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Genes in the GABA pathway increase in the lateral thalamus of Sprague Dawley rats during the proestrus/estrus phase

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Abstract

Pain can vary over the estrous cycle as a result of changes in estradiol concentration but the mechanism causing this variation is unclear. Because the thalamus is important in pain control, gene expression in the lateral thalamus (ventral posteromedial, ventral posterolateral, reticular thalamic nuclei) was screened at different phases of the estrous cycle. Gene expression changes in Sprague-Dawley rats were further analyzed by real-time PCR and ELISA and plasma estradiol levels were measured by RIAs at different phases of the estrous cycle. Our results indicated that both the RNA and protein expression of glutamate decarboxylase 1 and 2 (GAD1, GAD2), GABA(A) receptor-associated protein like 1 (GABARAPL1) and vesicular GABA transporter (VGAT) significantly increased in the lateral thalamus when plasma estradiol levels were elevated. Estradiol levels were elevated during the proestrus and estrus phases of the estrous cycle. Estrogen receptor α (ER α) was observed to be co-localized in thalamic cells and thalamic infusion of an ER α antagonist significantly reduced GAD1 and VGAT transcript. GAD1, GAD2, GABARAPL1 and VGAT have been shown to effect neuronal responses suggesting that modulation of pain during the estrous cycle can be dependent, in part, through estradiol induced changes in thalamic gene expression.

Introduction

Women often have a higher incidence of pain (Amandusson and Blomqvist, 2013; Fillingim and Maixner, 2000; Iacovides et al., 2015; Sherman and LeResche, 2006) and women report orofacial pain more often than men (Koopman et al., 2009). Polymorphisms in the estrogen receptor will increase the risk of women developing various orofacial pain conditions supporting a biological basis for estradiol (Kang et al., 2007; Ribeiro-Dasilva et al., 2009). Moreover, orofacial pain varies in intensity over the menstrual cycle (LeResche et al., 2003; Suenaga et al., 2001) and is marked by the attenuation of symptoms in post-menopausal women (Salonen et al., 1990; Von et al., 1988). However, the attenuation of symptoms in post-menopausal women is reversed when they take estrogen supplements (LeResche et al., 1994; LeResche et al., 1997). In support of a role for sex hormones, nociceptive responses are reduced in proestrus rats when estradiol levels are elevated (Fischer et al., 2008; Kramer

and Bellinger, 2009). Interestingly, elevated pain sensitivity at diestrus and metestrus has been linked to sex steroid modulation of GABA activity (Taherianfard and Mosavi, 2011).

Gamma-aminobutyric acid (GABA) receptors are found in the thalamus and GABA release in the thalamus has been shown to regulate pain responses (Olausson et al., 1994; Oliveras and Montagne-Clavel, 1994; Pirker et al., 2000; Reyes-Vazquez et al., 1986; Roberts et al., 1992). Estradiol has been shown to modulate pain responses by altering thalamic pain signaling (Naderi et al., 2014; Reed et al., 2009). Moreover, estradiol has been shown to modulate GABA production in the brain (Demling et al., 1985) where the estrogen receptor is present (Flugge et al., 1986; Herbison, 1994). Although estradiol has been shown to alter pain responses through altered GABA signaling centrally (Taherianfard and Mosavi, 2011) it is currently not clear estradiol's effect on thalamic GABA genes expression and the role of the estrogen receptor. To begin to address these questions gene expression was measured in the thalamus of cycling female rats.

During the estrous cycle gene expression was quantitated in the thalamus using a gene array, that includes >20,000 probes. Gene expression in the thalamus of proestrus and diestrus rats was compared. Of all the genes analyzed we focused on those with the greatest change in gene expression and on genes that had a known pain function. Using these criteria four GABA related genes (GAD1, GAD2, GABARAPL1, VGAT) showed significantly altered gene expression in the lateral thalamus. GAD1 and GAD2 are enzymes that produce GABA, VGAT transports GABA into vesicles and GABARAPL1 functions in GABA receptor signaling (Erlander et al., 1991; Karlsen et al., 1991; Mansuy et al., 2004; McIntire et al., 1997). The four candidate genes from the array were further analyzed by real time PCR and ELISA. The changes in transcript were then correlated to the level of plasma estradiol. Co-expression of these genes with ER α was analyzed and the role of ER α was further tested by administering an antagonist in the lateral hypothalamus.

Materials and Methods

Animal Husbandry

The Texas A&M University Baylor College of Dentistry Institutional Animal Care and Use Committee approved the experimental protocol. Female Sprague-Dawley rats (280–300 grams) from Harlan Industries, Houston, TX were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum. After a 4 day acclimation period the rats were smeared daily to determine the stage of the estrous cycle.

Tissue collection

Vaginal smears were performed for at least three consecutive days, the stage of the estrous cycle was determined and the rat sacrificed. In this study four different experiments were performed using the cycled female rats, in experiment #1, #2 and in experiment #3 the rats were sacrificed by exposure to CO₂ followed by decapitation. The brain was extracted using a rongeur and sliced on a slicer with 1 mm increments (Zivic, Pittsburgh, PA). Sections were 2 mm thick by skipping every other slot in the slicer and the sections between Bregma –3 to –5 were placed on glass slides and kept on dry ice. Lateral thalamic tissue was collected

with punches 2 mm in diameter, punches included the ventral posteromedial, ventral posterolateral and reticular thalamic nuclei. Tissue was stored in liquid nitrogen until RNA or protein could be isolated. In experiment #1 and #2 trunk blood was collected from each animal after decapitation and the thalamic tissue was used for RNA isolation, transcript quantitation or gene array studies. In the experiment #3 the thalamic tissue was used for protein isolation. Trunk blood was collected in half of the animals in experiment #3. In Experiment #4 the rats were perfused with 9% sucrose followed by 4% paraformaldehyde. The brain was extracted and stored in 25% sucrose at 4°C.

Array Analysis

Thalamic tissue from two diestrus and two proestrus rats was collected, pooled and the RNA isolated. The RNA was processed and analyzed using the Affymetrix Rat Gene 2.0 ST Array (Santa Clara, CA).

Cannulation and drug infusion

In experiment #1, adult female Sprague-Dawley rats (250–300 grams) were anesthetized with 2% isoflurane and an air flow of 2 liters per minute. Unilateral placement of a 22 gauge guide cannula (C313G, Plastics One, Roanoke, VA) was performed stereotaxically, with the tip placed in the lateral thalamic region (from Bregma posterior 3.6 mm, lateral 2.7 mm, depth 6.0 mm). After surgery analgesia was obtained with nalbuphine. At least one week following surgery proestrus was determined by a vaginal smear and 0.5 µl of the ER α antagonist MPP dihydrochloride (Tocris Bioscience, Bio-Techne, Minneapolis MN) was infused in the thalamus over a 5 minute period. MPP dihydrochloride was dissolved in 5% dextrose (30 nM solution) and control animals were infused with the same volume of 5% dextrose (i.e., vehicle). Infusion was performed using a 10 µl Hamilton syringe connected to PE-10 flexible tubing and a 28 gauge infusion cannula (C313I, Plastics One). After infusion the injection cannula was removed from the guide cannula and a dummy cannula cap was placed to seal off the opening (C313DC, Plastics One). Twenty-four hours after infusion a vaginal smear was completed to confirm estrus and the animal were euthanized by an overdose of CO₂. Blood plasma was collected and estradiol quantitated. The lateral thalamic tissue was isolated with a 2 mm punch and frozen in liquid nitrogen as previously described.

Radio Immuno Assay

In experiments #1 and #2 plasma 17 β -estradiol was quantitated in the trunk blood by first, centrifugation and collection of the upper plasma layer, vigorously mixing 0.5 ml of plasma with 5 ml of ether. Second, the solution was frozen in an ethanol, dry ice bath and the ether phase decanted. The ether phase contains the lipid soluble hormones. Third, the ether was evaporated by nitrogen gas and the remaining residue suspended in radioimmunoassay buffer supplied by the manufacturer and assayed according to the manufactures directions (Beckman Coulter, Pasadena, CA).

Real time PCR

In experiments #1 and #2 RNA extraction was performed using the RNA Lipid Tissue Kit from Qiagen (Valencia, CA). The RNA concentration in the resulting sample was

determined on a Nanodrop2000. A one-step reverse transcription PCR reaction was performed on BioRAD C1000 Thermal Cycler using the SYBR-Green 1-Step RT-PCR kit from Qiagen. The thermal protocol was 30 min @ 50 °C for the reverse transcription reaction, 15 min @ 95 °C for DNA pol activation and 40x (15 s @ 94 °C melting, 30 s @ 56 °C annealing, 30 s @ 72 °C extension). A melting curve was obtained thereafter for quality assurance. Sample amount was adjusted according to total RNA concentration to obtain 20 ng of total RNA per well in the final reaction mix. All reactions were run in triplicate. PCR runs that did not exhibit a proper amplification profile were discarded. For the real time PCR data a line tangent to the inflection point of each amplification curve was determined and the cycle number that this line intersected was labeled $C_{\gamma 0}$, as previously described in detail (Guescini et al., 2008). For each sample, the $C_{\gamma 0}$ for GAPDH was subtracted from the $C_{\gamma 0}$ of each gene of interest to normalize the results ($C_{\gamma 0}$). In the non-cannulated rats a $C_{\gamma 0}$ value at a particular estrous cycle phase was subtracted from the mean $C_{\gamma 0}$ metestrus value ($C_{\gamma 0}$ metestrus average – $C_{\gamma 0}$ (cycle phase) = $C_{\gamma 0}$). To get the fold change the $C_{\gamma 0}$ was raised to the second power ($2^{-C_{\gamma 0}}$). In the cannulated rats the $C_{\gamma 0}$ on the contralateral, non-cannulated side was subtracted from the $C_{\gamma 0}$ on the ipsilateral, cannulated side to calculate a $C_{\gamma 0}$ value for each gene of interest. This $C_{\gamma 0}$ was raised to the second power to determine the fold change. If the $2^{-C_{\gamma 0}}$ value was less than one we used the formula $(1/(2^{-C_{\gamma 0}}))$ to determine a negative fold change.

Enzyme-linked immunosorbent assay

In experiment #3 each thalamic tissue plug was stored separately in liquid nitrogen until analysis. For analysis the tissue was placed in 300 μ l of T-Per tissue protein extraction reagent containing Halt Protease Inhibitor and ground (Thermo Scientific, Rockford, IL). Ground samples were frozen and thawed, followed by centrifugation and decanting of the supernatant. Quantitation of protein in the supernatant was completed on duplicate 100 μ l samples of supernatant using an ELISA following the manufacturer's directions (MyBioSource, San Diego, CA). Total protein was determined in each sample using a BCA protein assay (Thermo Scientific, Waltham, WA). Values were given as the pg of GAD1, GAD2, VGAT or ng GABARAPL1 per mg of total protein.

Immuno-fluorescent Staining

In experiment #4 fixed whole brains stored in 25% sucrose (experiment #4) were frozen, cryo-sectioned and the 20 μ m sections placed on Histobond slides (VWR international, Radnor, PA). The tissue was then blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for 2 hours at room temperature. Following three rinses in PBS with 0.3% Triton-X 100 the slides were incubated in a primary antibody solution overnight at 4°C. Primary antibodies consisted of a 1:500 dilution of the mouse monoclonal ER α antibody (Millipore, Temecula, CA) and a 1:300 dilution of the rabbit polyclonal VGAT antibody (AB5062P, Millipore) or a 1:500 dilution of the rabbit polyclonal GABARAPL1 antibody (Millipore). Staining also included a 1:500 dilution of a rabbit polyclonal ER α antibody (C1355, Millipore) and a 1:300 dilution of the mouse-monoclonal GAD1 (AB26116 Abcam, Cambridge, MA) or a 1:500 dilution of the mouse-monoclonal GAD2 antibody (AB26113, Abcam). Primary antibodies were diluted with PBS, 5% BSA and 0.3% Triton X-100. After incubation in primary antibody the slides were rinsed three

times in PBS and Triton-X 100 for a total of 60 min and placed for 2 hours in a 1:500 dilution of secondary antibody in PBS and 0.3% Triton X-100. Secondary antibodies included a mixture of goat anti-mouse 488 and goat anti-rabbit 568 (Invitrogen, Carlsbad, CA). After rinsing the slides three times in PBS for a total of 60 min, the slides were then mounted with Fluoromount-G mounting medium containing Hoechst 33342 stain (Electron Microscopy Sciences, Hatfield, PA). The fluorescent signal was imaged using a Nikon fluorescent microscope and NIS-Elements imaging software and a Photometrics CoolSnap K4 CCD camera (Roper Scientific, Inc, Duluth, GA).

Statistics

The data were analyzed using ANOVA to determine significant estrous cycle or antagonist effects. When significant ($p < 0.05$), the test was followed by Tukey's post-hoc test. A two-tailed Spearman correlation statistic was used to compare the RIA and real time PCR data.

Results

When comparing thalamic transcript at proestrus and diestrus the genes that increased at least two fold on a large gene array were mostly related to GABA function (Table 1).

Measurement of plasma estradiol indicated rats in the proestrus or estrus phase of the estrous cycle had a significantly greater amount of estradiol in comparison to rats in either diestrus or metestrus. This elevated estradiol was observed in two groups of rats; one group in which transcript was measured (Fig. 1A) and another group in which protein was measured (Fig. 1B).

Transcript for the GABA related genes GAD1, GAD2, GABARAPL1 and VGAT was significantly elevated at the estrus phase of the estrous cycle (Fig. 2A, B, C and D).

In Figure 3 there was a direct relationship between the amount of transcript and the plasma estradiol. A significant correlation between GAD1 ($r = 0.35$, $p < 0.05$, Fig. 3A), GAD2 ($r = 0.36$, $p < 0.05$, Fig. 3B), VGAT ($r = 0.42$, $p < 0.01$, Fig. 3D) and estradiol was measured but no significant relationship was observed for GABARAPL1 and estradiol ($r = 0.25$, $p > 0.1$, Fig. 3C).

Protein levels were significantly greater in proestrus and estrus for GAD1 and GAD2 (Fig. 4A and B). GABARAPL1 and VGAT expression was greater in proestrus rats (Fig. 4C and D).

Immunostaining in the lateral thalamic region indicated that GAD2 and ER α co-localized to cells with neuronal morphology (Fig. 5A). Although GAD2 was observed in cells most of the GAD2 signal was present in varicosities (Fig. 5A, E, C and G). Thalamic ER α localized to the nucleus of most cells (Fig. 5B, F). Many ER α positive cells (Fig. 5B, F) were surrounded by GAD2 positive varicosities (Fig. 5A and E). There were cells that stained for GAD2 but did not stain for ER α (Fig. 5E). VGAT, GAD1 and GABARAPL1 also showed punctate staining along linear tracks, in between and adjacent to ER α positive cells, very similar to the GAD2 staining in Fig. 5 (data not shown).

Thalamic injection of the ER α antagonist MPP dihydrochloride lead to a significant, greater than 2 fold, decrease in VGAT and GAD1 transcript on the ipsilateral side in comparison to the non-injected contralateral side (Fig. 6). Thalamic injection of vehicle did not significantly alter gene expression when comparing the injected to the non-injected side, a value of 1 would indicate no change between the injected (ipsilateral) and non-injected (contralateral) sides (Fig. 6)

Discussion

Screening of thalamic genes in proestrus and diestrus rats revealed changes in expression for GAD1, GAD2, GABARAPL1 and VGAT. Expression of these four genes was studied further at each stage of the estrous cycle using real time PCR and ELISA assays. The results indicate that all four genes significantly increased at the proestrus or estrus stage, a period when estradiol is elevated. GAD1, GAD2, GABARAPL1 and VGAT show a similar pattern of change across the estrous cycle and the pattern of transcript expression correlated to the change in estradiol during the cycle. Co-localization of ER α was observed in cells of the lateral hypothalamus and thalamic infusion of an ER α antagonist significantly decreased VGAT and GAD1 transcript suggesting estradiol could, in part, modulate expression by binding this receptor. Importantly, elevated estradiol was associated with elevation of these GABA genes in the thalamus; consistent with the idea that estradiol can modulate pain responses by increasing activity in the GABA pathway.

In this study we demonstrate ER α was expressed in cells of the lateral hypothalamus and that this expression co-localized with GABAergic neurons. One explanation for the increased gene expression during proestrus and estrus would be that a higher concentration of estradiol bound to ER α and signaled increased gene expression. Results showing infusion of an ER α antagonist inhibits this gene expression further supports this idea. In previous reports estradiol has been shown modulate GABA production in the brain (Demling et al., 1985), particularly in regions where GAD co-localizes with estrogen and the estrogen receptor (Flugge et al., 1986; Herbison, 1994). GAD1 and GAD2 synthesize GABA in neurons (Martin and Rimvall, 1993) potentially through binding of the estrogen receptor to the GAD promoter (Hudgens et al., 2009). Estradiol will increase GAD expression in one brain nuclei and estradiol will decrease GAD expression in a different nuclei but the mechanism for these differences are unknown (McCarthy et al., 1995; Weiland, 1992). Alternatively, estradiol can alter GABA levels through enhancing expression of the GABA transporter VGAT (Herbison et al., 1995; Ottem et al., 2004). The effect of estradiol on VGAT has been hypothesized to result from estrogen receptor binding to an estrogen response element in the VGAT promoter (Herbison et al., 1995). GABARAPL1 plays a role in intracellular GABA(A) receptor trafficking (Mansuy et al., 2004) and estradiol has been shown to alter GABARAPL1 expression through binding an estrogen response element within the promoter (Vernier-Magnin et al., 2001).

Transcript levels for GAD1, GAD2 GABARAPL1 and VGAT were not significantly elevated in proestrus rats but the protein content was elevated at proestrus. One explanation for this result would be that the transcript was measured in early proestrus when estradiol was less. Whereas the protein was isolated from rats in late proestrus and plasma estradiol

was greater. The greater amount of estradiol could explain the higher amount of GAD1, GAD2, GABARAPL1 and VGAT protein versus transcript. Alternatively, estradiol did not have enough time to increase transcript in experiment #1 but did have sufficient time to increase protein content in experiment #3. Changes in gene expression typically takes several hours after binding ER α (Lee and Gorski; LeMeur et al.) and this delay, compounded with a lower level of estradiol, could have contributed to the lower amount of transcript observed at proestrus.

It is known that sex hormones (notably, estrogen) affect nociception and pain perception across mammalian species (Amandusson and Blomqvist, 2013; Fillingim and Maixner, 2000; Iacovides et al., 2015; Sherman and LeResche, 2006). For example, orofacial nociceptive responses are reduced in proestrus rats when estradiol levels are elevated (Fischer et al., 2008; Kramer and Bellinger, 2009). Elevated pain sensitivity at diestrus and metestrus has been linked to sex steroid modulation of GABA activity (Taherianfard and Mosavi, 2011). Viral introduction of GAD1 into the sciatic nerve has been shown to reduce neuropathic pain (Kanao et al., 2015). A recent study has shown that altering the activity of GAD1 positive cells in the cingulate cortex can effect nociceptive responses (Gu et al., 2015). Moreover, a link between GAD2 expression and pain was observed in the spinal cord (Lorenzo et al., 2014; Meisner et al., 2010). In mice where VGAT levels are reduced thermal nociception and inflammatory pain were enhanced (Yamada et al., 2012). Together these results suggest altered expression of GAD1, GAD2 and VGAT can affect pain responses.

Increased production of GABARAPL1, a GABAA receptor subunit recycling protein (Mansuy et al., 2004), during proestrus/estrus would increase turnover of the GABA receptor on the postsynaptic membranes. However, a higher turnover would decrease receptor density and reduce neuronal sensitivity to GABA resulting in attenuation of GABA's inhibitory effect.

In conclusion, during estrus and proestrus, periods of high estradiol, GAD1, GAD2, VGAT and GABARAPL1 were elevated. GAD1, GAD2, VGAT and GABARAPL1 all have estrogen response elements within their promoters (Herbison et al.; Hudgens et al.; Vernier-Magnin et al.) and co-localization of GAD2 and ER α in the thalamus, as well as, ER α antagonist studies suggests regulation could involve the estrogen receptor. Elevated GAD1, GAD2, and VGAT expression has been shown to attenuate pain responses (Gu et al., 2015; Kanao et al., 2015; Lorenzo et al., 2014; Meisner et al., 2010; Yamada et al., 2012) and previous evidence indicates that elevated estradiol will reduce orofacial pain (Fischer et al., 2008; Kramer and Bellinger, 2009) consistent with the idea that elevated GAD1, GAD2, and VGAT, at proestrus/estrus contributes, in part, to the reduced pain response.

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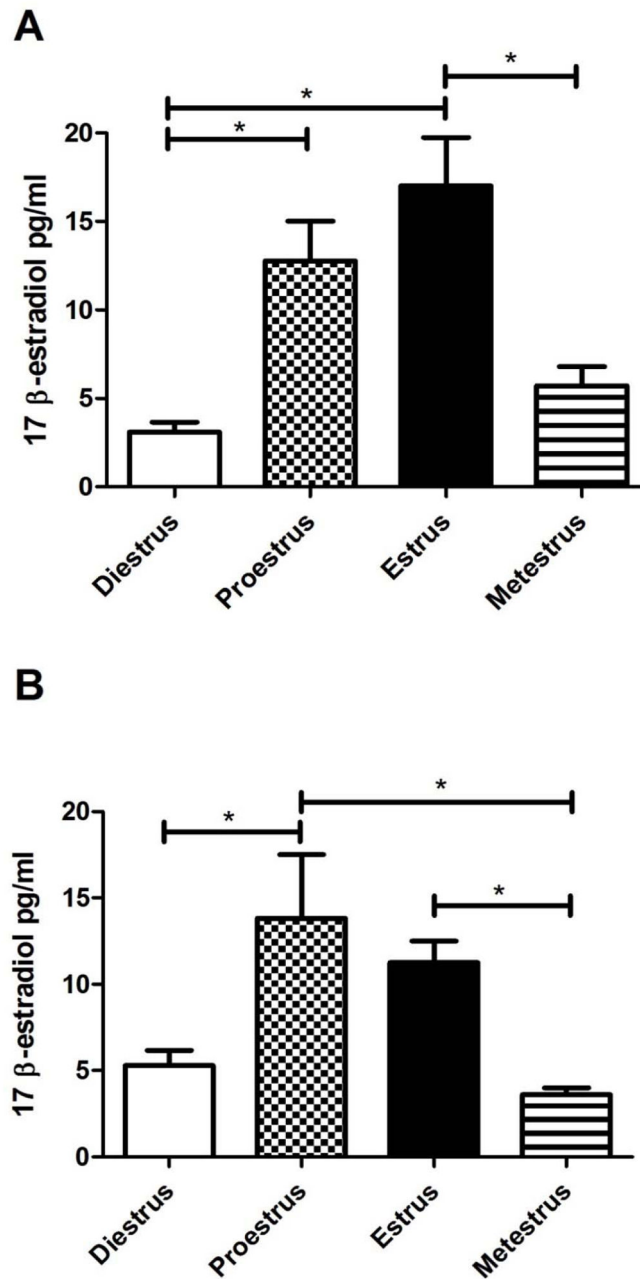


Figure 1. Plasma 17 β -estradiol concentration in rats during the estrous cycle
 Vaginal smears were performed and plasma was collected within one hour. Plasma estradiol was measured by RIA. Panel A is from Experiment #1 where transcript levels were measured in the thalamic tissue by RT-PCR. Panel B is from Experiment #3 where thalamic protein content was quantitated by ELISA. Significant differences of $p < 0.05$ are indicated by an asterisk. Seven animals were in each of the four estrous cycle phases.

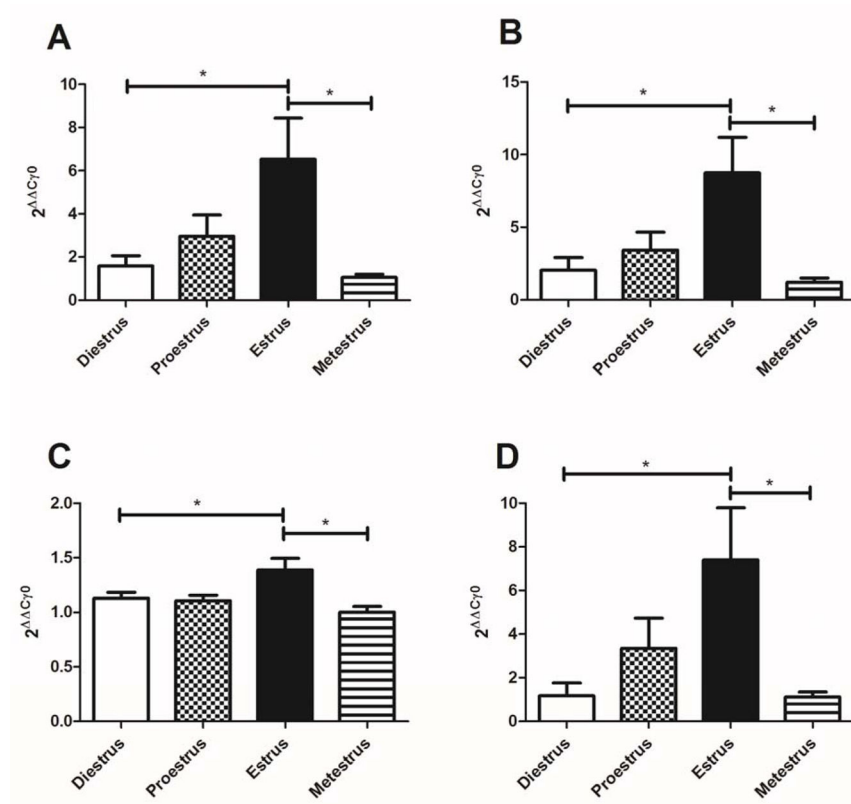


Figure 2. Transcript levels of GAD1, GAD2, GABARAPL1 and VGAT in the thalamus of cycling female rats

Estrous cycle phases were determined by vaginal smears and the thalamic tissue was isolated within one hour. RT-PCR analysis of the thalamic tissue was then performed. Panel A shows GAD1 transcript levels, Panel B shows GAD2 transcript expression, Panel C shows GABARAPL1 transcript expression and Panel D shows VGAT transcript expression. Significant differences of $p < 0.05$ are indicated by an asterisk. There were 7 animals in each of the three estrous phases except estrus where there were 8 animals.

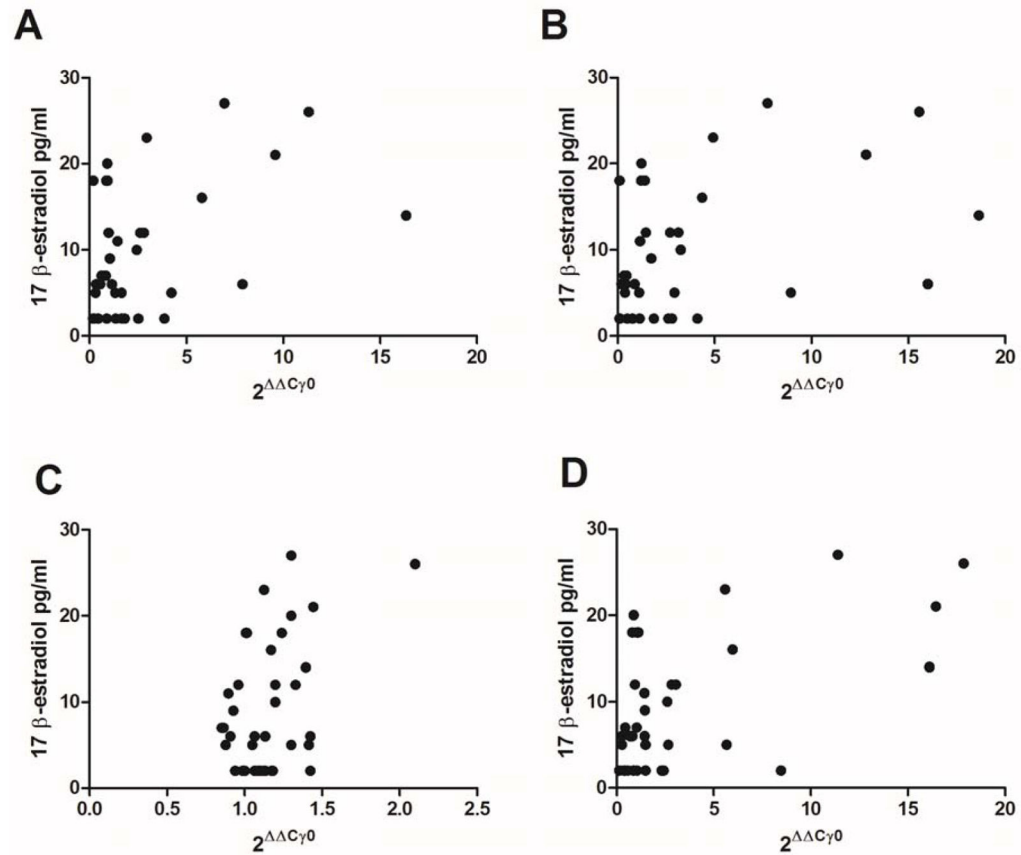


Figure 3. Thalamic gene expression plotted against the plasma estradiol concentration
 Panel A shows GAD1 transcript levels, Panel B shows GAD2 transcript expression, Panel C shows GABARAPL1 transcript expression and Panel D shows VGAT transcript expression plotted on the x-axis. The estradiol concentration was plotted on the y-axis. There were 7 animals in each of the three estrous phases except estrus where there were 8 animals.

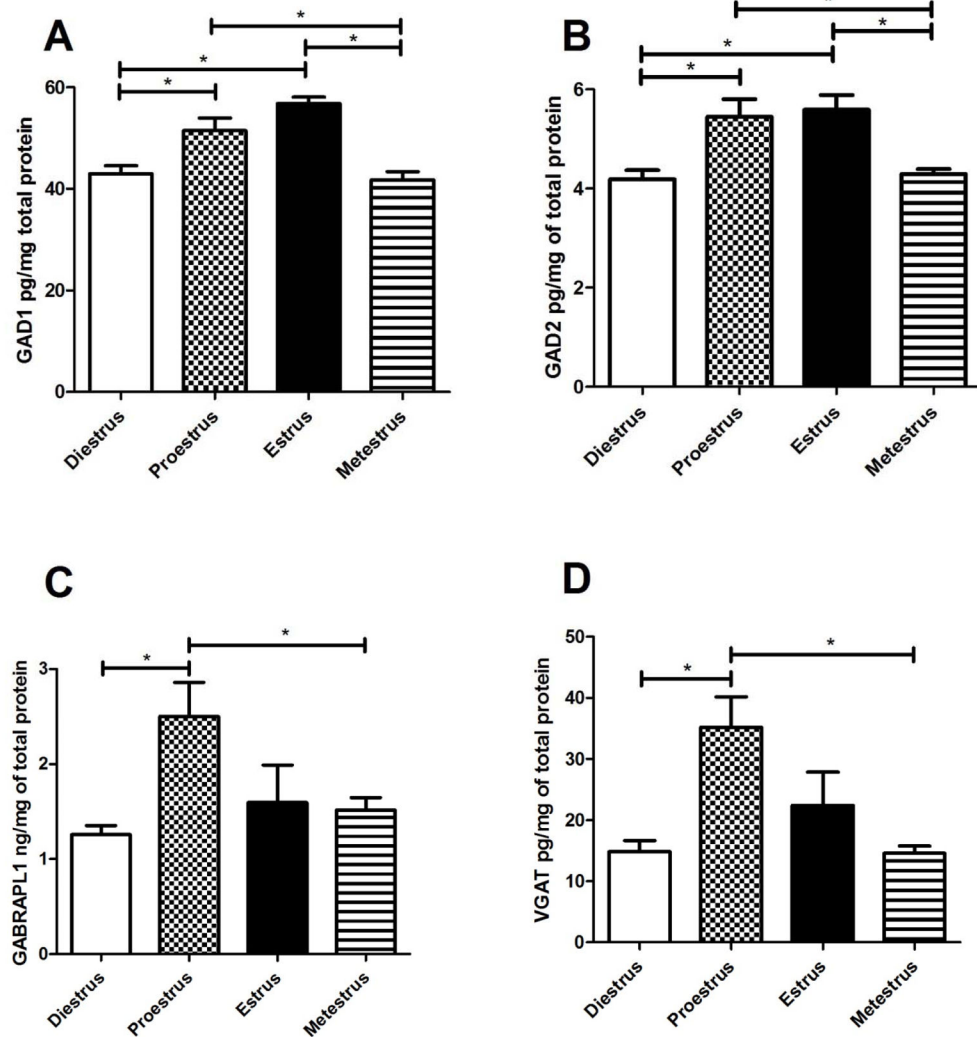


Figure 4. Protein content of GAD1, GAD2, GABARAPL1 and VGAT in the thalamus of cycling female rats

Estrous cycle phases were determined by vaginal smears and the thalamic tissue was isolated within one hour. Protein content was measured by ELISA. Panel A shows GAD1 protein levels, Panel B shows GAD2 protein expression, Panel C shows GABARAPL1 protein expression and Panel D shows VGAT protein expression. Significant differences of $p < 0.05$ are indicated by an asterisk. There were 5 animals in each of the 4 estrous phases.

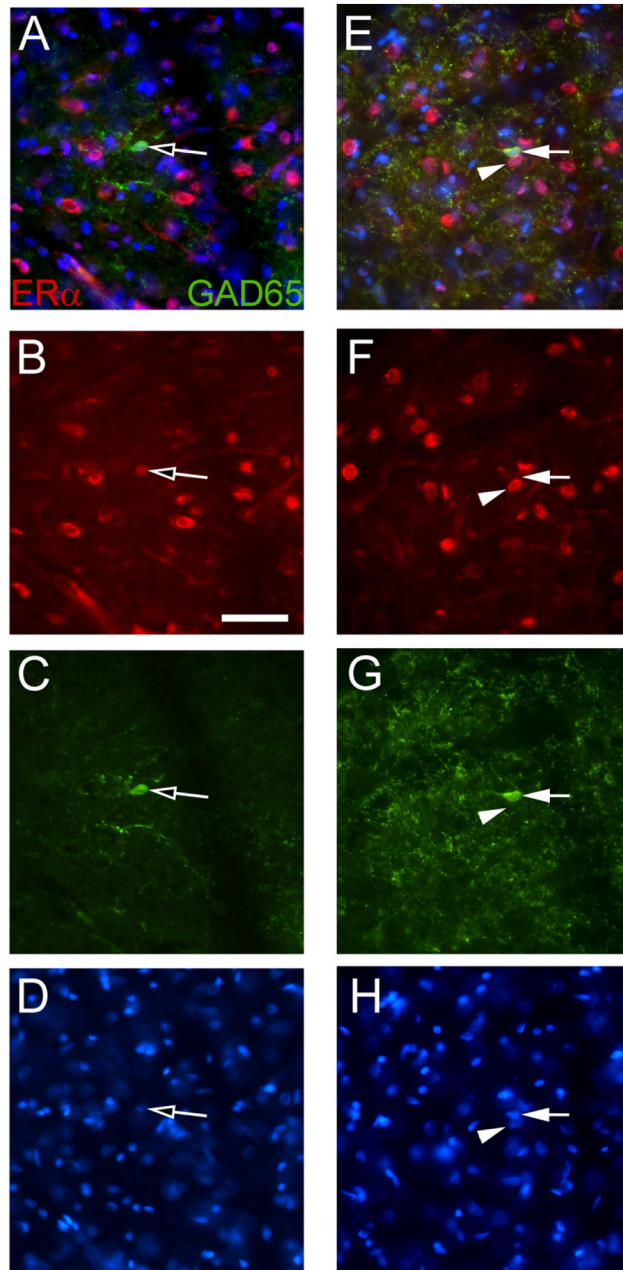


Figure 5. Lateral thalamic immuno-fluorescent staining for ER α and GAD2 (GAD65)

A frozen section from a diestrus rat was stained for ER α (red) and GAD2 (green). Panel A shows a cell with ER α and GAD2 co-localized (open arrow) and panel E is a different region on the same section showing a cell expressing ER α (arrowhead) and a cell expressing GAD2 (arrow). The ER α signal is shown in panels B and F, the GAD2 signal is shown in panels C and G and in panels D and H the nuclei are stained with Hoechst 33342. Image is taken from along the border of the reticular thalamic nucleus and the ventral posterolateral nucleus. Bar equals 50 μ m.

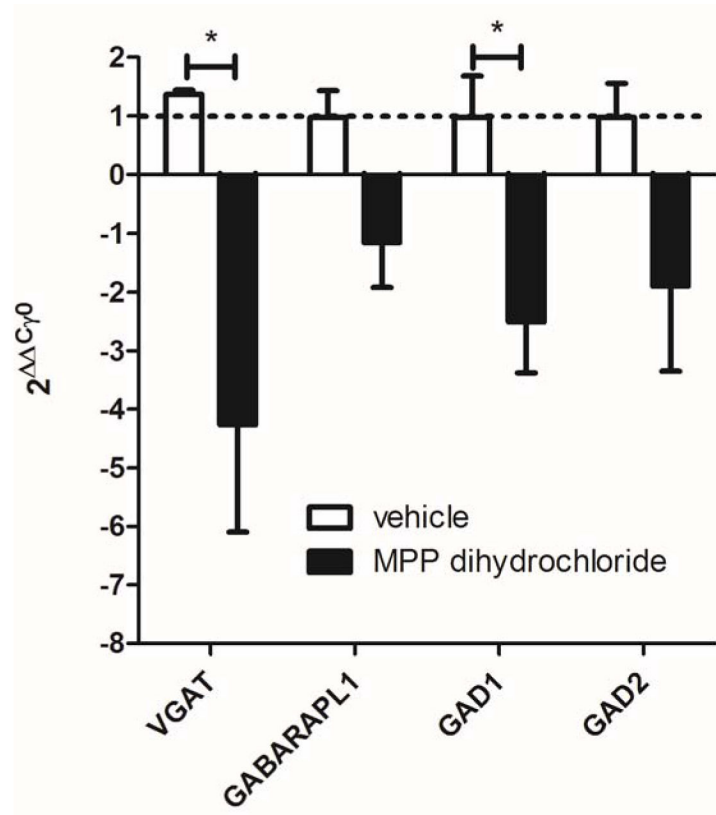


Figure 6. Thalamic injection of an ER α antagonist reduced expression of VGAT and GAD1 during estrus

The lateral region of the thalamus was cannulated unilaterally and two weeks after surgery vehicle or the ER α antagonist MPP dihydrochloride was infused on the day of proestrus. Rats were sacrificed the next day (i.e., estrus) and the injected thalamus (ipsilateral) and non-injected thalamus (contralateral) were isolated. The estrus phase was confirmed by measuring blood plasma estradiol levels and vaginal smears. Real time PCR was performed using the thalamic tissues and the amount of GAD1, GAD2, GABARAPL1 and VGAT was measured and reported as the fold change in transcript, contralateral thalamus versus ipsilateral thalamus. A value of less than 0 indicates that gene expression on the ipsilateral side was reduced versus the contralateral side. A value of 1 (dotted line) indicates the transcript levels were the same on the ipsilateral and contralateral sides. There were 4 vehicle injected rats and 4 MPP dihydrochloride injected rats.

Table 1

Thalamic gene expression changes over 2 fold between proestrus and diestrus

Fold change	Proestrus signal	Diestrus signal	Genbank#	Gene Description	Gene symbol
3.46	311.32	180.74		Similar to 40S ribosomal protein S3	RGDI560831
3.18	1519.41	678.48	M72422	Glutamate decarboxylase 2	Gad2
2.8	881.36	366.14	M34445	Glutamate decarboxylase 1	Gad1
2.05	502.57	275.51	AF030253	Solute carrier family 32 (GABA vesicular transporter) member1	Slc32a1 (vGAT)
2.05	392.20	145.97		Meishomeobox2	Meis2
2.03	103.05	197.57	FQ221570	Thyrotropin releasing hormone	Trh
2.01	222.72	185.77	FQ221553	Ubiquitin associated and SH3 domain containing. B	Ubash3b