Influence of 17β-estradiol and progesterone on GABAergic gene expression in the arcuate nucleus, amygdala and hippocampus of the rhesus macaque

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Abstract

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, and the responsiveness of neurons to GABA can be modulated by sex steroids. To better understand how ovarian steroids influence GABAergic system in the primate brain, we evaluated the expression of genes encoding GABA receptor subunits, glutamic acid decarboxylase (GAD) and a GABA transporter in the brains of female rhesus macaques. Ovariectomized adults were subjected to a hormone replacement paradigm involving either 17β-estradiol (E), or E plus progesterone (E+P). Untreated animals served as controls. Using GeneChip® microarray analysis and real-time RT-PCR (qPCR), we examined gene expression differences within and between the amygdala (AMD), hippocampus (HPC) and arcuate nuclei of the medial basal hypothalamus (MBH). The results from PCR corresponded with results from representative GeneChip® probesets, and showed similar effects of sex steroids on GABA receptor subunit gene expression in the AMD and HPC, and a more pronounced expression than in the MBH. Exposure to E+P attenuated *GAD1*, *GAD2* and *SLC32A1* gene expression in the AMD and HPC, but not in the MBH. GABA receptor subunit gene expression was generally higher in the AMD and HPC than in the MBH, with the exception of receptor subunits ε and γ2. Taken together, the data demonstrate differential regulation of GABA receptor subunits and GABAergic system components in the MBH compared to the AMD and HPC of rhesus macaques. Elevated ε and reduced δ subunit expression in the MBH supports the hypothesis that the hypothalamic GABAergic system is resistant to the modulatory effects of sex steroids.

Keywords

gamma-aminobutyric acid; GABA receptor; Macaca mulatta; microarray

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1. Introduction

Gamma-aminobutyric acid (GABA) is the most widespread inhibitory neurotransmitter in the vertebrate central nervous system (CNS) (Sivilotti and Nistri, 1991), where its release by both neurons (Zilberter et al., 1999) and glial cells (Angulo et al., 2008) affects various physiological functions. Changes in receptor subunit composition may alter properties and density of GABA receptor protein (Weiland and Orchinik, 1995) and may dramatically alter the effect the responsiveness of the cell to GABA (Steiger and Russek, 2004). Consequently, differential regulation of GABA receptor subtypes represents an important mechanism for maintaining or altering CNS excitation/inhibition (E/I) balance during homeostasis or in response to physiological challenge (Bethmann et al., 2008; Fritschy, 2008; Olsen and Sieghart, 2008). Fluctuations in the sex-steroid environment during reproductive cycles are highly associated with changes in GABA receptor subunit composition in the brain (Brussaard et al., 1997; Byrnes et al., 2007; Jorge et al., 2002; Lovick, 2006) and circulating steroids also affect expression of the rate limiting enzyme required for GABA synthesis, glutamic acid decarboxylase (GAD), (Herbison et al., 1992; McCarthy et al., 1995; Sagrillo and Selmanoff, 1997), as well as GABA transporters (Herbison et al., 1995).

The influence of ovarian steroids on GABA receptor subunit composition has been studied in detail in cellular models and in the rodent CNS, but not in primates. After ovulation, the corpus luteum (Stouffer, 2006) of rhesus macaque monkeys and humans, lasts much longer than in rodents and so continues to produce P for extended periods (Knobil, 1973; Weik et al., 1973), whereas in the rat and mouse, the ultra short-lived (Stouffer, 2006) corpus luteum is not well developed (Hilliard, 1973).

Progesterone can be metabolized in the brain to neuroactive metabolites which include 5α-dihydroprogesterone, 3α, 5α-tetrahydroprogesterone (allopregnanolone), and 3β, 5α-tetrahydroprogesterone (pregnanolone). These P derivatives are collectively referred to as THP (Compagnone and Mellon, 2000), and are potent positive modulators of $GABA_A$ receptors (Callachan et al., 1987; Gee et al., 1987). In addition to synthesis through modification of circulating steroids (Corpechot et al., 1983; Robel et al., 1987), these neuroactive steroids (neurosteroids) (Baulieu, 1998; Compagnone and Mellon, 2000) can also be synthesized de novo from cholesterol in rodent brains.

In humans, the links between P and neurosteroid levels may correlate anxiety and mood disorders with hormonal effects on the CNS during menstruation (Backstrom, 1976; Guille et al., 2008; N-Wihlback et al., 2006). The connections between hormonal fluctuations of reproductive cycles and neurosteroid modulation of GABAergic systems in primates are focal points in the study of human premenstrual syndrome (Rapkin et al., 1997).

The goal of the present study was to examine the effects of sex-steroids on GABAergic CNS physiology in a nonhuman primate, the rhesus macaque. Using Affymetrix® GeneChip® Rhesus Macaque Genome Arrays and quantitative real-time RT-PCR (qPCR), we examined how the expression of GABAergic genes in the amygdala (AMD), hippocampus (HPC) and arcuate nucleus of the medial basal hypothalamus (MBH) was affected by exposure to 17β-estradiol (E) or 17β-estradiol + progesterone (E+P); these hormone replacement paradigms mimic the follicular and luteal phases of the menstrual cycle, respectively.

2. Results

2.1 GeneChip® microarrays

A summary of the GeneChip® expression profiles for representative subunit probesets is provided in Table 1. Detailed information for all probesets annotated for GABA receptor

subunits, GAD, and the GABA transporter *SLC32A1* are provided in Table 2. Treatment-related expression differences within tissues were observed for genes encoding GABA$_A$ receptor subunits α1, α4, α5, β1, β3, γ2, and GABA$_B$ receptor subunits 1 and 2. Gene expression levels in the MBH were lower than in the AMD and HPC, with the exception of GABA$_A$ receptor subunit ε, where gene expression in the MBH was higher (Table 1). The most reliable probe sets for genes encoding GABA$_A$ receptor subunits α6, γ3, θ and π, as well as GABA$_C$ receptor subunits returned "absent" calls for multiple animals in multiple treatments, and were thus regarded as non-detectable using MAS 5.0 global scaling analysis. Expression of the gene encoding the vesicular GABA transporter *SLC32A1* was comparable across all three brain regions in OVX animals but tended to be reduced by E and E+P in the AMD and HPC. In the AMD, vesicular GABA transporter expression was significantly reduced with E+P treatment ($P < 0.01$). Similar results were observed with respect to expression of *GAD1* (67kDa) and *GAD2* (65 kDa) mRNA expression (Table 1).

2.2 Real-time RT-PCR (qPCR)

In general, gene expression patterns detected using the rhesus GeneChip® mirrored results from quantitative real-time RT-PCR (qPCR) analysis (Fig. 1). Effects of steroid hormone treatment on gene expression were observed in each brain region. In the MBH, E ($P < 0.01$) and E+P ($P < 0.05$)-treatment elevated *GABRE* expression above that of OVX controls. In the HPC, both E ($P < 0.05$) and E+P ($P < 0.01$) reduced the expression of *GABRA1*, and *GAD1* compared to OVX controls. E+P-treatment also reduced *GABRA4* expression ($P < 0.01$) in the HPC compared to OVX controls. In the AMD, E ($P < 0.01$) and E+P treatment reduced *GABRA1* expression compared to OVX controls and E+P treatment reduced *GAD1* expression ($P < 0.05$) relative to OVX controls (Fig. 2).

Within treatments, gene expression levels in the AMD and HPC tended to be more similar to each other than to expression levels in the MBH. In the OVX animals, *GABRA1*, *GABRA4* and *GABRD* gene expression in the AMD and HPC was generally higher than in the MBH. In E and E+P-treated animals, a similar regional expression pattern was observed for *GABRA4* and *GABRD*. Regional expression trends for *GABRE* showed an inverse pattern compared to other subunit receptors examined, with higher expression in the MBH than in the AMD and HPC. E+P- treated animals also showed higher *GAD1* gene expression in the MBH than in the AMD and HPC.

3. Discussion

In general, results from GeneChip® expression results were well corroborated by qPCR analysis, affirming the reliability of the expression data from selected probesets. However exceptions were noted for *GABRG2*, where AMD and HPC expression trends noted in the microarray analysis were not observed with qPCR. Similarly, E+P-treatment appeared to significantly change MBH expression levels for *GABRA1* and *GABRA4* in the microarray but not with qPCR analysis. Conversely for *GABRE*, MBH treatment effects detected with qPCR were not significantly different with microarray analysis.

In the current study, gene expression levels of most GABA receptor subunits tended to be lower in the MBH compared to the AMD and HPC. In a notable reversal of this trend, expression of the gene encoding the ε subunit was elevated in the MBH compared to expression in the AMD and HPC. Interestingly, elevated ε subunit expression in GABA receptors has been suggested as part of a mechanism by which the hypothalamus is buffered from neurosteroid fluctuations (Henderson, 2007). In the rat, hypothalamic neurons show high GABA$_A$ receptor ε subunit expression (Moragues et al., 2003; Sinkkonen et al., 2000; Whiting et al., 1997) and diminished responses to allopregnanolone compared to neurons with more typical ε subunit concentrations (Moragues et al., 2003; Sullivan and Moenter, 2003). Receptors incorporating the ε subunit

are also insensitive to the GABA-modulatory actions of benzodiazepines and pregnane steroids (Belelli et al., 2002; Davies et al., 1997), but mechanistic data from several studies are difficult to reconcile (reviewed in Lambert et al., 2003) and the specific action of ε subunit inclusion may be highly dependent on receptor stoichiometry (Thompson et al., 2002)

In contrast to the ε subunit, δ subunit expression in the current study was lower in the MBH than in the AMD and HPC. Similarly, a low expression level of the δ subunit has also been observed in the rat hypothalamus (Fritschy et al., 1994; Pirker et al., 2000). Mice lacking the δ subunit show reduced steroid sensitivity (Mihalek et al., 1999; Shen et al., 2007; Spigelman et al., 2003), and presence of the δ subunit confers susceptibility to steroid modulation (Belelli et al., 2002; Bianchi and Macdonald, 2003; Brown et al., 2002) by increasing the maximum effect of steroids acting on receptors which express it (Wohlfarth et al., 2002).

Because the ε subunit reduces the GABAergic response to steroids whereas δ subunit expression enhances this GABAergic response, elevated ε and reduced δ subunit expression in the MBH may be an effective expression combination for dampening GABAergic responses to steroid fluctuations in the arcuate nucleus. These findings support the hypothesis that differential GABA$_A$ receptor subunit regulation is part of a mechanism that protects the hypothalamic GABAergic system from the modulatory influence of sex steroids (Henderson, 2007). The significance of this buffering may be related to GABA$_A$ receptor mediation of both stimulatory and inhibitory actions of GABA on hypothalamic release of gonadotropin-releasing hormone (GnRH) (Adler and Crowley, 1986; Akema et al., 1990; Jarry et al., 1991; Lamberts et al., 1983; Moguilevsky et al., 1991) which modulates steroid release from the gonads. With respect to GABA effects on GnRH release, GABA containing terminals synapse directly with GnRH neurons in the rat (Leranth et al., 1985), whereas synaptic contact between GABAergic neurons and GnRH neurons has not been detected in the rhesus macaque (Thind and Goldsmith, 1995). In the macaque, it is possible that other mechanisms may intercede between GABA and GnRH (Windsor-Engnell et al., 2007). Estrogen regulation of regional GABA concentrations has long been implicated in the feedback action of estrogen on gonadotropin release (Mansky et al., 1982). As opposed to direct modulation of GABAergic neurons by neurosteroids, estrogen likely modulates GABAergic inputs that affect the functionality of the neural networks regulating LH secretion (Herbison et al., 1991; Herbison and Dyer, 1991).

HPC δ receptor subunit expression is correlated with P levels during the mouse estrous cycle with enhanced expression during late diestrus (when circulating P is high), and reduced expression when P levels were low (Maguire et al., 2005). However, P-induced increases in hippocampal δ subunit expression were not observed in the current study.

Sex-steroid effects on α4 subunit expression were observed in the current study with tendencies (Fig 1 and Fig 2) in the AMD and HPC toward expression reductions with E and E+P treatment groups compared to OVX controls. Direct E (Zhou and Smith, 2007) and P (Pierson et al., 2005) administration independently increase α4 mRNA expression in NT2-N neuronal cells. Exposure of rats to P transiently increases α4 subunit expression after 48–72 hrs (Gulinello et al., 2001; Hsu et al., 2003) but this increase is followed by a return to control levels after 4–5 days (Gulinello et al., 2001). Note also that P or THP withdrawal increases α4 subunit expression in the rat HPC (Smith et al., 1998a) and AMD (Gulinello et al., 2003), highlighting the possibility that steroid removal through ovariectomy may elevate expression of some GABA receptor subunits, although short-term steroid exposure may also produce transient expression increases. Such studies also raise the question of whether subunit expression changes may be used to adapt to hormonal conditions over time. Examinations of temporal changes in primate subunit expression will require more enhanced time-course experiments than used in the current study.

The α1β2γ2 GABA receptor is estimated to represent the most widespread subunit combination in the mammalian brain (Fritschy and Mohler, 1995; McKernan and Whiting, 1996; Somogyi et al., 1996). In the current study, although probeset data showing hippocampal reductions in α1 subunit expression with E and E+P treatment were strong, β2 subunit expression in 64% of the study animals was not safely above global background levels (absent call) (Table 2). Reductions in γ2 subunit expression seen with E+P treatment in the in the HPC on the array analysis were not corroborated with qPCR. The γ2 subunit influences postsynaptic clustering of GABA$_A$ receptors (Essrich et al., 1998) through palmitoylation, and represents an additional mechanism for regulation of trafficking, cell-surface expression and postsynaptic clustering (Keller et al., 2004; Rathenberg et al., 2004).

The metabotropic GABA$_B$ receptors mediate slow synaptic inhibition and consist of heterodimers of B1 and B2 subunits (Bettler et al., 2004) which share structural homology with class-III G protein-coupled receptors (Binet et al., 2004; Kaupmann et al., 1997). The only known molecular diversity of the GABA$_B$ receptors arises from two GABA$_{B1}$ isoforms (Kaupmann et al., 1998) whose few variants seem unable to explain the pharmacological heterogeneity of GABA$_B$ receptors observed in many functional studies (Huang, 2006; Raiteri, 2008). In the current study, E and E+P upregulation of the gene encoding the GABA$_B$ receptor subunit 1 in the AMD was not correlated to expression of the gene encoding subunit 2.

Ionotropic GABA$_C$ receptors are the least studied of the GABA receptor classes (Johnston et al., 2003), and expression of genes encoding GABA$_C$ receptor subunits were below the limits of microarray detection in the current study. The ρ subunits that make up the GABA$_C$ receptor have been identified in the retina (Enz et al., 1995), HPC (Enz et al., 1995; Rozzo et al., 2002) and pituitary (Boue-Grabot et al., 2000) of the rat.

Treatment with E downregulates GAD protein expression in rat hippocampal neurons (Murphy and Segal, 2000). Although this trend was noted for mRNA levels in the current study, significant reductions in *GAD1* expression were only noted in the AMD with E+P treatment.

Steroidogenic regulation of GABAergic systems has been extensively studied in cell culture and rodent models. However, comparisons between the primate and rodent models may require caution. Even if conversion of circulating steroids to neurosteroids in brains of primates and rodents were equal (this question requires investigation), sheer differences in circulating physiological concentrations between the two orders likely place respective brains under different physiological pressures. Compared to rhesus macaques, rats have higher levels of plasma P, with baseline concentrations of around 12ng/ml and peak concentrations of 55ng/ml. Moreover rats have a short (24 hour) peak to trough concentration oscillation (Freeman, 2006). In addition, the dominant circulating progestin in the rat is 20α-hydroxyprogesterone (Eto et al., 1962; Hashimoto et al., 1968; Piacsek et al., 1971), as opposed to P. In contrast, the serum P levels of the rhesus macaque are an order of magnitude lower than in the rat, with baseline concentrations of less than 0.5ng/ml and peak concentrations on the order of 5ng/ml across a 12 day oscillation (Downs and Urbanski, 2006; Knobil, 1974; Macdonald and Greep, 1972). Humans show similar P profiles to rhesus monkeys but with higher mean P levels, and sustained peaks in the range of 8–20 ng/ml (Hale et al., 2007; Neill et al., 1967; Vande Wiele et al., 1970; Veldhuis et al., 1988). However, short-term samples (taken at 10–15 minute intervals) show mid-luteal human P peaks as high as the 30–60 ng/ml range (Filicori et al., 1984; Rossmanith et al., 1990).

GABAergic effects of circulating steroids are likely mediated by direct conversion to neurosteroids in neurons. Examples such as rat hippocampal cultures that show a 60 fold increase in THP after 24 hrs of P treatment (Murphy and Segal, 2000), support the hypothesis that circulating P modulates GABA receptor composition through neurosteroid action

(Gulinello et al., 2001; Gulinello et al., 2003; Shen et al., 2005; Smith et al., 1998a; Smith et al., 1998b; Smith et al., 2007).

The THP P-metabolite, allopregnanolone, is the most abundant and efficacious endogenous positive allosteric modulator of the action of GABA at GABA$_A$ receptors (Pinna et al., 2008; Puia et al., 2003) and increases the efficacy of other positive allosteric modulators of GABA action (Belelli and Lambert, 2005; Pinna et al., 2000). Circulating levels of THP made by the corpus luteum parallel P levels throughout the menstrual cycle (Rapkin et al., 1997; Schmidt et al., 1994), and circulating steroids produced in the adrenals and ovaries are important modulators of GABAergic function (Paul and Purdy, 1992; Purdy et al., 1991; Reddy, 2003a; Reddy, 2003b). However, allopregnanolone is also produced in the brain from circulating progestins (Dong et al., 2001). In addition, production of allopregnanolone and other neurosteroids occurs in various brain regions of castrated adrenalectomized rats long after peripheral sources are removed (Cheney et al., 1995), and synthesis occurs in the brain through non-genomic mechanisms within seconds of behavioral stimulus (Frye et al., 2006).

In mice, THP concentrations in the brain are high enough to overwhelm potential peripheral contributions, and circadian fluctuations in brain concentrations overwhelm peripheral estrous cycle fluctuations (Corpechot et al., 1997). This suggests that the mouse may be more dependent on neurosteroid modulation of GABAergic inhibition than the primate (Smith et al., 2007). Assessing differences in the hormone physiology of primates and rodents may be made more complex because hormone bioavailability is modulated by steroid binding proteins which may be internalized into neuronal cells (Caldwell et al., 2006), and the interplay between hormone concentrations and steroid binding protein expression in the brain (Mopert et al., 2006; Sendemir et al., 2006) may differ between the two orders.

To a lesser extent, similar arguments of species-specific physiological considerations may be made regarding differences in estrogen physiology between primates and rodents. Rhesus macaque plasma E has baseline levels around 10–100 pg/ml with periovulatory concentrations exceeding 300 pg/ml (Downs and Urbanski, 2006; Freeman, 2006; Knobil, 1973; Weik et al., 1973). Rats show E baseline concentrations of 5–10 pg/ml with a proestrus peak just below 50 pg/ml (Freeman, 2006). Estradiol down regulates expression of brain-derived neurotrophic factor (BDNF) (Murphy et al., 1998b), which in turn down regulates GAD and GABA expression (Murphy et al., 1998a). This mechanism may explain some of estradiol's rapid anti-GABAergic properties (Murphy and Segal, 2000) and GABAergic inhibition through GAD regulation (McCarthy et al., 1995; Rudick and Woolley, 2001; Weiland, 1992)

In women, the AMD and hypothalamus are among the brain regions where concentrations of P and its neurosteroid metabolites, show variation according to ovarian steroid production (Bixo et al., 1997). Also in humans, circulating levels of THP made by the corpus luteum parallel P levels throughout the menstrual cycle (Rapkin et al., 1997; Schmidt et al., 1994), making the human cycle associated with a much longer period of THP exposure compared to short THP peaks in the mouse (Mannan and O'Shaughnessy, 1988). Effects of physiological hormone concentrations on GABAergic activity have been studied in humans in conjunction with certain medical conditions. For example, the condition known as catamenial epilepsy is influenced by steroid modulation of GABAergic activity (Reddy, 2009). Seizure likelihood increases at mid-cycle and during the luteal phase (Backstrom, 1976; Herzog et al., 1997) and rodent models show increased seizure susceptibility or severity with P or THP withdrawal (Frye and Bayon, 1998; Reddy and Rogawski, 2001; Rogawski, 2003; Smith et al., 1998a). Challenges using benzodiazepines as GABAergic agonists, and measures of CNS activity such as saccadic eye velocity have been used to evaluate GABAergic effects resulting from human cyclic hormone fluctuation (McAuley and Friedman, 1999; Rukstalis and de Wit, 1999; Sundstrom et al., 1997; Sundstrom et al., 1998; Sundstrom and Backstrom, 1998). However,

consensus regarding steroid-induced changes in GABA receptors has been complicated by difficulties in distinguishing effects due to changes in sensitivity to GABAergic drugs from effects due to changes in circulating hormone levels (reviewed in N-Wihlback et al., 2006). In addition, studies examining relationships between neuroactive steroid levels and premenstrual syndrome (PMS) or premenstrual dysphoric disorder (PMDD) have yielded discordant results (reviewed in Guille et al., 2008).

In light of physiological differences between primates and species typically used to study GABAergic system regulation, primate models may be most appropriate for elucidating effects of hormone cyclicity or hormone-replacement therapy on neurological changes in women. Findings in the rhesus macaque represented by data from the current study represent steps in establishing effects of physiological ovarian hormone concentrations on gene regulation of proteins modulating GABAergic function in brain areas influencing cognition and reproduction.

4. Experimental Procedure

4.1 Animals

Young (mean age = 9.67 years) adult rhesus macaques (*Macaca mulatta*) were obtained from the ONPRC Division of Animal Resources and were cared for in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* by the Division of Animal Resources at the Oregon National Primate Research Center (ONPRC). The animals were housed in a temperature-controlled environment and Primate Chow (Purina Mills Inc., St. Louis, MO) was made available to the animals twice daily, at 8:00 h and again at 15:00 h. This diet was supplemented with fresh fruit and vegetables, and the animals had continuous access to drinking water.

The animals were subjected to a hormone replacement paradigm designed to tightly mimic the sex-steroid changes that occur naturally across their menstrual cycle. All of the animals were bilaterally ovariectomized (OVX), and some of them then subjected to 17β-estradiol (E) replacement or E + progesterone (P) (E+P) replacement; the latter two paradigms (E and E+P) corresponded to the late follicular and mid luteal phases of the menstrual cycle respectively. On average (mean ± SEM) the animals were 9.4 ± 0.3 years old and weighed 5.5 ± 0.18 kg. The post-OVX interval was 6.7 ± 0.6 mo. Each of the three groups comprised four animals. Each animal received a SILASTIC implant (Dow Corning, Midland, MI), subcutaneously, in the periscapular area while under ketamine anesthesia (5 mg/kg, im; ketamine HCl, Parke Davis, Morris Plains, NJ). OVX control animals received an empty capsule. E- and E+P-treaded animals received SILASTIC capsule filled with crystalline 17β-estradiol (Steraloids, Wilton, NH). Fourteen days later, the E+P-treated animals received a SILASTIC capsule filled with crystalline P (Steraloids) (Kohama and Bethea, 1995). To verify the circulating hormone levels in these animals, serum samples were assayed for E and P. The OVX control group received no hormone replacement had undetectable serum E and P levels. The E group had serum concentrations of E = 117 ± 6.7 pg/ml, and the E+P group had serum E levels of 130 ± 9 pg/ml and P levels of 3.8 ± 0.4ng/ml. The animals were subjected to the E replacement paradigm for 4 weeks, with P treatment only in the final 2 weeks.

The animals were euthanized using sodium pentobarbital, and after a transcardial flush with 1 liter of 0.9% saline, their brains were immediately removed and cut into blocks. The brain blocks were preserved in RNA*later*® (Ambion; Austin, TX) and areas of interest (MBH, HPC and AMD) were subsequently dissected from the surrounding tissue, resulting in a total of 36 tissue samples.

4.2 RNA extraction and cDNA preparation

AMD, HPC and MBH were homogenized with a PowerGen rotor-stator homogenizer (Fisher Scientific, Pittsburgh, PA) and total RNA was isolated using a Qiagen RNeasy Mini Kit, according to the manufacturer's instructions (QIAGEN, Valencia, CA). Briefly, tissues were homogenized in RLT buffer with 1% β-mercaptoethanol. RNA was collected onto RNeasy mini columns and eluted in water. The concentration of RNA was measured by spectroscopy, with an expected A_{260}/A_{280} ratio close to 2, denoting an acceptably pure nucleic acid sample. RNA integrity and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA), and met Affymetrix requirements for hybridization to the Rhesus Genome GeneChip® microarray.

Using random hexamers, 500ng of RNA from each sample was converted to cDNA using manufacturer's instructions for the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™ life technologies, Carlsbad CA www.Invitrogen.com).

4.3 Primers and probes

Rhesus macaque sequences for *GAPDH* and *RPL13A* were confirmed in-house using RT-PCR primers against source sequences to amplify rhesus macaque hippocampal cDNA pooled from several individuals. These PCR products were sequenced using an ABI 3730×l DNA sequencer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions and corresponded highly to the annotated source sequences. qPCR primers and probes for prospective housekeeping genes *ALG9*, *GAPDH* and *RPL13A* as well as GABA subunit receptors *GABRA1*, *GABRA4*, *GABRG2*, *GABRD* and *GABRE* and the enzyme *GAD1* were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) from sequence sources listed described elsewhere (Noriega et al., submitted) To confirm that selected q PCR primers amplified the desired sequences, RT-PCR was conducted using Platinum® PCR SuperMix (Invitrogen™ life technologies, Carlsbad CA www.Invitrogen.com) and reaction products were analyzed on a 2% agarose gel.

4.4 qPCR

qPCR and data collection were conducted using the 7900HT Fast Real-Time PCR thermal cycler and sequence detection systems (Software version 2.2.1) from Applied Biosystems (Foster City, CA). No-template controls were included for each gene. Standard curves were constructed using cDNA pooled equally from all tissues and all animals. The curves were constructed from serial 5- fold serial dilutions and covered a cDNA dilution range of 0.2 to 0.0016 (larger fold dilutions produced unacceptably high cycle times for some genes in the study).

Reactions for qPCR were conducted in sealed 384-well optical plates in a total volume of 5 µl per well, using 1.0 µl of 1:5-diluted cDNA, 0.25 µM Taqman probe and 0.3µM each of forward and reverse primers. Reactions were conducted using thermal cycler conditions of: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15s at 95°C (DNA melting) and 1 min at 60°C (primer annealing/ extension). Baseline and threshold levels for amplification plots were determined automatically using the ABI sequence detection systems software version 2.2.1. Validation of the reference genes *GAPDH*, *RPL13A* and *ALG9* for normalization was conducted using geNorm, NormFinder and BestKeeper software as described elsewhere (Noriega et al., submitted). Normalization of linear quantity values from qPCR was performed using the arithmetic mean of linear quantity values for *GAPDH*, *RPL13A* and *ALG9*. i.e., we used a normalizer index comprised of multiple internal reference genes.

4.5 Gene microarrays

RNA quality was assessed using an Agilent 2100 Bioanalyzer. An assay performance assessment was conducted to confirm that the quality of the RNA used was acceptable and that genome hybridizations performed well. The performance assessment (conducted by AMC) was based on the GCOS analysis, and pair-wise scatter plots of Microarray Suite version 5.0 (MAS 5.0) absolute analyses. The RNA quality (35/36 samples) met the level recommended by Affymetrix for hybridization to the Rhesus Genome GeneChip® (micro)array, with the exception of an one sample (MBH from an OVX animal) that showed a left-ward shift in bioanalyzer profiles indicative of RNA degradation .

We examined gene expression in the MBH, AMD and HPC of rhesus macaques treated with a hormone replacement paradigm designed to test effects of E and E+P manipulation. In order to identify treatment-related changes in gene expression, we used the transcriptome (52,024 probe sets to monitor 47,000 transcripts) available on the GeneChip® Rhesus Macaque Genome Array which "leverages the homology of expressed sequences represented on the GeneChip® Human Genome U133 Plus 2.0 Array to annotate the Baylor School of Medicine's rhesus macaque whole-genome shotgun assembly" (Affymetrix, 2005)

Microarray assays were performed in the Affymetrix Microarray Core (AMC) of the OHSU Gene Microarray Shared Resource. Samples were prepared using the AMC One-cycle cDNA IVT (*in vitro* transcription) amplification/labeling protocol (Standard Labeling). Each sample target was hybridized to a Rhesus Genome GeneChip® microarray and image processing and expression analysis were performed using Affymetrix GeneChip® Operating Software (GCOS) version 1.2.

Absolute analyses were run using global scaling to an average target intensity of 200. In this way, scaling allowed for direct comparison of the hybridization values from the two targets. Statistical algorithms from the Affymetrix Microarray Suite version 5.0 (MAS 5.0) were used to generate signal metrics. The parameters α_1 and α_2, which set the point at which the probe set is called present, marginally present, or undetectable, were set to 0.05 and 0.065 (Affymetrix defaults) respectively. These parameters were used to determine expression levels for each probe set based on the detection P-value of the probe set.

We used Affymetrix probe set ID numbers to search the GeneChip® microarray for oligonucleotide signal intensities associated with genes for GABA receptor subunits (Dingledine et al., 1999). Probe set ID assignments were referenced according to listings for the Rhesus GeneChip® microarray and searched using the Affymetrix online Netaffx™ Analysis Center software (query function).

4.6 Statistics

We used Kruskal-Wallis one-way analysis of variance on GeneSifter™ software (VizX Labs, Seattle WA, http://www.genesifter.net/) to identify GABA subunit receptors showing differences in expression. This software incorporated Robust Multichip Analysis (RMA) algorithms to normalize expression data across all chips. In order to directly compare normalized expression values in finer detail than allowable in GeneSifter™, we used Affymetrix® Expression Console™ software (Affymetrix Inc Santa Clara, CA) to independently perform RMA where normalized expression values for each sample could be used to examine treatment-related differences in finer detail than allowable in GeneSifter™.

Kruskal-Wallis one-way non-parametric analysis of variance was used to compare logarithmically scaled RMA expression results within each tissue group (MBH, HPC or AMD) across the three treatments (OVX, E and E+P). Similarly, the Kruskal-Wallis test was used to compare linearly scaled relative quantity data from qPCR. Treatment-related differences in

mRNA expression were identified using Newman-Keuls post hoc tests. Tissue differences were assessed using one way parametric ANOVA (12 samples per group).

Abbreviations

ALG9, gene encoding asparagine-linked glycosylation 9 homolog

AMC, Affymetrix Microarray Core

AMD, amygdala

E, 17β-estradiol (treatment)

E+P, 17β-estradiol + progesterone (treatment)

GABA, gamma-aminobutyric acid

GABRA1, gene encoding GABA$_A$ receptor subunit alpha 1

GABRA4, gene encoding GABA$_A$ receptor subunit alpha 4

GABRD, gene encoding GABA$_A$ receptor subunit delta

GABRE, gene encoding GABA$_A$ receptor subunit epsilon

GABRG2, gene encoding GABA$_A$ receptor subunit gamma 2

GAD1, gene encoding glutamic acid decarboxylase 1 (67 kDa)

GAPDH, gene encoding glyceraldehyde 3-phosphate dehydrogenase

GCOS, GeneChip® Operating Software

HPC, hippocampus

MBH, arcuate nucleus of the medial basal hypothalamus

OHSU, Oregon Health & Science University

ONPRC, Oregon National Primate Research Center

OVX, ovariectomized

qPCR, quantitative real-time RT-PCR

RPL13A, gene encoding ribosomal protein L13a

SLC32A1, gene encoding solute carrier family 32 (GABA vesicular transporter), member 1, also known as VGAT

THP, neurosteroid derivatives of progesterone, 5α-dihydroprogesterone, namely, 3α, 5α-tetrahydroprogesterone (allopregnanolone), and 3β, 5α-tetrahydroprogesterone (pregnanolone)

Acknowledgments

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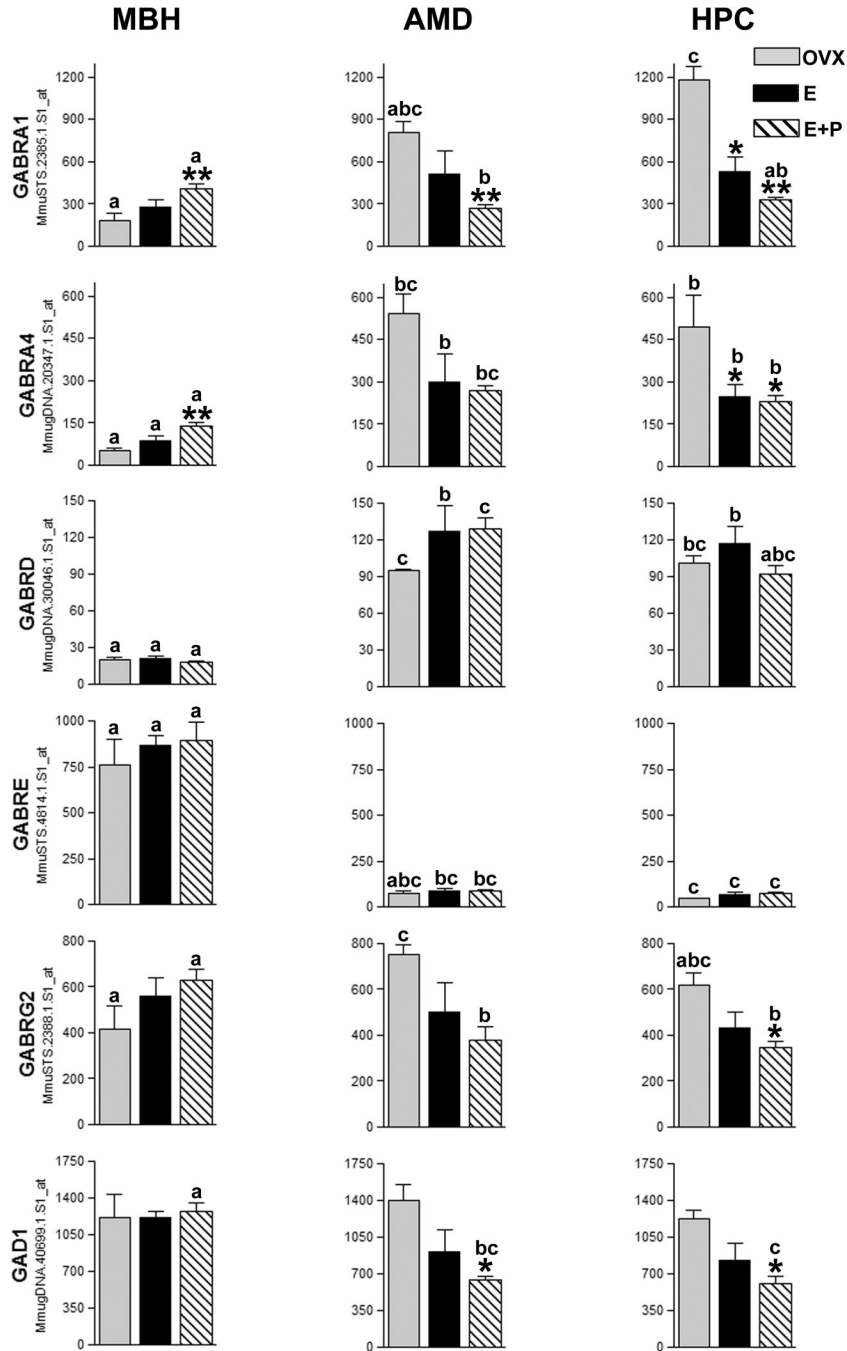**Figure 1. Relative expression of genes encoding GABA receptor subunits and *GAD1* in the rhesus macaque**

Histograms show expression data from representative GeneChip® Rhesus Genome Array probesets corresponding to genes examined using qPCR. Probesets from sequence tagged sites (MmuSTS designation) were prioritized. Otherwise A-grade annotations were given priority. Where annotation grades were equal, probesets showing the highest expression were selected. Histograms show antilogarithms of RMA-normalized (log2) expression values, however, statistical analyses were conducted using log-transformed data.

Tissues: MBH = arcuate nucleus of the medial basal hypothalamus; AMD = amygdala; HPC = hippocampus.

Hormone treatments: OVX = Ovariectomized; E = Estrogen; E+P = Estrogen + Progesterone
Asterisks denote statistical differences in expression for hormone effects within a brain region.
Post-hoc test P values are listed with respect to the OVX group for that tissue only. * = P <
0.05; ** = P < 0.01.
Letters denote statistical differences in expression between brain regions under the same
hormone treatment (compare bars with the same legend). Bars with the same legend sharing
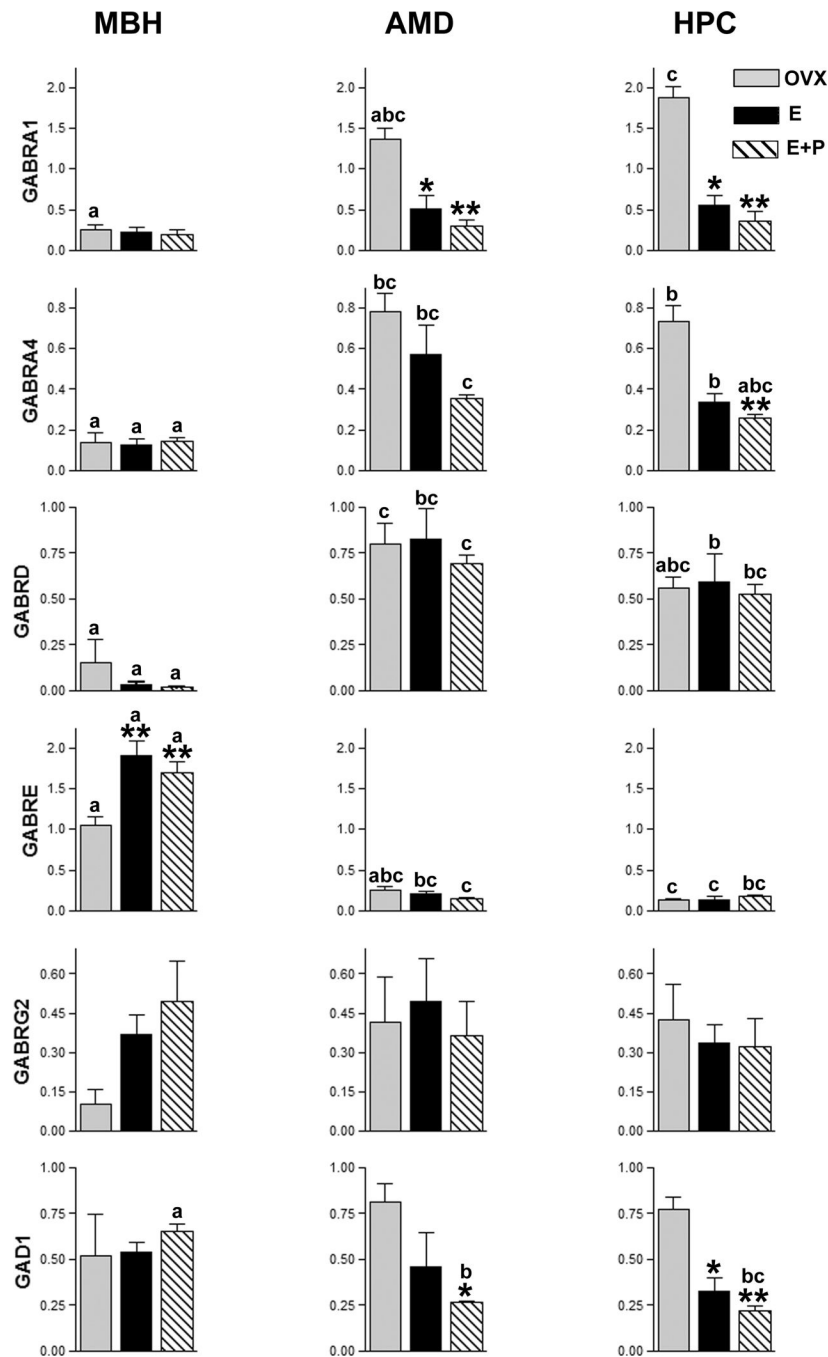the same letter do not differ. "a vs. b" = P < 0.05; "a vs. c" = P < 0.01.

Figure 2. qPCR expression data for genes encoding GABA receptor subunit and *GAD1* in the rhesus macaque

Histograms show mean and SEM from qPCR quantity values normalized using arithmetic means of quantity values for expression of *GAPDH*, *RPL13A* and *ALG9*.

Brain regions: MBH = arcuate nucleus of the medial basal hypothalamus; AMD = amygdala; HPC = hippocampus.

Hormone treatments: OVX = Ovariectomized; E = Estrogen; E+P = Estrogen + Progesterone. Asterisks denote statistical differences in expression for hormone effects within a brain region. Post-hoc test P values are listed with respect to the OVX group for that tissue only. * = P < 0.05; ** = P < 0.01.

Letters denote statistical differences in expression between brain regions under the same hormone treatment (compare bars with the same legend). Bars with the same legend sharing the same letter do not differ. "a vs. b" = $P < 0.05$; "a vs. c" = $P < 0.01$.

Table 1

Microarray analysis summary for expression of genes encoding GABAergic system components in the rhesus macaque.

non subunit Family	GABA Receptor subunit	Brain Region			Treatment			Probeset ID
		MBH	AMD	HPC	Ovx	E	E+P	
	α1	↑↑EP	↓↓EP	↓↓EP	↑↑H	---	↓A	MmuSTS.2385.1.S1_at
	α2	---	---	---	↑H, ↑↑A	---	---	MmuSTS.4812.1.S1_at
	α3	---	---	---	---	---	↓H	MmuSTS.4431.1.S1_at
	α4	↑↑EP	---	↓E, ↓EP	↑H, ↑A	↑H, ↑A	↑H, ↑↑A	MmugDNA.20347.1.S1_at
	α5	---	↑↑EP	---	↑H, ↑↑A	↑H, ↑A	↑H	MmugDNA.3506.1.S1_at
	α6	ND	ND	ND	ND	ND	ND	MmugDNA.20349.1.S1_at
	β1	---	↑↑EP	---	↑↑A	↑↑A	↑↑A	MmugDNA.18163.1.S1_at
	β2	---	---	---	↑H, ↑↑A	---	---	MmuSTS.2387.1.S1_at
A	β3	---	↓EP	---	↑↑A	↑↑A	↑↑H	MmugDNA.2520.1.S1_at
	γ1	---	---	---	↑A	---	---	MmugDNA.14456.1.S1_at
	γ2	---	---	↓EP	↑A	---	↓H, ↓A	MmuSTS.2388.1.S1_at
	γ3	ND	ND	ND	ND	ND	ND	MmugDNA.36967.1.S1_at
	δ	---	---	---	↑↑H, ↑A	↑H, ↑A	↑↑A	MmugDNA.30046.1.S1_at
	ε	---	---	---	↓↓H	↓↓H, ↓A↓↓H, ↓A	MmuSTS.4814.1.S1_at
	θ	ND	ND	ND	ND	ND	ND	MmuSTS.2391.1.S1_at
	π	ND	ND	ND	ND	ND	ND	MmuSTS.4815.1.S1_at
B	1	↑↑EP	---	---	↑↑A	↑↑A	↑↑A	MmugDNA.18163.1.S1_at
	2	↑EP	---	---	↑↑H	↑↑H, ↑A↑H, ↑↑A	MmugDNA.42052.1.S1_at
C	ρ1	ND	ND	ND	ND	ND	ND	MmugDNA.40686.1.S1_at
	ρ2	ND	ND	ND	ND	ND	ND	MmuSTS.4462.1.S1_at
GAD1		---	↓EP	↓EP	---	---	↓↓H, ↓A	MmugDNA.40699.1.S1_at
GAD2		---	↓↓EP	↓EP	---	---	↓↓H	MmugDNA.40703.1.S1_at
SLC32A1		---	↓EP	↓↓EP	---	---	↓↓H	MmugDNA.30515.1.S1_at

The table summarizes analyses of RMA-normalized gene expression using representative probesets on the GeneChip® Rhesus Macaque Genome Array. Arrows indicate Newman-Keuls post hoc test results from Kruskal-Wallis one-way analysis of variance. Selected probesets were prioritized by A-grade annotation and STS designation. For brain region, HPC = hippocampus; AMD = amygdala; and MBH= arcuate nucleus of the medial basal hypothalamus. For treatment, OVX = ovariectomized; E=estrogen treatment; and E+P = estrogen + progesterone treatment. For brain regions, differences are shown with respect to OVX animals. For Treatment, differences are shown with respect to the MBH. ↑= upregulation; ↓= downregulation. Number of arrows indicates post-hoc test P values with respect to the OVX or MBH group. One arrow indicates P < 0.05. Two arrows indicate P < 0.01. Dash (--) indicates no expression differences in a given category. ND = negative detection call (Affymetrix GCOS analysis) in multiple animals in at least two treatment groups.

Table 2

GABAergic system component sorted by probeset annotation.

Target description common names	Gene symbol (search)	Gene symbol (listed)	Probe set I.D.	AG	MBH				# Positive AMD				HPC			
					1	2	3	mean	1	2	3	mean	1	2	3	mean
GABA-A receptor α 1	Gabra1	none	MmuSTS.2385.1.S1_at	A	4	4	4	791	4	4	4	1348	4	4	4	1695
GABA-A receptor α 1	Gabra1	GABRA1	MmugDNA.15252.1.S1_at	B	4	4	4	700	4	4	4	1389	4	4	4	1845
GABA-A receptor α 2	Gabra2	GABRA2	MmugDNA.41722.1.S1_at	A	1	1	2	29	2	2	1	45	4	1	4	40
GABA-A receptor α 2	Gabra2	GABRA2	MmuSTS.4812.1.S1_at	B	4	4	4	784	4	4	4	1467	4	4	4	1238
GABA-A receptor α 3	Gabra3	LOC711230	MmuSTS.4431.1.S1_at	B	4	4	4	440	4	4	4	374	4	4	4	242
GABA-A receptor α 4	Gabra4	LOC704008	MmugDNA.20347.1.S1_at	A	4	4	4	282	4	4	4	1040	4	4	4	908
GABA-A receptor α 4	Gabra4	none	MmugDNA.6656.1.S1_at	A	3	2	2	36	3	3	3	97	4	3	4	68
GABA-A receptor α 5	Gabra5	GABRA5	MmuSTS.2386.1.S1_at	B	4	4	4	448	4	4	4	1437	4	4	4	1780
GABA-A receptor α 5	Gabra5	GABRA5	MmugDNA.3506.1.S1_at	A	3	2	3	75	4	2	4	306	4	4	4	353
GABA-A receptor α 5	Gabra5	GABRA5	MmugDNA.28557.1.S1_s_at	A	2	2	1	78	2	1	2	217	4	0	4	230
GABA-A receptor α 6	Gabra6	GABRA6	MmugDNA.20349.1.S1_at	A	0	0	1	25	1	1	0	35	0	0	0	23
GABA-A receptor β 1	Gabrb1	GABRB1	MmugDNA.24091.1.S1_at	A	4	4	4	3453	4	4	4	3680	4	4	4	3495
GABA-A receptor β 1	Gabrb1	none	MmugDNA.37267.1.S1_at	A	1	0	0	16	0	0	0	19	0	0	0	24
GABA-A receptor β 1	Gabrb1	GABRB1	Mmu.10481.1.S1_at	B	4	1	1	612	4	1	0	540	4	1	0	563
GABA-A receptor β 2	Gabrb2	LOC696767	MmuSTS.2387.1.S1_at	A	1	1	1	67	1	1	0	99	4	0	0	78
GABA-A receptor β 2	Gabrb2	none	MmugDNA.3290.1.S1_at	B	0	0	0	33	2	1	0	60	2	1	0	67
GABA-A receptor β 3	Gabrb3	GABRB3	MmuSTS.4813.1.S1_at	A	4	4	4	2337	4	4	4	3416	4	4	4	3586
GABA-A receptor β 3	Gabrb3	GABRB3	MmugDNA.2520.1.S1_at	A	4	4	4	1052	4	4	4	1535	4	4	4	1293
GABA-A receptor β 3	Gabrb3	GABRB3	MmugDNA.2522.1.S1_at	A	4	4	4	958	4	4	4	1896	4	4	4	1803
GABA-A receptor β 3	Gabrb3	none	MmugDNA.24108.1.S1_at	A	3	4	4	218	4	4	4	366	4	4	4	353
GABA-A receptor β 3	Gabrb3	none	MmugDNA.23997.1.S1_at	A	1	0	0	16	1	0	1	27	0	0	1	24
GABA-A receptor β 3	Gabrb3	GABRB3	MmugDNA.33250.1.S1_at	B	0	0	0	60	0	0	0	79	0	0	0	65
GABA-A receptor γ 1	Gabrg1	none	MmugDNA.14456.1.S1_at	A	4	4	4	247	4	4	4	253	4	4	4	181
GABA-A receptor γ 2	Gabrg2	LOC697248	MmuSTS.2388.1.S1_at	A	4	4	4	1787	4	4	4	1850	4	4	4	1583
GABA-A receptor γ 2	Gabrg2	LOC697248	MmugDNA.20370.1.S1_at	A	4	4	4	491	4	4	4	718	4	4	4	493
GABA-A receptor γ 3	Gabrg3	LOC720390	MmugDNA.36967.1.S1_at	A	2	2	0	46	0	0	0	33	0	0	0	40
GABA-A receptor δ	Gabrd	LOC710062	MmugDNA.30046.1.S1_at	A	0	1	0	34	4	4	4	390	1	4	4	313
GABA-A receptor δ	Gabrd	LOC710062	MmugDNA.20351.1.S1_at	A	0	0	0	15	4	4	4	307	4	4	3	235
GABA-A receptor ε	Gabre	GABRE	MmuSTS.4814.1.S1_at	A	4	4	4	2000	4	4	4	253	4	4	4	194
GABA-A receptor θ	Gabrq	LOC705252	MmuSTS.2391.1.S1_at	A	0	0	2	47	0	0	0	38	0	0	0	22
GABA-A receptor π	Gabrp	GABRP	MmuSTS.4815.1.S1_at	A	0	0	0	10	0	0	0	7	0	0	0	9
GABA-B receptor 1	Gabbr1	LOC708987	MmugDNA.18163.1.S1_at	A	4	4	4	1535	4	4	4	4081	4	4	4	3108
GABA-B receptor 1	Gabbr1	none	MmugDNA.40991.1.S1_at	R	3	4	4	89	3	4	4	112	4	4	4	108
GABA-B receptor 2	Gabbr2	GABBR2	MmugDNA.42052.1.S1_at	A	4	4	4	1985	4	4	4	5440	4	4	4	5901.12
GABA-B receptor 2	Gabbr2	GABBR2	MmuSTS.2471.1.S1_at	A	4	4	4	1220	4	4	4	3485	4	4	4	3634.03
GABA-B receptor 2	Gabbr2	GABBR2	MmugDNA.42052.1.S1_s_at	A	4	4	4	896.3	4	4	4	2884	4	4	4	3011.51
GABA-B receptor 2	Gabbr2	GABBR2	MmugDNA.13130.1.S1_s_at	A	4	3	3	191.1	4	4	4	925	4	4	3	962.675
GABA-B receptor 2	Gabbr2	GABBR2	MmugDNA.29020.1.S1_at	A	4	3	3	106.8	4	4	4	475	4	4	3	518.683
GABA-C receptor ρ 1	Gabrr1	GABRR1	MmugDNA.40686.1.S1_at	A	0	0	0	28	0	0	0	13	0	0	0	14
GABA-C receptor ρ 2	Gabrr2	GABRR2	MmuSTS.4462.1.S1_at	A	0	0	0	34	0	0	0	46	0	1	0	32
GABA(A) receptor-associated protein	GABARAP	GABARAP	MmugDNA.2269.1.S1_at	A	4	4	4	2851	4	4	4	3742	4	4	4	3060.24
GABA receptor-associated protein-like 1	GABARAPL1	LOC702869	MmugDNA.9557.1.S1_at	B	4	4	4	4370	4	4	4	3002	4	4	4	3952.11
glycine cleavage system protein H	GABARAPL1	LOC698520	MmugDNA.9557.1.S1_s_at	A	4	4	4	3252	4	4	4	4484	4	4	4	4023.68
GABA receptor-associated protein-like 1	GABARAPL1	LOC702869	MmugDNA.14486.1.S1_at	B	4	4	4	1692	4	4	4	2448	4	4	4	2131.4
glycine cleavage system protein H	GABARAPL1	LOC698520	Mmu.3418.2.S1_at	A	3	4	4	1525	4	4	4	1928	4	4	4	1695.14
GABA receptor-associated protein like 2	GABARAPL2	LOC714413	MmugDNA.18777.1.S1_at	A	4	4	4	7882	4	4	4	6647	4	4	4	6617.59
GABA receptor-associated protein	GABARAPL2	LOC714413	Mmu.11093.1.S1_at	A	4	4	4	4050	4	4	4	4094	4	4	4	3503.16

Target description common names	Gene symbol (search)	Gene symbol (listed)	Probe set I.D.	AG	MBH				# Positive AMD				HPC			
					1	2	3	mean	1	2	3	mean	1	2	3	mean
like 2																
coiled-coil domain containing 23	SLC32A1	LOC698308	MmugDNA.30515.1.S1_at	A	4	4	4	650.8	4	4	4	512.4	4	4	4	321.483
solute carrier family 6	SLC6A11		MmugDNA.19358.1.S1_at	A	2	3	3	101.1	0	0	0	70.66	0	0	0	50.85
	SLC6A11		MmugDNA.3052.1.S1_at	A	0	0	0	13.56	1	0	2	23.1	0	0	0	12.525
	SLC6A11		MmugDNA.31612.1.S1_at	B	0	0	0	22.92	0	0	0	13.29	0	0	1	14.2667
	SLC6A1		MmuSTS.3877.1.S1_at	A	4	4	4	2314	4	4	4	3351	4	4	4	3241.03
multiple coiled-coil GABABR1-BP	JAKMIP1		MmugDNA.30250.1.S1_at	B	4	4	4	761.5	4	4	4	990	4	4	4	1061.1
glutamate decarboxylase 1	GAD1	GAD1	MmugDNA.40699.1.S1_at	A	4	4	4	3501	4	4	4	2869	4	4	4	2663.33
glutamate decarboxylase 1	GAD1	GAD1	MmuAffx.22.1.S1_at	B	3	3	3	331.3	4	2	1	263	2	2	0	180.9
glutamate decarboxylase 1	GAD1	GAD1	MmugDNA.20165.1.S1_at	A	3	4	4	235.1	4	3	2	245.4	4	3	3	165.375
poly(A) binding protein	GAD2	LOC721857	MmugDNA.40703.1.S1_at	A	4	4	4	2084	4	4	4	2225	4	4	4	1708.69
Glutamate decarboxylase 2	GAD2		MmugDNA.34507.1.S1_at	A	4	4	4	634.4	4	4	4	390	4	4	4	445.408
Glutamate decarboxylase 2	GAD2	GAD2	MmugDNA.12677.1.S1_at	A	2	1	2	119.3	3	2	2	115.9	2	2	1	117.4
poly(A) binding protein	GAD2	LOC721857	MmugDNA.3230.1.S1_at	B	2	4	3	95.1	4	1	3	73.57	4	2	2	80.825
	GAD2		MmugDNA.33087.1.S1_at	C	2	3	3	54.56	3	0	1	30.36	3	1	0	37.15
poly(A) binding protein	GAD2	LOC721857	MmugDNA.6977.1.S1_at	B	0	0	1	48.1	1	0	0	35.5	1	0	0	31
	GAD2		MmugDNA.9669.1.S1_at	A	0	0	0	8.817	0	0	0	12.37	0	0	0	14.1417

The table summarizes MAS 5.0 analysis of GeneChip® Rhesus Macaque Genome Arrays showing mean detection signals and number of animals with positive detection calls for genes encoding GABAergic system components. The search gene symbol is the text string used to retrieve annotation information in the Affymetrix NetAffx analysis center. The listed gene symbols are NetAffx annotations for specific probe sets retrieved under the gene symbol search. "AG" = Annotation grade (NetAffx); "# Positive" = number of animals showing positive detection for the given gene; "MBH" = arcuate nucleus of the medial basal hypothalamus; "HPC" = Hippocampus; "AMD" = Amygdala; "1" = OVX; "2" = E; "3" = E+P; "mean" = mean detection signal across the three treatment groups for the given tissue (MBH, HPC or AMD).