin column (Boehringer Mannheim, Indianapolis, IN), and the radioactivity was estimated using a scintillation counter (1.5-3.1 x 10^6 cpm/μl). The purified ^{35}S-labeled cRNA probes were diluted in hybridization buffer and preserved in 1-ml fractions. Each ml of hybridization solution contained 500 μg yeast tRNA, 10 μm dithiothreitol (DTT) and 5 million cpm of probe. Sections on each slide received 80 μl of hybridization solution, and the slide was covered with a glass coverslip and sealed with DPX (Electron Microscopy Sciences, Ft. Washington, PA) before incubation for 20 h at 58°C. Following hybridization, slides were washed four times in 4X SSC prior to RNase digestion (20 μg/ml for 30 min at 37°C) then rinsed at room temperature in decreasing concentrations of SSC that contained 1 mM DTT (2X, 1X, 0.5X; 10 min each) to a final concentration of 0.1 X SSC at 65°C for 30 min. Sections were dehydrated in increasing concentrations of ethanol, exposed to Dupont Cronex X-ray film with autoradiographic ^{14}C microscales (Amersham, Arlington Heights, IL) for 5 days and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). After 14 days the autoradiograms were developed with a Kodak D-19 developer (Eastman Kodak Co., Rochester, NY), and the sections were counter-stained with thionin. For hybridization controls, sense RNA probe for NET was used in identical procedures for select brainstem sections. No positive autoradiographic signals were observed in any tested section.

Data Processing and Analysis

The expression of NET mRNA was examined throughout the entire A1, A2 and A6 areas within the caudal-rostral confinement of each area. Anatomically identified sections (25) at the caudal, central and rostral portion of each noradrenergic area were selected for image analysis (6, 30) according to the distribution pattern of NET mRNA in the brainstem of unmated rabbits (30). Briefly, positive radioactive signals were analyzed under darkfield illumination by a Leica DM/LS III microscope. The selected images were captured and digitized by a Leica DC 200 CCD camera, and the digitized images were recorded with Adobe Photoshop Software (Adobe Systems Inc., San Jose, CA) and stored in a PC computer. For each level in each of the three noradrenergic cell groups (A1, A2 and A6), we analyzed the optical density in a constant area of anatomically matched sections across the 12 animals with the NIH image software program adapted to the Windows 95 operation system for IBM PCs by the Scion Image Corporation (downloaded from www.scionimages.com). The data, expressed in optical density/unit area, in each level (n=3) of the three noradrenergic areas were analyzed by a one-way analysis of variance for time effects, and the difference in group means was compared by the post hoc LST test (Statistix for Windows, Analytical Software, Tallahassee, FL). Then data from the three levels (n=9) of A1, A2 or A6 were combined, analyzed by one-way

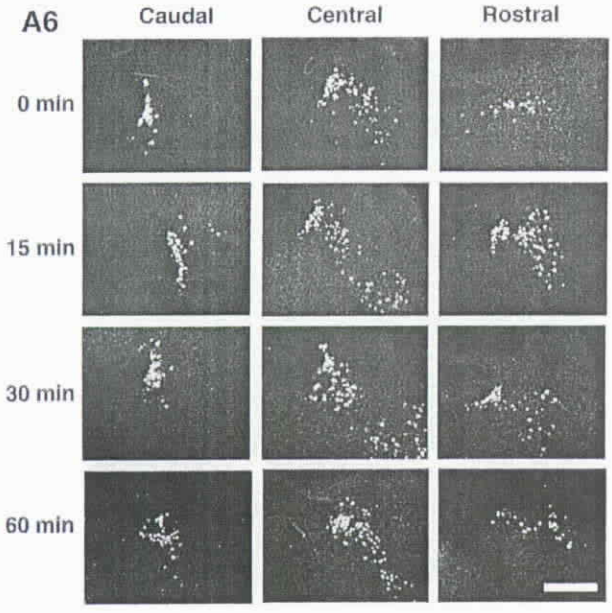

Fig. 3. Darkfield images of NET mRNA expression in the caudal, central and rostral portion of the A6 noradrenergic areas of one representative female rabbit before (0 min) and at 15, 30 and 60 min after coitus. Bar length = 100 mm. See Fig. 1 legend for details.

ANOVA and compared by the Tukey post hoc test. A value of $P<0.05$ was considered significant.

Results

The expression of NET mRNA before and after coitus in the A1 noradrenergic area, shown as darkfield images, is presented in Figure 1. Compared to nonmated rabbits (0 min), NET mRNA expression increased at the caudal A1 level within 15 min and remained higher ($P<0.05$) at 30 and 60 min postcoitus, whereas the NET mRNA expression in the central and rostral level of the A1 area was higher only at 15 min postcoitus (Fig. 4). When data from the three caudal-rostral levels of the A1 area were combined, the expression of NET mRNA was found to be higher ($P<0.05$) at 15, but not at 30 or 60 min after coitus (Fig. 4). In the A2 area (Fig. 2), NET mRNA expression in the caudal and central portion did not significantly increase until 30 min postcoitus. Thereafter, the enhanced NET gene expression was maintained until 60 min postcoitus (Fig. 4). No significant ($P>0.05$) difference in NET gene expression at any time point was observed in the rostral A2 area. When data from the three caudal-rostral levels of the A2 area were combined, the NET mRNA expression was found to be higher ($P<0.05$) at 30 and 60 min after coitus (Fig. 4). In contrast to the A1 and A2 areas, NET mRNA expressions did not increase in any of the three caudal-rostral levels in the A6 area (Figs. 3 and 4).

Discussion

The present data by *in situ* hybridization suggest that the expression of NET mRNA in different noradrenergic cell populations (i.e., A1, A2 and A6) followed different patterns and time courses in response to coital stimuli. Coitus increased NET gene expression in the A1 and A2 areas, but not in the A6 area. Moreover, transcriptional signals of NET increased sooner in the A1 area (15 min postcoitus) than in the A2 area (30 min postcoitus). However, the NET transcriptional activity lasted longer in the A2 area (at 60 min postcoitus) than in the A1 area (significant only at the 15-min time point). These results, in conjunction with previous findings that 1) hypothalamic release of NE increases following coitus in female rabbits (22, 41), 2) treatment with adrenergic antagonists attenuates the coitus-induced GnRH/LH surge (43) and blocks ovulation (33) and 3) coitus increases c-fos and TH gene expressions within 30 min in the A1 and A2 areas (6, 39), support the general hypothesis that brainstem NE neurons are involved in the relay of coital sensory stimuli (29, 36) or estrogen signals (8, 16) to hypothalamic GnRH-secreting signals.

In a previous study (6), we found that there is an increase in c-fos mRNA in the A1 and A2 brainstem areas relative to unmated controls by 30 min after coitus, and that this increase in transcriptional activity leads to the production of Fos protein in select populations of DBH-staining neurons. We hypothesized that coital neural signals are transported via the spinal cord to the caudal brainstem where the signals activate gene transcription/translation processes in A1 and/or A2 noradrenergic cells. One of the coitus-induced transcription/translation products is TH since the expression of TH mRNA in both the A1 and A2 areas increased concomitantly with the increase in c-fos mRNA expression.

We report here, for the first time, that another coitus-induced transcriptional product in the brainstem is NET (Figs. 1-4). There is similarity in the location and intensity between NET mRNA-expressing and TH mRNA-expressing neurons in the A1 and A2 areas either before or after coitus (6), suggesting that TH and NET mRNAs are expressed in the same cell, although this has not been confirmed with double-labeling techniques. However, this is likely the case, as shown in adjacent sections in which the distribution pattern

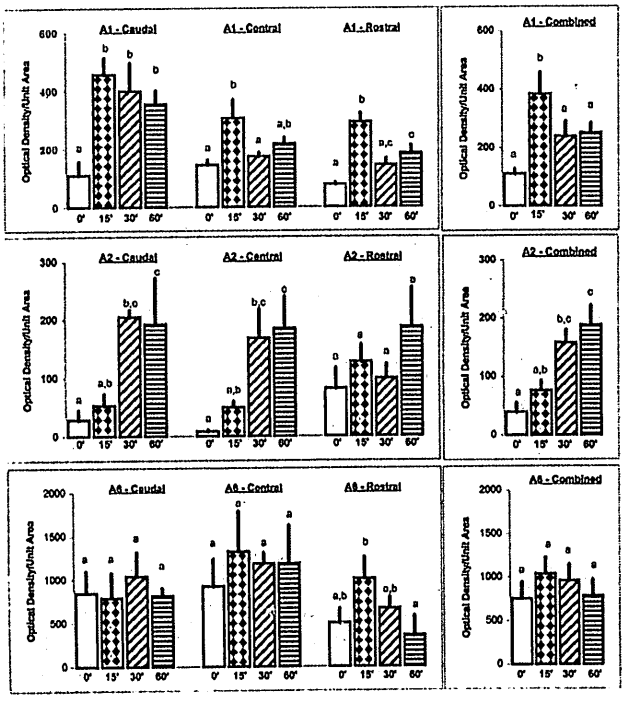

Fig. 4. Comparison of NET mRNA expressions, indicated by optical density/unit area, in three brainstem noradrenergic areas (A1, A2 and A6) before (0 min) and at 15, 30 and 60 min after coitus (n=3 in each time group). The three caudal-rostral levels of each noradrenergic area was analyzed and presented individually and combined (i.e., caudal + central + rostral). NET expression at different time points after coitus was compared to NET expression before coitus, and the difference in mean values is indicated by different letters (i. e., a, b or c).

of the two genes was determined in rabbits (30) and rodents (24). The cellular localization and expression of TH and NET impacts directly on the function of NE as it is synthesized, released and taken up by presynaptic NE terminals. Basically, an increase in TH activity increases, and an increase in NET activity decreases, NE concentrations in the synaptic cleft of NE terminals.

The physiological implication of the increase in both TH and NET gene expressions in the A1 and A2 areas after coitus cannot be resolved by this and other studies (6, 42). However, several postulations can be offered. First, the coitus-induced NET transcription is not translated into increased NET protein at the presynaptic NE terminals. This remains an attractive hypothesis from the standpoint that the concentration of NE in median eminence microdialysate increases within 20 min postcoitus (41). This event can be achieved by an increase in TH production and NE release without changing the activity of NET. Second, the dynamic expression of NE synthesis, release and reuptake is always under the dual control of TH and NET activity, even during the intensified release of NE/GnRH shortly after coitus. This postulation assumes that the effect of enhanced TH/NE activity supercedes the effect of enhanced NET activity, thus resulting in a net effect of increased NE reaching post-synaptic GnRH neurons. The current results would support this postulation. Third, it would then follow that the primary role for a coitus-induced NET mRNA increase is to keep the size of the NE surge in check and ultimately to terminate the NE surge. Both NE and GnRH peak at approximately 60 min after coitus (22, 36, 41). Thereafter, both the neurotransmitter and peptide gradually decline. Interestingly, TH gene expression in the A1 and A2 area was highest at 30 min after coitus (6). However, by 60 min the expression of TH had already declined (6). In contrast, NET gene expression increased as early as 15 min after coitus in the A1 area and 30 min after coitus in the A2 area. The heightened NET mRNA level was maintained at 60 min postcoitus, at least in the A2 area (this study). These results extend our hypothesis on the coitus-induced GnRH surge as follows: Coital signals are transmitted via the spinal cord to the A1 and A2 noradrenergic areas, with the possibility that the coital signals are transmitted to the A1 area first before they are transmitted to the A2 and A6 areas. This relay of coital signals is transformed into enhanced transcriptional/translational activity of at least TH and NET, which provide the resources for enhancing NE synthesis, release and reuptake, resulting in a cumulative effect of rising NE and hence GnRH concentration in the mediobasal hypothalamus (MBH). TH gene expression, however, starts to decline 30 min after coitus, whereas NET gene expression continues to increase beyond 60 min. This results in a reduction of NE synthesis and/or release in the presence of a maintained level of NET activity after coitus, thus the initiation of the decline of the NE/GnRH surge.

Partial support of this concept can be found in rodent studies. For example, the injection of the retrograde tracers fluorogold and fluororuby in the preoptic region retrogradely labeled 11.8% of DBH neurons in the brainstem A1 area, 7.6% in the A2 area and less than 1% in the A6 area (40). This result was partially confirmed recently using green fluorescent latex microspheres injected into the rostral preoptic area (35). Retrogradely labeled DBH neurons were noted exclusively in the A1 and A2 areas, but not in the A6 area. Furthermore, the above-mentioned studies agree with electrophysiological evidence regarding projections from the A1 region to the preoptic area (18). While a direct noradrenergic neural connection between A1/A2 neurons and the hypothalamus would strongly support the concept of a brainstem relay between coitus and

hypothalamic surges of NE and GnRH, it is unclear as to how coital signals are processed in the brainstem before reaching the hypothalamus. In a previous study (42), we reported that mRNA expression of TH and NET genes were increased in the A6 brainstem region 15 to 120 min after mating in female rabbits, but that levels of mRNA were unchanged in postcoital males. In those studies (42), levels of mRNA were quantified by solution hybridization assays in frozen tissue punches of the A6 region, but not in those of the A1 or A2 regions. In this study, we utilized the *in situ* hybridization technique, and we did not observe changes in the expression of NET mRNA in the A6 area. Whereas quantification of mRNA levels are more accurate and sensitive by the solution hybridization technique, individual cell identity and multiple labels within the same cell were feasible with the current utilized techniques. Because A6 is the largest noradrenergic cell group in the brainstem and neural connections between the locus coeruleus and forebrain (including the hypothalamus) have been demonstrated in the rabbit and other species using retrograde tracing techniques (14, 38), it is feasible to hypothesize that the A6 is a secondary relay site (indirect input from A1 and/or A2) where noradrenergic transmission to the forebrain and hypothalamus (including TH and NET) is activated via a separate path. It is also possible that the A6 is a secondary relay station when coital signals are transmitted to several brain areas that are responsible for postcoital changes in episodes of paradoxical sleep, licking, eating and drinking patterns (21). Some of these postcoital changes may involve peptides, including neuropeptide Y (11) and galanin (14), which have been found to be colocalized with DBH or TH in these A6 neurons.

It is almost certain that NE is not the only neurotransmitter system involved in the relay of the coitus-induced GnRH surge. In addition to dibenamine, an adrenergic antagonist, ovulation in the rabbit can also be blocked by atropine, a cholinergic antagonist (33). Moreover, intra-hypothalamic infusion of prazosin, a selective α-adrenergic antagonist, fails to completely block the coitus-induced GnRH surge (43). Cells producing acetylcholine (Ach) are located in the brainstem; some are located in the rostral A6 area (the dorsolateral tegmental nucleus [nDLT]) where Ach cells are overlapped with noradrenergic cells in several species, including the rabbit (17, 23, 28, 32, 37). The nDLT and the ventrolateral tegmental area (VLT) just rostral to the A1 area exhibited enhanced mRNA expression of choline acetyltransferase, the rate-limiting enzyme for acetylcholine synthesis, after coitus (28). We hypothesize that part of the coital signals may be transformed into acetylcholine signals either directly from the spinal cord to the nDLT/VLT or indirectly via A1/A2, which then stimulate hypothalamic GnRH release in concert with the stimulation by NE. Intraventricular administration of α-bungarotoxin, a nicotinic cholinergic antagonist, attenuates pulsatile LH secretion in the rat (19). We observed that the coitus-induced LH release in estrogen-treated, ovariectomized rabbits was attenuated either by α-bungarotoxin or scopolamine, a muscarinic Ach antagonist (26).

Both NE and Ach innervate the hypothalamus, including the anteroventral periventricular area (AVPV) and the ventrolateral hypothalamic area (VLH). These two hypothalamic areas, and that of the encapsulated portion of the bed nucleus of the stria terminalis (BNSTe), are the three major areas in the hypothalamus that exhibit intensified c-fos mRNA expression after coitus (6). Moreover, the number of Fos/GnRH double-labeled neurons near the AVPV, BNSTe and VLH also increases after coitus (7). Thus, we hypothesize that coital NE and Ach signals are transmitted from the brainstem to the AVPV, VLH or BNSTe, where NE and/or Ach signals stimulate MBH-GnRH release, either directly via GnRH neurons adjacent to the AVPV, BNSTe and/or VLH areas, or indirectly via non-GnRH neurons within the three hypothalamic areas.

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References

1. Barraclough, C.A. and Wise, P.M. The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. *Endocr. Rev.* 3:91-119, 1982.
2. Blessing, W.W., Chalmers, J.P. and Howe, P.R.C. Distribution of catecholamine-containing cell bodies in the rabbit central nervous system. *J. Comp. Neurol.* 179:407-423, 1978.
3. Blessing, W.W., Howe, P.R.C., Joh, T.H., Oliver, J.R. and Willoughby, J.O. Distribution of tyrosine hydroxylase and neuropeptide Y-like immunoreactive neurons in rabbit medulla oblongata, with attention to colocalization studies, presumptive adrenaline-synthesizing perikarya, and vagal preganglionic cells. *J. Comp. Neurol.* 248:285-300, 1986.
4. Bonisch, H., Paulus, G., Martiny-Baron, G., Lingen, B. and Coppeneur, D. Molecular aspects of the neuronal noradrenaline transporter. *J. Neural Transm. Suppl.* 34:11-17, 1991.
5. Caba, M., Silver, R., Gonzalez-Mariscal, G., Jimenez, A. and

Beyer, C. Oxytocin and vasopressin immunoreactivity in rabbit hypothalamus during estrus, late pregnancy, and postpartum. *Brain Res.* 720:7-16, 1996.

6. Caba, M., Bao, J., Pau, K.Y.F. and Spies, H.G. Molecular activation of noradrenergic neurons in the rabbit brainstem after coitus. *Brain Res. Mol. Brain Res.* 77:222-231, 2000.
7. Caba, M., Pau, K.Y.F., Beyer, C., Gonzalez, A., Silver, R. and Spies, H.G. Coitus-induced activation of c-fos and gonadotropin-releasing hormone in hypothalamic neurons in female rabbits. *Brain Res. Mol. Brain Res.* 78:69-79, 2000.
8. Conde, G.L., Bicknell, R.J. and Herbison, A.E. Changing patterns of Fos expression in brainstem catecholaminergic neurons during the rat oestrous cycle. *Brain Res.* 672:68-76, 1995.
9. Cox, K.H., DeLeon, D.V., Angerer, L.M. and Angerer, R.C. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* 101:485-502, 1984.
10. Dahlström, A. and Fuxe, K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand. Suppl.* 232:1-55, 1964.
11. Everitt, B.J., Hökfelt, T., Terenius, L., Tatemoto, K., Mutt, V. and Goldstein, M. Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. *Neuroscience* 11:443-462, 1984.
12. Gore, A.C. and Roberts, J.L. Regulation of gonadotropin-releasing hormone gene expression in vivo and in vitro. *Front. Neuroendocrinol.* 18:209-245, 1997.
13. Halliday, G.M. and McLachlan, E.M. A comparative analysis of neurons containing catecholamine-synthesizing enzymes and neuropeptide Y in the ventrolateral medulla of rats, guinea-pigs and cats. *Neuroscience* 43:531-550, 1991.
14. Holets, V.R., Hökfelt, T., Rokaeus, A., Terenius, L. and Goldstein, M. Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. *Neuroscience* 24:893-906, 1988.
15. Jennes, L., Beckman, W.C., Stumpf, W.E. and Grzanna, R. Anatomical relationships of serotoninergic and noradrenalinergic projections with the GnRH system in septum and hypothalamus. *Exp. Brain Res.* 46:331-338, 1982.
16. Jennes, L., Jennes, M.E., Purvis, C. and Nees, M. c-fos expression in noradrenergic A2 neurons of the rat during the estrous cycle and after steroid hormone treatments. *Brain Res.* 586: 171-175, 1992.
17. Jones, B.E. and Beaudet, A. Distribution of acetylcholine and catecholamine neurons in the cat brainstem: A choline acetyltransferase and tyrosine hydroxylase immunohistochemical study. *J. Comp. Neurol.* 261:15-32, 1987.
18. Kaba, H., Saito, H., Otsuka, K., Seto, K. and Kawakami, M. Effects of estrogen on the excitability of neurons projecting from the noradrenergic A1 region to the preoptic and anterior hypothalamic area. *Brain Res.* 274:156-159, 1983.
19. Kalash, J., Romita, V. and Billiar, R.B. Third ventricular injection of alpha-bungarotoxin decreases pulsatile luteinizing hormone secretion in the ovariectomized rat. *Neuroendocrinology* 49:462-470, 1989.
20. Kanner, B.I. and Schuldiner, S. Mechanism of transport and storage of neurotransmitters. *CRC Crit. Rev. Biochem.* 22:1-38, 1987.
21. Kawakami, M. and Sawyer, C.H. Induction of behavioral and electroencephalographic changes in the rabbit by hormone administration or brain stimulation. *Endocrinology* 65:631-643, 1959.
22. Kaynard, A.H., Pau, K.Y.F., Hess, D.L. and Spies, H.G. Gonadotropin-releasing hormone and norepinephrine release from the rabbit mediobasal and anterior hypothalamus during the mating-induced luteiniziing hormone surge. *Endocrinology* 127: 1176-1185, 1990.
23. Kimura, H., McGeer, P.L. and Peng, J.H. Choline acetyltransferase-containing neurons in the rat brain. In: Handbook of Chemical Neuroanatomy. Classical Transmitters and Transmitter Receptors in the CNS, Part II, edited by Björklund, A., Hökfelt, T. and Kuhar, M.J. New York, NY: Elsevier, 1984, vol. 3, pp. 51-67.
24. Lorang, D., Amara, S.G. and Simerly, R.B. Cell-type-specific expression of catecholamine transporters in the rat brain. *J. Neurosci.* 14:4903-4914, 1994.
25. Meessen, H. and Olszewski, J. Cytoarchitektonischer Atlas des Rautenhirns des Kaninchens. Basel, Switzerland: S. Karger, 1949.
26. Pau, K.Y.F. Cholinergic regulation of the coitus-induced luteinizing hormone (LH) and prolactin release in ovariectomized, estrogen-treated rabbits. The 34th Annual Meeting of the Society for the Study of Reproduction, Ottawa, Ontario, Canada, July 28-August 1, 2001, Abstract #27.
27. Pau, K.Y.F. and Spies, H.G. Estrogen-dependent effects of norepinephrine on hypothalamic gonadotropin-releasing hormone release in the rabbit. *Brain Res.* 399:15-23, 1986.
28. Pau, K.Y.F. and Spies, H.G. Cholinergic acetyltransferase and acetylcholine transporter mRNA expression after coitus in New Zealand White rabbits. The 3rd Annual Northwest Reproductive Sciences Symposium, Corvallis, Oregon, USA, April 20-21, 2001, Abstract #5.
29. Pau, K.Y.F. and Spies, H.G. Neuroendocrine signals in the regulation of gonadotropin-releasing hormone secretion. *Chin. J. Physiol.* 40:181-196, 1997.
30. Pau, K.Y.F., Ma, Y.J., Yu, J.H., Yang, S.P., Airhart, N. and Spies, H.G. Topographic comparison of the expression of norepinephrine transporter, tyrosine hydroxylase and neuropeptide Y mRNA in association with dopamine beta-hydroxylase neurons in the rabbit brainstem. *Brain Res. Mol. Brain Res.* 48:367-381, 1997.
31. Pacholczyk, T., Blakely, R.D. and Amara, S.G. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature* 350:350-354, 1991.
32. Satoh, K., Armstrong, D.M. and Fibiger, H.C. A comparison of the distribution of central cholinergic neurons as demonstrated by acetylcholinesterase pharmacohistochemistry and choline acetyltransferase immunohistochemistry. *Brain Res. Bull.* 11: 693-720, 1983.
33. Sawyer, C.H., Markee, J.E. and Hollinshead, W.H. Inhibition of ovulation in the rabbit by the adrenergic-blocking agent dibenamine. *Endocrinology* 41:395-402, 1947.
34. Sawyer, C.H., Markee, J.E. and Townsend, B.F. Cholinergic and adrenergic components in the neurohumoral control of the release of LH in the rabbit. *Endocrinology* 44:18-37, 1949.
35. Simonian, S.X., Spratt, D.P. and Herbison, A.E. Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. *J. Comp. Neurol.* 411:346-358, 1999.
36. Spies, H.G., Pau, K.Y.F. and Yang, S.P. Coital and estrogen signals: a contrast in the preovulatory neuroendocrine networks of rabbits and rhesus monkeys. *Biol. Reprod.* 56:310-319, 1997.
37. Sutin, E.L. and Jacobowitz, D.M. Immunocytochemical localization of peptides and other neurochemicals in the rat laterodorsal tegmental nucleus and adjacent area. *J. Comp. Neurol.* 270:243-270, 1988.
38. Swanson, L.W. and Hartman, B.K. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. *J. Comp. Neurol.* 163:467-505, 1975.
39. Wersinger, S.R. and Baum, M.J. Sexually dimorphic activation of midbrain tyrosine hydroxylase neurons after mating or exposure to chemosensory cues in the ferret. *Biol. Reprod.* 56:1407-1414, 1997.
40. Wright, D.E. and Jennes, L. Origin of noradrenergic projections to GnRH perikarya-containing areas in the medial septum-diagonal band and preoptic area. *Brain Res.* 621:272-278, 1993.

41. Yang, S.P., Pau, K.Y.F., Hess, D.L and Spies, H.G. Sexual dimorphism in secretion of hypothalamic gonadotropin-releasing hormone and norepinephrine after coitus in rabbits. *Endocrinology* 137:2683-2693, 1996.
42. Yang, S.P., Pau, K.Y.F. and Spies, H.G. Tyrosine hydroxylase and norepinephrine transporter mRNA levels increase in locus coeruleus after coitus in rabbits. *J. Mol. Endocrinol.* 19:311-319, 1997.
43. Yang, S.P., Pau, K.Y.F., Airhart, N. and Spies, H.G. Attenuation of gonadotropin-releasing hormone reflex to coitus by alpha1-adrenergic receptor blockade in the rabbit. *Proc. Soc. Exp. Biol. Med.* 218:204-209, 1998.