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Brain region- and sex-specific alterations in DAMGO-stimulated [^{35}S]GTPγS binding in mice with *Oprm1* A112G

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Abstract

The A118G single nucleotide polymorphism (SNP) of the human μ fopioid receptor (MOPR) gene (OPRM1) was associated with heightened dopamine release by alcohol intake, better treatment outcome for nicotine and alcohol addiction and reduced analgesic responses to morphine. A mouse model that possesses the equivalent substitution (A112G) in the oprm1 gene was generated to delineate the mechanisms of the impact of the SNP. Mice homozygous for the G allele (G/G) displayed lower morphine-induced antinociception than A/A mice, similar to humans. In this study, we examined whether A112G SNP affected MOPR-mediated G protein activation in the mouse model. We compared A/A and G/G mice in the MOPR selective agonist DAMGO-stimulated [^{35}S]GTPγS binding in brain regions by autoradiography. When the data of males and females were combined, G/G mice exhibited lower DAMGO-stimulated [^{35}S]GTPγS binding in the VTA than A/A mice, in accord with previously reported reduced morphine-induced hyperactivity and locomotor sensitization in G/G mice. In the NAc core, female G/G mice displayed lower DAMGO-stimulated [^{35}S]GTPγS binding than female A/A mice, which is consistent with previously reported deficiency in morphine-induced conditioned place preference in female G/G mice. In G/G mice, males showed higher DAMGO-stimulated [^{35}S]GTPγS binding than females in the cingulate cortex, CPu, NAc core, thalamus and amygdala. Thus A112G SNP affects DAMGO-stimulated [^{35}S]GTPγS binding in region- and sex-specific manners.

Keywords

A118G; mu opioid receptor; single nucleotide polymorphism; [^{35}S]GTPγS binding autoradiography

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Author contribution

Participated in research design: Liu-Chen, Wang, Huang, Blendy
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Introduction

The A118G single nucleotide polymorphism (SNP) of the human μ opioid receptor gene (OPRM1) is the most common SNP in the coding region of the MOPR (Bergen, Kokoszka, Peterson et al., 1997; Bond, LaForge, Tian et al., 1998). This SNP results in a change of amino acid at position 40 from asparagine to aspartate (N40D) in the N-terminal domain, which removes one of five potential N-linked glycosylation sites of the receptor (Bergen et al., 1997; Bond et al., 1998). Recently we reported that this SNP reduced the N-glycosylation and stability of the MOPR protein (Huang, Chen, Mague et al., 2012). The A118G allele frequency varies among ethnic groups: 1–3% in African Americans, 10–14% in both Caucasians and Hispanics, 35–49% in Asians and 8–21% in other populations [reviewed in (Kreek, Bart, Lilly et al., 2005; Mague and Blendy, 2010)]. Clinical studies revealed that among alcohol dependent patients treated with naltrexone, those with one or two copies of the G118 allele had better outcome (Oslin, Berrettini, Kranzler et al., 2003; Kim, Kim, Choi et al., 2009). Among individuals using transdermal nicotine for smoke cessation, those of the A/G and G/G genotype had better responses (Lerman, Wileyto, Patterson et al., 2004). Ramchandani, Umhau, Pavon et al. (2011) reported that alcohol intake induced dopamine release in carriers of the G118 allele, but not in A118 homozygotes. In addition, subjects with G118 allele needed higher morphine doses to attain adequate pain control for acute postoperative pain compared to those homozygous for the A118 allele subjects (Chou, Wang, Liu et al., 2006; Chou, Yang, Lu et al., 2006; Sia, Lim, Lim et al., 2008; Hayashida, Nagashima, Satoh et al., 2008; Campa, Gioia, Tomei et al., 2008). Studies on the role of G118 allele in alcohol or drug dependence have reported positive, negative, or no associations [for reviews, see (Kreek et al., 2005; Mague and Blendy, 2010)].

Mague, Isiegas, Huang et al. (2009) generated a mutant mouse line that possessed the mouse equivalent (A112G, N38D) of the human SNP for studying the mechanisms underlying the changes associated with OPRM1 A118G SNP in humans. The mice homozygous for G112 allele (G/G mice) displayed reduced antinociceptive responses to morphine than those homologous for A112 allele (A/A mice) (Mague et al., 2009), similar to the results in humans. In addition, G/G mice showed reduced morphine-induced hyperactivity and locomotor sensitization (Mague et al., 2009). Moreover, female, not male, G/G mice failed to show a conditioned place preference to morphine-paired environments (Mague et al., 2009). Recently, Ramchandani et al. (2011) have also generated a mouse model of the OPRM1 A118G SNP by replacing the mouse exon 1 with the human exon 1 carrying the A118 or G118 allele. They found that homozygous G118 mice showed a 4-fold greater peak dopamine response to an alcohol challenge as homozygous A118 mice. Furthermore, Mahmoud, Thorsell, Sommer et al. (2011) reported that the potency and efficacy of morphine in modulating Ca^{2+} channels were reduced in sensory neurons of mice expressing the 118 GG gene compared with those expressing the 118AA version.

We have demonstrated that mice with oprm1 A112G displayed lower MOPR expression in some, but not all, brain regions (Wang, Huang, Ung et al., 2012). The effects of A112G on MOPR protein expression have sex differences in some brain regions (Wang et al., 2012). In the mouse brains A112G reduced N-glycosylation of the MOPR and in cultured cells

A118G in the human MOPR decreased N-glycosylation and protein stability (Huang et al., 2012). The reduction in MOPR expression in brain regions by the A112G (Wang et al., 2012) is likely to be involved in attenuated morphine-induced antinociception, hyperactivity and locomotor sensitization (Mague et al., 2009). The observed behavioral changes in A112G mice may also be due to alterations in MOPR-G protein coupling, in addition to changes in MOPR level. In the present study, we investigated whether A112G SNP affected MOPR-mediated G protein activation among brain regions by quantitative in vitro autoradiography of DAMGO-stimulated [^{35}S]GTPγS binding.

Materials and methods

Animals

A112G-MOPR knock-in mice were generated on C57BL/6 genetic background in Dr. Blendy's laboratory. The animals (8–15 weeks, 18–30 g) were housed in the University of Pennsylvania animal facility and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Animals were used in accordance with the methods approved by University of Pennsylvania Animal Care and Use Committee. Mice homozygous for the A112 or G112 allele are used for the study.

Materials

DAMGO, GDP and GTPγS were purchased from Sigma Chemical Co (St. Louis, MO). [^{35}S]GTPγS (1250 Ci/mmole) and phosphor screens were obtained from Perkin Elmer (Boston, MA). [^{14}C]microscales were purchased from GE healthcare, formerly Amersham Biosciences (Arlington Heights, IL). All other reagent-grade chemicals were obtained from Sigma Chemical Co (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

In vitro autoradiography of DAMGO-stimulated [^{35}S]GTPγS binding

The experiments were conducted according to our published procedures, which were adapted from those of (Sim and Childers, 1997; Sim-Selley, Selley, Vogt et al., 2000). Coronal sections (20 μm) of regions of interest were cut on a cryostat (Leica CM3050 S) maintained at −20°C, thaw-mounted onto gelatin-subbed slides, and stored at −80°C until processed. Sections were incubated with 2 mM GDP in [^{35}S]GTPγS binding buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) for 15 min at 25°C, and then incubated for 2 hr at 25°C in [^{35}S]GTPγS binding buffer with 0.04 nM [^{35}S]GTPγS and 2 mM GDP with or without the μ agonist DAMGO (10 μM). After incubation, sections were rinsed twice (2 minutes each) in cold 50 mM Tris-HCl buffer, pH 7.4, once (30 seconds) in deionized H_2O, and then dried thoroughly and exposed to phosphor screens for 48 hr in cassettes along with [^{14}C]microscales for densitometric analysis.

Autoradiography data analysis—Images captured on phosphor screens were visualized using a Cyclone Storage Phosphor Scanner (Packard Bioscience) and data were analyzed using the OptiQuant program associated with scanner. Images were quantified by densitometric analysis with [^{14}C] standards. A brain paste standard assay was performed to determine the correction factor necessary to calculate nCi/g for [^{35}S] from nCi/g for [^{14}C], as previously described (Sim and Childers, 1997). Net agonist-stimulated [^{35}S]GTPγS

binding was calculated by subtracting basal binding (obtained in the absence of agonist) from agonist-stimulated binding. Data were reported as mean values ± S.E.M.

Statistical analysis

Two-way ANOVA was used to determine statistical significant differences between groups, with sex and genetic factor as main factors. For comparison of two groups within one factor, two-tailed Student's *t* test was performed. *$p<0.05$, **$p<0.01$ was the level of significance in all statistical analyses.

Results

DAMGO-stimulated [^{35}S]GTPγS binding in A/A and G/G mouse brains

DAMGO-stimulated [^{35}S]GTPγS binding, a measure of MOPR-mediated G protein activation (Traynor and Nahorski, 1995; Sim, Selley, and Childers, 1995), was performed on coronal sections of both the A/A and G/G mouse brains at 3 anatomical levels. There is no difference between A/A and G/G on the overall anatomical distribution of G protein activation; therefore, representative autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding in male A/A mice are shown in Fig. 1. High levels of DAMGO-stimulated [^{35}S]GTPγS binding were observed in the cortices, CPu, NAc in the telencephalon (Fig. 1A) and in the thalamus, hypothalamus and amygdala in the diencephalon (Fig. 1B). In the midbrain, DAMGO-stimulated [^{35}S]GTPγS binding were found in the substantial nigra, PAG and VTA, albeit at lower levels (Fig. 1C).

We compared the levels and distribution of the DAMGO-stimulated [^{35}S]GTPγS binding between A/A and G/G mice brains. Representative pseudo-color autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding in male A/A and G/G mice are shown in Fig. 2. A/A and G/G mice displayed similar regional distribution (Fig. 2). Quantitation of [^{35}S]GTPγS binding in the presence and absence of DAMGO was performed by measuring the density of autoradiograms in brain regions. Net DAMGO-stimulated [^{35}S]GTPγS binding was obtained by subtracting the basal binding from the binding in the presence of DAMGO. Density was converted to radioactivity using calibration curves generated from the [^{14}C] standards. Table 1 shows the combined data of male and female mice of the same genotype and analyses of basal and net DAMGO-stimulated [^{35}S]GTPγS binding. In the VTA, A/A mice displayed significantly lower basal [^{35}S]GTPγS binding, but higher net DAMGO-stimulated [^{35}S]GTPγS binding than G/G mice. In all the other brain regions, there are no significant differences between A/A and G/G mice in basal and net DAMGO-stimulated [^{35}S]GTPγS binding, but G/G mice show a trend of higher basal [^{35}S]GTPγS binding than A/A mice. Moreover, there are large regional variations in basal and net DAMGO-stimulated [^{35}S]GTPγS binding.

Sex Differences in net DAMGO-stimulated [^{35}S]GTPγS binding

To explore if there are sex differences in basal or DAMGO-stimulated [^{35}S]GTPγS binding in A/A and G/G mice, the data of males and females were analyzed separately. There were no sex differences in basal [^{35}S]GTPγS binding in A/A or G/G mice in any of the brain regions. As shown in Fig. 3, males G/G mice showed significantly higher net DAMGO-

stimulated [^{35}S]GTPγS binding than female G/G mice in the cingulate cortex, CPu, NAc core, thalamus and amygdala. In contrast, there are no sex differences in A/A mice. In addition, in the NAc core, female A/A mice displayed significantly higher DAMGO-stimulated [^{35}S]GTPγS binding than female G/G mice (Fig. 4).

Discussion

The current studies demonstrated that A112G SNP led to lower DAMGO-stimulated [^{35}S]GTPγS binding in the VTA. In addition, A112G SNP resulted in reductions in DAMGO-stimulated [^{35}S]GTPγS in the NAc core in females, but not in males. These biochemical changes resulting from A112G SNP may correlate with alterations in morphine-induced in vivo pharmacological effects, which are discussed below.

Our results are different from those of (Ramchandani et al., 2011) on the A118G mice they generated. They found that there were no differences between G/G and A/A mice in DAMGO-stimulated [^{35}S]GTPγS binding in the dorsal striatum, NAc and VTA. The discrepancy may be due to different mouse models used. The mice used in the present study has A112G substitution in the mouse MOPR sequence, where those in the Ramchandani et al. (2011) study have the first exon of the human MOPR gene with A118 and G118 in A/A and G/G mice, respectively.

Reduced DAMGO-stimulated [^{35}S]GTPγS binding by A112G SNP in the VTA may be related to decreased morphine-induced hyperactivity and locomotor sensitization

VTA plays an important role in morphine-induced hyperactivity and locomotor sensitization (Bunney, Massari, and Pert, 1984; Kalivas and Duffy, 1987). Our observations that A112G SNP led to reduced DAMGO-stimulated [^{35}S]GTPγS binding in VTA are consistent with the findings that G/G mice showed decreased morphine-induced hyperactivity and locomotor sensitization (Mague et al., 2009). In addition, we have demonstrated that A112G SNP results in reduced MOPR expression in the VTA (Wang et al., 2012), which may play a role as well.

Reduced DAMGO-stimulated [^{35}S]GTPγS binding in the NAc core of female G/G mice may contribute to impaired morphine-induced conditioned place preference

The NAc is a key neural substrate involved in rewarding properties of drugs of abuse, including morphine (Koob, Sanna, and Bloom, 1998). We found that in the NAc core female G/G mice displayed significantly lower DAMGO-stimulated [^{35}S]GTPγS binding than female A/A mice; however, there is no difference between male A/A and male G/G mice. These differences are likely to contribute to the previous report that female G/G mice failed to show a morphine-induced conditioned place preference, in contrast to female A/A, male A/A and male G/G mice (Mague et al., 2009).

Effects of A112G SNP on DAMGO-stimulated [^{35}S]GTPγS binding have sex differences

We found that among G/G mice males displayed higher DAMGO-stimulated [^{35}S]GTPγS binding than females in the cingulate cortex, CPu, thalamus, NAc core and amygdala; however, no sex differences were observed among A/A mice. These results indicate that the

sex differences are limited to the G/G mice in these regions. In future studies, it may be important to examine if sex differences are present in the G/G mice in behavioral endpoints mediated by the MOPR in these brain regions.

We have found that male A/A mice showed higher [^{3}H]DAMGO binding than G/G mice in more brain regions than female A/A over G/G (Wang et al., 2012). DAMGO-stimulated [^{35}S]GTPγS binding did not show a similar trend as [^{3}H]DAMGO binding. There are several possible contributing factors for the discordance between DAMGO-stimulated [^{35}S]GTPγS binding and [^{3}H]DAMGO binding to the MOPR, as we previously discussed (Wang et al., 2012). N-linked glycosylation (Knezevic, Polasek, Gornik et al., 2009; Stanta, Saldova, Struwe et al., 2010; Huang et al., 2012), regional differences in MOPR-G protein coupling efficiency (Wang, Rasakham, Huang et al., 2011), hormonal state (Loyd, Wang, and Murphy, 2008), and epigenetic regulation [reviewed in (Jessen and Auger, 2011)] may influence sex-specific protein expression. It appears that these factors affect G/G mice, but not A/A mice, of which the underlying mechanisms remain to be investigated.

Lack of correlation between DAMGO-stimulated [^{35}S]GTPγS binding and receptor levels

We found that A112G SNP led to reduced DAMGO-stimulated [^{35}S]GTPγS binding only in the VTA when data of male and female mice were combined. In contrast, our previous studies revealed that A112G SNP resulted in lower MOPR expression in several brain regions, including VTA, cortices, NAc, hypothalamus, amygdala, PAG, and superficial layers of the superior colliculus (Wang et al., 2012). Our data indicate that changes in DAMGO-stimulated [^{35}S]GTPγS binding levels do not correlate with alterations in MOPR levels. A similar lack of a close correlation has been observed between mu, kappa or cannabinoid receptor and G protein activation (Breivogel, Sim, and Childers, 1997; Vogt, Sim-Selley, Childers et al., 2001; Wang et al., 2011). The mechanisms underlying these discrepancies are not yet clear, but some factors may be involved. The ratios of receptor to G proteins may vary among brain regions. MOPRs have been shown to activate different types of G proteins (Chakrabarti, Prather, Yu et al., 1995), which may lead to different numbers of G proteins activated per receptor in different regions. It is also possible that some MOPRs may not be coupled functionally, due to their intracellular location (Wang, Van Bockstaele, and Liu-Chen, 2008).

Regional differences in basal [^{35}S]GTPγS binding

In the VTA, G/G mice (males and females pooled) exhibited significantly higher basal [^{35}S]GTPγS binding than A/A mice. In most of the other regions, G/G mice showed a trend of higher basal levels, but they did not reach statistical significance. Previously Mague et al. (2009) reported lower MOPR protein levels by immunoblotting and lower B_{max} value of [^{3}H]DAMGO binding to the MOPR in the brains of G/G mice, compared to A/A mice. We also found that A/A mice displayed higher MOPR expression than G/G mice in both males and females (Wang et al., 2012). Therefore, the higher basal [^{35}S]GTPγS binding in G/G mice is not due to differences in the MOPR level. Basal [^{35}S]GTPγS binding may represent constitutive (agonist-independent) activation of Gα subunits of trimeric G proteins and many other guanine nucleotide binding proteins present in the brain. Differences in levels and activities of these guanine nucleotide binding proteins may contribute to the differences

in basal [^{35}S]GTPγS binding between A/A and G/G mice. It has also been shown that [^{35}S]GTPγS binding to NDPK accounts for part of the high basal binding (Zhang, Li, Chen et al., 1999).

In addition, there is a marked variation in basal [^{35}S]GTPγS binding across different brain regions. For example, in the A/A mice brain, the basal [^{35}S]GTPγS binding ranged from 1127 nCi/g in the VTA to 5028 nCi/g in the superficial layers of the superior colliculus. Our findings are consistent with those of Sim, Selley, Dworkin et al. (1996). GDP is required to reduce basal [^{35}S]GTPγS binding (Traynor and Nahorski, 1995) and the concentration required varies among cells and tissues (Huang, Xu, Yoon et al., 2007). It is conceivable that brain regions may require different GDP concentrations to achieve similar basal [^{35}S]GTPγS binding. However, we had to use one concentration of GDP (2 mM) in the experiment for all brain regions, which may contribute to varying levels of basal [^{35}S]GTPγS binding. Thus, the net DAMGO-stimulated [^{35}S]GTPγS binding as % of basal binding is consequently variable across brain regions and is quite low in some regions, which are consistent with the finding of (Wang et al., 2011) and Sim et al. (1996).

In summary, A112G affected DAMGO-stimulated [^{35}S]GTPγS binding in region- and sex-specific manners. A112G SNP led to reduced DAMGO-stimulated [^{35}S]GTPγS binding in the VTA, which may be associated with lower morphine-induced hyperactivity and locomotor sensitization. There are sex differences in the effects of A112G SNP in NAc core that may contribute to lack of morphine-induced conditioned place preference in female G/G mice. In G/G mice males showed significantly higher net DAMGO-stimulated [^{35}S]GTPγS binding than females in some brain areas, of which the in vivo pharmacological consequences remain to be examined.

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ABBREVIATIONS

A/A	homozygous for A112 allele
G/G	homozygous for G112 allele
MOPR	mu opioid receptor
OPRM1* and *oprm1	human and mouse MOPR genes, respectively
SNP	single nucleotide polymorphism
CPu	caudate putamen
NAc	nucleus accumbens
PAG	periaqueductal gray
VTA	ventral tegmental area

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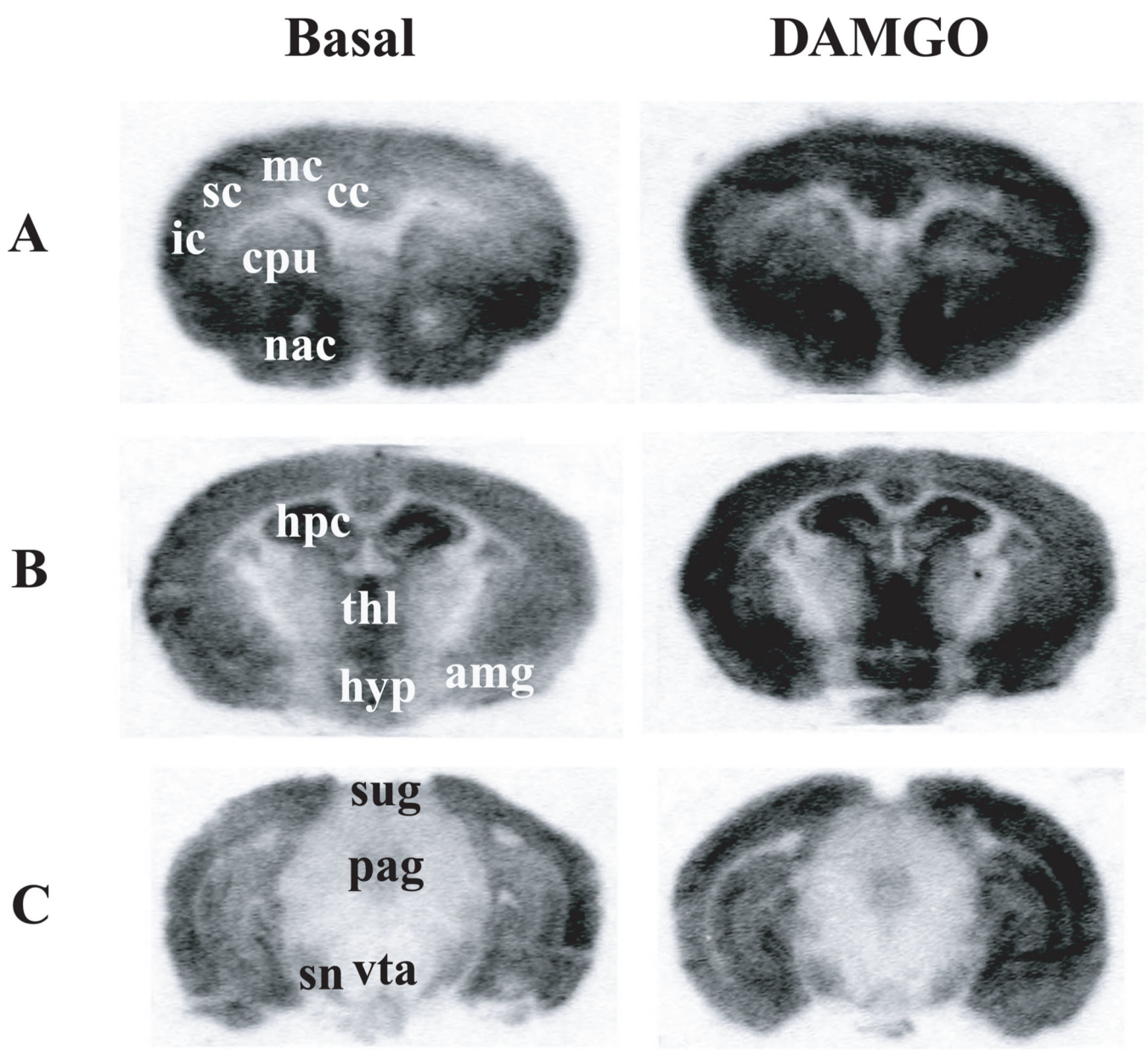

Figure 1. Autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding in selected coronal sections of male A/A mouse brains at the telencephalon (A), diencephalon (B) and midbrain (C) levels

Sections were incubated with ~0.04 nM [^{35}S]GTPγS and 2 mM GDP in the absence (basal) or presence of 10 μM DAMGO for 2 hr at 25°C, washed, dried, and exposed to phosphor screens for 48 hr. Abbreviation: cc, cingulate cortex; mc, motor cortex; sc, somatosensory cortex; ic, insular cortex; cpu, caudate putamen; acb, nucleus accumbens; hpc, hippocampus; hyp, hypothalamus; thl, thalamus; amg, amygdala; pag, periaqueductal gray; sn, substantial nigra; sug, superficial grey of superior colliculus; vta, ventral tegmental area.

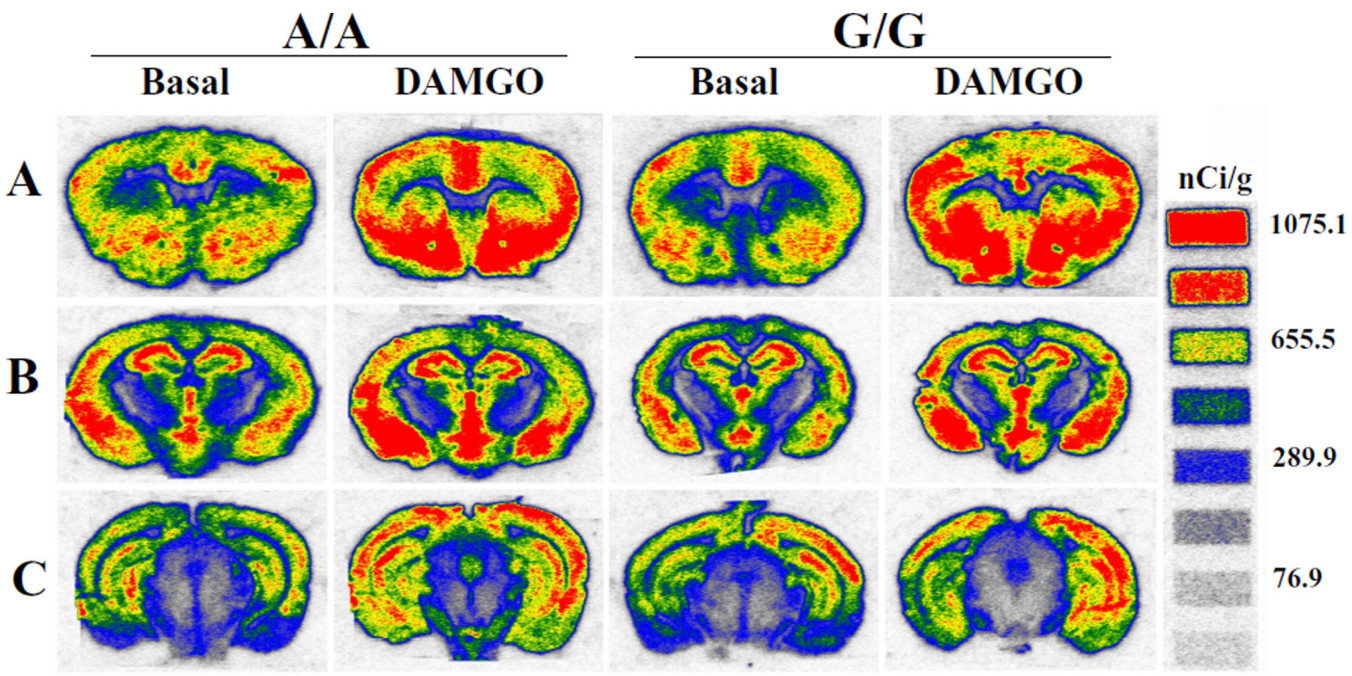

Figure 2. Pseudo-color autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding to the MOPR in selected coronal sections of A/A and G/G male mouse brains at 3 anatomical levels (A–C)

Experiments were carried out as described in Fig. 1 legend.

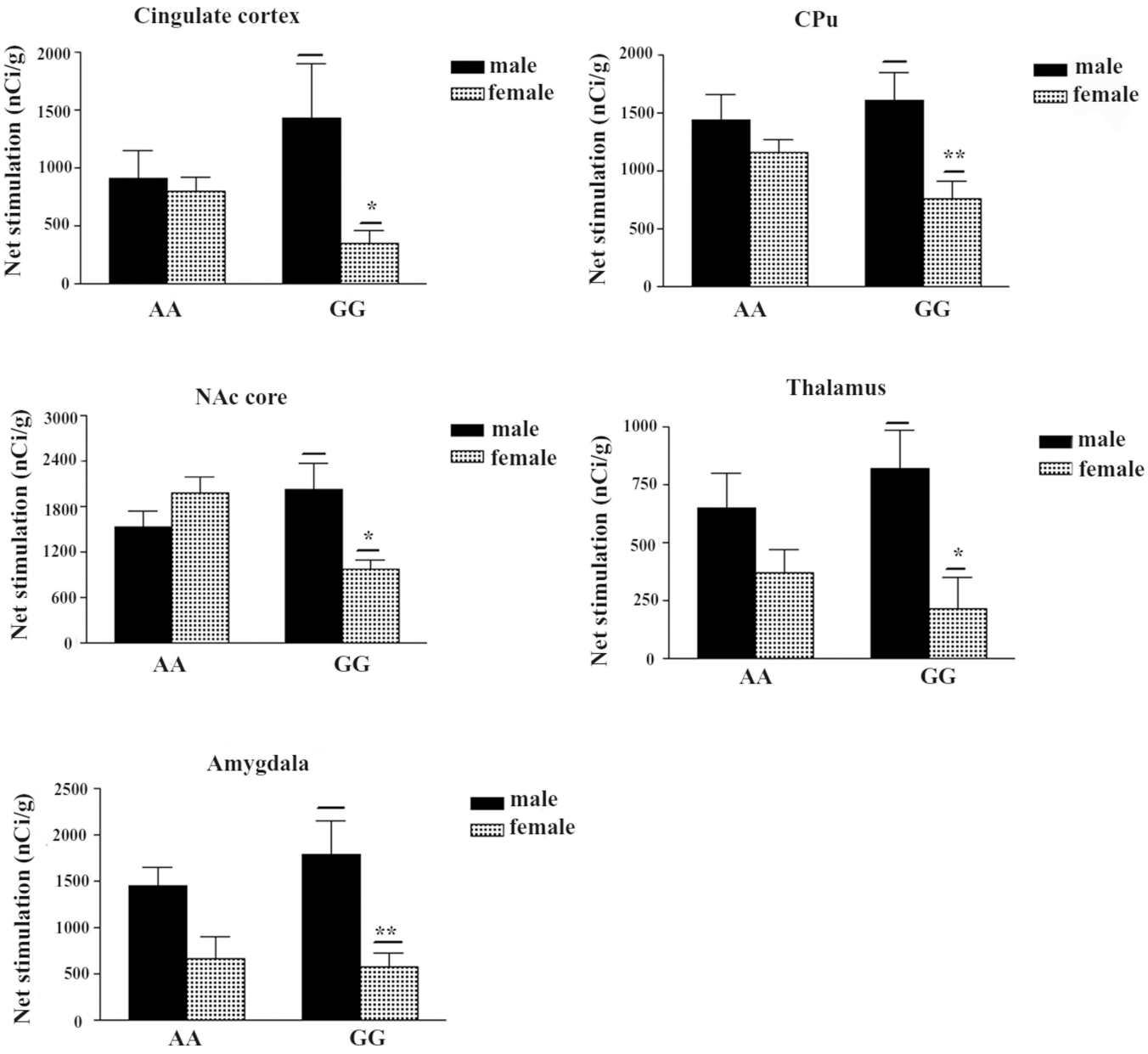

Figure 3. Brain regions that show significant sex differences in DAMGO-stimulated [^{35}S]GTPγS binding in G/G mice

Data are expressed at mean ± S.E.M. of five to six animals, three or four sections from each brain. *p < 0.05 different from G/G male by the non-paired two-tailed Student's *t* test.

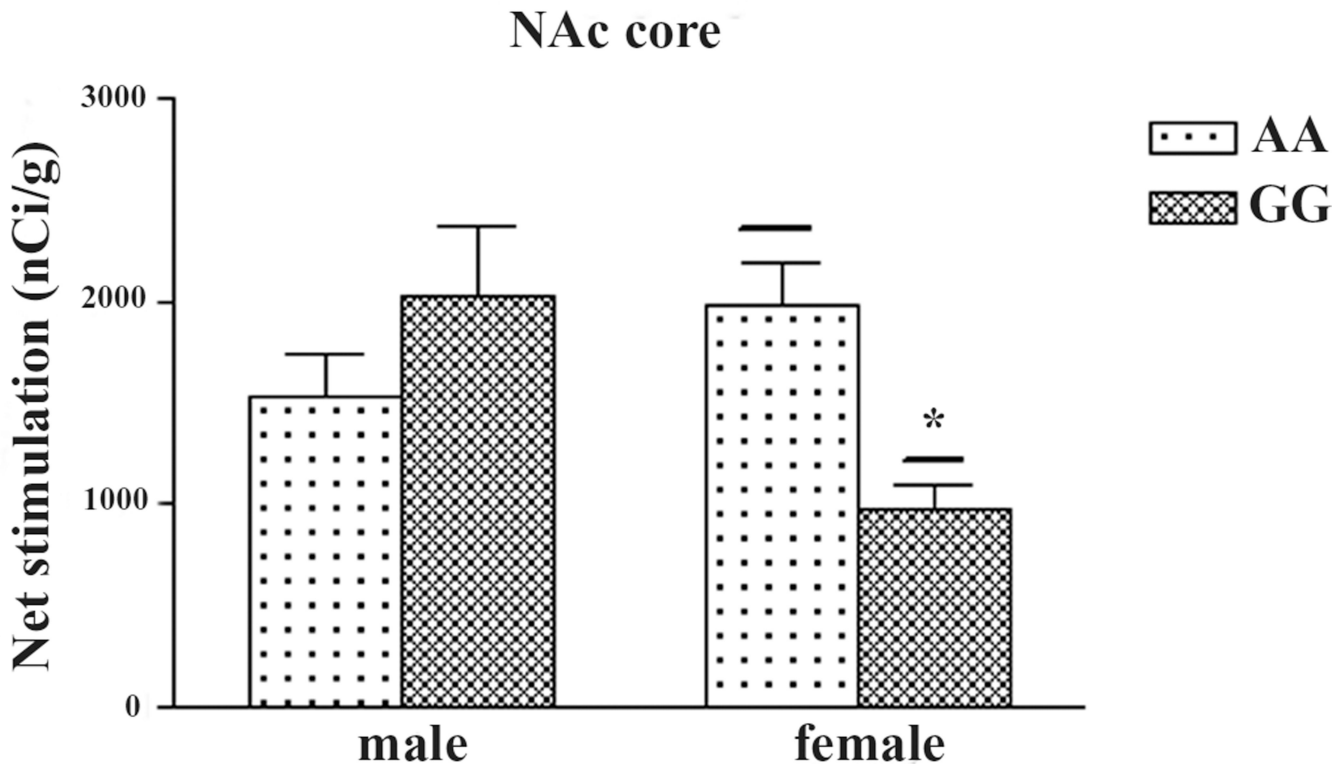

Figure 4. In the nucleus accumbens core, there is significant genotype difference in DAMGO-stimulated [^{35}S]GTPγS binding in female mice

Data are expressed at mean ± S.E.M. of five to six animals, three or four sections from each brain. *p < 0.05, **p < 0.01 different from A/A female by the non-paired two-tailed Student's *t* test.

Table 1

Quantitation of in vitro autoradiography of DAMGO-stimulated [^{35}S]GTPγS binding in brains of A/A and G/G mice.

Region	Basal [^{35}S]GTPγS binding (pCi/mg)		Net DAMGO-stimulated [^{35}S]GTPγS binding (pCi/mg)	
	A/A	G/G	A/A	G/G
Cingulate Cortex	3644 ± 147	3933 ± 255	861 ± 134	888 ± 282
Motor Cortex	3206 ± 179	3562 ± 170	453 ± 100	569 ± 225
Somatosensory Cortex	3386 ± 211	3839 ± 105	328 ± 102	481 ± 251
Insular Cortex	3916 ± 206	4408 ± 158	599 ± 173	663 ± 187
CPu	2822 ± 179	2965 ± 80	1300 ± 121	1187 ± 184
NAc				
Core	4714 ± 259	4971 ± 237	1727 ± 160	1542 ± 254
Shell	4135 ± 245	4606 ± 150	1512 ± 150	1511 ± 217
Hippocampus	4108 ± 198	4713 ± 193	277 ± 89	308 ± 129
Amygdala	4016 ± 138	4192 ± 260	1090 ± 192	1177 ± 262
Thalamus	2959 ± 79	3073 ± 190	520 ± 101	519 ± 135
Hypothalamus	3514 ± 212	4332 ± 357	937 ± 130	996 ± 256
PAG	2432 ± 119	2278 ± 184	714 ± 93	600 ± 152
Substantial nigra	2730 ± 161	2892 ± 133	538 ± 88	445 ± 90
VTA	1127 ± 96	1419 ± 74*	693 ± 161	339 ± 86*
Superifical layers of the superior colliculus	5028 ± 249	6382 ± 592	1279 ± 272	706 ± 244

Quantitation was made from both left and right sides of each section. Data are expressed as mean ± S.E.M. of eleven to twelve animals, three to four sections from each brain.

* $p<0.05$, different from A/A mice by the non-paired two-tailed Student's *t* test