Published in final edited form as:
Brain Res. 2012 August 27; 1470: 35–44. doi:10.1016/j.brainres.2012.06.002.

Glutamic acid decarboxylase 65 and 67 expression in the lateral septum is up-regulated in association with the postpartum period in mice

Changjiu Zhao[a,*], **Terri Driessen**[a], and **Stephen C. Gammie**[a,b]

[a]Department of Zoology, University of Wisconsin-Madison, 1117 West Johnson Street, Madison, WI 53706, USA

[b]Neuroscience Training Program, University of Wisconsin-Madison, Madison, WI 53706, USA

Abstract

The postpartum period in mammals undergoes a variety of physiological adaptations, including metabolic, behavioral and neuroendocrine alterations. GABA signaling has been strongly linked to various emotional states, stress responses and offspring protection. However, whether GABA signaling may change in the lateral septum (LS), a core brain region for regulating behavioral, emotional and stress responses in postpartum mice has not previously been examined. In this study, we tested whether the expression of two isoforms of glutamic acid decarboxylase (GAD), GAD65 (GAD2) and GAD67 (GAD1), the rate-limiting enzyme for GABA synthesis, exhibits altered expression in postpartum mice relative to nonmaternal, virgin mice. Using microdissected septal tissue from virgin and age-matched postpartum females, quantitative real-time PCR and Western blotting were carried out to assess GAD mRNA and protein expression, respectively. We found both protein and mRNA expression of GAD67 in the whole septum was up-regulated in postpartum mice. By contrast, no significant difference in the whole septum was observed in GAD65 expression. We then conducted a finer level of analysis using smaller micro-dissections and found GAD67 to be significantly increased in rostral LS, but not in caudal LS or medial septum (MS). Further, GAD65 mRNA expression in rostral LS, but not in caudal LS or MS was also significantly elevated in postpartum mice. These findings suggest that an increased GABA production in rostral LS of the postpartum mice via elevated GAD65 and GAD67 expression may contribute to multiple alterations in behavioral and emotional states, and responses to stress that occur during the postpartum period. Given that rostral LS contains GABA neurons that are projection neurons or local interneurons, it still needs to be determined whether the function of elevated GABA is for local or distant action or both.

Keywords

Glutamic acid decarboxylase; GAD1; GAD2; Lateral septum; Lactation; Mice

1. Introduction

The postpartum period in mammals is associated with the emergence of a number of maternal behaviors, including offspring care and offspring protection (Lonstein and Fleming, 2002; Numan and Insel, 2003) as well as changes in emotional state and stress reactivity (Carter et al., 2001; Neumann, 2001). Behavioral responses are altered during the

[*]*Corresponding author*. Fax: +1 608 262 9083. czhao23@wisc.edu (C. Zhao).

postpartum period. In rodents, maternal behaviors are initiated following birth whereby a dam builds a nest, retrieves the pups to the nest site, licks the pups, and adopts a nursing posture over them to permit suckling (Dollinger et al., 1980; Rosenblatt and Lehrman, 1963). The dams can also display high levels of protective behaviors. In contrast, virgin females do not exhibit this suite of behaviors and can even act antagonistically towards pups (Fleming and Luebke, 1981; McCarthy, 1990). Gene expression changes in specific brain regions support such physiological adaptations that occur during the postpartum period (Broad et al., 1993; Brunton et al., 2008; Ottinger et al., 1995; Smith, 1993). The inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), has been strongly linked to aggressive behavior, emotional state, including anxiety, depression, and reactivity to stressors (Cole and Sawchenko, 2002; Cullinan, 1998; de Almeida et al., 2005; Earnheart et al., 2007; Lonstein, 2007; Sanders and Shekhar, 1995). GABA is also linked to offspring protection as the activation of $GABA_A$ receptor with various benzodiazepines ($GABA_A$ receptor agonists) elevated protective behavior (Lee and Gammie, 2007; Mos and Olivier, 1989; Yoshimura and Ogawa, 1991). Further, antagonism of $GABA_A$ receptor with bicuculline prominently disrupted maternal aggression while leaving other components of maternal behavior relatively intact in lactating females (Hansen and Ferreira, 1986; Lee and Gammie, 2009, 2010). GABA is synthesized from its immediate precursor glutamate by the rate-limiting enzyme glutamic acid decarboxylase (GAD). The adult brain expresses two isoforms of GAD, GAD65 (GAD2) and GAD67 (GAD1), which are encoded by two independently regulated genes and differ in cellular expression and synthesis of GABA (Erlander et al., 1991; Soghomonian and Martin, 1998). GABA-positive neurons containing both local inter-neurons and projection neurons are abundantly expressed in the lateral septum (LS) (Castaneda et al., 2005; Garrido Sanabria et al., 2006; Panula et al., 1984). Further, expression of GAD is an accurate reflection of GABA synthesis and activity (Lindefors, 1993; Mason et al., 2001; Segovia et al., 1990).

LS is a core brain region linked to emotional state (e.g., anxiety, fear and depression) and stress reactivity (Sheehan et al., 2004; Singewald et al., 2011). For example, exposure to a variety of stressful and anxiogenic stimuli increases the activity of LS neurons (Cullinan et al., 1995; Silveira et al., 1993, 1995) and stimulation of neural activity in LS reduces fear and anxiety (Thomas, 1988; Thomas and Evans, 1983). Site-directed injection of a $5\text{-}HT_{1A}$ receptor agonist into the LS produces antidepressant-like effects (Martin et al., 1990; Schreiber and De Vry, 1993). LS has also been linked to offspring protection. The magnitude of oxytocin receptor binding in the LS positively correlates with the intensity of maternal defense (Caughey et al., 2011) and recent site directed injection studies, including GABA and norepinephr-ine manipulations, indicate a critical role for LS in protective behavior (D'Anna and Gammie, 2009; Lee and Gammie, 2009; Scotti et al., 2011). Although LS is linked to emotional state and aspects of maternal care, it is still not known whether changes in GABA occur in LS in association with the post-partum period.

In this study, we first used quantitative real-time PCR (qPCR) and Western blotting approaches to determine whether changes occur in the expression of GAD65 and GAD67 in the whole septum (lateral and medial) during the postpartum period. Because the whole septum includes LS and medial septum (MS), and because LS has subdivisions with different known actions and projections, we extended this study by also evaluating expression changes of GAD65 and GAD67 in rostral LS (LSr), caudal LS (LSc) and MS.

2. Results

2.1. GAD65 and GAD67 gene expression in the whole septum by qPCR analysis

Expression of GAD67 mRNA in the whole septum was up-regulated in lactating versus virgin mice ($p = 0.031$). By contrast, no significant difference was observed in GAD65 mRNA expression (Fig. 1).

2.2. GAD65 and GAD67 protein expression in the whole septum by Western blot analysis

In parallel with the expression changes of mRNA, GAD67 protein was increased ($p = 0.025$, Fig. 2C and D), while GAD65 protein was without detectable alteration in postpartum females compared to the virgin mice (p 0.88, Fig. 2A and B).

2.3. GAD65 and GAD67 gene expression in the subdivisions of septum by qPCR analysis

GAD67 mRNA expression in LSr was dramatically up-regulated in lactating mice compared to virgin females ($p < 0.001$, Fig. 3A), whereas no significant difference was observed in LSc ($p = 0.54$) and MS ($p = 0.30$, Fig. 3B and C). It should be noted that the up-regulation of GAD67 in LSr ($p < 0.001$) was more prominent than that in the whole septum ($p < 0.025$). Interestingly, GAD65 mRNA in LSr ($p < 0.031$, Fig. 3A) was significantly elevated as well, which was not detected in the whole septum. Similar to the GAD67 mRNA expression, GAD65 mRNA was not altered in either LSc ($p = 0.48$) or MS ($p = 0.88$, Fig. 3B and C).

3. Discussion

This study was designed to investigate the working hypothesis that altered GABA signaling in LS may be a mechanism for changing behavioral responses or emotional reactivity during the postpartum state. The initial finding of the present study was the increased expression of GAD67 protein and mRNA in the whole septum, suggesting that GAD67 expression is regulated at both transcriptional and translational levels. To further clarify whether the increase in GAD67 was global or subdivision-specific within the septum, we separated the whole septum into LSr, LSc and MS. We found that both GAD65 and GAD67 expression were elevated only in the LSr of postpartum mice, whereas no apparent changes were observed in the LSc or MS. These findings strongly suggest that heightened GAD67 expression in the whole septum is mainly derived from LSr. One possible explanation for a lack of change in GAD65 expression in the whole septum but an increase in the LSr is a dilution effect as increased GAD65 was only found in LSr, but not in the other two subdivisions (e.g., LSc and MS).

Our present findings are consistent with previous studies showing that lactation induces an increased GABA release and activity in the hypothalamus (Kendrick et al., 1992; Kornblatt and Grattan, 2001; Rodriguez et al., 2004). For example, GABA release was increased in the medial preoptic area and the bed nucleus of the stria terminalis (BNST) of sheep during lactation (Kendrick et al., 1992). GABA activity was significantly elevated in the cingulate cortex and the ventrolateral preoptic area of lactating rats compared with diestrous virgin females (Kornblatt and Grattan, 2001). As elevated GABA signaling in LS produces an anxiolytic effect (Drugan et al., 1986; Pesold and Treit, 1996), and anxiety and fear are reduced during lactation (Neumann et al., 2000), it is possible that heightened GABA signaling in LS may contribute to altered fear and anxiety responses during the post-partum period.

The mammalian septum is characterized as a heterogeneous forebrain region that is divided into two main divisions, the MS and LS. MS is densely interconnected with the

hippocampus (Sheehan et al., 2004) and functions mainly as a region that modulates processes related to learning, attention and memory (Brioni et al., 1990; Easton et al., 2011; Johnson et al., 2002; Kanju et al., 2012). LS has been classically divided into three major parts: rostral (LSr), caudal (LSc), and ventral (LSv) subdivisions in terms of chemoarchitecture and neu-roanatomical connections (Goodson et al., 2004; Risold and Swanson, 1996, 1997a,b). LSr is intensely interconnected with medial hypothalamus, and is closely involved in the regulation of reproductive, agonistic and aggressive behaviors (Ricci et al., 2005; Sheehan et al., 2004). Specifically, LSr projects to: anterior hypothalamus (AH), medial preoptic area, BNST, lateral preoptic region, lateral hypothalamus (LH), paraven-tricular nucleus (PVN), amygdala, premammillary nucleus, and periaqueductal grey. By contrast, LSc is densely interconnected with LH, and is typically implicated in controlling locomotor activation and motivation related to rewarding stimuli (Sheehan et al., 2004). LSc also projects to AH, nucleus of the diagonal band, BNST, amygdala, ventral tegmental area, and supramammillary nucleus. Our finding of selective up-regulation of GAD in LSr subdivision of maternal brain supports the idea that altered GABA signaling in LSr plays an important role in changes in behavioral profiles during the postpartum period as LSr has high connectivity with regions previously implicated in maternal defense and emotional state, including periaqueductal gray, amydgala, BNST, and PVN (Consiglio et al., 2005; Consiglio and Lucion, 1996; Lee and Gammie, 2010; Lonstein and Gammie, 2002; Lonstein and Stern, 1997; Sheehan et al., 2004). The LSr contains neurons that express mRNA for neurotensin and enkephalin, while the LSc contains neurons that express mRNA for somatosta-tin, mineralocorticoid receptor and androgen receptor (Risold and Swanson, 1996, 1997a,b). It is reasonable to speculate that the distinct patterns in chemoarchitecture between the LSr and LSc may contribute to the behavioral differences linked to these two subdivisions as neuropeptides and steroid hormone receptors expressed are implicated in the regulation of various different behaviors. In accordance with neuroana-tomical connections, recent work from our lab supports an important role for LSr in regulating maternal defense. Injection of CDP at a dose that elevated defense triggered significant c-Fos decreases in LSr (Lee and Gammie, 2007). Site-directed injection of the β-adrenergic receptor antagonist, propranolol, in the LSr significantly elevated protective behavior in lactating female mice (Scotti et al., 2011), while injection of the $GABA_A$ receptor antagonist, bicuculline, reduced offspring protection (Lee and Gammie, 2009).

Regulation of GAD expression is complex and involves multiple mechanisms at both genomic and nongenomic levels. In previous studies, special attention has been dedicated to the effect of steroid hormones, including estrogen, progesterone and anabolic–androgenic steroid (Grattan et al., 1996; Grimes et al., 2003; Herbison, 1997; Leigh et al., 1990; Weiland, 1992). For example, administration of estradiol and progesterone potently inhibited GAD activity in the arcuate nucleus, ventromedial hypothalamus, and corticomedial amygdala in ovariectomized female rats (Wallis and Luttge, 1980). Estradiol treatment increased GAD65 mRNA as measured by in situ hybridization, but decreased GAD67 mRNA in the magnocellular preoptic area (McCarthy et al., 1995). However, this treatment produced an opposite effect in the dorsal medial nucleus of the hypothalamus, with an increase in GAD67 but a decrease in GAD65 mRNAs. An example of a regulatory effect of progesterone is that GAD67 mRNA levels in the preoptic area were suppressed after progesterone treatment (Unda et al., 1995). In addition to hormones, physical contact with the pups modifies GAD synthesis and activity in the maternal brain (Kornblatt and Grattan, 2001; Qureshi et al., 1987). For example, the CSF concentration of GABA was markedly elevated in dams with pup removed for 6 h after pups were reunited for 24 h (Qureshi et al., 1987), although a role for hormones cannot be excluded. Conversely, GAD activity was increased in the main and accessory olfactory bulbs of lactating mothers with pups removed for 4 h, while was reinstated to the levels of nondeprived mothers after pups were reunited and physically interacted with the deprived mothers (Munaro, 1990). These

findings indicate that pup stimuli (e.g., suckling, olfactory cues) play an important role in regulating GAD expression. In addition, maternal care in turn affects GAD expression of the offspring brain. Adult male offspring nurtured by high pup licking/ grooming mothers expressed higher levels of GAD67 mRNA in the hippocampus compared with those of low pup licking/ grooming dams (Zhang et al., 2010). These findings suggest that epigenetic modifications play an important role in gene expression regulation. Given that dramatic fluctuations of steroid hormones are associated with mating, gestation, parturition and lactation, and close mother–pup interaction that occurs during lactation, it is highly likely that multiple mechanisms act in concert to achieve the heightened GAD expression in the LSr of maternal brain.

In this study, the virgin females differ in several respects from the postpartum mice. First, they were pair-housed with a female conspecific while postpartum mice were housed with a male conspecific during mating. Second, the postpartum mice underwent pregnancy, parturition and lactation, whereas the virgin females did not. Further, the virgin females were housed alone for one week without pup contact at all while the postpartum mice were housed with their pups for one week. It should be pointed out that all these differences may contribute to the observed changes in GAD gene expression during the postpartum period. As has been well documented, a number of factors contribute to the formation of the maternal brain, including the experience of mating, pregnancy, parturition, lactation, and the sensory input from the pups (Bridges, 1996; Ciofi et al., 1993; Nahi and Arbogast, 2003; Numan and Insel, 2003; Svare, 1990). All these factors orchestrate many of changes in gene expression that occur during the postpartum period. This study seeks to explore the results of this constellation of experiences by examining the differences in gene expression between virgin mice, who have never been exposed to any of these experiences, and lactating mice, who have been exposed to all of them. Thus, the observed changes in GAD65 and GAD67 gene expression in postpartum female mice reflect the integral results of multiple factors.

4. Conclusions

This study demonstrated that GAD65 and GAD67 gene expression in rostral LS of postpartum mice was up-regulated. The heightened GAD protein expression reflects its transcriptional up-regulation and/or posttranscriptional stabilization of its mRNA as changes of protein paralleled expression changes of mRNA. These findings indicate for the first time that elevated GAD65 and GAD67 in rostral LS in maternal females may contribute to behavioral, emotional and stressful changes that emerge during the postpartum period. In this study, we were unable to address the issue of where specifically in LS GAD mRNA changes were occurring and whether such changes colocalized with other markers, such as oxytocin receptor. Future studies are warranted to address these issues using in situ hybridization approaches.

5. Experimental procedures

5.1. Animals

Age-matched virgin and lactating mice previously selected for high maternal defense (original stock was outbred hsd:ICR mice) (*Mus domesticus*) (Harlan, Madison, WI) were used. Selected mice were used because they exhibit the reliable emergence of a number of maternal characteristics, including nursing and offspring protection, and thus provide a solid platform for comparing maternal and nonmaternal brains. All mice were ~70 day old at the time of brain collection. Female mice were housed with breeder males (hsd:ICR strain) for 2 weeks. At the same time, virgin females were pair-housed to provide similar social milieu. When breeder males were removed, all females including pregnant and virgin were housed individually and provided precut nesting material until brain collection. The timing of

housing and isolation was performed to minimize the effects of isolation-induced stress. Polypropylene cages were changed once weekly, but when pups were born (postpartum day 0), cages were not changed for the postpartum female or the age-matched virgin control for the remainder of the experiment. All animals were housed in the same room and cages of virgin and lactating females were alternated with one another on the same shelves. Female mice were given ad lib access to breeder chow (Harlan) and tap water. All mice were housed on a 14:10 light/dark cycle with lights on at 06:00 h CST. All procedures followed the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the University of Wisconsin.

5.2. Staging of virgin females

Following removal of brains, virgin females were examined for stage of estrous cycle using a vaginal lavage (Drazen et al., 1999; Marcondes et al., 2002). As a default, female mice in vaginal proestrus were not used because this stage is associated with mating in mice.

5.3. Tissue collection from the whole septum for qPCR and Western blotting

On the day of birth (parturition was designated as postpartum day 0), litters were culled to 11 pups and female mice with litters fewer than 9 pups were excluded from the study. On postpartum days 6–8 between 10:00 and 12:00 h, lactating and virgin mice were lightly anaesthetized with isoflurane and then decapitated. The whole brain was removed, snap frozen in isopentane on dry ice, and then stored at −80 °C until sliced. Sections at a thickness of 300 μm were sliced on a cryostat (Leica, CM1850, Bannock-burn, IL, USA) and mounted on glass slides. Target tissue was removed using a micropunch technique. Microdissection of frozen brain sections was made with Brain Punch Set from Stoelting (Stoelting, Wood Dale, IL, USA) under a dissecting microscope. The whole septum including lateral and medial parts was collected bilaterally from Bregma 1.045 to 0.02 and pooled (Fig. 4), so that each mouse provided one sample of septum. Microdissections were flash frozen on dry ice and stored at −80 °C until processed. An N of 10 for RNA and 8 for protein in each of the two groups (lactating versus virgin) was used for expression comparisons. Virgin mice were in the following stages: diestrus (N = 8) and estrus (N = 2) for qPCR array; diestrus (N = 5) and estrus (N = 3) for Western blotting analysis.

5.4. Tissue collection from the rostral LS, caudal LS, and MS for qPCR

To determine the extent to which each subdivision of the septum contributes to the GAD gene expression changes associated with the postpartum period, we divided the whole septum into three subdivisions: LSr, LSc and MS. Micro-punches from LSr, LSc and MS were collected bilaterally from Bregma 1.045 to 0.02. For a given brain region and individual, tissue was pooled. LSr was captured from Bregma 1.045 to 0.445 and LSc was removed from Bregma 0.245 to 0.02 (Fig. 4). Each mouse provided one sample of each subdivision of the septum. An N of 12 for each of the two groups (all virgin females were diestrus) was used for gene expression comparison.

5.5. Gene expression analysis with qPCR

Gene expression for GAD65 and GAD67 was analyzed using qPCR. Total RNA was extracted with an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA) according to the manufacturer's specifications. Following extraction, RNA integrity was assessed using Agilent RNA 6000 Nano Chips with Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Concentration of total RNA was determined using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). With purified RNA, a Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was

used to reverse transcribe 100 ng of RNA to cDNA in an Eppendorf MasterCycler Personal PCR machine (Eppen-dorf, Hamburg, Germany). The cDNA was then amplified using a SsoFast EvaGreen Supermix kit (Bio-Rad, Hercules, CA) in a StepOnePlus real-time PCR system (Applied Biosys-tems, Foster City, CA). The amplification mixtures (20 μL) contained 1 × SsoFast EvaGreen Supermix, 160 ng template cDNA, and 500 nM forward and reverse primers. Each sample was run in triplicate and standard amplification procedures were used. The cycling profile is as follows: an initial melting step at 95 °C for 30 s followed by 40 cycles of a 95 °C melting step for 5 s, a 58 °C annealing step except GAD65 (at 57 °C) for 20 s, and a 72 °C elongation step for 20 s. Primers for GAD genes and reference genes (Table 1) were designed and screened for specificity using NCBI Primer-BLAST. Ywhaz and CycA were used as reference genes, as they have been found to be among the most stable genes in rodent brain (Bonefeld et al., 2008; Nelissen et al., 2010). Following amplification, a standard curve was generated to assess the empirical PCR reaction efficiency, and a dissociation curve analysis was performed to insure specificity of PCR products. C_t values were calculated using the StepOnePlus software. The expression ratio of mRNA of genes in lactating relative to virgin (normalized against two reference genes Ywhaz and CycA) was calculated using a relative expression software tool REST 2009, which corrects for empirical PCR efficiency, allows for the use of multiple reference genes, and utilizes a randomization test of significance (Pfaffl et al., 2002).

5.6. Western blotting

Micropunched tissue was homogenized with ice-cold lysis buffer (1.0 mL/100 mg of tissue) in pre-chilled centrifuge tubes. Lysis buffer consisted of 50 mM Tris-HCl, pH 7.4, 0.5% Na-deoxycholate, 1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail (P8340, Sigma, St Louis, MO, USA) and phosphatase inhibitor cocktail (P0044, Sigma). Following tissue homogenization, samples were centrifuged at 12,000 rpm for 10 min at 4 °C to sediment unbroken cells and nuclei. The supernatant fraction was collected, and protein concentration determined using BCA Protein Assay (Pierce Chemical Co., Rockford, IL, USA). Twenty micrograms of total protein from each mouse were gel electrophoresed using a 4–20% precast Tris-HCl gel (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane. Membranes were washed briefly in 0.1 M Tris buffered saline containing 0.05% Tween 20 (TBST) and blocked for one hour in 0.1 M TBST containing 5% nonfat dry milk with constant agitation at room temperature. The membrane was incubated with a primary antibody recognizing either GAD65 (rabbit anti-GAD65, 1:1000; AB5082, Millipore, Billerica, MA, USA) or GAD67 (mouse anti-GAD67, 1:5000; MAB5406, Millipore) in TBST containing 5% nonfat dry milk overnight at 4 °C with agitation, and washed 3 times for 5 min each in TBST. Following washes, the PVDF membrane was incubated in a HRP-linked secondary antibody (goat anti-rabbit IgG, 1:10,000 for GAD65, Cell Signaling Technology, Beverly, MA, USA; horse anti-mouse IgG, 1:10,000 for GAD67, Cell Signaling) for 1 h at room temperature with agitation, and washed 3 times for 5 min each in TBST. Immunoreactive bands were detected using a chemiluminescence kit (ECL Plus, Amersham, Arlington Heights, IL, USA) and exposed to film (Hyperfilm-ECL, Amersham). Membranes were stripped of antibodies, and re-blotted with β-actin (1:2000; Abcam, Cambridge, MA) as an internal control. Films for the immunoblots were developed using an AGFA CP1000 X-ray film processor (AGFA, Nunawading, Australia), digitized using a GS-800 Imaging Densitometer (Bio-Rad, Hercules, CA, USA), and analyzed using the Quantity One program (Bio-Rad). Values obtained are ratios of band volume, which takes both the optical density and the area of spread of the band into account, between GAD65 or GAD67 and β-actin.

5.7. Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The ratio data of Western blot were expressed as mean ± SEM and analyzed using Independent-Samples *T* test. $p < 0.05$ was considered statistically significant.

Acknowledgments

This research was supported by National Institutes of Health Grant R01 MH 085642 to S.C.G., the authors wish to thank Sharon Stevenson for excellent technical and administrative assistance, Dr. Anthony P. Auger for immunoblot development, Dr. Greg Sabat for image analysis of Western blotting and Kate Skogen and Jeff Alexander for animal care.

Abbreviations

AH	anterior hypothalamus
BNST	bed nucleus of the stria terminalis
CycA	Cyclophilin A
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAD65 (GAD2)	glutamic acid decarboxylase 65
GAD67 (GAD1)	glutamic acid decarboxylase 67
LH	lateral hypothalamus
LS	lateral septum
LSc	caudal lateral septum
LSr	rostral lateral septum
MS	medial septum
PVN	paraventricular nucleus
qPCR	quantitative real-time polymerase chain reaction
Ywhaz	tyrosine 3-monooxygenase/tryptophan, 5-monooxygenase activation protein, zeta polypeptide

REFERENCES

Bonefeld BE, Elfving B, Wegener G. Reference genes for normalization: a study of rat brain tissue. Synapse. 2008; 62:302–309. [PubMed: 18241047]

Bridges, RS. Biochemical basis of parental behavior in the rat. In: Rosenblatt, JS.; Snowden, CT., editors. Parental Care: Evolution, Mechanisms, and Adaptive Significance. San Diego: Academic Press; 1996. p. 215-237.

Brioni JD, Decker MW, Gamboa LP, Izquierdo I, McGaugh JL. Muscimol injections in the medial septum impair spatial learning. Brain Res. 1990; 522:227–234. [PubMed: 2224525]

Broad KD, Kendrick KM, Sirinathsinghji DJ, Keverne EB. Changes in oxytocin immunoreactivity and mRNA expression in the sheep brain during pregnancy, parturition and lactation and in response to oestrogen and progesterone. J. Neuroendocrinol. 1993; 5:435–444. [PubMed: 8401567]

Brunton PJ, Russell JA, Douglas AJ. Adaptive responses of the maternal hypothalamic-pituitary-adrenal axis during pregnancy and lactation. J. Neuroendocrinol. 2008; 20:764–776. [PubMed: 18601699]

Caldwell KK, Sheema S, Paz RD, Samudio-Ruiz SL, Laughlin MH, Spence NE, Roehlk MJ, Alcon SN, Allan AM. Fetal alcohol spectrum disorder-associated depression: evidence for reductions in

the levels of brain-derived neurotrophic factor in a mouse model. Pharmacol. Biochem. Behav. 2008; 90:614–624. [PubMed: 18558427]

Carter CS, Altemus M, Chrousos GP. Neuroendocrine and emotional changes in the post-partum period. Prog. Brain Res. 2001; 133:241–249. [PubMed: 11589134]

Castaneda MT, Sanabria ER, Hernandez S, Ayala A, Reyna TA, Wu JY, Colom LV. Glutamic acid decarboxylase isoforms are differentially distributed in the septal region of the rat. Neurosci. Res. 2005; 52:107–119. [PubMed: 15811558]

Caughey SD, Klampfl SM, Bishop VR, Pfoertsch J, Neumann ID, Bosch OJ, Meddle SL. Changes in the intensity of maternal aggression and central oxytocin and vasopressin V1a receptors across the peripartum period in the rat. J. Neuroendocrinol. 2011; 23:1113–1124. [PubMed: 21929717]

Ciofi P, Crowley WR, Pillez A, Schmued LL, Tramu G, Mazzuca M. Plasticity in expression of immunoreactiv-ity for neuropeptide Y, enkephalins and neurotensin in the hypothalamic tubero-infundibular dopaminergic system during lactation in mice. J. Neuroendocrinol. 1993; 5:599–602. [PubMed: 8680430]

Cole RL, Sawchenko PE. Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J. Neurosci. 2002; 22:959–969. [PubMed: 11826124]

Consiglio AR, Borsoi A, Pereira GA, Lucion AB. Effects of oxytocin microinjected into the central amygdaloid nucleus and bed nucleus of stria terminalis on maternal aggressive behavior in rats. Physiol. Behav. 2005; 85:354–362. [PubMed: 15935410]

Consiglio AR, Lucion AB. Lesion of hypothalamic para-ventricular nucleus and maternal aggressive behavior in female rats. Physiol. Behav. 1996; 59:591–596. [PubMed: 8778839]

Cullinan WE. Evidence for a PVN site of action of GABA in the regulatory control of the rat stress axis. Physiologist. 1998; 41:353.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience. 1995; 64:477–505. [PubMed: 7700534]

D'Anna KL, Gammie SC. Activation of corticotropin-releasing factor receptor 2 in lateral septum negatively regulates maternal defense. Behav. Neurosci. 2009; 123:356–368. [PubMed: 19331459]

de Almeida RM, Ferrari PF, Parmigiani S, Miczek KA. Escalated aggressive behavior: dopamine, serotonin and GABA. Eur. J. Pharmacol. 2005; 526:51–64. [PubMed: 16325649]

Dollinger MJ, Holloway WR, Denenberg VH. Parturition in the rat (Rattus norvegicus): normative aspects and the temporal patterning of behaviours. Behav. Processes. 1980; 5:21–37.

Drazen DL, Klein SL, Burnett AL, Wallach EE, Crone JK, Huang PL, Nelson RJ. Reproductive function in female mice lacking the gene for endothelial nitric oxide synthase. Nitric Oxide. 1999; 3:366–374. [PubMed: 10534440]

Drugan RC, Skolnick P, Paul SM, Crawley JN. Low doses of muscimol produce anticonflict actions in the lateral septum of the rat. Neuropharmacology. 1986; 25:203–205. [PubMed: 3703171]

Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, Luscher B. GABAergic control of adult hippo-campal neurogenesis in relation to behavior indicative of trait anxiety and depression states. J. Neurosci. 2007; 27:3845–3854. [PubMed: 17409249]

Easton A, Fitchett AE, Eacott MJ, Baxter MG. Medial septal cholinergic neurons are necessary for context-place memory but not episodic-like memory. Hippocampus. 2011; 21:1021–1027. [PubMed: 20842629]

Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxy-lases. Neuron. 1991; 7:91–100. [PubMed: 2069816]

Fleming AS, Luebke C. Timidity prevents the virgin female rat from being a good mother: emotionality differences between nulliparous and parturient females. Physiol. Behav. 1981; 27:863–868. [PubMed: 7323193]

Garrido Sanabria ER, Castaneda MT, Banuelos C, Perez-Cordova MG, Hernandez S, Colom LV. Septal GABAergic neurons are selectively vulnerable to pilocarpine-induced status epilepti-cus and chronic spontaneous seizures. Neuroscience. 2006; 142:871–883. [PubMed: 16934946]

Goodson JL, Evans AK, Lindberg L. Chemoarchitectonic subdivisions of the songbird septum and a comparative overview of septum chemical anatomy in jawed vertebrates. J. Comp. Neurol. 2004; 473:293–314. [PubMed: 15116393]

Grattan DR, Rocca MS, Strauss KI, Sagrillo CA, Selmanoff M, McCarthy MM. GABAergic neuronal activity and mRNA levels for both forms of glutamic acid decarboxylase (GAD65 and GAD67) are reduced in the diagonal band of Broca during the afternoon of proestrus. Brain Res. 1996; 733:46–55. [PubMed: 8891247]

Grimes JM, Ricci LA, Melloni RH Jr. Glutamic acid decarboxylase (GAD65) immunoreactivity in brains of aggressive, adolescent anabolic steroid-treated hamsters. Horm. Behav. 2003; 44:271–280. [PubMed: 14609549]

Hansen S, Ferreira A. Effects of bicuculline infusions in the ventromedial hypothalamus and amygdaloid complex on food intake and affective behavior in mother rats. Behav. Neurosci. 1986; 100:410–415. [PubMed: 3730149]

Herbison AE. Estrogen regulation of GABA transmission in rat preoptic area. Brain Res. Bull. 1997; 44:321–326. [PubMed: 9370195]

Johnson DA, Zambon NJ, Gibbs RB. Selective lesion of cholinergic neurons in the medial septum by 192 IgG-saporin impairs learning in a delayed matching to position T-maze paradigm. Brain Res. 2002; 943:132–141. [PubMed: 12088847]

Kanju PM, Parameshwaran K, Sims-Robinson C, Uthayathas S, Josephson EM, Rajakumar N, Dhanasekaran M, Suppir-amaniam V. Selective cholinergic depletion in medial septum leads to impaired long term potentiation and gluta-matergic synaptic currents in the hippocampus. PLoS One. 2012; 7:e31073. [PubMed: 22355337]

Kendrick KM, Keverne EB, Hinton MR, Goode JA. Oxyto-cin, amino acid and monoamine release in the region of the medial preoptic area and bed nucleus of the stria terminalis of the sheep during parturition and suckling. Brain Res. 1992; 569:199–209. [PubMed: 1540826]

Kornblatt JJ, Grattan DR. Lactation alters gamma-ami-nobutyric acid neuronal activity in the hypothalamus and cerebral cortex in the rat. Neuroendocrinology. 2001; 73:175–184. [PubMed: 11307036]

Lee G, Gammie SC. GABA enhancement of maternal defense in mice: possible neural correlates. Pharmacol. Bio-chem. Behav. 2007; 86:176–187.

Lee G, Gammie SC. GABA(A) receptor signaling in the lateral septum regulates maternal aggression in mice. Behav. Neurosci. 2009; 123:1169–1177. [PubMed: 20001101]

Lee G, Gammie SC. GABAA receptor signaling in caudal periaqueductal gray regulates maternal aggression and maternal care in mice. Behav. Brain Res. 2010; 213:230–237. [PubMed: 20457185]

Leigh AJ, Carter ND, Horton R, Silverligh JJ, Wilson CA. Ovarian steroid regulation of glutamic acid decarbox-ylase gene expression in individual hypothalamic nuclei. J. Neuroendocrinol. 1990; 2:433–438. [PubMed: 19215370]

Lindefors N. Dopaminergic regulation of glutamic acid decarboxylase mRNA expression and GABA release in the striatum: a review. Prog. Neuropsychopharmacol. Biol. Psychiatry. 1993; 17:887–903. [PubMed: 8278600]

Lonstein JS. Regulation of anxiety during the postpartum period. Front. Neuroendocrinol. 2007; 28:115–141. [PubMed: 17604088]

Lonstein JS, Fleming AS. Parental behaviors in rats and mice. Curr. Protoc. Neurosci. 2002; 8.15:1–26.

Lonstein JS, Gammie SC. Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. Neu-rosci. Biobehav. Rev. 2002; 26:869–888.

Lonstein JS, Stern JM. Role of the midbrain periaqueductal gray in maternal nurturance and aggression: c-Fos and electrolytic lesion studies in lactating rats. J. Neurosci. 1997; 17:3364–3378. [PubMed: 9113892]

Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz. J. Biol. 2002; 62:609–614. [PubMed: 12659010]

Martin P, Beninger RJ, Hamon M, Puech AJ. Antide-pressant-like action of 8-OH-DPAT, a 5-HT1A agonist, in the learned helplessness paradigm: evidence for a postsynaptic mechanism. Behav. Brain Res. 1990; 38:135–144. [PubMed: 2141988]

Mason GF, Martin DL, Martin SB, Manor D, Sibson NR, Patel A, Rothman DL, Behar KL. Decrease in GABA synthesis rate in rat cortex following GABA-transaminase inhibition correlates with the decrease in GAD(67) protein. Brain Res. 2001; 914:81–91. [PubMed: 11578600]

McCarthy MM. Oxytocin inhibits infanticide in female house mice (Mus domesticus). Horm. Behav. 1990; 24:365–375. [PubMed: 2227849]

McCarthy MM, Kaufman LC, Brooks PJ, Pfaff DW, Schwartz-Giblin S. Estrogen modulation of mRNA levels for the two forms of glutamic acid decarboxylase (GAD) in female rat brain. J. Comp. Neurol. 1995; 360:685–697. [PubMed: 8801259]

Mos J, Olivier B. Quantitative and comparative analyses of pro-aggressive actions of benzodiazepines in maternal aggression of rats. Psychopharmacology (Berl). 1989; 97:152–153. [PubMed: 2567023]

Munaro NI. Maternal behavior: glutamic acid decarbox-ylase activity in the olfactory bulb of the rat. Pharmacol. Biochem. Behav. 1990; 36:81–84. [PubMed: 2349273]

Nahi F, Arbogast LA. Prolactin modulates hypothalamic preproenkephalin, but not proopiomelanocortin, gene expression during lactation. Endocrine. 2003; 20:115–122. [PubMed: 12668876]

Nelissen K, Smeets K, Mulder M, Hendriks JJ, Ameloot M. Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR. J. Neurosci. Methods. 2010; 187:78–83. [PubMed: 20036692]

Neumann ID. Alterations in behavioral and neuroendo-crine stress coping strategies in pregnant, parturient and lactating rats. Prog. Brain Res. 2001; 133:143–152. [PubMed: 11589127]

Neumann ID, Torner L, Wigger A. Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behaviour in virgin, pregnant and lactat-ing rats. Neuroscience. 2000; 95:567–575. [PubMed: 10658637]

Numan, M.; Insel, TR. The Neurobiology of Parental Behavior. New York: Springer; 2003.

Ottinger MA, Rosewell KL, Weiland NG, Margaretten KT, Wise PM. Effect of lactation on hypothalamic preproen-kephalin gene expression. J. Neuroendocrinol. 1995; 7:341–346. [PubMed: 7550279]

Panula P, Revuelta AV, Cheney DL, Wu JY, Costa E. An immunohistochemical study on the location of GABAergic neurons in rat septum. J. Comp. Neurol. 1984; 222:69–80. [PubMed: 6365983]

Pesold C, Treit D. The neuroanatomical specificity of the anxiolytic effects of intra-septal infusions of midazolam. Brain Res. 1996; 710:161–168. [PubMed: 8963655]

Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002; 30:e36. [PubMed: 11972351]

Qureshi GA, Hansen S, Sodersten P. Offspring control of cerebrospinal fluid GABA concentrations in lactating rats. Neurosci. Lett. 1987; 75:85–88. [PubMed: 2883612]

Ricci LA, Grimes JM, Knyshevski I, Melloni RH. Repeated cocaine exposure during adolescence alters glutamic acid dec-arboxylase-65 (GAD65) immunoreactivity in hamster brain: correlation with offensive aggression. Brain Res. 2005; 1035:131–138. [PubMed: 15722053]

Risold PY, Swanson LW. Structural evidence for functional domains in the rat hippocampus. Science. 1996; 272:1484–1486. [PubMed: 8633241]

Risold PY, Swanson LW. Chemoarchitecture of the rat lateral septal nucleus. Brain Res. Rev. 1997a; 24:91–113. [PubMed: 9385453]

Risold PY, Swanson LW. Connections of the rat lateral septal complex. Brain Res. Rev. 1997b; 24:115–195. [PubMed: 9385454]

Rodriguez C, Guillamon A, Pinos H, Collado P. Post-partum changes in the GABAergic system in the bed nucleus of the accessory olfactory tract. Neurochem. Int. 2004; 44:179–183. [PubMed: 14568561]

Rosenblatt, JS.; Lehrman, DS. Maternal behavior in the laboratory rat. In: Rheingold, HL., editor. Maternal Behavior in Mammals. New York: John Wiley and Sons; 1963. p. 8-57.

Sanders SK, Shekhar A. Regulation of anxiety by GABAA receptors in the rat amygdala. Pharmacol. Biochem. Behav. 1995; 52:701–706. [PubMed: 8587908]

Schreiber R, De Vry J. Neuroanatomical basis for the antidepressant-like effects of the 5-HT(1A) receptor agonists 8-OH-DPAT and ipsapirone in the rat forced swimming test. Behav. Pharmacol. 1993; 4:625–636. [PubMed: 11224231]

Scotti MA, Lee G, Gammie SC. Maternal defense is modulated by beta adrenergic receptors in lateral septum in mice. Behav. Neurosci. 2011; 125:434–445. [PubMed: 21480688]

Segovia J, Tillakaratne NJ, Whelan K, Tobin AJ, Gale K. Parallel increases in striatal glutamic acid decarboxylase activity and mRNA levels in rats with lesions of the nigros-triatal pathway. Brain Res. 1990; 529:345–348. [PubMed: 2126483]

Sheehan TP, Chambers RA, Russell DS. Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res. Rev. 2004; 46:71–117. [PubMed: 15297155]

Silveira MC, Sandner G, Di Scala G, Graeff FG. c-Fos immunoreactivity in the brain following electrical or chemical stimulation of the medial hypothalamus of freely moving rats. Brain Res. 1995; 674:265–274. [PubMed: 7796106]

Silveira MC, Sandner G, Graeff FG. Induction of Fos immunoreactivity in the brain by exposure to the elevated plusmaze. Behav. Brain Res. 1993; 56:115–118. [PubMed: 8397853]

Singewald GM, Rjabokon A, Singewald N, Ebner K. The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses. Neuropsychopharmacology. 2011; 36:793–804. [PubMed: 21160468]

Smith MS. Lactation alters neuropeptide-Y and proopio-melanocortin gene expression in the arcuate nucleus of the rat. Endocrinology. 1993; 133:1258–1265. [PubMed: 8365368]

Soghomonian JJ, Martin DL. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol. Sci. 1998; 19:500–505. [PubMed: 9871412]

Svare, B. Maternal aggression: hormonal, genetic, and developmental determinants. In: Krasnegor, NA.; Bridges, RS., editors. Mammalian Parenting: Biochemical, Neurobiologi-cal, and Behavioral Determinants. New York: Oxford University Press; 1990. p. 118-132.

Thomas E. Forebrain mechanisms in the relief of fear: the role of the lateral septum. Psychobiology. 1988; 16:36–44.

Thomas E, Evans GJ. Septal inhibition of aversive emotional states. Physiol. Behav. 1983; 31:673–678. [PubMed: 6665056]

Unda R, Brann DW, Mahesh VB. Progesterone suppression of glutamic acid decarboxylase (GAD67) mRNA levels in the preoptic area: correlation to the luteinizing hormone surge. Neuroendocrinology. 1995; 62:562–570. [PubMed: 8751281]

Wallis CJ, Luttge WG. Influence of estrogen and progesterone on glutamic acid decarboxylase activity in discrete regions of rat brain. J. Neurochem. 1980; 34:609–613. [PubMed: 7354335]

Weiland NG. Glutamic acid decarboxylase messenger ribonucleic acid is regulated by estradiol and progesterone in the hippocampus. Endocrinology. 1992; 131:2697–2702. [PubMed: 1446611]

Yoshimura H, Ogawa N. Ethopharmacology of maternal aggression in mice: effects of diazepam and SM-3997. Eur. J. Pharmacol. 1991; 200:147–153. [PubMed: 1685120]

Zhang TY, Hellstrom IC, Bagot RC, Wen X, Diorio J, Meaney MJ. Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. J. Neurosci. 2010; 30:13130–13137. [PubMed: 20881131]

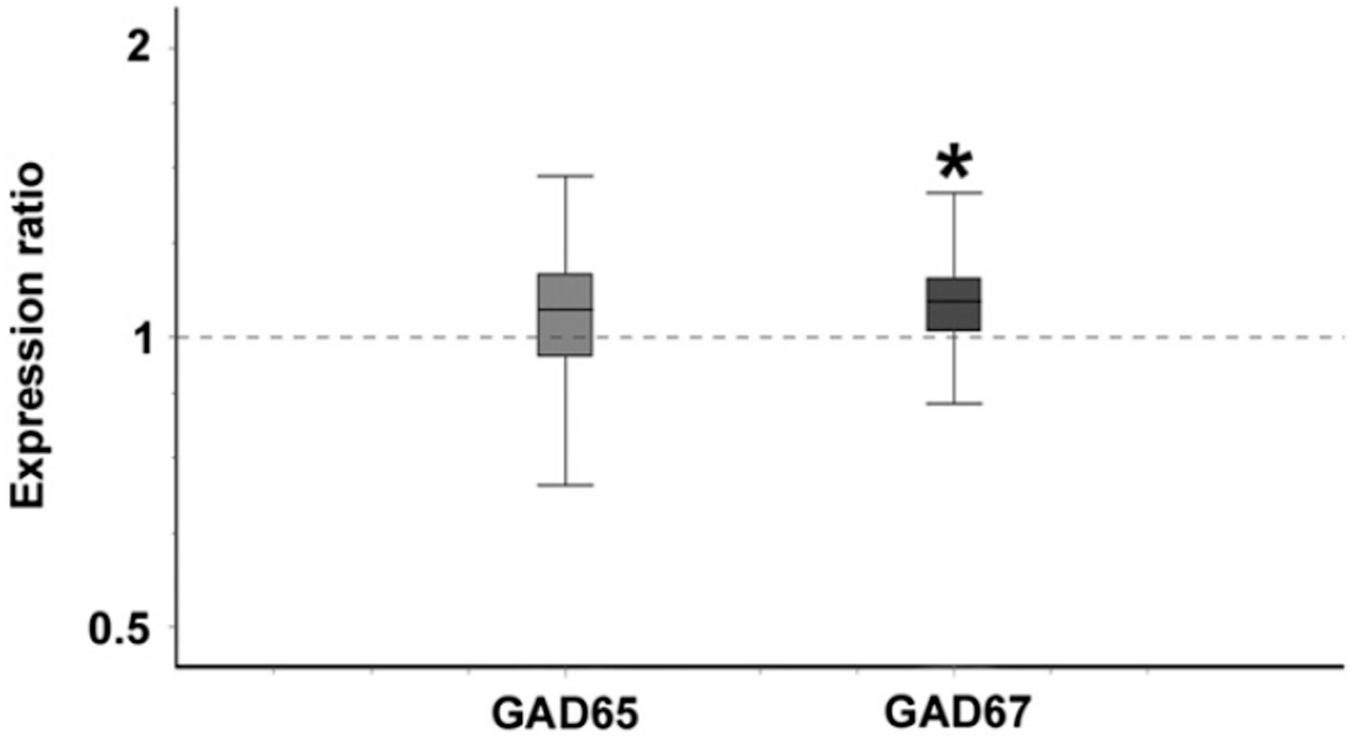

Fig. 1.

Quantitative real-time PCR analysis of GAD65 and GAD67 gene expression in the whole septum. Relative expression distribution of mRNA (Y-axis) represented as a ratio of lactating versus virgin mice (n = 10/group), was normalized against two reference genes CycA and Ywhaz, and shown by box-and-whisker plot as medians (solid lines), interquartile range (boxes) and ranges (whiskers). Ratios over one indicate genes that are more highly expressed in lactating than in virgin mice. Note that GAD67 mRNA in the whole septum was up-regulated in lactating mice compared to virgin females, while no significant change was observed in GAD65 mRNA. *$p < 0.05$ lactating versus virgin control.

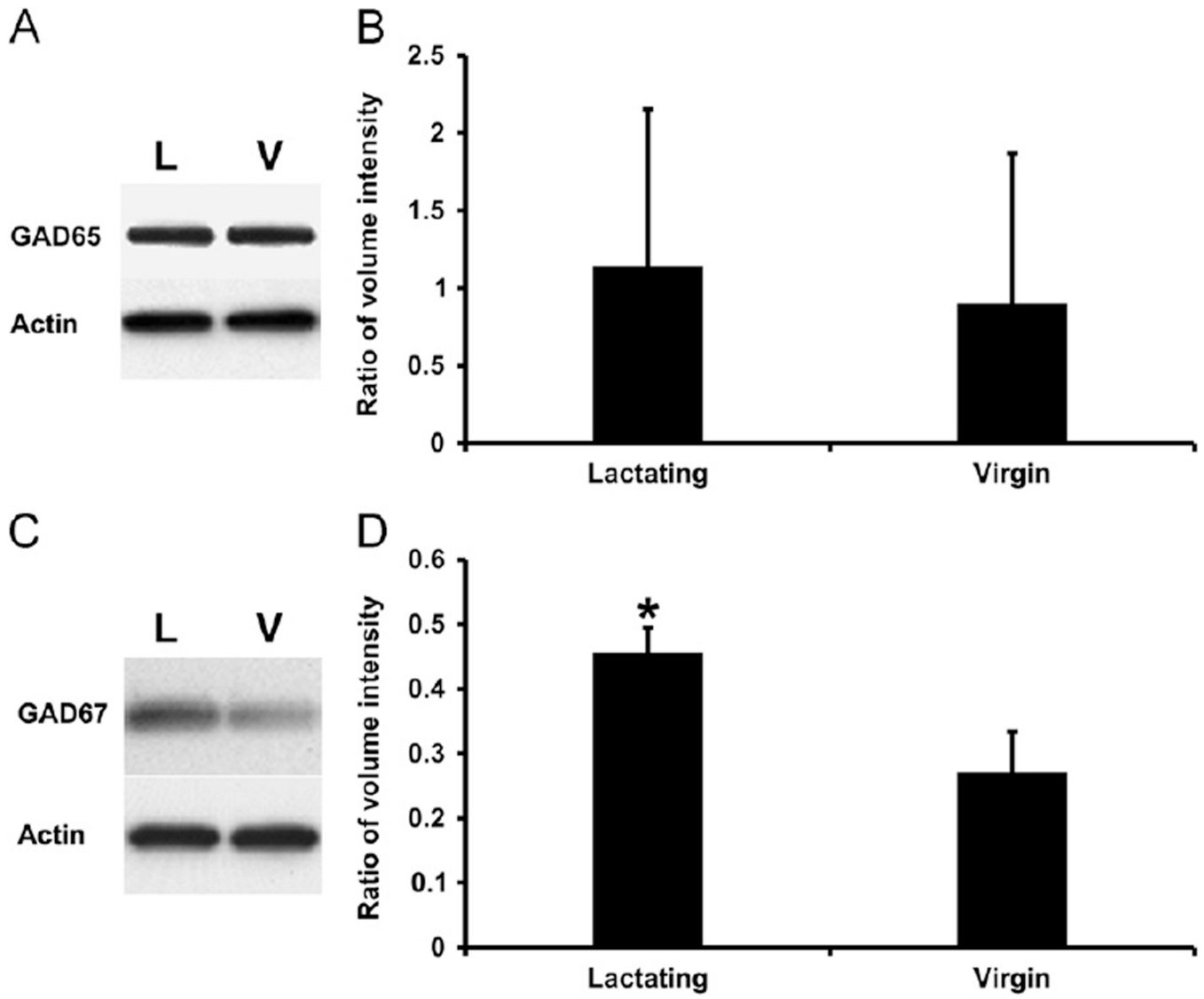

Fig. 2.

Western blot analysis of GAD65 and GAD67 protein expression in the whole septum of lactating and virgin mice ($n = 8$/group). Representative Western immunoblot bands represent GAD65 (A) and GAD67 (C) protein expression with normalization against housekeeping protein, β-actin. Densitometric analysis of immunoreactive bands shows a ratio of mean band volume intensity of GAD65 (B) or GAD67 (D) to β-actin. *$p < 0.05$ relative to virgin mice.

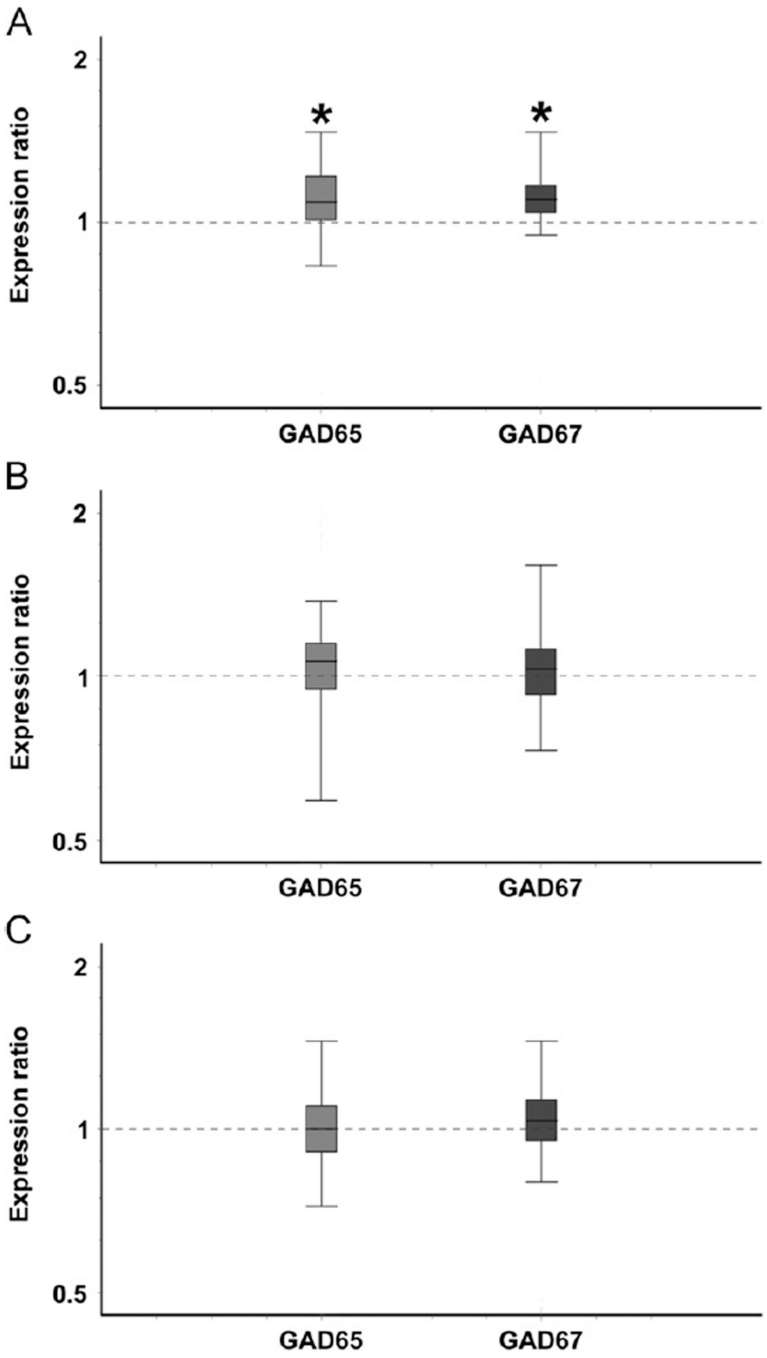

Fig. 3.

Quantitative real-time PCR analysis of GAD65 and GAD67 gene expression in the rostral LS (A), caudal LS (B) and medial septum (C). Relative expression distribution of mRNA (Y-axis) represented as a ratio of lactating versus virgin mice (n = 12/group), was normalized against two reference genes CycA and Ywhaz, and shown by box-and-whisker plot as medians (solid lines), interquartile range (boxes) and ranges (whiskers). Ratios over one indicate genes that are more highly expressed in lactating than in virgin mice. Note that mRNAs of both GAD65 and GAD67 in rostral LS (A) was elevated in lactating mice compared to virgin females, while no significant change in GAD65 and GAD67 was observed in caudal LS (B) and medial septum (C).*p< 0.05 lactating versus virgin control.

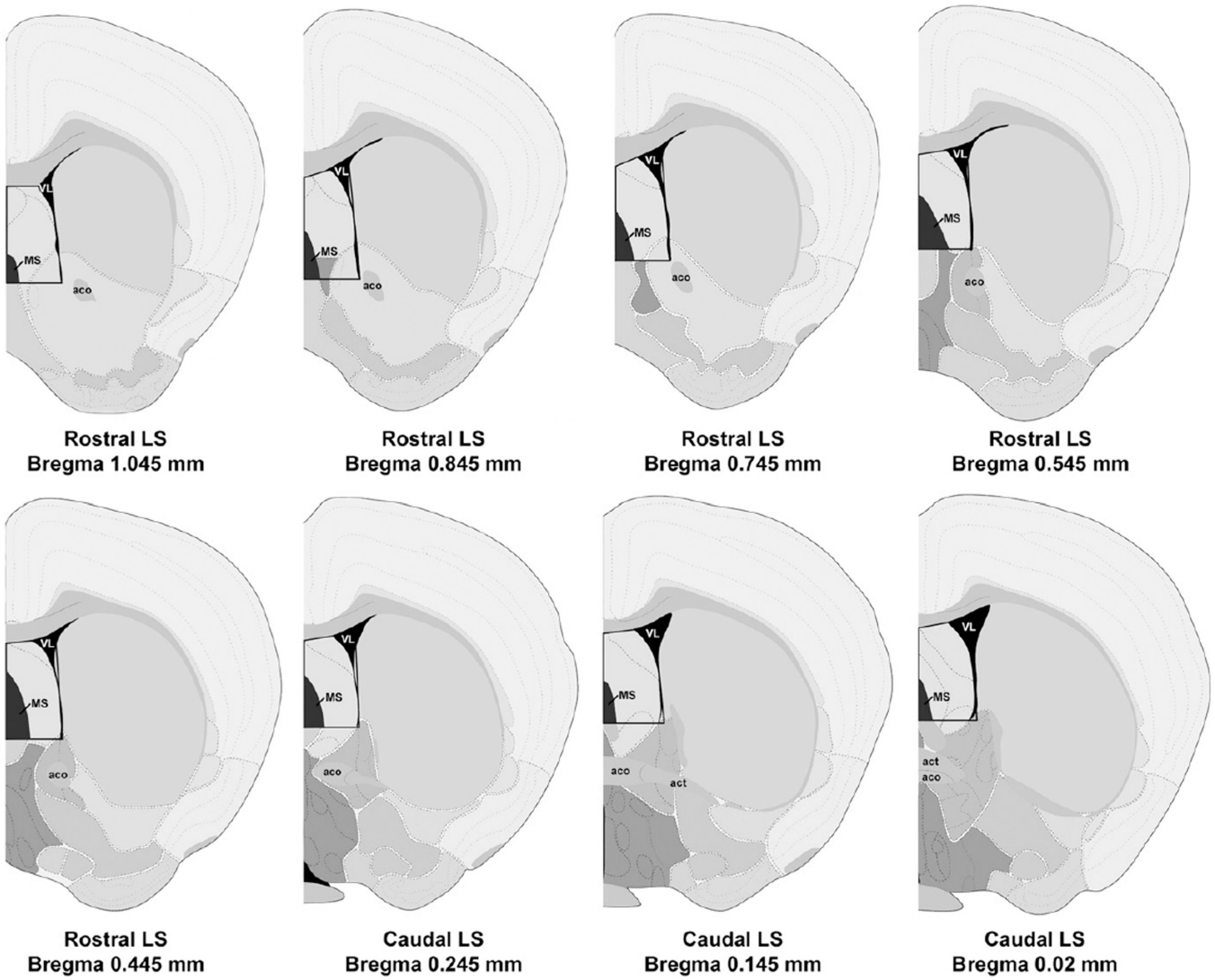

Fig. 4.

Schematic representation of the whole septum (boxed areas) dissected for gene and protein analysis. Black areas marked with MS were micropunched and collected for MS sampling, with the remaining boxed areas for LS analysis. Reprinted and modified from The Allen Mouse Brain Atlas (reference atlas version 1, 2008). Distance relative to Bregma is indicated in each section. Abbreviations: aco, anterior commissure, olfactory limb; act, anterior commissure, temporal limb; MS, medial septum; VL, lateral ventricle.

Table 1

Primer sequences for target and reference genes.

Symbol	NCBI accession number	Full name	Forward primer	Reverse primer
GAD65	NM_008078.2	Glutamic acid decarboxylase 65	5’-TCAACTAAGTCCCACCCTAAG-3’	5’-CCCTGTAGAGTCAATACCTGC-3’
GAD67	NM_008077.4	Glutamic acid decarboxylase 67	5’-CTCAGGCTGTATGTCAGATGTTC-3’	5’-AAGCGAGTCACAGAGATTGGTC-3’
CycA	NM_008907.1	Cyclophilin A	5’-TGCTGGACCAAACACAAACG-3’	5’-GCCTTCTTTCACCTTCCCAAA-3’
Ywhaz	NM_011740.3	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	5’-TCCTTATTCCCTCTTGGCAG-3’	5’-ATGGAAGCTACATTAGCGGTTT-3’

CycA primer sequences were described in the published literature (Caldwell et al., 2008).