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REGIONALLY SELECTIVE EFFECTS OF GABA ON HYPOTHALAMIC GABA$_A$ RECEPTOR mRNA *IN VITRO*

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Abstract

We tested whether GABA$_A$ receptor (R) subunit mRNA levels are homeostatically influenced by short-term exposure to GABA in two adjacent regions of the posterior hypothalamus. mRNA levels for seven GABA$_A$R subunits and GABA-synthesizing enzyme (GAD) were quantified in the perifornical (PF) and dorsomedial (DM) hypothalamus following superfusion of slices for 90 min with a drug-free medium, GABA uptake blocker with or without GABA$_A$R antagonist, gabazine, or GABA$_A$R agonist with tetrodotoxin. Increasing endogenous GABA decreased mRNAs for all seven GABA$_A$R subunits in the PF, and for three also in the DM, region; gabazine antagonized these effects in the PF region only and increased GAD-65 mRNA. Stimulation of GABA$_A$Rs in the presence of tetrodotoxin decreased mRNA for one GABA$_A$R subunit (β_1). We conclude that, in the PF region where GABA facilitates sleep, increased GABA release may limit GABA$_A$R-mediated inhibition, whereas in the DM region, GABA-induced changes are mainly mediated by non-GABA$_A$ receptors.

Keywords

GABA$_A$ receptors; gabazine; GAD; hypothalamus; muscimol; RT-PCR; sleep homeostasis; synaptic plasticity

INTRODUCTION

GABA$_A$ receptors (GABA$_A$Rs) control excitability of neurons by opening Cl channnels, thereby causing a rapid hyperpolarization. Functional GABA$_A$Rs are pentamers assembled from at least 20 subunits [1]. Inhibition mediated by GABA$_A$Rs can be modulated by exogenous and endogenous compounds through alterations in the receptors' sensitivity, trafficking and assembly. Such a plasticity of GABAergic inhibition occurs in cortical and hippocampal neurons, as well as neuronal cell cultures, following treatments lasting several days [2-7], and has been discussed in the context of homeostatic mechanisms that regulate synaptic strength in relation to the magnitudes of synaptic excitation and inhibition [8]. However, it is not clear whether such a plasticity also contributes to short-term regulation of GABA$_A$Rs in relation to the control of normal physiologic functions. Here, we focused on the potential for GABA$_A$R regulation at the mRNA level in the posterior hypothalamus, where manipulations with GABAergic transmission have powerful effects on sleep and wakefulness.

The perifornical (PF) region of the posterior hypothalamus has dense GABAergic innervation [9,10]. GABA$_A$R-mediated inhibition exerted in this region may impact several homeostatically regulated systems, including those for feeding, metabolism and motor activity, but its role in the control of sleep is especially well documented. Endogenous GABA levels

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increase in the PF region during slow-wave sleep (SWS) [11], local microinjections of $GABA_AR$ agonists facilitate sleep [12], and $GABA_AR$ antagonists promote wakefulness [13, 14]. Importantly, the sleep-promoting effects of GABA in the posterior hypothalamus appear to be strengthened following anterior hypothalamic lesions that eliminate an important source of GABAergic input to the posterior hypothalamus and cause insomnia [12]. These findings prompted us to hypothesize that the potency of GABAergic inhibition in the PF region may increase following a period of reduced stimulation of $GABA_ARs$. Such a mechanism could contribute to the homeostatic regulation of sleep by facilitating sleep after a period of wakefulness and by reducing sleep propensity ("sleepiness") following a period of rest, thus acting over a time-scale compatible with the normal sleep-wake cycle [15]. To begin addressing this hypothesis, we developed an *in vitro* model that allows one to quantify the local effects of GABA on mRNAs relevant for GABAergic transmission. We determined that a 90 min-long increase in GABAergic inhibition within the posterior hypothalamus decreases mRNA levels for $GABA_AR$ subunits in a regionally selective and $GABA_AR$-dependent manner. A preliminary report has been published [16].

EXPERIMENTAL PROCEDURES

Animals

Eighteen adult male Sprague-Dawley rats (300-370 g) were used. The procedures for animal handling followed the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

In vitro exposure of hypothalamic slices to GABAergic drugs

Rats were deeply anaesthetized with isoflurane (3.5%) and decapitated. Transverse slices, 400 μm thick, were cut, and one was selected from the region just caudal to the decussation of the optic tract and divided along the midline. Each half-slice was initially placed in a separate perfusion chamber (0.4 ml) and superfused (1ml/min) for 1 h at room temperature with O_2-saturated artificial cerebrospinal fluid (ACSF, see [17] for composition; the same medium was previously used to record from hypothalamic neurons [18]).

Gabazine (SR-95531), a selective $GABA_AR$ antagonist; muscimol, a selective agonist of $GABA_ARs$, and NO-711, a selective blocker of type 1 GABA transporter (uptake blocker) [19], were purchased from Sigma-Aldrich (Saint Louis, MO), and tetrodotoxin (TTX) from Tocris (Ellisville, MO). All drugs were freshly dissolved in ACSF. The concentration of gabazine (20 μM) was sufficient to fully and selectively block and functionally antagonize $GABA_ARs$ in hypothalamic slices *in vitro* [20,21].

After stabilization in ACSF, to increase endogenous GABA levels, one half-slice was superfused with ACSF containing 20 μM NO-711 only and the other with 20 :M gabazine and NO-711, both for 90 min at 34°C. In another series, one half-slice was superfused with 10 :M muscimol and 1 μm TTX, and the other with TTX only. TTX was used to suppress synaptic interactions among cells in the slice, with 1 μm being sufficient to block synaptic transmission in hypothalamic slices [18]. To determine mRNA levels under baseline conditions, separate half-slices were superfused with ACSF for 90 min at 34°C.

All exposures were concluded with 10 min superfusion with ACSF at 34°C, and then two 700 μm circular punches were cut from each half-slice, one from the perifornical (PF) region and the other from the dorsomedial/paraventricular (DM) region (see Fig. 1A and [17] for anatomical definitions of PF and DM punches). To verify punch locations, slices were fixed in formalin, cut into 25 μm sections, mounted and stained with Neutral red.

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Quantitative RT-PCR, quality control and data analysis

RNA extraction, its quantification, and subsequent quantitative RT-PCRs were performed as described previously [17]. Total RNA was extracted from each punch, and one half of each sample reverse-transcribed (PowerScript reverse transcriptase, BD Biosciences-Clontech, Palo Alto, CA). PCRs were conducted and calibrated using LightCycler® system (Roche Diagnostics, Indianapolis, IN), with primer sets for $GABA_AR$ subunits and GAD isoforms designed using Vector NTI software (Invitrogen Bioinformatics, Carlsbad, CA) (see Supplementary Material for primer sequences and reaction conditions). Individual transcripts were quantified as cDNA copy numbers per 1 ng (or 1 pg) of total RNA extracted from the sample.

To control for amplification of genomic DNA, 34 DNase-treated but not reverse-transcribed RNA samples were submitted to PCRs with different primer sets. None of those reactions was positive. Differences in mRNA levels were examined using one-way ANOVA with Bonferroni's correction (Analyse-It Software, UK). To minimize the effect of variability in slice conditions, paired tests were used to assess the difference in transcript levels between two halves of the same slice simultaneously subjected to different treatments.

RESULTS AND DISCUSSION

Regional differences in mRNA levels

To assess the sensitivity and regional selectivity of our methodology, we verified that we could detect one known regional difference in basal mRNA levels between the PF and DM region. Consistent with prior *in situ* hybridization study [22], in slices superfused with ACSF, the mRNA levels for ε subunit of $GABA_AR$ were significantly higher in the DM than the PF region (120±28 (SE) copies/ng of total RNA vs. 21±5, $p<0.006$; Fig. 1B). The basal mRNA levels for the remaining six $GABA_AR$ subunits studied did not significantly differ between the two regions.

Effects of endogenous GABA and $GABA_AR$ antagonist on $GABA_AR$ subunit and GAD mRNA

We quantified mRNA levels for seven $GABA_AR$ subunits that are expressed in the posterior hypothalamus (α_1, α_2, α_3, β_1, β_2, β_3 and ε) [22-24] in two anatomically adjacent but functionally different hypothalamic regions following three *in vitro* treatments: superfusion of slices with plain ACSF, GABA uptake blocker (NO-711), or the latter combined with the $GABA_AR$ antagonist, gabazine.

When compared to incubation in ACSF, NO-711 reduced the mRNA levels of all seven studied $GABA_AR$ subunits in the PF region and for three also in the DM region (α_1, α_2 and ε). Figure 2 shows the changes in mRNA levels for those $GABA_AR$ subunits that were significantly reduced by NO-711 in the PF region only, whereas Fig. 3 shows those $GABA_AR$ subunit mRNAs that were significantly reduced by NO-711 in both regions (and in contrast to those illustrated in Fig. 2, were not sensitive to gabazine - see below). In the PF region, the effect of NO-711 varied from a ~2-fold decrease for the α_3 and β_2 subunits (Fig. 2A and C) to ~24-fold decrease for the α_1 subunit (Fig. 3A).

Unique to the PF region was that incubation with gabazine in the presence of NO-711 entirely abolished, or at least significantly attenuated, the decreases resulting from superfusion with NO-711 only. The effect was significant for five subunits (α_2,α_3, β_1,β_3 and ε) and the remaining two (α_1 and β_2) exhibited the same trend (Figs. 2 and 3). The mRNAs for the α_2, α_3, β_1, β_3 and ε subunit were ~2-fold higher following combined exposure to gabazine and NO-711 than to NO-711 only. The level of α_2 subunit mRNA was 400±130 (SE) copies/ng of total RNA in NO-711 only and 780±70 ($p<0.01$) in gabazine with NO-711. The corresponding levels for the

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remaining four significantly affected subunits were: 3,200±560 vs. 6,400±800 ($p<0.02$) for the α_3, 1,200±400 vs. 2,100±300 ($p<0.04$) for β_1, 6,700±1,400 vs. 11,900±900 ($p<0.04$) for β_3, and 8±1 vs. 23±3 ($p<0.01$) for ε subunit. The mRNA levels for three subunits, α_3, β_2 and ε, measured following combined exposure to NO-711 and gabazine were not significantly different from those in ACSF, suggesting that the entire effect of NO-711 on these mRNAs was mediated by $GABA_A$Rs. In a stark contrast to the PF region, in the DM region, gabazine had no effect when compared to NO-711 only (Figs. 2 and 3), suggesting that, in this region, the effects of the GABA reuptake blocker were not mediated by $GABA_A$Rs.

These results show that increased local GABA levels reduce mRNA levels for all seven posterior hypothalamic $GABA_A$R subunits studied. With the regard to our hypothesis, this finding supports the concept that increased $GABA_A$R stimulation may lead to a reduced $GABA_A$R expression, thus limiting the potential for GABAergic inhibition mediated by these receptors. Importantly our exposures were relatively short (90 min), and thus compatible with the timing of physiologic changes in GABA levels. Comparable periods of sleep or lack thereof cause measurable behavioral and electrophysiological changes [15].

The sharp difference in the effects of gabazine between the PF and DM region is one of the most remarkable findings of this study. Only three out of the seven studied subunits were affected by the GABA uptake blocker in the DM region, and none was significantly affected by gabazine even though the basal levels of all but one subunit were not different between the two regions. This potentially important regional selectivity may be related to the cellular composition and functions of the two regions, and our ability to detect the difference to our use of exposures lasting less than 2 hrs, rather than days.

The lack of gabazine effects in the DM region suggests that this region has a relatively large pool of non-$GABA_A$ type GABA receptors. Indeed, although $GABA_B$Rs are present in both regions [25], at least the type 1 $GABA_B$R-like immunoreactivity is higher in the DM than PF region [26]. Thus, actions mediated by $GABA_B$Rs may be considered among the mechanisms underlying the gabazine-insensitive effect of NO-711 in the DM region. The regional differences in the effects of GABA and gabazine are probably also related to differences in the functions and cellular compositions of the two regions. The PF region contains two neuronal groups, orexin (ORX) and melanin-concentrating hormone (MCH), involved in the control of sleep and wakefulness [27-29]. The DM region, comprising the hypothalamic dorsomedial and paraventricular nuclei, may also contribute to the circadian control of sleep [30], but is primarily involved in neuroendocrine and autonomic homeostasis, including the responses to stress [31]. In the PF region, GABA, MCH and ORX neurons have α_2 or α_3 subunits [24], ORX and MCH cells have subunits [21], and may also express β_2 and/or β_3 subunits [25]. So, some of the mRNA changes that we found could occur in ORX, MCH and/or GABAergic PF neurons. Nevertheless, studies of $GABA_A$Rs at the single cell level are needed to further elucidate the basis for the regional differences found in this study.

Our results pose the question as to whether the changes observed at the mRNA level translate into corresponding changes in functional $GABA_A$Rs and magnitude of resulting inhibition. While this remains to be determined, current concepts of up- and downregulation of GABAergic inhibition at the level of a single synapse suggest that regulation of receptor numbers is the main mechanism [4,8]. In cultured hippocampal slices, the expression of $GABA_A$R subunit mRNAs and their proteins increased with the level of neuronal activity [32], and a positive correlation between $GABA_A$R subunit mRNA and the corresponding protein was observed [33]. Thus, the relatively large and involving many subunits mRNA changes that we found are likely to lead to the corresponding changes in the numbers of functional $GABA_A$Rs.

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Effects of endogenous GABA on GAD mRNA—Regulation of GABAergic transmission by GABA may also involve changes in GABA synthesis. However, compared to ACSF, the effects of NO-711 on mRNA levels for GAD-65 or GAD-67 were not significant in either region (Fig. 4A and B). In contrast, the combined exposure to gabazine and NO-711 resulted in a significant increase in both regions of the GAD-65 mRNA when compared to its levels in ACSF (61±14 (SE) copies/pg of total RNA vs. 26±5 ($p<0.04$) for the PF, and 116±31 vs. 53±4 ($p<0.05$) for the DM region (Fig. 4A). Thus, the mRNA for the GAD isoform proposed to be readily responsive to the need for GABA as a transmitter [34] was affected in a manner whereby increased cellular activity could lead to increased inhibition that then could limit that activity [8]. However, unlike the effects of gabazine on $GABA_AR$ subunits, those on GAD-65 mRNA had no regional selectivity.

Effects of $GABA_AR$ agonist, muscimol, with synaptic interactions suppressed with TTX —The effects observed in our experiments could be secondary to changes in the level of local cell activity [6-8]. To test whether the changes described in the preceding sections could be mediated by stimulation of $GABA_AR$ in the absence of cellular activity and local synaptic interactions, we measured mRNA levels in half-slices of which one was superfused with muscimol and TTX and the other with TTX only. Most transcripts had reduced mRNA levels in TTX compared to ACSF (data not shown) and were not further reduced by the combined exposure to muscimol and TTX. However, mRNA for one $GABA_AR$ subunit (β_1), and in the PF region only, was significant reduced in muscimol with TTX when compared to TTX only (3,300±480 (SE) copies/ng of total RNA vs. 4,500±450, $p<0.05$) (Fig. 4C).

The absence of the effects of muscimol on most $GABA_AR$ subunits and GAD in TTX, suggests that most of the changes observed in response to NO-711 and gabazine were related to the level of cellular activity. However, the decrease of the β_1 subunit mRNA following $GABA_AR$ stimulation in TTX shows that GABA may negatively regulate the expression of $GABA_ARs$ in relation to the use of the receptor. Notably, this effect is autologous because it occurred in synaptically isolated neurons. Similarly, stimulation of $GABA_ARs$ altered transcription in cells of the mammalian circadian clock in the absence of cellular activity [35], and downregulated a promoter of the human β_1 subunit of $GABA_AR$ [36].

Potential physiologic role

Our results demonstrate that mRNA levels of multiple $GABA_AR$ subunits, and hence potentially also synthesis of new $GABA_ARs$, decrease with increased magnitude of GABAergic stimulation. The effect occurs after relatively short-lasting exposures and has different underlying mechanisms in the two compared hypothalamic regions.

Neurons in the PF region are directly involved in critical functions such as feeding, motivation and motor activity that are closely associated with sleep and vigilance [27,28]. This places the regulation of sleep in the center of interest with the regard to mRNA changes that we found in the PF region. In our separate *in vivo* study [37], 6 hrs of sleep deprivation increased the β_1, β_3 and ε subunit mRNA levels in the PF region only similarly to the effects of gabazine in the present study. Also of interest with the regard to our hypothesis is the finding that, in humans, a mutation in the gene for the $\beta_3$subunit is associated with insomnia [38]. Since appropriate mRNA changes occur mainly in the PF region, it will be of interest to identify the mechanisms that regulate the magnitude of $GABA_AR$-mediated inhibition in neurochemically distinct PF neurons.

Supplementary Material

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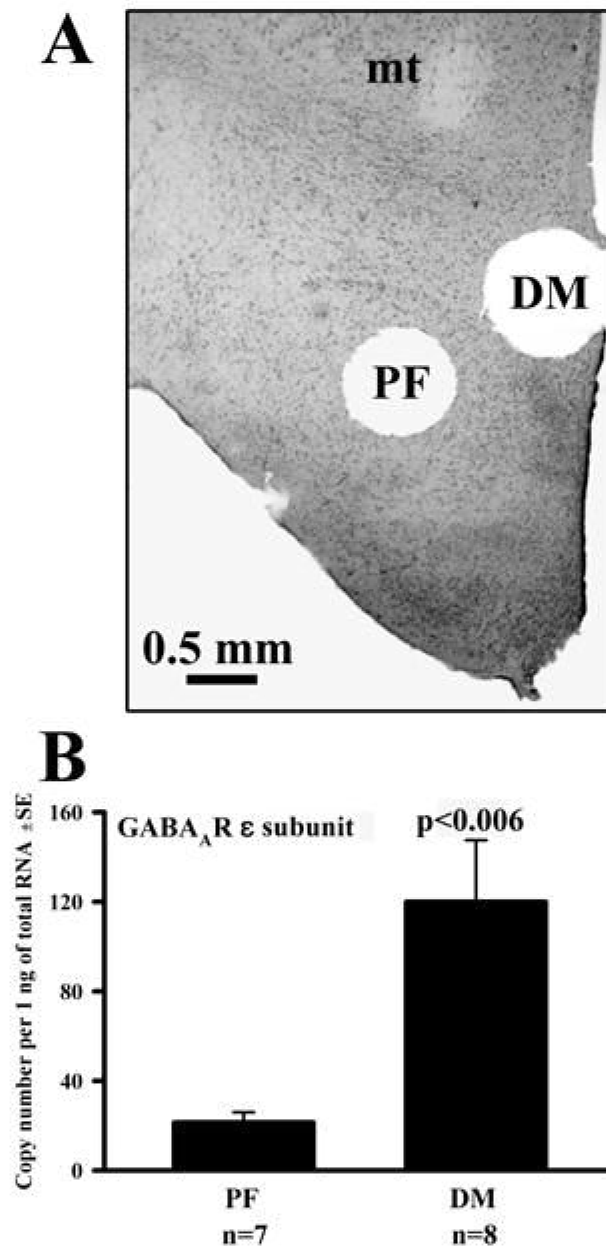

Figure 1.

(A) Example of tissue punches extracted from the perifornical (PF) and dorsomedial (DM) regions of a hypothalamic half-slice (mt, mammillothalamic tract). (B) Basal levels of the mRNA for ε subunit of $GABA_A$ receptor were higher in the DM than PF region.

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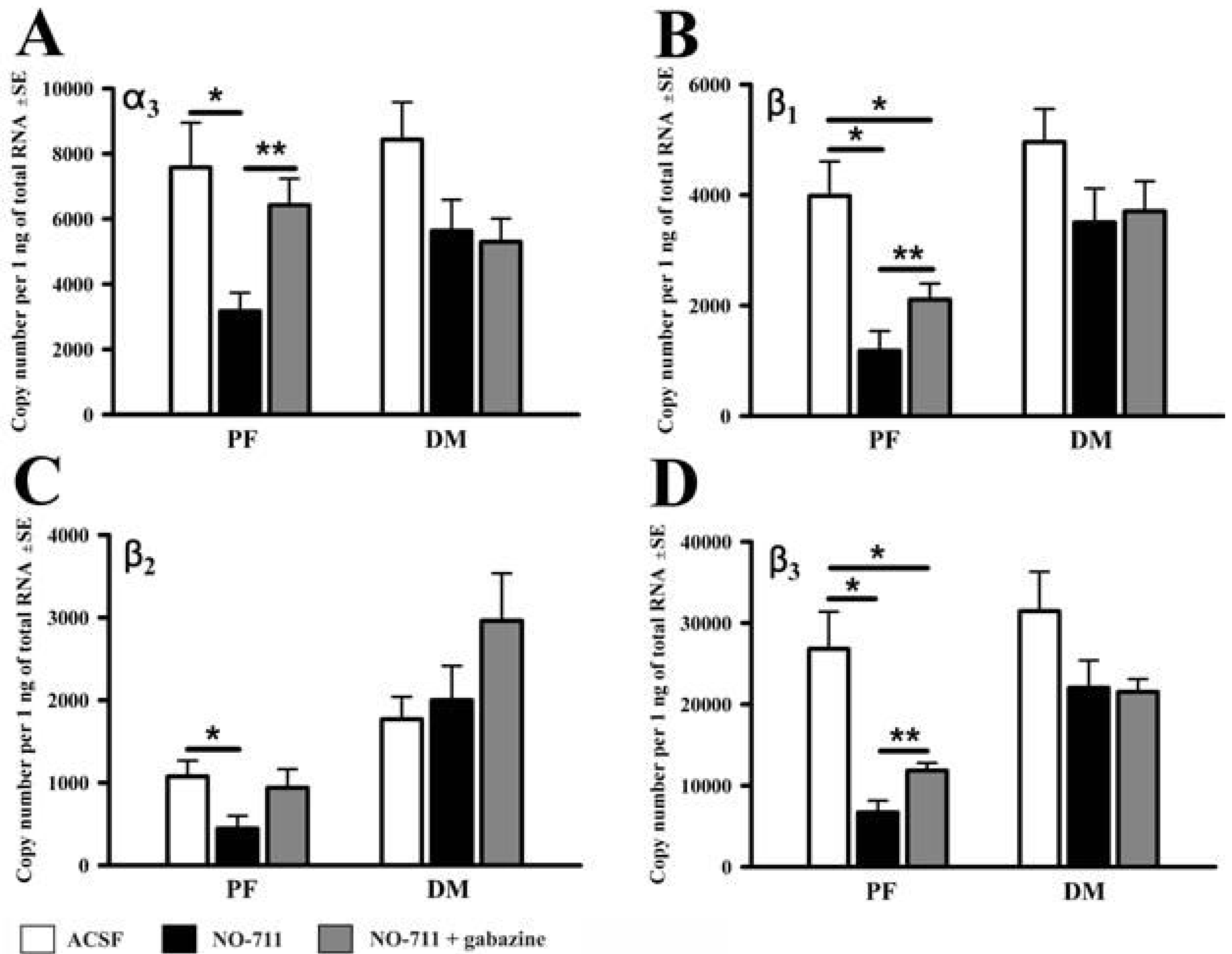

Figure 2.
mRNA levels of those $GABA_A$ receptor subunits that were significantly reduced by the GABA uptake blocker (NO-711) in the PF region only. For these subunits, NO-711 and gabazine exerted opposite effects in the PF region, whereas their effects in the DM region were not significant. The data were obtained from 8 half-slices superfused with neutral medium (ACSF), and 6 pairs of half-slices of which one was superfused with NO-711 and the other with NO-711 +gabazine. * $p<0.05$, one-way ANOVA; ** $p<0.05$, paired t-test.

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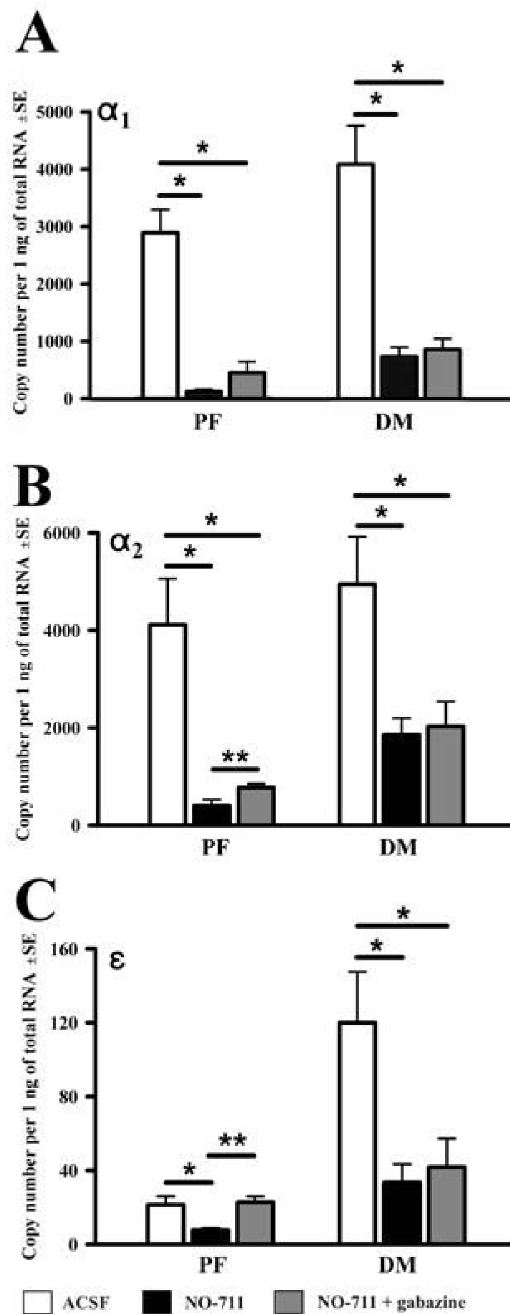

Figure 3.

mRNA levels for those $GABA_A$ receptor subunits that were significantly reduced by the GABA uptake blocker (NO-711) in both the PF and DM region (but with the effects of gabazine being significant in the PF region only). The data were obtained from the same half-slices as in Fig. 2. * $p<0.05$, one-way ANOVA; ** $p<0.05$, paired t-test.

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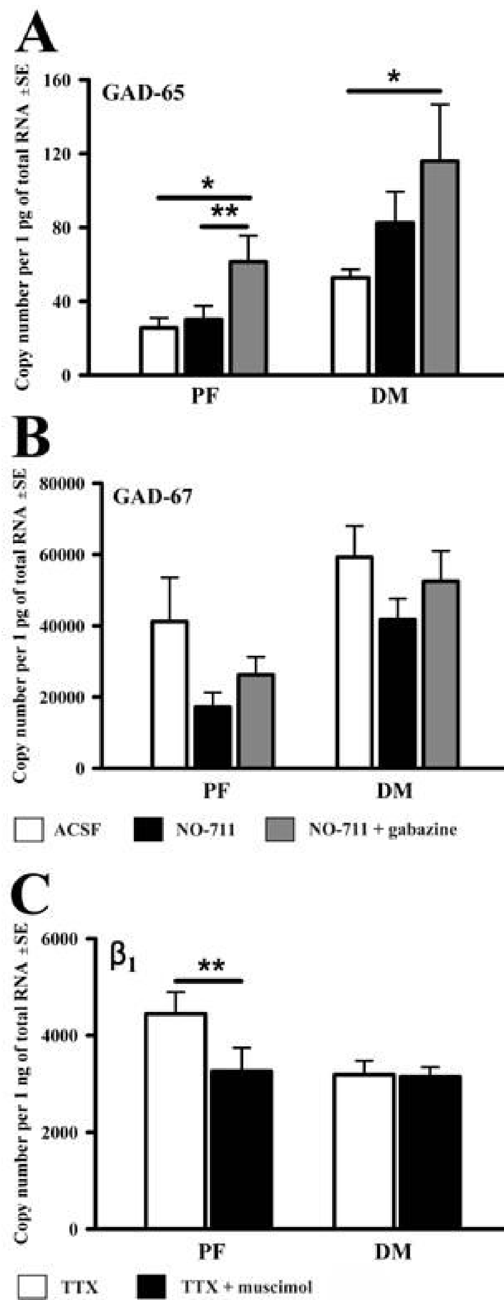

Figure 4.
Effects of the GABA uptake blocker (NO-711) with or without gabazine on GAD-65(A) and GAD-67 (B) mRNA levels, and the effect of the $GABA_AR$ agonist, muscimol, in the presence of tetrodotoxin (TTX) on the $GABA_AR$ β1 subunit mRNA (C) in the PF and DM regions. The data in (A and B) are from the same half-slices as in Fig. 2. The data in (C) are from six PF and five DM pairs of punches extracted from half-slices that were simultaneously superfused with TTX only or TTX+muscimol. * $p<0.05$, one-way ANOVA; ** $p<0.05$, paired t-test.