$GABA_A$ receptor modulation of 5-HT neuronal firing in the median raphe nucleus: implications for the action of anxiolytics.

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Abstract

5-HT neurones in the median raphe nucleus (MRN) are involved in anxiety and the sleep/wake cycle. Here, using in vitro electrophysiology, we examined if the firing of MRN 5-HT neurones is regulated by $GABA_A$ receptors. The $GABA_A$ receptor agonists THIP and muscimol caused concentration dependent inhibition of MRN 5-HT neurones. The $GABA_A$ receptor antagonist bicuculline blocked the responses to THIP and muscimol. Bicuculline alone increased the basal firing activity. Responses to THIP were enhanced by the Z hypnotic zolpidem at concentrations selective for the α_2/α_3 subunits of the $GABA_A$ receptor (0.2 and 1 μM) but not at a concentration selective for the α_1 subunit (0.02 μM). Consistent with these functional data, 5-HT neurones have been shown to express the α_3 (but not α_2) subunit. The anxiolytic effects of $GABA_A$ receptor modulators are reportedly mediated by α_3-containing receptors. Hence the MRN 5-HT system may be a target for anxiolytic drugs.

Keywords: α_1 subunit, α_3 subunit, electrophysiology, rat, zolpidem

Introduction

Evidence suggests that the 5-HT neurones of the midbrain median raphe nucleus (MRN) are involved in the sleep/wake cycle. Thus, 5-HT projections from the MRN have been shown to regulate activity patterns in the hippocampus involved in sleep (see Vertes and Kocsis, 1997) and modulate the suprachiasmatic nucleus (SCN) - considered to contain the mammalian circadian clock-(Meyer-Bernstein and Morin, 1999; Muscat et al., 2005). Evidence also suggests that 5-HT neurones in the MRN are involved in anxiety. Thus, 5-HT levels in MRN projection areas are reported to increase with anxiety (Matsuo et al., 1996; Andrews et al., 1997) and inhibition or ablation of MRN 5-HT neurones reduces anxiety-like behaviour in several anxiety models (Carli and Samanin, 1988; Andrews et al., 1994, 1997; File et al., 1996; Andrade et al., 2004; Dos Santos et al., 2005).

It is well established that enhancing GABA neurotransmission in the brain can be both anxiolytic and sedative/hypnotic. Some data indicate that anxiolysis and sleep are mediated, at least in part, by increased activation of $GABA_A$ receptors in the MRN. Thus, intra-MRN application of $GABA_A$ receptor agonists or modulators have been reported to reduce anxiety-like behaviour (Gonzalez et al., 1998; Dos Santos et al., 2005) and induce hippocampal activity patterns associated with sleep (Li et al., 2005).

Given that MRN 5-HT neurones are implicated in both sleep/wake and anxiety behaviours (see above), it is possible that the effects of systemically and intra-MRN administered, $GABA_A$ agonists and modulators on sleep and anxiety is mediated by the inhibition of 5-HT neurones in the MRN. Indeed, 5-HT-immunoreactive neurones in the MRN have been shown to express $GABA_A$ receptor α subunit–immunoreactivity (Gao et al., 1993) and intra-MRN injections of $GABA_A$ agonists have been shown to suppress 5-HT release in MRN innervated forebrain regions (Tao et al., 1996; Shim et al., 1997; Glass et al., 2003). However, to date GABAergic regulation of 5-HT neuronal activity in the MRN has not been directly demonstrated.

Recent data suggest that anxiolysis and sedation may be mediated by $GABA_A$ receptors of different subunit composition. Thus pharmacological studies indicate

that anxiolysis is mediated by $GABA_A$ receptors containing the α_3 subunit (Atack et al., 2005 a,b) whilst ataxia and decreased locomotion (indices of sedation) are mediated by $GABA_A$ receptors containing the α_1 subunit (Platt et al., 2002; Rowlett et al., 2005). However, the brain regions mediating the anxiolytic and sedative effects of $GABA_A$ receptor subunit selective drugs have not to date been examined.

In the present study we used in vitro single unit electrophysiological recording in a rat midbrain slice preparation to examine the $GABA_A$ receptor regulation of 5-HT neuronal activity in the MRN. We also examined the potential modulation of the response to a $GABA_A$ receptor agonist by the Z hypnotic zolpidem. Due to its different affinities, this drug can be used to distinguish between α_1, α_2/α_3 and α_5 subunit containing $GABA_A$ receptors (Pritchett and Seeburg, 1990; Araujo et al., 1999).

Experimental procedures

Experimental subjects

All experiments carried out had been reviewed and approved by the University of Newcastle Ethical Review Panel and were in accordance with the UK Animals (Scientific procedures) act of 1986 and the European Community Council Directive of 24th November 1986 (86/609/EEC). All efforts were made to minimize any pain or discomfort of the animals. Male hooded Lister rats (Charles River, Kent, UK) (200-400 g), were group housed under controlled conditions of temperature and humidity in a 12 h light/dark cycle (lights on 7 am). All animals were allowed to acclimatise to the holding facilities for at least one week before use.

Electrophysiology

Animals were decapitated (without prior anaesthesia) between 8.30 and 10.00 am. The brain was quickly removed and submerged in oxygenated (95% O_2-5% CO_2) sucrose slush (composition (mM): sucrose: 200, HEPES 10, $MgSO_4$ 7, NaH_2PO_4 1.2, KCl 2.5, $NaHCO_3$ 25, $CaCl_2$ 0.5, D-glucose 10, pH 7.4) for 5 minutes. The caudal portion of the brain was then mounted on a block using cyanoacrylate glue and submerged in oxygenated sucrose slush in the chamber of a vibrating microtome (Vibratome 1000, Vibratome, St. Louis, USA). Coronal slices of the midbrain (350

μm thick), containing the MRN, were cut and then transferred to a Petri dish containing oxygenated artificial cerebrospinal fluid (aCSF) (composition (mM): NaCl 124, $MgSO_4$ 2.4, KH_2PO_4 1.25, KCl 3.25, $NaHCO_3$ 26, $CaCl_2$ 2, D-glucose 10, pH 7.4). Slices between Bregma -7.6 and -8.0 mm (Paxinos and Watson, 1998) were trimmed and individual slices were placed in interface perfusion chambers and perfused with aCSF (0.5 ml/min) at 36˚C. The aCSF contained the adrenoceptor agonist norepinephrine (NE; 30 μM) in order to evoke 5-HT neuronal firing.

Extracellular recordings were made from neurones in the MRN using glass microelectrodes (1.5 mm OD, Clarke Electromedical, Reading, UK; 1-3 MΩ in vitro impedance) filled with 2 M NaCl. Signals were amplified (x1000) with an AC differential preamplifier and were fed to a PC via a computer interface (1401 or micro1401, CED, Cambridge, UK) and collected using Spike2 software (version 4, CED, Cambridge, UK). The microelectrode was advanced slowly through the slice (approximately 150 μm per minute) to decrease the probability of missing neurones with slow firing rates. Neurones were identified on the basis of their location (in the MRN), basal electrophysiological characteristics of slow regular firing and an inhibitory response to 5-HT (25-50μM) (see Results).

Following a period of recording of basal firing activity, drugs were applied via the perfusion medium at intervals of at least 4 minutes. 5-HT, 4,5,6,7-Tetrahydrisoxazolo[5,4-c]pyridin-3-ol (THIP) and muscimol, were applied for two minutes. Bicuculline was applied for 3 min before and 2 min during reapplication of the agonist. Zolpidem was applied for 6 min before and 2 min during reapplication of the agonist. Control experiments demonstrated that responses to the $GABA_A$ receptor agonists were quickly reversible and were not desensitised by repeated application, so it was possible to record more than one neurone in a slice. However, since bicuculline and zolpidem (>1 μM) were found to only wash off slowly, recordings from subsequent neurones in the same slice were delayed until at least 45 minutes after zolpidem and 90 minutes and after bicuculline application. Between 1 and 6 neurones per slice were recorded and either 1 or 2 slices from each animal were used.

Materials

NaCl, $MgSO_4$, KH_2PO_4, KCl, $NaHCO_3$ and sucrose were purchased from BDH Laboratory Supplies (Dorset, UK). Bicuculline methiodide, $CaCl_2$, D-(+)-glucose, HEPES, muscimol, (±)-norepinephrine, 5-HT hydrochloride were purchased from Sigma. THIP hydrochloride and zolpidem were purchased from Tocris. Stock solutions (10 mM) of 5-HT, muscimol and THIP were made in aCSF. A 1 mM stock solution of zolpidem was made in dimethyl sulfoxide (DMSO) (maximum final DMSO concentration 0.5%).

Data analysis and statistics

Electrophysiological data were analysed offline from the firing rate data collected on Spike 2. Basal firing was determined in a 120 s period at the start of the recording and is expressed in Hz. Inhibitory responses to 5-HT and the $GABA_A$ receptor agonists were determined as the firing rate in a 120s period covering the maximum change in baseline and expressed as a percentage change (decrease) relative to the firing rate in the 120s immediately before the application of the drug. A change in firing rate of 5% or more over the period of application of the drug was considered to be a 'response'. The effect of $GABA_A$ receptor modulators was assessed by comparison of the response to the agonist in the absence of and in the presence of the modulator. An increase in the response by more than 10% was considered to be an 'enhancement'. Data presented are individual examples or the mean ± SEM. Within neurone differences were analysed by paired t-test, Wilcoxon signed ranks test or one-way repeated measures ANOVA and between group differences were analysed by unpaired t-test or two-way repeated measures ANOVA.

Results

Firing activity of MRN neurones

In the presence of the adrenoceptor agonist, norepinephrine (30 μM), 112 slow firing (0.29 -4.63 Hz) neurones were recorded in the MRN. All neurones were tested with 5-HT (25-50 μM) and those which showed an inhibitory response to 5-HT were considered to be 5-HT neurones (n = 101). The basal firing rate of these 5-HT neurones ranged from 0.29 – 3.16 Hz (mean basal firing rate; 1.09 ± 0.07 Hz, n =

101). Of these 101 neurones 99 showed an inhibitory response to 25 μM 5-HT (mean inhibition 55.6 ± 3.2 %; range 9.7 to 100 %). The firing activity of the 2 neurones insensitive to 25μM 5-HT, was inhibited by 50 μM 5-HT.

Of the remaining 11 neurones, 8 neurones were excited and 3 were insensitive to 5-HT. The basal firing rate of the non-5-HT neurones ranged from 0.93 – 4.63 Hz (mean basal firing rate; 1.97 ± 0.33 Hz, n = 11).

Response of MRN 5-HT neurones to $GABA_A$ receptor agonists

The firing activity of 5-HT neurones in the MRN (56/59) was inhibited by the $GABA_A$ receptor agonist THIP (10-25 μM) (Fig. 1A). Of these 56 neurones 49 showed an inhibitory response to THIP (10 μM) (mean inhibition 20.2 ± 2.7 %, n = 49; range 5.1 to 100 %). The firing activity of those neurones which were insensitive to 10 μM THIP, decreased, when tested with 25 μM THIP (n = 7). 5-HT neurones (n = 7) tested with increasing concentrations of THIP (10, 25, 50 μM) showed a concentration dependent inhibitory response (Fig. 1B). Repeated applications of THIP produced inhibitory responses that differed by less than 10% (range -8.1 to 9.4%, n =10). There was no statistical difference between the responses to the first and second application (mean difference 1.4 ± 2.1 %, n = 10; p = 0.53, paired t-test).

The $GABA_A$ receptor agonist muscimol (1-10 μM) inhibited the firing rate of all neurones tested (n = 15, Fig. 1C). Of these 15 neurones, 13 showed an inhibitory response to muscimol (3 μM) (mean inhibition 56.7 ± 10.1 %, n = 13; range 9.3 to 100 %). The two neurones not inhibited by 3 μM muscimol were inhibited by 10 μM muscimol. 5-HT neurones (n = 7) tested with increasing concentrations of muscimol (1, 3, 10 μM) showed a concentration dependent inhibitory response (Fig. 1D). Interestingly, there was a strong positive correlation between the responses to 5-HT (25 μM) and muscimol (3 μM) (n = 13, Pearson correlation = 0.83, p = 0.001) (Fig. 2).

The inhibitory responses to both THIP (50 μM) and muscimol (3 μM) were blocked by the $GABA_A$ receptor antagonist bicuculline (50 μM) (Fig. 3). Thus the mean

inhibitory response to THIP in the absence of bicuculline was 82.4 ± 7.4 % compared to just 6.1 ± 5.6 % (n = 9) in the presence of the antagonist ($p < 0.001$, paired t-test, Fig. 3A). Similarly, the mean inhibitory response to muscimol in the absence of bicuculline was 49.5 ± 16.2 % (n = 6) compared to a response of -2.9 ± 1.5 % in the presence of bicuculline ($p < 0.05$, Wilcoxon signed ranks test, Fig. 3B,C).

The $GABA_A$ receptor antagonist, bicuculline alone increased the basal firing rate of the 5-HT neurones (see Fig. 3C). In the presence of bicuculline (50 μM) alone, the basal firing rate increased by 36.0 ± 6.2 % (0.91 ± 0.11 to 1.23 ± 0.15 Hz, n = 15; $p < 0.001$, paired t-test).

Modulation of $GABA_A$ receptor response by zolpidem

The effect of the Z hypnotic zolpidem (0.02 – 5 μM) on a submaximal response to THIP (10 – 25 μM) was examined. Zolpidem 0.02 μM enhanced the response to THIP in 1/11 neurones tested (9%). The remaining 10 neurones showed no consistent change in the magnitude of the response to THIP. The mean inhibitory response to THIP was not significantly altered by 0.02 μM zolpidem (mean inhibitory response to THIP 32.2 ± 7.5 vs 35.6 ± 8.7 %, n = 11; $p = 0.23$, paired t-test; Fig. 4A,B). A concentration of 0.2 μM zolpidem enhanced the response to THIP in 10 of 15 neurones tested (67%) and had no effect on the response in the remaining 5 neurones. In this group of 15 neurones the mean response to THIP was significantly enhanced by zolpidem (0.2 μM) (mean inhibitory response to THIP 26.6 ± 4.2 vs 43.2 ± 6.8 %; $p < 0.05$, paired t-test; Fig. 4A,C). Application of 1 μM zolpidem enhanced the response to THIP in all 9 neurones tested, which included the 5 neurones which failed to show a response to 0.2 μM zolpidem. Analysis of the group data showed a significant effect of zolpidem at this concentration (mean inhibitory response to THIP 17.9 ± 2.0 vs 52.6 ± 7.5 %; $p < 0.001$, paired t-test, Fig. 4D). The highest concentration of zolpidem (5 μM) enhanced the response to THIP in all 5 neurones tested. Again the mean response to THIP was significantly enhanced by zolpidem (mean inhibitory response to THIP18.2 ± 2.9 vs 67.4 ± 10.2 %; (n = 5; $p < 0.05$, paired t-test, Fig. 4E).

Zolpidem alone had no effect on the basal firing rate of the 5-HT neurones at any of the concentrations tested (0.02 μM, n = 11; 0.2 μM, n = 15; 1 μM, n = 9; 5 μM, n = 5; paired t-test).

Discussion

Using extracellular electrophysiology in the in vitro slice preparation we examined the regulation of 5-HT neuronal activity in the MRN by $GABA_A$ receptors. Furthermore, we assessed the modulation of the neuronal responses to $GABA_A$ receptor activation by a Z hypnotic, which has differential affinity for $GABA_A$ receptors containing α_1, α_2/α_3 and α_5 subunits.

Identity of neurones recorded

The MRN neurones recorded in this study fired slowly and regularly in the presence of the adrenoceptor agonist, noradrenaline. The vast majority of these neurones was inhibited by 5-HT. We have previously shown that the 5-HT inhibition of MRN neurones is greatly attenuated by WAY100635 (Judge and Gartside, 2005), indicating mediation by 5-HT_{1A} receptors. As it has been shown that only 5-HT-containing neurones in the MRN are hyperpolarized by a 5-HT_{1A} receptor agonist (Beck et al., 2004), we can be confident that the neurones inhibited by 5-HT in the present study are 5-HT-containing.

$GABA_A$ receptor regulation

The selective $GABA_A$ receptor agonists THIP and muscimol (Kemp et al., 1986; Krogsgaard-Larsen and Falch, 1981) inhibited the firing activity of 5-HT neurones in the MRN. Although THIP has been reported to show a preference for δ containing $GABA_A$ receptors (Krogsgaard-Larsen et al., 2002), it is unlikely that at the concentrations used in the present study such a preference was demonstrated. The inhibitory responses were concentration dependent and attenuated by the selective $GABA_A$ receptor antagonist bicuculline (Bowery et al., 1984). These data indicate that MRN 5-HT neurones are inhibited by $GABA_A$ receptor activation. This is consistent with the indirect evidence from microdialysis studies in which it has been shown that intra-MRN injections of $GABA_A$ agonists suppress 5-HT release in the

hippocampus and SCN (Shim et al., 1997; Glass et al., 2003), whilst $GABA_A$ antagonists stimulate 5-HT release in SCN (Glass et al., 2003).

It is of note that we found that the responses of the MRN 5-HT neurones to 5-HT and muscimol were positively correlated. This may simply be due to the baseline membrane potential of the neurone or alternatively it could indicate that the expression or function of $GABA_A$ and $5\text{-}HT_{1A}$ receptors on MRN 5-HT neurones are somehow linked. However, the response to THIP was not correlated with the response to 5-HT. It is unclear why there is this discrepancy.

Modulation of $GABA_A$ receptors and subunit composition

We found that the inhibitory responses to THIP were enhanced by the Z hypnotic zolpidem. This drugs acts at the benzodiazepine binding site to enhance transmission (Arbilla et al., 1986). In the present study we used the Z hypnotic zolpidem to examine the α subunit composition of the $GABA_A$ receptors on MRN 5-HT neurones. This drug has Ki values of 0.02, 0.4 and 5 μM for α_1, α_2/α_3 and α_5 -containing $GABA_A$ receptors, respectively (Pritchett and Seeburg, 1990; Araujo et al., 1999) and hence can be used to make inferences about the α-subunit composition of the receptors. We found that 0.2 μM, zolpidem enhanced the response to THIP in two-thirds of MRN 5-HT neurones tested, whilst all showed enhancement at 1 μM. These concentrations are one twenty-fifth and one fifth, respectively of the Ki for α_5 -containing $GABA_A$ receptors. Therefore we conclude that the $GABA_A$ receptors on MRN 5-HT neurones do not contain the α_5 subunit. Since these concentrations of zolpidem (0.2 μM and 1 μM) are one half and 2.5 times the reported Ki for α_2/α_3 -containing $GABA_A$ receptors, the data are consistent with the majority of 5-HT neurones in the MRN possessing α_2/α_3 -containing $GABA_A$ receptors. Finally we observed clear enhancement of the THIP response in 1 neurone (9%) after the lowest concentration of zolpidem (0.02 μM) was applied. This is equal to the Ki for α_1, -containing $GABA_A$ receptors and suggests that a small proportion of MRN 5-HT neurones possess α_1-containing $GABA_A$ receptors. Taken together our functional data are entirely consistent with the immunocytochemical evidence that the majority of 5-HT-immunoreactive neurones in the raphe nuclei are immunoreactive for the α_3

$GABA_A$ receptor subunit with only a small minority immunoreactive for the α_1 and α_2 $GABA_A$ receptor subunit (Gao et al., 1993; Rodriguez-Pallares et al., 2001).

Tonic GABAergic regulation in the slice preparation

Here we found that application of the $GABA_A$ antagonist bicuculline increased the basal firing rate of 5-HT neurones in the MRN suggesting that the MRN is under tonic GABAergic tone in the slice preparation. We have also recently shown that 5-HT neurones in the dorsal raphe nucleus (DRN) are inhibited by $GABA_A$ receptor activation (Judge et al., 2004) but in contrast to the present data, we found no effect of $GABA_A$ antagonists on 5-HT neuronal firing in the DRN. The effect of bicuculline in the MRN suggests that MRN 5-HT neurones are innervated by a population of GABAergic neurones which are present in the midbrain slice. A few GABAergic neurones are located in the midline of the DRN and MRN amongst the 5-HT neurones, but a much greater number are found in the lateral wings of the DRN and adjacent areas such as the ventral tegmental nucleus and periaqueductal grey area (Serrats et al., 2003; Day et al., 2004). Consistent with our data showing GABAergic tone, recent in vivo studies have shown that $GABA_A$ receptor antagonists increase 5-HT release in MRN innervated areas (Glass et al., 2003; Li et al., 2005). In other brain regions GABAergic tone is mediated by α_4 containing $GABA_A$ receptors (Jia et al., 2005; Mangan et al., 2005). As application of zolpidem alone did not decrease basal firing rate at even the highest concentration (5μM), it is probable that GABAergic tone in the MRN is also mediated by this receptor subtype.

Implications for the actions of anxiolytic and sedative drugs

It has been shown previously that application of benzodiazepines directly into the MRN is anxiolytic (Gonzalez et al., 1998). Given that anxiolysis is reportedly mediated by α_3 subunit–containing $GABA_A$ receptors (Atack et al., 2005 a,b), our data showing enhancement at α_2/α_3 subunit–selective concentrations, indicate that 5-HT neurones in the MRN may be involved in the action of systemically applied anxiolytic drugs. In contrast, sedation is reported to be mediated by α_1 subunit–containing $GABA_A$ receptors (Crestani et al., 2000; Rowlett et al., 2005). Our data indicate that only a small proportion of the 5-HT neurones in the MRN possess this particular type

of $GABA_A$ receptor and we conclude that sedation is not likely to be mediated by inhibition of 5-HT neurones in the MRN.

Summary

We have shown that the firing activity of 5-HT neurones in the MRN is regulated by $GABA_A$ receptors. Taken together with immunocytochemical data (Gao et al., 1993; Rodriguez-Pallares et al., 2001), our data also indicate that the majority of the $GABA_A$ receptors contain α_3 subunits which have been shown to mediate anxiolytic effects of $GABA_A$ receptor modulators. Thus GABAergic inhibition of MRN 5-HT neurones may be involved in the action of anxiolytic drugs.

Acknowledgements

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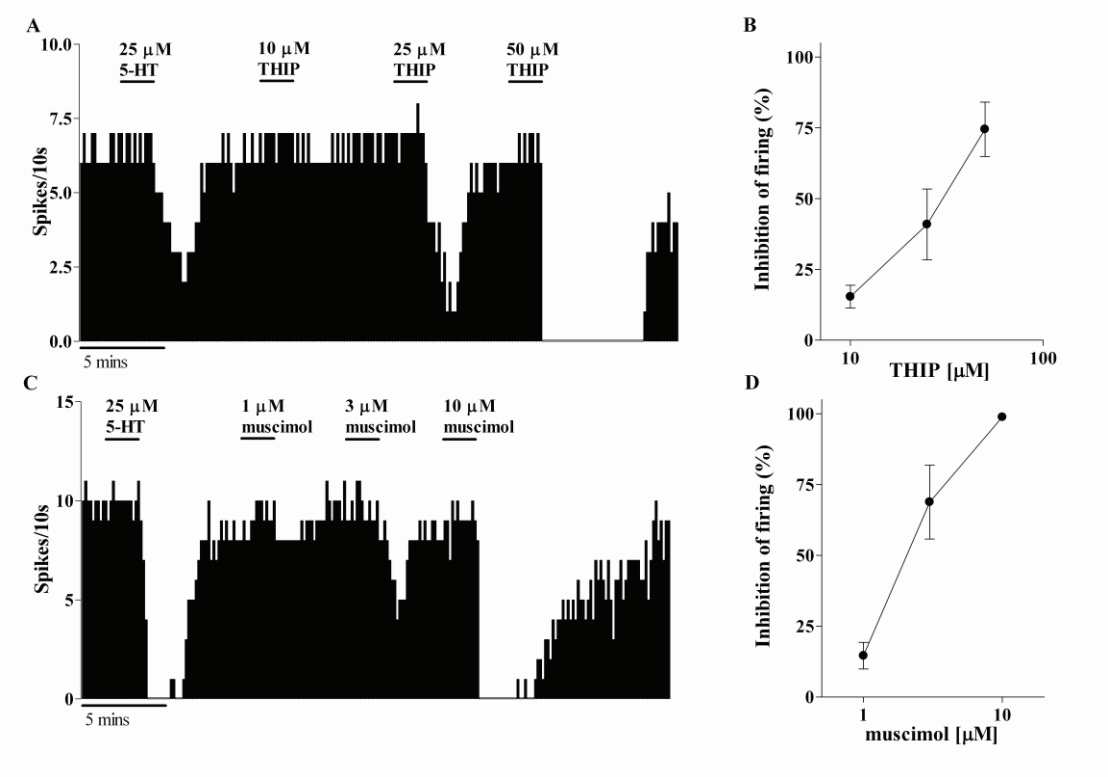

Figure 1. Inhibitory responses of 5-HT neurones in the MRN to $GABA_A$ receptor agonists. (A) Example of a recording showing responses to 5-HT (25 μM) and increasing concentrations of THIP of an individual neurone. THIP (10, 25 and 50 μM) was applied for periods of 2 min as indicated by the bars. Note that the recording has not been corrected for the perfusion lag (approximately 2 min). Basal activity was recorded in the continuous presence of 30 μM NE. (B) Concentration-response relationship for inhibitory responses to THIP in MRN neurones (n = 7). (C) Example of a recording from an individual neurone showing responses to 5-HT (50 μM) and increasing concentrations of muscimol (1, 3 and 10 μM) applied for periods of 2 min as indicated by bars. (D) Concentration-response relationship for inhibitory responses to muscimol in MRN neurones (n = 7). Data shown are mean ± SEM.

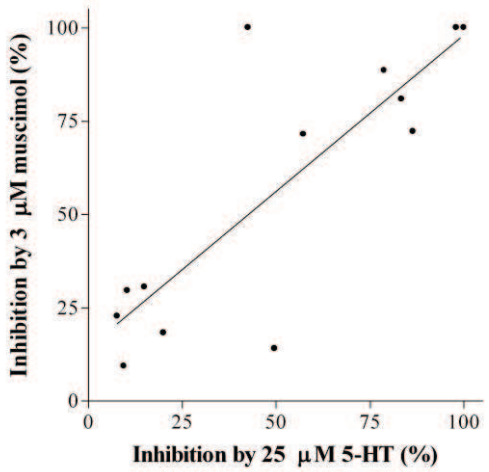

Figure 2. Correlation between inhibitory responses of individual MRN 5-HT neurones to 5-HT (25 μM) and muscimol (3 μM). The regression line is also shown.

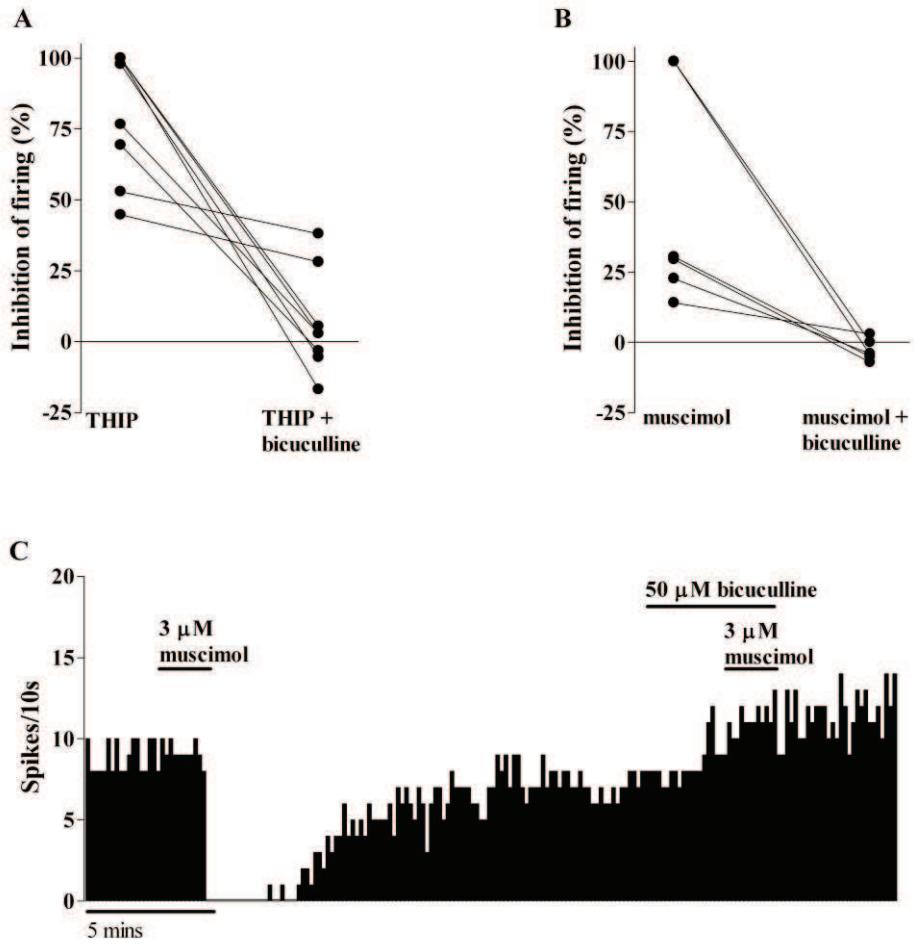

Figure 3. Inhibitory responses of MRN 5-HT neurones to (A) THIP (50 μM) and (B) muscimol (3 μM) in the absence and presence of the $GABA_A$ receptor antagonist bicuculline (50 μM). The responses from the same neurones are joined by a line. (C) Example of a recording showing responses of an individual neurone to muscimol (3 μM) in the absence and presence of bicuculline. Bicuculline was applied for 3 min before and 2 min during reapplication of muscimol as indicated by the bars.

Note that the basal firing rate increased following the application of bicuculline alone.

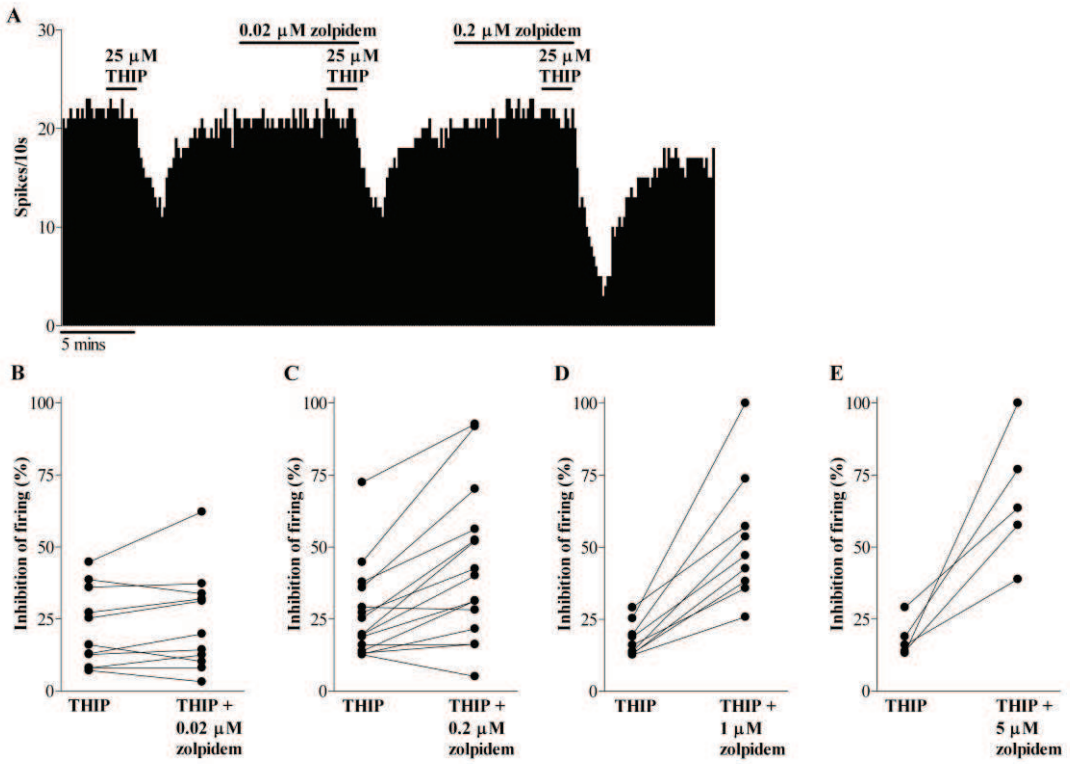

Figure 4. Inhibitory responses of MRN 5-HT neurones to THIP (10-25 μM) in the absence and presence of the Z hypnotic zolpidem. (A) Example of a recording showing responses of an individual neurone to THIP (25 μM) in the absence and presence of increasing concentrations of zolpidem (0.02 and 0.2 μM). Zolpidem was applied for 6 min before and 2 min during reapplication of THIP as indicated by the bars. (B-E) Inhibitory responses of individual MRN 5-HT neurones to THIP (10-25 μM) in the absence and presence of (B) 0.02 μM, (C) 0.2 μM, (D) 1 μM and (E) 5 μM zolpidem. The responses from the same neurones are joined by a line.