The excitatory peptide kisspeptin restores the luteinizing hormone surge and modulates amino acid neurotransmission in the medial preoptic area of middle-aged rats

Abbrev Title: Kp-10 rescues LH surges in middle-aged rats

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Precis: Middle-aged female rats with impaired LH surges exhibit deficits in estradiol induction of Kiss1 mRNA expression in the anteroventral periventricular region of the hypothalamus, and infusion of kisspeptin into the medial preoptic area rescues LH surge amplitude while decreasing GABA and increasing glutamate release.

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Abstract

Reproductive success depends upon a robust and appropriately timed preovulatory luteinizing hormone (LH) surge. The LH surge, in turn, requires ovarian steroid modulation of gonadotropin releasing hormone (GnRH) neuron activation by the neuropeptide kisspeptin and by glutamate and γ-aminobutyric acid (GABA) neurotransmission in the medial preoptic area (mPOA). Middle-aged females exhibit reduced excitation of GnRH neurons and attenuated LH surges under estrogen positive feedback conditions, in part, due to increased GABA and decreased glutamate neurotransmission in the mPOA. This study tested the hypothesis that altered kisspeptin regulation by ovarian steroids plays a role in age-related LH surge dysfunction. We demonstrate that middle-aged rats exhibiting delayed and attenuated LH surges have reduced levels of Kiss1 mRNA in the anterior hypothalamus under estrogen positive feedback conditions. Kisspeptin application directly into the mPOA rescues total LH release and the LH surge amplitude in middle-aged rats and increases glutamate and decreases GABA release to levels seen in the mPOA of young females. Moreover, the N-methyl-D-aspartate receptor antagonist MK801 blocks kisspeptin reinstatement of the LH surge. These observations suggest that age-related LH surge dysfunction results, in part, from reduced kisspeptin drive under estrogen positive feedback conditions and that kisspeptin regulates GnRH/LH release, in part, through modulation of mPOA glutamate and GABA release.

Introduction

Initiation of a robust and appropriately timed preovulatory luteinizing hormone (LH) surge is achieved by estradiol and progesterone regulation of excitatory and inhibitory inputs onto gonadotropin releasing hormone (GnRH) neurons. Kisspeptin, a potent activator of GnRH neurons (1-4), is essential for LH surges (for review (5)). Afferent inputs from the anteroventral periventricular nucleus (AVPV) (6, 7) modulate the amplitude and frequency of GnRH secretion and generation of the LH surge (8). The expression of kisspeptin and of Kiss1 mRNA increases in the AVPV on the day of proestrus and under estradiol positive feedback conditions (9, 10). Kisspeptin neurons in the AVPV and GnRH neurons both express cFos at the time of the LH surge (1, 9, 10). Lastly, kisspeptin antibody attenuates the LH surge when infused into the preoptic area (11). Hence AVPV kisspeptin neurons projecting to GnRH neurons in the medial preoptic area (mPOA) are thought to be critical for estradiol positive feedback regulation of the LH surge (12, 13).

Estradiol initiates positive feedback by modulating many neurotransmitter inputs to GnRH neurons (for review (14, 15)). The LH surge in young female rats is accompanied by increased glutamate and decreased γ-aminobutyric acid (GABA) neurotransmission in mPOA, where most GnRH neurons are located (16, 17). Glutamate, glutamate agonists (18), and $GABA_A$ antagonists (19-21) stimulate GnRH synthesis and LH release. GABA inhibits GnRH neurons (22), and glutamate receptor antagonists (23, 24) and $GABA_A$ receptor agonists (25) block GnRH/LH release. Recent studies suggest that kisspeptin increases glutamatergic (26, 27) and decreases GABAergic (28) input to GnRH neurons. These data imply that kisspeptin directly and indirectly affects GnRH neurons (3, 26).

An early and consistent marker of reproductive aging in female rats is a delayed and attenuated preovulatory LH surge (for review (14)), resulting in part from a reduced ability of steroids to increase glutamatergic and decrease GABAergic neurotransmission in the mPOA (29-33). We recently showed that LH surge amplitude in middle-aged rats is rescued by simultaneously increasing synaptic glutamate and decreasing GABA and $GABA_A$ receptor activation in the mPOA (21). Others have suggested that reduced excitatory input from the AVPV may underlie age-related LH surge changes (34). Although the role of kisspeptin in reproductive senescence has not been investigated, Kiss1 mRNA expression is altered in the medial basal hypothalamus of aged human and non-human primates (35, 36). Therefore, we tested the hypothesis that age-related GnRH/LH surge impairment results from reduced expression of kisspeptin in the AVPV under estrogen positive feedback conditions, and that this may result in altered mPOA glutamate and GABA release.

Materials and Methods

Animals

Young (3-4 months) and middle-aged (9-11 months, retired breeders) female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed individually and maintained on a 14-h light:10-h dark cycle with lights off at 2000 h. Because we wish to identify changes in hypothalamic neurotransmitters that occur in the early stages of reproductive aging, while females still exhibit normal estrous cycles, only rats with at least two normal 4-day estrous cycles were used.

Drugs

Estradiol benzoate and progesterone were purchased from Steraloids, Inc. (Newport, RI), dissolved in peanut oil and injected subcutaneously (sc). Mouse kisspeptin-10 (110-119)-NH_2 (Kp-10; Phoenix Pharmaceutical, Belmont, CA), the rodent analog of human C-terminal kisspeptin decapeptide (112-121)-NH_2 was chosen because of equivalent affects on LH release as compared to the full length peptide (37). Kp-10 was dissolved at 10 nM in artificial cerebrospinal fluid (ACSF) and dialyzed into the mPOA. The molecular weight of Kp-10 is 1.3 kDa, well below the 30 kDa molecular cut-off for the microdialysis membrane. MK801 (0.3 mg/kg; Sigma, St. Louis, MO), a non-competitive NMDA receptor antagonist, was given sc at a dose previously shown to block LH

surges in young females (23, 38). Cetrorelix acetate (100 µg/0.1 ml; EMD Serono, Inc., Rockland, MA), a competitive GnRH receptor antagonist, was suspended in 5% mannitol and administered sc 24 hr before and just prior to progesterone injection (39-41). ACSF included 124 mM sodium chloride, 5 mM potassium chloride, 1.2 mM monopotassium phosphate, 10 mM magnesium sulfate, 1.8 mM calcium chloride, 26 mM sodium bicarbonate, 10 mM dextrose, pH 7.4 and were from Fisher Scientific (Pittsburgh, PA).

Stereotaxic surgery and jugular vein catheterization

Rats were ovariohysterectomized (OVX) under ketamine (80 mg/kg) and xylazine (4 mg/kg) anesthesia; stereotaxic placement of the microdialysis guide cannula into the mPOA occurred during the same surgical session (21). Using Bregma as a landmark and stereotaxic coordinates from Pellegrino *et al.* (42) (dorsal/ventral -8.6, anterior/posterior +2.0 and medial/lateral ±0.6), a unilateral guide cannula was implanted in the mPOA. Guide cannulae and concentric dialysis probes (2 mm dialysis surface, 340 µm outer diameter) were purchased from BASi (West Lafayette, IN). Eight days later, rats received an intra-atrial jugular vein catheter for serial blood sampling (31). Catheters were kept patent with daily infusion of heparinized saline (50 IU). All animal protocols were approved by the Institutional Animal Care and Use Committee and adhered to NIH Guidelines for the Care and Use of Laboratory Animals.

Steroid priming

All rats were primed with estradiol and progesterone for LH surge induction (31). At 0900 hr on the day of catheterization, rats were injected with 2 µg of estradiol benzoate; a second injection was given 24 hr later. A single injection of 500 µg of progesterone was given at 0900, 48 hr after the first estradiol benzoate injection (43). This protocol produces an LH surge in more than 80% of rats.

Microdialysis and plasma sampling

Microdialysis samples were collected at 30 min intervals from freely moving rats beginning at 0800 hr (31). Microdialysis of the mPOA with Kp-10 (10 nM, 1.25 µl/min) began 1.5 hr before progesterone injection and continued throughout the experiment. Controls were dialyzed with ACSF alone. Blood sampling began 1 hr before or at the time of progesterone injection and continued every 1-2 hr for 12 hr. Blood was collected into tubes containing heparinized saline (10 IU), refrigerated overnight and centrifuged at 10,000 x g for 20 min. Plasma was stored at -70°C until assayed for LH. An equal volume of warmed saline was infused to avoid hypovolemia. Microdialysis samples were collected, flash frozen, and stored until glutamate and GABA were determined (21). Animals were overdosed with ketamine, decapitated, and the brain rapidly frozen for later histological assessment of probe placement.

Hypothalamic dissection, RNA purification, reverse transcription and real time PCR

Independent groups of OVX, young and middle-aged rats were primed with peanut oil (control) or estradiol benzoate and progesterone as described above. Rats were killed 4 or 7 hr after the progesterone or final oil injection. The entire hypothalamus and preoptic area were dissected, then transected just posterior to the optic chiasm. The anterior hypothalamus, which includes the AVPV, was immediately frozen on dry ice and kept at -80ºC for later determination of Kiss1 mRNA expression. Although this tissue fragment includes cell groups in addition to the AVPV, the only Kiss1 mRNA expressing cells are those in the AVPV (for review (44)).

DNA-free total RNA was purified using the RNeasy Lipid Mini kit from Qiagen (Valencia, CA) including a DNase step. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA) using 500 ng of RNA per 20 µl of RT reaction. Gene expression was measured by real time PCR using TaqMan Gene Expression Assays and Master Mix (Applied Biosystems) according to manufacturer's instructions. The final reaction mix contained proprietary TaqMan probes and primers for the normalizer (rat GAPDH endogenous control, VIC®/MGB probe, RefSeq Rn01775763_g1, context sequence NM_017008.3) and specific target

(Kiss1, Fam probe, RefSeq Rn00710914_m1, context sequence NM_181692.1). Real-time PCR was performed using an ABI PRISM 7900HT (Applied Biosystems) in multiplex conditions using 50 ng of cDNA per 20 µl of total reaction mix. Amplified transcripts for Kiss1 were quantified using the comparative threshold cycle method and GAPDH as normalizer. The fold change in Kiss1 expression was then calculated as $2^{-\Delta\Delta CT}$ where CT = threshold cycle, ΔCT = CT (Kiss1) - CT (GAPDH), ΔΔCT = ΔCT (experimental) - ΔCT (reference). ΔCT (reference) was calculated using the mean of the ΔCT for the anterior hypothalamus of OVX animals treated with oil.

LH assay

LH was assayed at Northwestern University using rat LH radioimmunoassay reagents provided by the National Hormone and Peptide Program. The lower limit of the assay was 0.2 ng/ml, and the intra- and interassay coefficients of variation were 7.6 and 5.8 %, respectively. LH is reported as ng/ml/hr^{-1} (area under the curve, AUC) or ng/ml. A LH surge was defined as an increase in plasma LH equal to or greater than 1.5 times baseline (average LH value between 0800 and 1000 h) for at least two consecutive samples. Equivalent percentages of young (13/16) and middle-aged (30/34) rats with verified probe placements exhibited a LH surge.

Analysis of glutamate and GABA

Amino acids were separated by HPLC and their content in microdialysis samples was quantified as previously described (21). Amino acid identification and quantification were achieved by comparing peak retention times and heights in samples to known standards (Sigma). Amino acid content is reported as pmol/µl or pmol/µl//hr^{-}1 (AUC). The lower limit for detection of glutamate and GABA was 0.08 pmol. The recovery rate for glutamate and GABA is approximately 15% and is consistent across probes, based on in vitro calibrations of randomly selected probes.

Histological verification of probe placements

Every third 40 µm section throughout the extent of the dialysis probe track was stained with thionin to map probe placement in the mPOA (Figure 1). Only rats with a confirmed LH surge and appropriate probe placement were included in the data analysis. Two to four rats from each age group were discarded due to inaccurate probe placement and/or clogging of the probe.

Statistical analysis

The AUC for total glutamate and GABA and serum LH release was calculated using Sigma Plot 10.0 (Systat Software, Inc, Chicago, IL). Two-way ANOVA (age x treatment) was used to determine differences in Kiss1 mRNA, total and peak glutamate, GABA and LH levels. Total LH, glutamate and GABA in middle-aged rats treated with ACSF, Kp-10, Kp-10 + cetrorelix, and Kp-10 + MK801 were analyzed by one-way ANOVA. $P \leq 0.05$ was considered statistically significant. Latency to LH surge onset was evaluated with Kruskal-Wallis. Bonferonni or Newman Keuls post-hoc tests were performed as appropriate.

Results

Estradiol induces less Kiss1 mRNA in middle-aged females.

Figure 2A shows the effect of age and hormone treatment on the expression of Kiss1 mRNA in the anterior hypothalamus. Young rats were killed 4 hr after the progesterone or oil injection. Middle-aged rats were killed at both 4 and 7 hr after progesterone or oil injection. These time points reflect the onset time of the LH surge in young (4 hr) and middle-aged (7 hr) rats. There were no differences in Kiss1 mRNA levels in middle-aged rats killed at 4 or 7 hr after progesterone, therefore, these data were pooled. Hormone treatment significantly increased Kiss1 mRNA levels in both age groups (Figure 2A). However, hormone treatment induced significantly less Kiss1 mRNA in middle-aged than young rats. Age did not affect the expression of Kiss1 mRNA in control OVX rats.

Kisspeptin infusion restores LH surge amplitude in middle-aged rats

Kp-10 was dialyzed into the mPOA of OVX, steroid-primed rats beginning at 0730 hr on the day of the LH surge (Figure 2B-F). Control middle-aged rats exhibited significantly less total and peak LH release than all other groups

(Figure 2B-F). Kp-10 administration to middle-aged females significantly increased peak and total LH release to levels equivalent to young controls. Kp-10 had no effect on LH release in young rats. The LH surge was delayed in control middle-aged rats compared to young rats. Kp-10 did not affect LH surge onset in either age group. To verify that Kp-10 acted on GnRH neurons rather than on pituitary gonadotrophs (45), additional middle-aged rats infused with Kp-10 were also treated with the GnRH receptor antagonist cetrorelix (Figure 3). Cetrorelix blocked the LH surge in middle-aged rats treated with Kp-10.

Kisspeptin modulates extracellular glutamate and GABA

Total and peak glutamate release from the mPOA on the day of the LH surge in control middle-aged rats were less than 30% of those in young controls (Figure 4A-D). Kp-10 treated, middle-aged rats released significantly more glutamate than middle-aged controls and as much as young controls. In contrast, Kp-10 significantly reduced glutamate by more than 60% in young rats. Total and peak mPOA GABA on the day of the LH surge in control middle-aged rats was significantly greater than in all other groups (Figure 4E-HG). Kp-10 reduced total and peak GABA release in middle-aged rats to levels that were equivalent to young controls. Kp-10 did not affect GABA in young rats.

MK801 receptor blockade

There is good evidence that the NMDA subtype of glutamate receptor is essential for the generation of LH surges in young females in that the NMDA receptor antagonist MK801 blocks the LH surge (23, 46). Because Kp-10 restoration of the LH surge in middle-aged rats was associated with increased glutamate release in the mPOA, we asked whether activation of NMDA receptors was necessary for Kp-10 rescue of the LH surge (Figure 5). Systemic administration of the NMDA receptor antagonist MK801 blocked Kp-10-induced restoration of LH surges in middle-aged rats. MK801 also increased mPOA glutamate release but did not significantly affect GABA release.

Discussion

These data suggest that age-related attenuation of LH surge amplitude results from impaired hypothalamic sensitivity to estrogen positive feedback, resulting in reduced excitatory input to GnRH neurons. Specifically, age-related changes in LH surge amplitude may be causally linked to reduced Kiss1 mRNA expression in the AVPV, reduced mPOA glutamate and increased mPOA GABA release in response to estradiol and progesterone priming. Kisspeptin infusion into the mPOA of hormone-primed rats rescues GnRH/LH release and elevates local glutamate and decreases local GABA release, thereby restoring the balance of local excitatory and inhibitory amino acid release in the mPOA of middle-aged rats to levels typical of young females. Thus, age-related LH surge changes most likely result from reduced kisspeptin availability and/or release rather than reduced Kiss1r expression or compromised Kiss1r function in GnRH neurons.

Middle-aged females have compromised endocrine (LH) and neural (Kiss1 mRNA) responses to estradiol

A hallmark of impending reproductive failure in middle-aged rats is a delayed and attenuated LH surge, which is not due to reductions in GnRH neuron number or pituitary dysfunction (for review (14)). Instead, age-related LH surge dysfunction reflects compromised excitatory input to GnRH neurons under estradiol positive feedback conditions (21, 29, 33, 47). On the day of the surge, fewer GnRH neurons in middle-aged females express cFos, a marker of neuronal activation, than in young females (34, 48). AVPV neurons, which provide excitatory afferent input to GnRH neurons (6, 49, 50), also exhibit reductions in the percentage of cFos-positive neurons in middle-aged rats (34). Moreover, unilateral electrolytic lesions of the AVPV in young females produce ipsilateral reductions in cFos expression in GnRH neurons and LH release that resemble those of middle-aged female rats (34). Therefore, we hypothesized that age-related LH surge dysfunction may reflect reduced excitatory input to GnRH and other mPOA neurons from AVPV kisspeptin neurons. We first determined if

estradiol increases Kiss1 mRNA levels in the anterior hypothalamus of middle-aged rats. Although our dissection includes cells in addition to those in the AVPV, the only Kiss1-expressing cells in the dissection would be in the AVPV (for review (51)). In the absence of ovarian steroids, OVX young and middle-aged rats express equivalent levels of Kiss1 mRNA. Estradiol treatment significantly increased Kiss1 mRNA levels in both young and middle-aged rats; however, the response to estradiol was significantly reduced in middle-aged rats.

This finding suggested that the delayed and attenuated LH surge may result from reduced excitatory drive from AVPV kisspeptin neurons. If so, we hypothesized that infusion of Kp-10 directly into the mPOA (52) might rescue LH surges in middle-aged rats. Moreover, because unilateral electrolytic lesions in young females attenuate LH release (34), we predicted that unilateral infusion of kisspeptin might rescue the LH surge. Consistent with this reasoning, unilateral infusion of Kp-10 into the mPOA rescued the LH surge in middle-aged rats. Therefore, age-related LH surge changes most likely result from reduced kisspeptin availability.

Kisspeptin is permissive for generation of the LH surge.

The delayed onset of the LH surge in middle-aged females is proposed to result from altered circadian inputs (for review (53)). AVPV kisspeptin neurons are hypothesized to be the node through which circadian signals initiate the LH surge (10). Therefore, we expected that Kp-10 infusion into the mPOA would advance the LH surge in both young and middle-aged rats. Interestingly, continuous infusion of Kp-10 beginning at 0730 hr did not change the onset of the LH surge nor did it produce an immediate LH response in either age group. Our data are consistent with Roa et al. who also showed no change in the onset of the LH surge when Kp-10 was infused intraventricularly in cycling females on the day of proestrus (54). These data suggest that under steroid positive feedback conditions, kisspeptin release in the mPOA modulates the activity of GnRH neurons and of other hormone-sensitive afferent inputs to GnRH neurons (55), especially GABA and glutamate neurons (Figure 6). These findings also imply that kisspeptin plays a permissive role in generation of the LH surge.

Our findings do not dispute the possibility that circadian inputs activate Kiss1 neurons on the day of the LH surge. The suprachiasmatic nucleus (SCN) is considered the circadian timekeeper driving LH surges (56). SCN neurons (e.g., that express vasoactive intestinal polypeptide (VIP) or arginine vasopressin) send projections both to neurons in the AVPV and to GnRH neurons and other mPOA neurons (57). Immunoneutralization of VIP with intraventricular VIP antiserum (58), infusion of VIP antisense oligonucleotides into the SCN (59) or thermal ablation of VIP neurons in the SCN (60) of young rats produces delayed and attenuated LH surges that resemble middle-aged rats. Additionally, GnRH and AVPV neurons and VIP neurons in the SCN of middle-aged rats exhibit reduced cFos expression on the day of the surge (34, 48, 59, 61, 62). Thus, both kisspeptin and GnRH neurons may receive compromised circadian inputs from VIP neurons located in the SCN of middle-aged rats (61).

It has been hypothesized that kisspeptin neurons may receive and/or transmit circadian signals to GnRH neurons (10). This hypothesis is buttressed by the observation that kisspeptin immediately induces LH release in OVX rats under negative feedback conditions (without steroid priming) or when infused into the ventricles (9, 54, 63). Short latency LH release observed after intravenous or ventricular infusions of kisspeptin might reflect actions on GnRH terminals in the median eminence (2, 51) and/or on pituitary gonadotropes (45). In contrast, direct application of Kp-10 into the mPOA is unlikely to reach these sites. Hence, our data are not in conflict with the hypothesis that kisspeptin neurons located in the AVPV receive and then transmit circadian signals to GnRH neurons. However, our data suggest that kisspeptin does not independently drive the timing of the LH surge.

Our experiments are the first to evaluate the effects of continuous infusion of Kp-10 into the mPOA on the LH surge under positive feedback conditions. Although Roa et al. (54) evaluated Kp-10 effects on the LH surge in cycling rats on proestrus, they injected a single

bolus of Kp-10 into the lateral ventricle at 1200 h, close to the onset of the LH surge in young rats. It is impossible to predict if an earlier injection of Kp-10 on the day of proestrus would have stimulated immediate LH release. These investigators also injected a single bolus of Kp-10 into the lateral ventricle after a 4-day combined estrogen plus progesterone protocol. We cannot directly compare that study to ours because Kp-10 was injected into the lateral ventricle in a negative gonadal steroid feedback environment.

Kisspeptin infusion modulates extracellular GABA and glutamate in the mPOA

Our laboratory (21) and others (for review (66)) provide strong evidence that age-related LH surge dysfunction involves reduced excitatory input to GnRH neurons under estrogen positive feedback conditions. We recently showed that attenuated LH surges in middle-aged rats are causally related to reduced glutamate and increased GABA release in the mPOA. When we increased glutamate on a background of reduced $GABA_A$ receptor activation in the mPOA (21), GnRH neurons in middle-aged females appeared to maintain responsiveness to these neurotransmitters and produced a robust LH surge that was comparable to that of young females.

It is unclear why middle-aged rats release more GABA and less glutamate in the mPOA than young rats under positive feedback conditions (21). Because mounting evidence suggests that kisspeptin modulates amino acid neurotransmission (26-28, 67), we hypothesized that kisspeptin may also affect GnRH/LH release through modulation of local glutamate and GABA release. Our microdialysis results demonstrate that infusion of Kp-10 into the mPOA of middle-aged rats restores the altered balance between glutamate and GABA release on the day of the LH surge to levels observed in young controls. These findings are consistent with earlier work showing that peak and total LH release in middle-aged rats can be rescued by increasing glutamate and decreasing GABA and $GABA_A$ neurotransmission in the mPOA (21).

Restoration of LH surge amplitude in middle-aged rats by Kp-10 was associated with an elevation of mPOA glutamate. Because NMDA receptors are expressed by GnRH neurons, and they are critical in generation of the LH surge in young females (24, 68), we assessed whether activation of NMDA receptors contributed to Kp-10 facilitation of GnRH/LH release in middle-aged females. The NMDA receptor antagonist MK801 completely blocked the LH surge in middle-aged female rats infused with Kp-10. MK801 blockade of the LH surge most likely represents postsynaptic actions of the drug, because MK801 did not reverse the effects of Kp-10 on extracellular glutamate or GABA in the mPOA. This observation is consistent with other evidence that neurotransmitter systems in addition to kisspeptin are required for generation of the LH surge (15, 55, 69, 70). For example, there is evidence that the Kiss1r is not essential for estradiol-induced LH surges (55). Other studies also support the hypothesis that kisspeptin indirectly affects GnRH/LH release by acting on glutamate neurons situated proximal to GnRH neurons (26). Most important, our data strongly suggest that Kiss1r activation, while necessary, is not sufficient for steroid-induced GnRH/LH surges in middle-aged rats and other neurotransmitters such as glutamate are critical.

Delayed and attenuated LH surges in middle-aged rats are also associated with increased GABA in the mPOA relative to young rats (21, 32). Rescue of LH surge amplitude in middle-aged rats by kisspeptin and maintenance of the LH surge in control and kisspeptin-treated young rats correlated with low levels of extracellular GABA in the mPOA. This is consistent with our previous report that high levels of mPOA GABA are associated with low amplitude LH surges in middle-aged rats and that pharmacological antagonism of $GABA_A$ neurotransmission increases the magnitude of the LH surge (21). Although direct application of GABA onto GnRH neurons induces $GABA_A$ receptor-mediated excitation (71), we posit that the net effect of increased extracellular GABA within the mPOA is to inhibit GnRH neuron activation, and that GnRH neurons are not the only target of the GABA detected by microdialysis (72). Interestingly, kisspeptin blocks $GABA_B$ receptor-mediated inhibition of GnRH neurons (28). Therefore, when the

$GABA_B$ receptors are inhibited and GABA levels in the mPOA are reduced by Kp-10, the expected net result would be increased activation of GnRH neurons and enhanced GnRH/LH release.

Unexpectedly, Kp-10 infusion in young hormone-primed rats significantly decreased mPOA glutamate levels. However, this reduction in glutamate did not significantly affect the LH surge. We do not know why Kp-10 has different effects on glutamate in young and middle-aged rats. Extinction of LH release is observed after 48 hours of continuous kisspeptin exposure in female rats (73). Thus, continuous infusion of exogenous Kp-10 into the mPOA of young females, which are kisspeptin-replete, could reduce the ability of the peptide to activate nearby glutamate neurons, thereby decreasing synaptic glutamate levels.

There are several reasons why young females with reduced mPOA glutamate still exhibit a normal LH surge. Neurotransmitters other than glutamate, especially norepinephrine, contribute to the LH surge and are implicated in LH surge dysfunction in middle-aged females (14). Because these neurotransmitter systems are intact and respond normally to estradiol in young females (Figure 6), they may be sufficient to generate a robust LH surge despite reduced glutamate. Alternatively, LH surges in young females may have been normal because the reduction in glutamate occurred on a background of elevated Kp-10 and low GABA. In other words, as long as GABA levels are low and other excitatory neurotransmitter systems are not compromised, as they are in middle-aged rats (14), GnRH neuron excitation in young rats may be sufficient to generate a normal LH surge even if glutamate levels are reduced. GnRH neurons of middle-aged females also have altered NMDA receptor subunit stoichiometry, which may render young rats more sensitive to glutamate (29, 74, 75). Alternatively, glutamate neurotransmission may be more critical in generation of the LH surge in middle-aged than in young rats.

Is kisspeptin restoring the LH surge in an artificial manner?

Kisspeptin activates GnRH neurons and Kp-10 induces GnRH/LH release independent of steroid exposure (26, 54, 76). Therefore, one might hypothesize that because Kp-10 can induce GnRH/LH release in the absence of ovarian steroids, then the rescue of GnRH/LH release in middle-aged rats by Kp-10 may not reflect the physiological mechanisms that normally drive the LH surge in young animals. However, we do not believe that this is the case. First, estrogen receptor-alpha (ER-α) and estradiol priming of the hypothalamic-pituitary axis are essential for the LH surge (for review (77)). Consistent with this concept, Roa et al., recently demonstrated that a selective ER-α antagonist completely blocked LH surges in control and kisspeptin-treated young rats on proestrus but did not inhibit GnRH-induced LH release (54). Additionally when kisspeptin was infused into the ventricles of OVX rats that were not treated with estradiol, LH release was brief and did not resemble LH surges of young (9, 54, 63) or middle-aged rats (31). Moreover, if Kp-10 effects on GnRH/LH release do not require ovarian hormones and are unrelated to the normal LH surge mechanism, then Kp-10 should also have the same effect on GnRH/LH release in all females regardless of age. Lastly, the effects of Kp-10 on LH release and mPOA glutamate and GABA levels are consistent with our previous work demonstrating rescue of LH surge amplitude in middle-aged rats when the balance of glutamate and GABA is restored in the mPOA (21).

Kp-10 does not desensitize GnRH neurons

Several studies suggest that continuous application of Kp-10 desensitizes GnRH neurons (26, 27, 67) and consequently reduces GnRH/LH release (64, 73). Electrophysiology studies in OVX mice treated with estradiol (26) or intact mice killed during diestrus (67) suggest that desensitization of GnRH neurons occurs after a brief exposure to high concentrations of kisspeptin. Another study evaluated GnRH/LH release during 7 days of intraventricular Kp-10 infusion in cycling rats and suggested that GnRH neuron desensitization emerges only after two days of continuous Kp-10 exposure (73). We saw no evidence that Kp-10 infusion throughout the day of the LH surge desensitizes GnRH/LH release. Perhaps if we continued Kp-

10 for 48 h we would have observed desensitization (73).

Kisspeptin did not act on gonadotropes

Although unlikely, it is possible that Kp-10 diffused to the pituitary, where it acted on gonadotopes to stimulate LH release (45). Therefore, we treated Kp-10-infused, middle-aged rats with the GnRH antagonist cetrorelix. Kp-10 rescue of LH release in middle-aged rats was completely blocked by cetrorelix. These data are consistent with other studies showing that GnRH receptor antagonists block LH release induced by Kp-10 and -54 (78, 79).

Summary

We demonstrate that estradiol induction of Kiss1 mRNA expression is reduced in the anterior hypothalamus of middle-aged rats and that Kp-10 infusion into the mPOA under estrogen positive feedback conditions rescues LH surge amplitude and restores the balance of glutamate and GABA release. Kp-10 effects are blocked by a GnRH receptor antagonist, indicating that Kp-10 affects GnRH neurons (80). The NMDA receptor antagonist MK801 also blocked Kp-10 rescue of the LH surge. Taken together, our data strongly suggest that age-related changes in the LH surge reflect, in part, reduced excitatory input from AVPV kisspeptin neurons to GnRH neurons and other mPOA neurons under estrogen positive feedback conditions. Age-related LH surge changes result from reduced Kiss1 availability rather than reduced Kiss1r expression or compromised Kiss1r function in GnRH neurons. Our findings imply that kisspeptin directly and indirectly affects GnRH neuron activity by modulating local glutamate and GABA release in the mPOA (Figure 6).

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Figure Legends

Figure 1. (A) *Illustration of microdialysis probe placements in the medial preoptic area*. (A) The diagram corresponds to a coronal section at approximately 0.0 mm relative to Bregma (Plate 33) in the atlas of Paxinos and Watson (81). Medial preoptic area (mPOA); third ventricle (3V); och (optic chiasm); ventromedial preoptic nucleus (VMPO), ventrolateral preoptic nucleus (VLPO); anteroventral periventricular area (AVPV); supraoptic nucleus (SO); Alar nucleus (Al); Strial part preoptic nucleus (StA); (B) Photomicrograph of thionin-stained coronal section showing a representative probe placement between Plates 32 and 33 in Paxinos and Watson (80). Magnification at 40X shows the approximate location of a microdialysis probe. The arrow indicates the site of probe tip.

Figure 2: *The attenuated LH surge is rescued by Kp-10 and correlates with reduced production of Kiss1 mRNA under estradiol positive feedback conditions.* (A) Kiss1 mRNA in the anterior hypothalamus, which includes the AVPV. Data are expressed as mean ± SEM from OVX young (Y) rats primed with estradiol and progesterone (E2+P; N=4) or oil (N=4) and OVX middle-aged (MA) rats primed with E2+P or oil and killed at 4 h (oil; N=4, E2+P; N 4) or 7 h (oil; N=4, E2+P; N=4) after the P or last oil injection. There was no statistical difference in Kiss1 mRNA levels in E2-primed MA rats killed at 4 (n =4) or 7 hr (n=4) after P; therefore, these data were pooled. The same was true for MA rats primed with oil (N=4/time point). There was a significant main effect of hormone treatment [$F=44$, $P<0.0001$], age [$F=5$, $P<0.03$] and an interaction between hormone treatment and age [$F=7$, $P<0.01$]. [a] $P<0.005$ vs. all E2+P groups; [b] $P<0.05$ vs. Y E2+P. (B-F) Plasma LH levels are expressed as mean ± SEM from OVX and E2+P primed young control rats (Y; N=6) and middle-aged control rats (MA; N=7) dialyzed with ACSF and young (Y+Kp-10; N=6) and middle-aged rats (MA+Kp-10; N=10) dialyzed with 10 nM Kp-10.

Progesterone was injected at 0900 h (time 0). (B) LH surge in control rats. (C) LH surge in Kp-10 treated rats. (D) Total LH (AUC); there was a main effect of age [F=11, P<0.01] and an interaction between age and Kp-10 [F=12, P<0.001]. (E) Peak LH; there was a main effect of age [F=11, P<0.005] and an interaction between age and Kp-10 [F=5.5, P<0.05]. (F) LH surge onset (h relative to P injection) [Kruskal Wallis=21.8, P<0.005]. [a] P<0.05 vs Y; [b] P<0.05 vs. Y+Kp-10; [c] P<0.01 vs. MA+Kp-10.

Figure 3. *Kisspeptin acts on the hypothalamus to enhance LH surges in middle-aged rats.* Data are means ± SEM from OVX, estradiol and progesterone replaced middle-aged control (MA; N=4) and MA rats dialyzed with 10 nM Kp-10 alone (Kp-10; N=4), with 10 nM Kp-10 and injected with vehicle (Veh; N=4) or with 10 nM Kp-10 and injected with 100 µg of the GnRH receptor antagonist cetrorelix both 24 hr prior to and immediately before the progesterone injection (Kp-10 + cetrorelix; N=4). Progesterone was injected at 0900 h (time 0). (A) LH surges. (B) Peak LH; F=24.5, P<0.0001. (C) Total LH (AUC); F= 15.7, P<0.001. (D) LH surge onset (h relative to P injection) [a] P<0.001 vs. all other groups

Figure 4. *Kisspeptin differentially affects glutamate and GABA release in the mPOA of young and middle-aged rats on the day of the LH surge.* Data are means ± SEM and are from the same animals shown in Figure 2. Time course of extracellular glutamate (Glu) (A-B) and GABA (E-F) levels in the mPOA of control and Kp-10-treated young (Y) and middle-aged rats (MA). (C) Total Glu (AUC); there was an interaction between age and Kp-10 [F=25.12, P<0.0001]. (D) Peak Glu release; there was an interaction between age and Kp-10 [F=14.4, P<0.001]. (G) Total GABA; there were main effects of age [F=7.7, p<0.01] and treatment [F=11.8, P<0.005] and an interaction between age and Kp-10 [F=24.9, P<0.001]. (H) Peak GABA release; there were main effects of age [F=8.5, P<0.01] and treatment [F=7 P<0.02] and an interaction between age and

Kp-10 [F=11.8, P<0.003]. [a] P<0.05 vs. Y control; [b] P<0.01 vs MA+Kp-10; [c] P<0.001 vs. Y control; [d] P<0.01 vs. Y+ Kp-10 vs. MA+ Kp-10.

Figure 5. *Kisspeptin facilitation of LH release in middle-aged rats requires activation of NMDA receptors*. Data are means ± SEM. (A) Time course of LH release from control (N=4), Kp-10 (N=7) and Kp-10+MK801-treated (N=6) middle-aged rats (MA). (B) Total LH release [F=13.9, P<0.005]. (C) Total glutamate (Glu) release [F=74.8, P<0.001]. (D) Peak Glu [F=43.2, P<0.005]. (E) Total GABA release [F=13.9, P<0.01]. (F) Peak GABA [F=9.2, P<0.005]. [a]P<0.01 vs. MA; [b] P<0.001 vs. MA+Kp-10+MK801.

Figure 6. *Proposed model for direct and indirect actions of kisspeptin on GnRH/LH release in young and middle-aged rats under estradiol positive feedback conditions*. (A) Young adult rats: in the presence of estradiol (E2) positive feedback environment, increased kisspeptin in AVPV neurons directly activate GnRH neurons and indirectly affects the GnRH/LH surge by increasing glutamate and decreasing GABA release in the mPOA. Kisspeptin can also inhibit $GABA_B$ receptors (28) on GnRH neurons. GnRH neurons in young females also receive E2-regulated inputs that include but are not limited to norepinephrine neurons (for review (14)). Estradiol positive feedback is mediated by estrogen receptor-α (ER-α), which is expressed in the neurons indicated. (B) Middle-aged rats: we propose that middle-aged rats have age-related changes in responsiveness to E2 in all ER-α expressing neurons shown. E2 induces less Kiss1 mRNA in the AVPV of middle-aged females, which may attenuate GnRH/LH release and contribute to increased GABA and GABA mediated inhibition, and decreased glutamate in the mPOA. The increase in GABA may reduce both glutamate and norepinephrine release (82) . The altered balance of glutamate and GABA neurotransmission, along with reductions in norepinephrine (83, 84) and alterations of other neurotransmitters in response to E2 positive feedback reduces

activation of GnRH neurons on the day of the LH surge (for review (14)). Kisspeptin infusion into the mPOA rescues LH surge amplitude by direct actions on GnRH neurons and by restoring the balance of glutamate and GABA release to levels typical of young females. Black arrows: excitatory actions; Gray arrows: inhibitory actions. Large arrows represent robust input and small arrows indicate reduced input. (**+**) and (**-**) indicate relative amounts of afferent excitatory and inhibitory input, respectively.

Figure 1

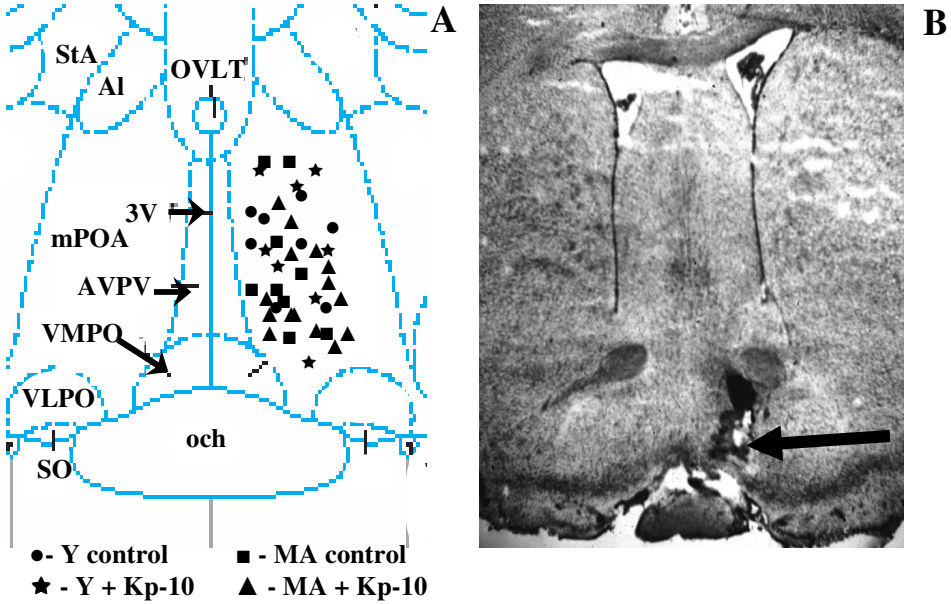
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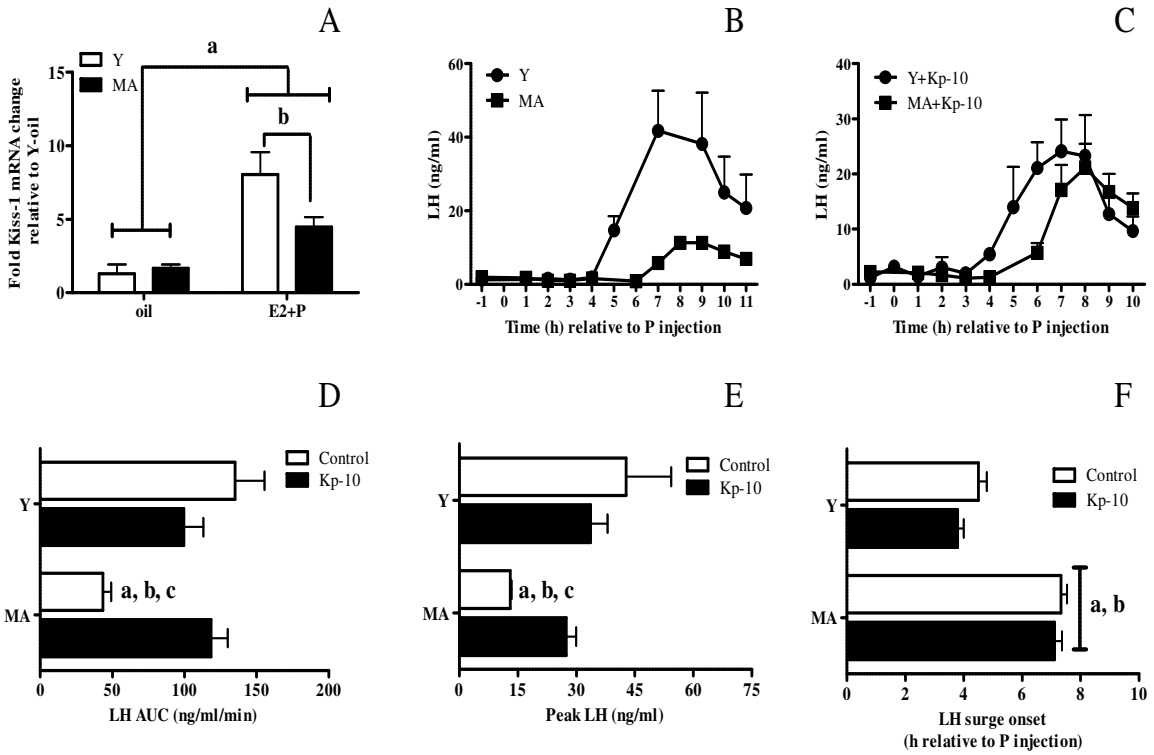
Figure 2


A
B
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MA
a
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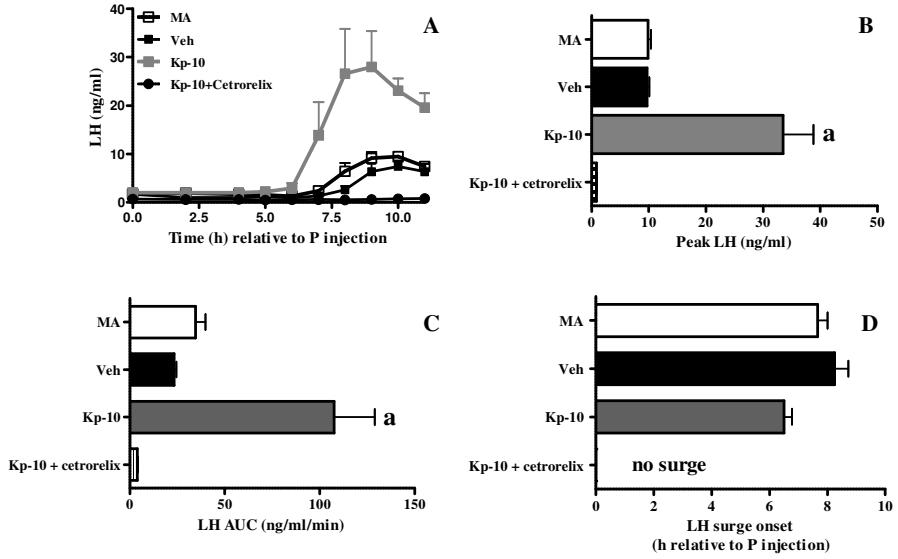
Fold Kiss-1 mRNA change relative to Y-oil
oil
E2+P
LH (ng/ml)
Time (h) relative to P injection
Y+Kp-10
MA+Kp-10
D
E
F
Control
Kp-10
a, b, c
LH AUC (ng/ml/min)
Peak LH (ng/ml)
a, b
LH surge onset
(h relative to P injection)

Figure 3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LH (ng/ml)
Time (h) relative to P injection
B
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Veh
Kp-10
Kp-10 + cetrorelix
a
Peak LH (ng/ml)
C
MA
Veh
Kp-10
Kp-10 + cetrorelix
a
LH AUC (ng/ml/min)
D
MA
Veh
Kp-10
Kp-10 + cetrorelix
no surge
LH surge onset
(h relative to P injection)

Figure 4

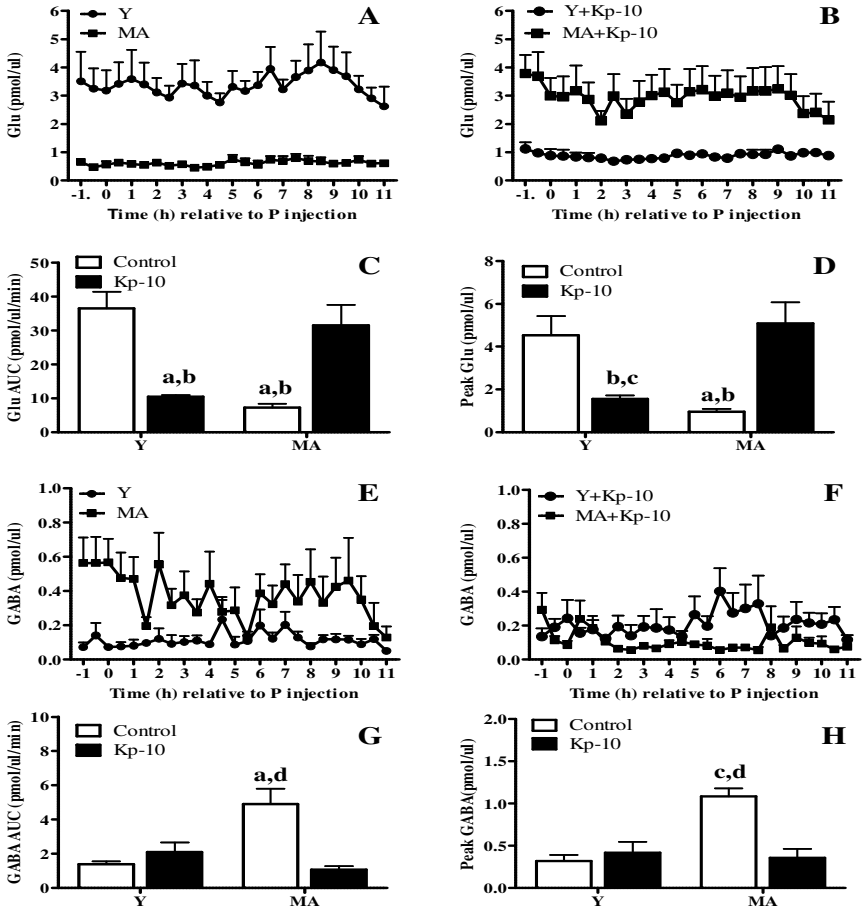

A
Y
MA
Glu (pmol/ul)
Time (h) relative to P injection
B
Y+Kp-10
MA+Kp-10
Glu (pmol/ul)
Time (h) relative to P injection
C
Control
Kp-10
Glu AUC (pmol/ul/min)
a,b
a,b
Y
MA
D
Control
Kp-10
Peak Glu (pmol/ul)
b,c
a,b
Y
MA
E
Y
MA
GABA (pmol/ul)
Time (h) relative to P injection
F
Y+Kp-10
MA+Kp-10
GABA (pmol/ul)
Time (h) relative to P injection
G
Control
Kp-10
GABA AUC (pmol/ul/min)
a,d
Y
MA
H
Control
Kp-10
Peak GABA(pmol/ul)
c,d
Y
MA

Figure 5

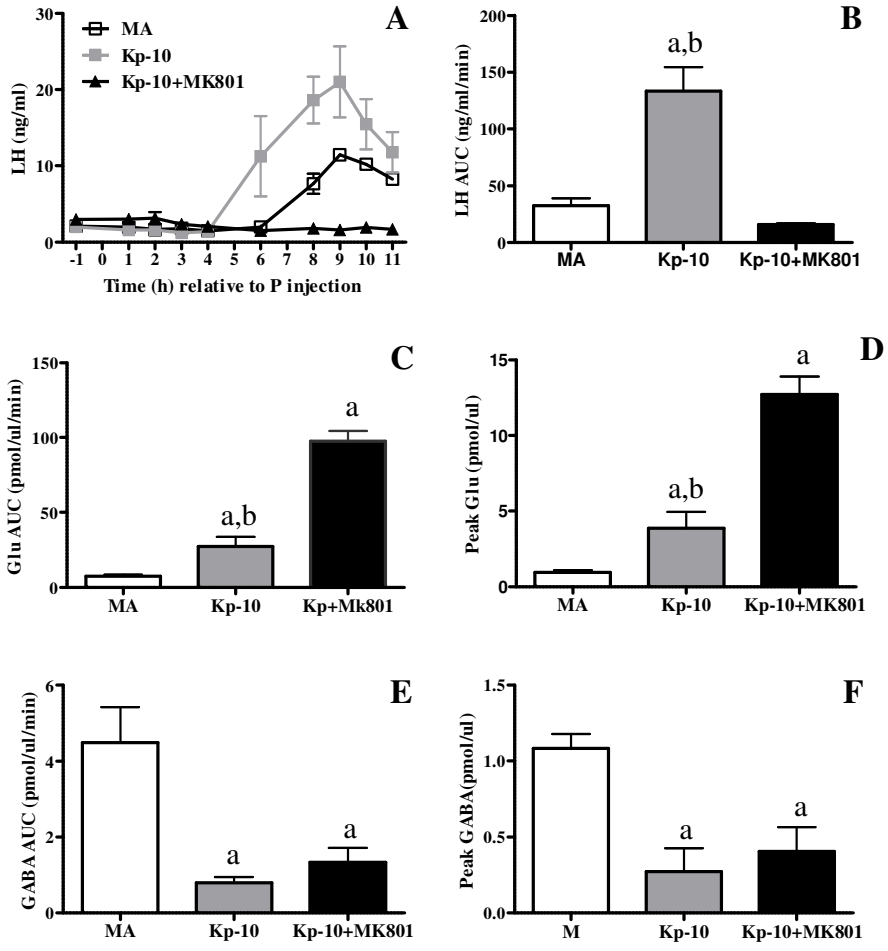
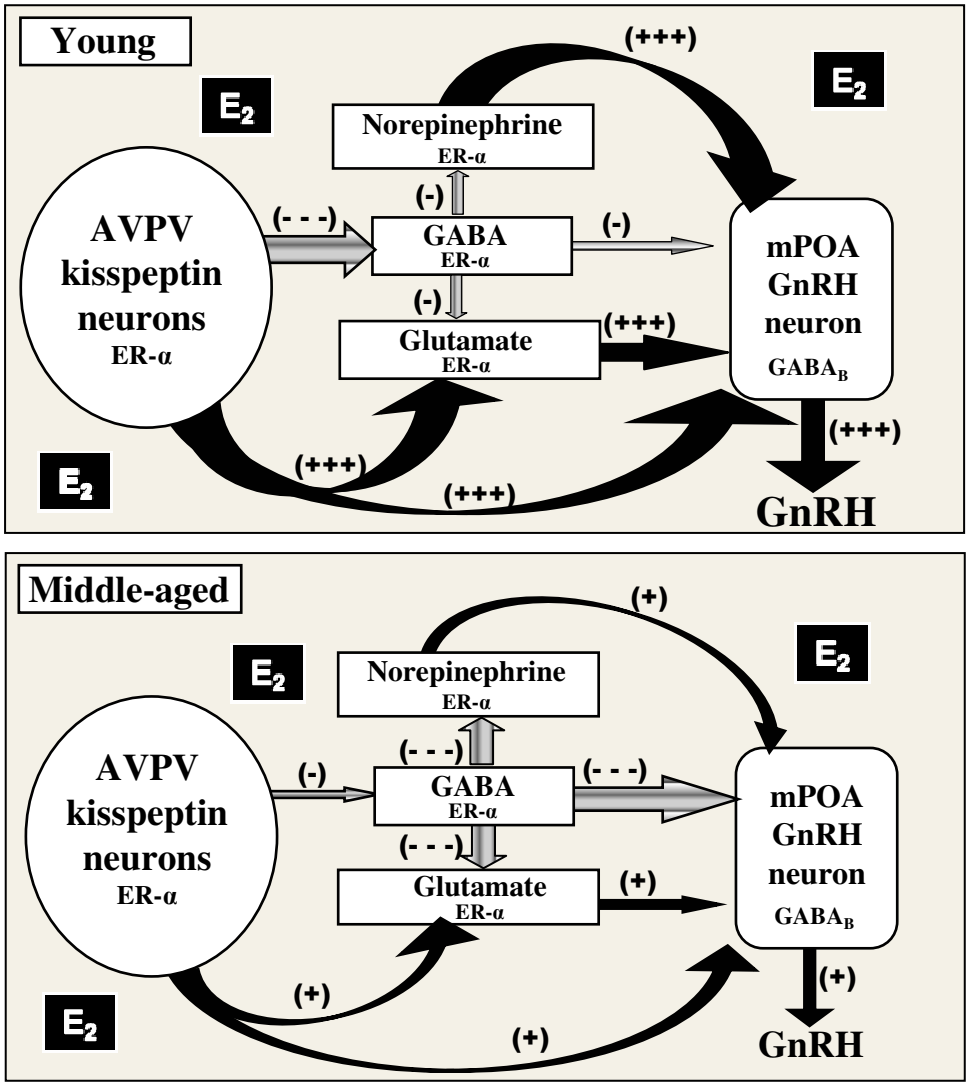

A
MA
Kp-10
Kp-10+MK801
LH (ng/ml)
Time (h) relative to P injection
B
LH AUC (ng/ml/min)
a,b
MA
Kp-10
Kp-10+MK801
C
Glu AUC (pmol/ul/min)
a
a,b
MA
Kp-10
Kp+Mk801
D
Peak Glu (pmol/ul)
a
a,b
MA
Kp-10
Kp-10+MK801
E
GABA AUC (pmol/ul/min)
a
a
MA
Kp-10
Kp-10+MK801
F
Peak GABA(pmol/ul)
a
a
M
Kp-10
Kp-10+MK801

Figure 6


Young
E2
(+++)
E2
Norepinephrine
ER-α
(-)
AVPV
kisspeptin
neurons
ER-α
(- - -)
GABA
ER-α
(-)
mPOA
GnRH
neuron
GABAB
(-)
Glutamate
ER-α
(+++)
(+++)
(+++)
E2
(+++)
GnRH
Middle-aged
(+)
E2
E2
Norepinephrine
ER-α
(- - -)
AVPV
kisspeptin
neurons
ER-α
(-)
GABA
ER-α
(- - -)
mPOA
GnRH
neuron
GABAB
(- - -)
Glutamate
ER-α
(+)
(+)
(+)
E2
(+)
GnRH