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Methylene blue inhibits GABA_A receptors by interaction with GABA binding site

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Abstract

Methylene blue (MB) is commonly used in diagnostic procedures and is also used to treat various medical conditions. Neurological effects of MB have been reported in clinical observations and experimental studies. Thus the modulation of GABA_A receptor function by MB was investigated. Whole-cell GABA-activated currents were recorded from HEK293 cells expressing various GABA_A receptor subunit configurations. MB inhibition of GABA currents was apparent at 3 μ M, and it had an IC₅₀ of 31 μ M in human α 1 β 2 γ 2 receptors. The MB action was rapid and reversible. MB inhibition was not mediated via the picrotoxin site, as a mutation (T6'F of the β 2 subunit) known to confer resistance to picrotoxin had no effect on MB-induced inhibition. Blockade of GABA_A receptors by MB was demonstrated across a range of receptors expressing varying subunits, including those expressed at extrasynaptic sites. The sensitivity of α 1 β 2 receptors to MB was similar to that observed in α 1 β 2 γ 2 receptors, indicating that MB's action via the benzodiazepine or Zn²⁺ site is unlikely. MB-induced inhibition of GABA response was competitive with respect to GABA. Furthermore, mutation of α 1 F64 to A and β 2 Y205 to F in the extracellular N-terminus, both residues which are known to comprise GABA binding pocket, remarkably diminished MB inhibition of GABA currents. These data suggest that MB inhibits GABA_A receptor function by direct or allosteric interaction with the GABA binding site. Finally, in mouse hippocampal CA1 pyramidal neurons, MB inhibited GABA-activated currents as well as GABAergic IPSCs. We demonstrate that MB directly inhibits GABA_A receptor function, which may underlie some of the effects of MB on the CNS.

Graphical abstract

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Keywords

recombinant receptors; memory enhancing; hippocampus; IPSC

1. Introduction

The neurotransmitter γ -aminobutyric acid (GABA), the predominant fast inhibitory neurotransmitter of the adult central nervous system (CNS), acts principally via Cl^- selective ligand-gated ion channels (GABA_A receptors). Activation of GABA_A receptors results in membrane hyperpolarization and neuronal inhibition in adults. GABA_A receptors are pentameric membrane proteins comprising seven different classes of subunits, many with multiple variants (α 1-6, β 1-3, γ 1-3, ρ 1-3, δ , ϵ , π and θ) (Barnard et al. 1998; Whiting 1999). Within the GABA_A receptor family, the combination of α 1 β 2 γ 2 is the most abundantly expressed member in brain (Fritschy and Brünig, 2003). Dysfunction of GABA_A receptor-mediated neurotransmission can shift the balance of inhibitory and excitatory forces in the CNS, resulting in pathological consequences. Abnormal GABAergic neurotransmission is implicated in the pathophysiology of epilepsy, depression, seizure disorders and anxiety disorders. Correspondingly, enhancement or modulation of GABA-induced currents an important mechanism of action of a large number of existing and developing therapeutic agents.

Methylene blue (MB) has been used in humans for more than 120 years. It is clinically used in diagnostic procedures and also used to treat a number of medical conditions such as carbon monoxide poisoning, methemoglobinemia and cyanide poisoning (Clifton and Leikin, 2003). Due to its high lipophilicity, MB has a remarkably high permeability through the blood-brain barrier and can reach concentrations up to hundreds of μM in brain after intravenous or oral administration (Peter et al., 2000; O'Leary et al., 2010). In recent years, MB has attracted great interest as it is proposed to be a new neurobiological approach to improve normal memory and to treat memory impairment and neurodegeneration associated with mitochondrial dysfunction. The memory-enhancing effect of MB has been investigated in phase II clinical trials on patients with Alzheimer disease (ID: NCT02380573, ClinicalTrials.gov) and post-traumatic stress disorder (ID: NCT01188694). MB has multiple

cellular and molecular targets in the CNS that appear to underlie its neurological effects (Oz et al., 2011; Rojas et al., 2012). Here we identified GABA_A receptors as a pharmacological target for the CNS actions of MB.

2. Materials and methods

2.1. Brain slice preparation

Transverse hippocampal slices were acutely prepared from postnatal day (P) 0–15 (day of birth = P1) C57BL/6J mouse (either sex). The mouse was anesthetized with isoflurane and rapidly decapitated. All procedures were conducted in accordance with the *National Institutes of Health Guide for Care and Use of Laboratory Animals*. All stages of brain dissection and tissue slicing were conducted in ice-cold (~4 °C) artificial cerebral-spinal fluid (ACSF) of the following composition (in mM): 124 NaCl, 5.0 KCl, 1.3 MgSO₄, 26 NaHCO₃, 1.24 KH₂PO₄, 2.4 CaCl₂ and 10 glucose; 300 mOsm and pH ~7.4 after equilibration with a 95% O₂/5% CO₂ gas mixture (carbogen). Thin hippocampal slices (200 µm) were cut with a vibratome VT1000S (Leica Biosystems, Inc). Slices were submerged in ACSF (22–25 °C) aerated with the carbogen gas mixture. Slices were stored at room temperature in carbogen bubbled ACSF until used.

2.2. Cloned GABA_A receptors

Cells stably expressing human $\alpha 1\beta 2\gamma 2$ receptors were studied in the present investigation. Cells transiently expressing various human or rat GABA_A subunit configurations were also studied. Human $\alpha 1$, $\beta 2$ and $\gamma 2L$ (long isoform of the $\gamma 2$ subunit) subunit cDNA in the vector pCDM8 were generously supplied by Dr. Nancy Leidenherimer (Louisiana State University, LA, USA). Rat GABA_A receptor $\alpha 1$, $\beta 2$ and $\gamma 2$ were generously provided by Dr. Cynthia Czajkowski (Wisconsin University at Madison, WI) and subcloned into mammalian transfection vector pCDNA3.1 (Invitrogen). Human embryonic kidney cells (HEK293) were transfected using PolyJet^{TM} DNA *In Vitro* transfection reagent (SignaGen Laboratories, Rockville, MD) to achieve transient expression of $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$, $\alpha 4\beta 2\delta$ or the corresponding mutants. Briefly, HEK cells were plated onto coverslips and transfected with wild-type or mutant subunits. Typically, GABA_A receptor subunit cDNA in 1:1:3 ratio for $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits or 1:1:5 for $\alpha 4$, $\beta 2$ and δ was added to cells growing exponentially on one coverslip placed in a 35 mm culture dish. cDNA ratios for each subunit are based on previous studies (Boileau et al., 2003; Meera et al., 2009) and our own experience. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection.

2.3. Site-Directed-Mutagenesis

Mutations of receptor subunit cDNA were performed using commercially available QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with commercially produced mutagenic primers (Integrated DNA Technologies). All mutants were verified by DNA sequencing (Biotechnology Core Facility, Texas Tech University, Lubbock, TX).

2.4. Electrophysiology

All whole-cell patch recordings were made at room temperature (22–25°C) at a holding potential of –60 mV. Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 7–8 MΩ. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. For brain slice studies, a single slice was transferred to a recording chamber (~2 ml) and superfused continuously (5–7 ml/min) with external solution consisting of the following (in mM): 140 NaCl, 3.0 KCl, 2 MgCl₂, 10 HEPES, 2.4 CaCl₂, 10 glucose, 330 mOsm and pH 7.3. Individual CA1 pyramidal neurons within the hippocampal slice were visualized using an upright, fixed stage microscope (Nikon Optiphot-2UD) equipped with standard Hoffman modulation contrast (HMC) optics and a video camera system (Sony model XC-75 CCD video camera module, DOT-X monitor). Miniature GABAergic inhibitory postsynaptic currents (mIPSCs) were isolated and recorded in the whole-cell configuration in the presence of glutamate receptor antagonists (10 μM CNQX and 50 μM AP-5) and tetrodotoxin (TTX, 0.3 μM). For studies on cloned receptors, a coverslip containing cultured cells was placed in a small chamber (~1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5–8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 MgCl₂, 3.0 CaCl₂, 10 HEPES, 10 D-glucose, pH 7.3. The currents (GABAergic mIPSCs, GABA-activated Cl[–] currents) from the whole-cell configuration were obtained using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) equipped with a CV201A headstage. Signals were filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and sampled at 30 kHz using a Digidata 1200 and pClamp software (pClamp 6.0, Axon Instruments) and stored on a computer for offline analysis. The series resistance (R_s) was compensated online by 60–70% in voltage-clamp mode to reduce voltage errors. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored the current response to a 5 mV voltage pulse on our digital oscilloscope. This stored trace was continually referenced throughout the recording. The cells in which access resistance or baseline showed instability during recording period were not included in the analysis.

2.5. Experimental protocol

GABA was prepared in the extracellular solution, and was applied from independent reservoirs by gravity flow for 10 sec to cells using a Y-shaped tube positioned adjacent to the cell. With this system, the 10–90% rise time of the junction potential at the open tip averages approximately 30 ms (Huang and Dillon, 1999). Once a control response was determined, the effect of MB was examined by co-applying it with a roughly EC₃₀ concentration of GABA. Because recovery from the drug-induced effect was readily obtained, effects of multiple concentrations of MB could generally be obtained from same cell. Current-voltage (I–V) relationship of GABA currents was assessed from a single cell using a ramp stimulus protocol. A transmembrane voltage ramped from –60 to +60 mV over 0.5 s time course was applied prior to (passive conductance phase) and during GABA application. The difference of these two current ramps (pA) represented the GABA-induced current, and was plotted as

a function of applied holding potential (mV) to yield the I–V curve of GABA-induced currents.

2.6. Chemicals

MB was purchased from American Reagent, Inc (Shirley, NY) and directly diluted in saline to the final concentrations. Tetrodotoxin (TTX) was purchased from Tocris (Bristol, UK) and prepared in ddH₂O. 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and 2-amino-5-phosphonopentanoic acid (AP-5) were provided from NIMH Chemical Synthesis and Drug Supply Program, and prepared in ddH₂O. Picrtoxin (PTX) was purchased from Sigma and prepared in DMSO. The stock solutions were diluted in saline so that the final DMSO concentration (v/v) was < 0.1%.

2.7. Data analysis

The GABA-activated currents were analyzed with Clampfit 6.0 (Axon Instruments). The miniature synaptic events were detected and analyzed using MiniAnalysis 6.03 (Synaptosoft, Decatur, GA). IPSC events were sampled for a 5-min continuous recording period for each neuron under control, MB and washout conditions. IPSC events were detected automatically using the threshold criteria of root mean square (RMS) noise and then inspected visually for any aberrant currents. GABA-dependent IPSCs were analyzed by the rise time (10–90%), peak amplitude and charge transfer (area under IPSC waveform) parameters of the MiniAnalysis. The time constants of current decay were calculated with biexponential function fitting of the averaged trace of grouped IPSC events.

GABA concentration-response profiles were fitted to the following equation: $I/I_{\max} = [GABA]^n / (EC_{50}^n + [GABA]^n)$, where I and $I_{\max}$ represent the normalized GABA-induced current at a given concentration and the maximal current induced by a saturating concentration of GABA, respectively, EC_{50} is 50% effective GABA concentration, and n is the Hill coefficient.

Concentration-response profiles for MB action were evaluated using approximately the EC_{30} GABA concentration. Concentration-response curves for MB inhibitory effect were fitted to the following equation: $I/I_{\max} = [MB]^n / ([MB]^n + IC_{50})$, where I is Cl^- current amplitude at the end of drug application normalized to control at a given MB concentration, IC_{50} is the half-blocking concentration, and n is the Hill coefficient. A minimum of three individual experiments was conducted for each paradigm.

MB facilitated current decay rate under a number of conditions. The percentage of decay (%D) was calculated according to the formula $\%D = 100 \times (I_{\text{peak}} - I_{10s}) / I_{\text{peak}}$, where I_{peak} is the current at the peak of the ligand (GABA or GABA plus MB) response and I_{10s} is the current remaining after 10 sec of continuous application. All data are presented as means $\pm$ SEM. Student's t-test (paired or unpaired) or one-way ANOVA followed by Holm-Sidak *post hoc* test was used to determine statistical significance ($p < 0.05$).

3. Results

Table 1 illustrates concentration-response profiles for the GABA_A receptor configurations assessed in the present studies. The EC₃₀ agonist (GABA or muscimol) concentrations were calculated and used for each configuration in subsequent investigation of potential MB effects.

3.1. MB inhibition in human $\alpha 1\beta 2\gamma 2$ GABA_A receptors

We first evaluated potential MB-induced effects on HEK293 cells stably expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors using whole-cell patch clamp technique. When co-applied with EC₃₀ GABA (10 μ M), MB (1–300 μ M) inhibited GABA-activated currents in a concentration-dependent manner (Fig. 1). The inhibitory effects of MB were rapid in onset and fully reversible within a one to three min washout period (Fig. 1A). MB exhibited its inhibitory action as low as 3 μ M, reducing GABA initial peak currents to 92 ± 2.6 of control ($p < 0.05$, paired t-test, compared to control). MB inhibited initial peak as well as end-application currents (Fig. 1A). The IC₅₀ values were 28 ± 6.1 and 31 ± 5.8 μ M for initial peak and end-application GABA-gated currents, respectively ($n = 6$, $p > 0.05$, paired t-test). Moreover, percentage of decay (%D) was increased by MB at concentrations above 100 μ M (%D: from $23 \pm 3.3\%$ at control to $48 \pm 4.3\%$ at 100 μ M MB; from $19 \pm 2.6\%$ at control to $69 \pm 3.4\%$ at 300 μ M MB, $n = 6$, $p < 0.05$, paired t-test, compared to control). As a result, 300 μ M MB decreased GABA currents to $25 \pm 3.9\%$ and $9.7 \pm 1.9\%$ of control for initial peak and end-application current, respectively ($p < 0.05$, paired t-test). As enhanced current decay reflects inhibitory action via desensitization or channel block and better represents MB-inhibitory action, we quantified MB inhibition using end-application currents (Table 1). Next, we examined the effect of pre-applied 10 μ M MB for 2 min on the subsequent response to 10 μ M MB co-applied with 10 μ M GABA in human $\alpha 1\beta 2\gamma 2$ receptors. As shown in Fig. 2, pre-equilibration of MB did not alter the ability of MB to block GABA response. On average, MB inhibited GABA-activated currents to $78 \pm 2.7\%$ via co-application and $78 \pm 2.1\%$ with pre-treatment ($n = 5$).

To further distinguish whether MB primarily acts on GABA molecules or GABA_A receptors, we examined the MB effect on the currents gated by another GABA_A receptor agonist, muscimol, which is structurally different from GABA but acts at the GABA binding site (Amin and Weiss, 1993). As shown in Fig. 3, MB (1–1000 μ M) resulted in a concentration-dependent inhibition on the currents activated by EC₃₀ muscimol (2.5 μ M) in human $\alpha 1\beta 2\gamma 2$ receptors. The IC₅₀ for MB inhibition was 29 ± 2.8 μ M and Hill coefficient was 1.3 ± 0.1 ($n = 5$). Similar MB effect on currents activated by two agonists with different structure favors MB action on the receptors.

3.2. Dependence of inhibition by MB on the GABA concentration

MB may modulate GABA-activated currents by affecting the affinity of receptors for GABA and/or the efficacy of GABA at the receptors. To distinguish between these possibilities, the effect of MB on concentration-response curves was studied in human $\alpha 1\beta 2\gamma 2$ receptors stably expressed in HEK cells. As shown in Fig. 2, MB (30 μ M) had greater inhibitory effect on the currents activated by low [GABA] but had little effect on the response to saturating

[GABA] in $\alpha 1\beta 2\gamma 2$ receptors. As a result, the EC_{50} for GABA shifted from $34 \pm 4.9 \mu\text{M}$ in control to $49 \pm 5.2 \mu\text{M}$ in the presence of $30 \mu\text{M}$ MB ($n=8$, $p<0.05$, paired t-test), whereas the maximum current and Hill coefficient were not significantly altered (Fig. 4A). To further examine dependence of MB inhibition on [GABA], we examined MB inhibitory curves at different [GABA]. As shown in Fig. 4B and Table 2, the blocking effect of MB depends on the concentration of GABA used. IC_{50} values were $19 \pm 2.2 \mu\text{M}$ (with $5 \mu\text{M}$ GABA), $31 \mu\text{M} \pm 1.8$ ($10 \mu\text{M}$ GABA) and $218 \pm 28 \mu\text{M}$ ($30 \mu\text{M}$ GABA) ($p<0.05$, one-way ANOVA test). These data suggest that MB blocks GABA currents in a competitive manner.

3.3. Effect of MB on the GABA current-voltage relationships

Because a voltage-dependent effect of a drug may shed some light on possible sites of action, we assessed the extent to which the effect of MB on $GABA_A$ receptors may be influenced by membrane voltage. We tested MB's ability to modulate the current-voltage (I-V) relationship of GABA-induced currents recorded from human $\alpha 1\beta 2\gamma 2$ receptors. In the absence of MB, the I-V relationship displayed outward rectification (Fig. 5A), consistent with our previous report (Huang and Dillon, 1999). In the presence of $30 \mu\text{M}$ MB, the reversal potential of GABA-activated current was not altered (Fig 5A). However, the MB-induced inhibition of the GABA response displayed a modest but significant voltage-dependent effect (Fig. 5A). The average inhibitory effect of $30 \mu\text{M}$ MB was $58 \pm 3.2\%$ at -60 mV and $45 \pm 4.5\%$ at $+60 \text{ mV}$ ($n=13$, $p<0.05$, paired t-test) (Fig. 5B).

3.4. Subunit-dependent activity of MB at $GABA_A$ receptors

$GABA_A$ subunit composition determines pharmacological properties (Huang et al., 2006). For example, the $\gamma 2$ subunit is required for sensitivity to the benzodiazepine (BDZ) family of compounds (Sigel and Buhr, 1997). δ -subunit containing $GABA_A$ receptors are insensitive to BDZ but are a preferred receptor subtype for gaboxadol (THIP) and neurosteroids at low (nanomolar) concentrations (Stell et al., 2003; Krosgaard-Larsen et al., 2004). Thus we investigated whether MB preferentially interacts with specific subunit configurations of $GABA_A$ receptors. HEK293 cells were stably expressing rat $\alpha 3\beta 2\gamma 2$ rat $\alpha 6\beta 2\gamma 2$ or transiently transfected with rat $\alpha 1\beta 2$, human $\alpha 5\beta 2\gamma 2$ and rat $\alpha 4\beta 2\delta$ receptors. Currents activated using equipotent [GABA] (EC_{30} of corresponding $GABA_A$ receptors, Table 1) were recorded with co-application of varying [MB] ($1\text{--}300 \mu\text{M}$). As shown in Fig. 6 and Table 2, MB inhibited GABA-gated currents of all $GABA_A$ receptor configurations tested in a concentration-dependent manner. Furthermore, $\alpha 5\beta 2\gamma 2$ receptors had higher sensitivity to MB while $\alpha 6\beta 2\gamma 2$ had relatively less sensitivity to MB compared to human $\alpha 1\beta 2\gamma 2$ receptors (Table 2). Whereas maximal efficacy was not established in all cases due to insolubility issue of MB at concentrations $> 1 \text{ mM}$, in most cases residual currents at end-application of $300 \mu\text{M}$ MB were 10–30 % of control.

3.5. Effect of $\beta 2(T6'F)$ mutation on MB inhibition

MB had inhibitory effects on all receptor subunit configurations examined, suggesting a conserved site for this action. A pore-lining threonine residue (T'6) within the second transmembrane domain (TM2), which is highly conserved among all $GABA_A$ subunits, confers sensitivity of $GABA_A$ receptors to picrotoxin (PTX) (Gurley et al., 1995; Zhang et al., 1995), pentylentetrazole (Dibas and Dillon, 2000), ethanol (Johnson et al., 2012) and

4'-ethynyl-4-n-propylbicycloorthobenzoate (EBOB) (Hisano et al., 2007). Thus, we examined whether this residue is also involved in MB's inhibitory effect. The effect of MB on GABA currents activated by an EC₃₀ GABA was assessed in wild type $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 2(T6'F)\gamma 2$ receptors transiently transfected in HEK293 cells. The ability of a $\beta 2$ T6'F mutation to block sensitivity to PTX (Gurley et al., 1995) was confirmed here (data not shown). As shown in Fig. 7 and Table 2, mutation of $\beta 2$ T6'F had little influence on MB-mediated inhibition of GABA-activated currents. On average, IC₅₀ values were 34 ± 2.5 and 35 ± 6.0 μ M for wild type and mutant receptors, respectively. The inhibitions of GABA response caused by 300 μ M MB were 70 ± 5.9 and 77 ± 6.8 % for wild type and $\alpha 1\beta 2(T6'F)\gamma 2$, respectively ($p > 0.05$, unpaired t-test). These data suggest that MB-induced inhibition of GABA_A receptors is not mediated via T6' at the TM2 region.

3.6. Effect of mutations of GABA binding residues on MB inhibition

The above results indicate MB inhibits GABA-activated currents in a competitive manner. This led to the possibility that MB may interact at the GABA binding site. Based on previous investigations (Amin and Weiss, 1993; Boileau et al., 1999; Holden and Czajkowski, 2002; Huang et al., 2004), we mutated two key residues within the GABA binding pocket in the N-terminal domains of human GABA_A receptors: F64A in the $\alpha 1$ subunit and Y205F in the $\beta 2$ subunit. The mutant $\alpha 1$ or $\beta 2$ subunits were co-expressed with wild type $\beta 2$ and $\gamma 2$ or $\alpha 1$ and $\gamma 2$ subunits, respectively. The ternary receptors containing mutant $\alpha 1$ or $\beta 2$ subunits greatly shifted the GABA concentration-response curve to the left, resulting in approximately 47–4,280 folds increase in GABA EC₅₀ compared to wild type $\alpha 1\beta 2\gamma 2$ receptors (Table 1) (Huang et al., 2004). The effect of MB on GABA-activated currents was assessed using EC₃₀ GABA. Single mutation of Y205F at $\beta 2$ subunits significantly attenuated both potency and efficacy of MB inhibitory effect, resulting in a 3-fold increase in IC₅₀ (from 31 ± 1.8 μ M in wild type to 93 ± 8.6 μ M in mutant) and 27% reduction of MB efficacy measured with 300 μ M MB (from 90 ± 1.9 % in wild type to 66 ± 5.1 % in mutant receptors, $p < 0.05$, unpaired t-test) (Table 2, Fig. 8). The $\alpha 1$ F64A mutation dramatically diminished MB sensitivity in $\alpha 1\beta 2\gamma 2$ configuration. The efficacy for MB inhibition was reduced by 83% [15 ± 5.3 % in $\alpha 1(F64A)\beta 2\gamma 2$] compared to wild type. Double point mutations of $\alpha 1(F64A)\beta 2(Y205F)\gamma 2$ essentially abolished MB effect (Fig. 8).

3.7. MB inhibition of hippocampal GABA_A receptors

To ensure that blockade of GABA_A receptors was not unique to recombinant receptors, we assessed its effect on GABA currents recorded from pyramidal neurons in the mouse hippocampal CA1 area. CA1 pyramidal neurons express synaptic GABA_A receptors consisting of $\alpha 1$ -3, $\beta 1$ -3 and $\gamma 1$ -3 subunits, and extrasynaptic GABA_A receptors containing either $\alpha 4/\alpha 5$ or δ subunits (Brunig et al., 2002; Stell et al., 2003; Caraiscos et al., 2004). As shown in Fig. 9, MB also reversibly and dose-dependently blocked the whole-cell currents activated by 10 μ M GABA. The ability of MB to block native GABA_A receptors was comparable to that observed in the recombinant receptors. To further investigate whether MB modulated GABAergic neurotransmission, we assessed the effect of MB on miniature GABAergic IPSCs (mIPSCs) recorded with whole-cell configuration from mouse CA1 pyramidal neurons. GABAergic mIPSCs were isolated by blockade of ionotropic glutamate receptors (50 μ M AP-5 and 10 μ M CNQX) and Na⁺ channels (0.3 μ M TTX), at a holding

potential of -60 mV. Once the baseline was stable, mIPSCs were collected over a 5-min period before, during and after the perfusion of $10\text{ }\mu\text{M}$ MB. As shown in Fig. 10, in all recorded cells ($n=7$), MB decreased amplitude, frequency and charge transfer of mIPSCs. The mean amplitude of mIPSCs was reduced from 39 ± 3.3 pA in control to 33 ± 2.5 pA in the presence of MB ($p<0.01$, paired t-test). The 10–90% rise time was increased by MB while decaying phase of mIPSCs was not altered. Moreover, MB modulatory effect on GABA_A receptor-mediated synaptic currents was reversible (Fig. 10).

4. Discussion

To our knowledge, we demonstrate here for the first time that MB inhibits GABA_A receptors at clinically relevant concentrations (Peter et al., 2000). Our data show that MB reversibly and dose-dependently blocks GABA-activated currents from recombinant GABA_A receptors and hippocampal CA1 pyramidal neurons. Theoretically, MB may modulate GABA-activated currents by reducing effective [GABA] as a result of MB chemically reacting with the GABA molecule, acting on GABA_A receptors and/or influencing receptor phosphorylation via intracellular pathways. Several lines of evidence presented here suggest that MB directly acts on the receptors. First, enhanced current decay at high [MB] is inconsistent with reduced effective [GABA] due to direct reaction of MB with the GABA molecule. Similar inhibition of MB on the currents activated by GABA and the structurally distinct GABA agonist muscimol also supports MB action on the receptors instead of the ligands. Second, MB action is rapid in onset and washout, which is incompatible with an effect mediated via changes in intracellular signaling. Finally, results from mutagenesis studies provide direct evidence to support MB interaction with GABA_A receptors.

We attempted to map the action site for MB inhibition with investigation of different GABA subunit compositions and site-directed mutagenesis. MB blockade of the GABA response was demonstrated in all subunit configurations we tested here. Preservation of MB inhibition in BZD-insensitive receptors ($\alpha 1\beta 2$ and $\alpha 6\beta 2\gamma 2$) excludes the BZD site for MB action. GABA_A receptors containing δ -subunits are generally thought to be more sensitive to THIP, neurosteroids and Zn^{2+} than those containing γ subunits (Korpi et al., 2002; Stell et al., 2003; Krosgaard-Larsen et al., 2004). We found that the sensitivity of δ -containing receptors to MB is similar to that of other subunit configurations, suggesting that MB action is unlikely mediated via THIP, neurosteroids or Zn^{2+} sites. The fact that mutation of T6'F in the $\beta 2$ subunit has little influence on MB's inhibitory effect eliminates likelihood of MB action via the site associated with the channel blocker picrotoxin. Competitive antagonism of MB with GABA supports the notion that MB may interact with the GABA binding site. Based on $4\text{ }\text{\AA}$ resolution of acetylcholine binding protein (AChBP) (Miyazawa et al., 2003; Unwin, 2005) and $3\text{ }\text{\AA}$ resolution of GABA_A receptor $\beta 3$ crystal structures (Miller and Aricescu, 2014), the ligand binding site is formed by loops (designated loops A–F) at the inter-subunit interfaces (Corringer et al. 2000). We have identified two residues ($\beta 2$ Y205 of loop C and $\alpha 1$ F64 of loop D) of the GABA binding pocket that confer MB sensitivity. Previous studies have shown that the Y205 residue of the $\beta 2$ subunit is complementarily facing F64 of the $\alpha 1$ subunit within the binding pocket (Newell and Czajkowski, 2003; Miller and Aricescu, 2014). Mutation of $\beta 2$ Y205F and/or $\alpha 1$ F64A caused remarkable shifts in both GABA and MB sensitivity. Furthermore, the reduction of MB sensitivity

appears to be in parallel with the shifts of GABA EC₅₀ in the following sequence: $\alpha 1(\text{F64A})\beta 2(\text{Y205F})\gamma 2 > \alpha 1(\text{F64A})\beta 2\gamma 2 > \alpha 1\beta 2(\text{Y205F})\gamma 2$. It has been reported that the ability of ligands to bind to GABA_A receptors is inhibited by protons (Huang et al., 2004; Wang et al., 2005). Notably, the pK_a of MB is about 0 to -1; hence effectively 100% of MB is in the protonated form at physiological pH (DiSanto and Wagner, 1972). However, whether MB directly or allosterically interacts with GABA in the agonist binding site requires further investigations using radioligand binding and computer modeling. Voltage-dependence would not be expected for a ligand interacting at the GABA binding site. However, this effect was nominal, and in fact might relate as much to relative channel open probability than voltage per se (O'Toole and Jenkins, 2012). Outward rectification is relatively enhanced at lower level of open probability in the presence of MB. As a result, MB-induced inhibition at positive potentials is less than at negative potentials.

MB at a dose of 1–4.0 mg/kg is safe in humans (Peter et al., 2000; Walter-Sack et al., 2009). Pharmacokinetic studies show that the half-life of MB in human body is approximately 10 hours and bioavailability is ~73%. Depending on the route of administration (i.e., oral vs i.v.), the peak concentration of MB in blood ranges from 2.2–19 μM with typical dosage (1–2 mg/kg) commonly used in clinical treatments as well as clinical trials as memory-enhancing intervention (Peter et al., 2000; Walter-Sack et al., 2009). MB can accumulate rapidly in the brain and its concentration is 10–20 times higher in the brain than in the blood one hour following systemic administration, reaching brain concentration up to hundreds of μM (Peter et al., 2000). In the present studies, we show MB started to inhibit GABA_A receptors at concentrations as low as 3 μM . MB was able to inhibit $\gamma 2$ -containing synaptic receptors as well as extrasynaptic receptors with IC₅₀ values from 19 to 49 μM and inhibitory efficacy from 74–96%. In hippocampal slices, MB was also shown to inhibit GABA-evoked currents and GABAergic mIPSCs in CA1 pyramidal neurons. The decreased frequency of mIPSCs in the presence of MB is in a good agreement with inhibition of presynaptic $\alpha 7$ nicotinic ACh receptors by MB (Al Mansouri et al., 2012). Since MB competitively inhibits GABA_A receptor function, reduction of mIPSC amplitude by MB seems contradictory to the view that synaptic GABA levels were thought to be saturating. However, The studies from the last two decades indicate that the actual concentration of neurotransmitter at postsynaptic receptors is not invariably saturating, being influenced by cleft surface area, number of neurotransmitter vesicles released, cleft configuration and time (Mody and Pearce, 2004; Barberis et al., 2011). A substantial number of postsynaptic receptors are exposed to subsaturating [GABA] (Hill et al., 1998). Inhibition of mIPSC amplitude in the hippocampal neurons by MB could be at least partially attributed to dynamic fluctuation of [GABA] at postsynaptic receptors and/or reduced GABA release from presynaptic neurons caused by MB inhibition on nACh $\alpha 7$ receptors (Al Mansouri et al., 2012). Modulation of GABAergic mIPSC amplitude has also been reported with zolpidem (Defazio and Hablitz, 1998; Chen et al., 2007) and protons (Mozrzymas et al., 2003; Chen and Huang, 2014). Collectively, our data suggest that MB inhibits GABA_A receptor-mediated neurotransmission at clinically relevant concentrations.

The memory-enhancing effects of low-dose methylene blue were first shown in rodents 30 years ago, and have been demonstrated in short-term or long-term memory tasks in rodents and humans (Callaway et al., 2004; Gonzalez-Lima and Bruchey, 2004; Rojas et al., 2012;

Rodriguez et al., 2016a; Rodriguez et al., 2016b). MB has recently been investigated as a memory-enhancing drug to treat neurodegenerative diseases associated with impaired mitochondrial oxidative metabolism in animal models and clinical trials (Lin et al., 2012; Poteet et al., 2012; Rojas et al., 2012; Zhao et al., 2016). The beneficial memory effects of MB are traditionally linked to its remarkable antioxidant, metabolic-enhancing properties in mitochondria and inhibition of acetylcholinesterase (AChE) (Oz et al., 2011; Rojas et al., 2012). The results presented here reveal GABA_A receptors as a novel target for MB's neurological effects. The GABA_A receptor mediates most central nervous system inhibition and plays a critical role for synaptic plasticity and learning behaviors. The involvement of GABAergic inhibitory interneurons during long-term potentiation (LTP) induction is well documented (Kullmann and Lamsa, 2011). Decreased GABA-mediated synaptic inhibition in the hippocampus generally results in an enhanced performance in learning and memory. For example, blockade of GABA blockers dramatically facilitated the induction of LTP (Wigstrom and Gustafsson, 1983; Wigstrom and Gustafsson, 1985). The benzodiazepine antagonist flumazenil or partial inverse agonists facilitated place learning in the Morris water-maze (Lal and Forster, 1990; Brioni et al., 1991; McNamara and Skelton, 1993). In addition, recent studies have demonstrated that elevated GABA inhibitory tone in the hippocampus impairs cognitive function in animal models of Alzheimer's disease and in AD patients (Jo et al., 2014; Wu et al., 2014). Our results combined with these prior findings implicate GABA_A receptors in the memory-enhancing effects of MB. The therapeutic window for this effect may be relatively narrow, however. Although MB is safe when used at therapeutic doses (2 mg/kg), higher doses can cause adverse CNS effects such as anxiety, confusion and dizziness, and a single bolus injection of 5 mg/kg MB in adult rats can cause neuronal death (Vutskits et al., 2008). The adverse CNS effects noted above as well as the neurotoxic effects could be explained by extension of MB's therapeutically beneficial, memory-enhancing effects on GABAergic neurotransmission.

In summary, we demonstrated that MB directly blocks GABA_A receptors as well as GABAergic neurotransmission at clinically relevant concentrations, which may contribute to the neurological effects of MB. Furthermore, our data suggest that MB inhibits GABA_A receptor function by direct or allosteric interaction with GABA binding sites.

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Abbreviations

AChBP	acetylcholine binding protein
AP-5	2-amino-5-phosphonopentanoic acid
BDZ	benzodiazepine
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione

DMSO	dimethyl sulfoxide
GABA	γ -aminobutyric acid
HEK293	human embryonic kidney cell line
LTP	long-term potentiation
MB	methylene blue
MUS	muscimol
mIPSCs	miniature inhibitory postsynaptic currents
PTX	picrotoxin
TM2	second transmembrane domain
TTX	tetrodotoxin

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Highlights

- Methylene blue inhibits recombinant and native GABA_A receptors at clinically relevant concentrations
- Methylene blue blocks synaptic and extrasynaptic GABA_A receptors
- The inhibitory action of methylene blue is mediated by interaction with GABA_A receptors at the agonist binding site

Figure 1. Concentration-response relationship for MB-mediated inhibition of human $\alpha 1\beta 2\gamma 2$ receptors stably expressed in HEK293 cells

A, Representative traces showing whole-cell currents activated by EC_{30} GABA recorded from HEK293 cells stably expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors. MB (1–300 μM) was co-applied with EC_{30} GABA (10 μM) for 10 sec. Note that MB caused reversible and concentration-dependent inhibition of GABA currents. **B**, A plot of the MB concentration-response relationship. All currents are normalized to the response to GABA without MB. The IC_{50} values and Hill coefficients (in parentheses) were 28 ± 6.1 (1.2 ± 0.3) and 31 ± 5.8 (1.2 ± 0.3) for initial peak and end-application GABA-gated currents, respectively. The curve shows the fit of the mean of at least 5 cells as described in the methods.

Figure 2. Effect of pre-applied MB on the response of $\alpha 1\beta 2\gamma 2$ receptors to MB

A, Representative traces showing whole-cell currents activated by 10 μ M GABA recorded from HEK293 cells stably expressing human $\alpha 1\beta 2\gamma 2$ receptors. MB (10 μ M) was co-applied with GABA for 10 sec to the cell without or with 2-min pre-treatment with MB (10 μ M). **B**, Summary data on MB-induced inhibition of GABA response from the same cells without or with 2-min MB pre-treatment. $n=5$.

Figure 3. Concentration-response relationship for MB-mediated inhibition of the currents activated by muscimol

A, Representative traces showing whole-cell currents activated by EC₃₀ muscimol (MUS) recorded from HEK293 cells stably expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors. MB (1–1000 μM) was co-applied with 2.5 μM muscimol for 10 sec. **B**, A plot of the MB concentration-response relationship. All currents are normalized to the response to control without MB. The IC₅₀ value was $29 \pm 2.8 \mu\text{M}$ and Hill coefficient 1.3 ± 0.1 , $n=5$.

Figure 4. Dependence of MB blocking effect on GABA concentration

A, The currents activated by varying [GABA] (1–1000 μM) were recorded from the same cell in the absence and presence of 30 μM MB. MB increased the EC_{50} for GABA from 34 ± 4.9 to 49 ± 5.2 μM ($n=8$, $p<0.05$, paired t-test) but did not significantly affect the maximum of GABA currents. **B**, Concentration-response curves for MB blocking effect at varying [GABA]. The curve at 10 μM GABA was re-plotted from Fig. 1B for direct comparison. Increase in [GABA] caused a right shift in MB blocking curving with IC_{50} shifted from 19 to 218 μM with 5 μM or 30 μM, respectively.

Figure 5. Voltage-dependence of MB effect on stable human GABA_A $\alpha 1\beta 2\gamma 2$ receptors MB (30 μ M) was co-applied with 10 μ M GABA. The current response to a voltage ramp from -60 to $+60$ mV (0.23 mV/ms) at control, during MB application and washout. **A**, Summary data from 13 cells. Note that MB did not affect the reversal potential of GABA response. **B**, Summary data for MB-induced inhibition of GABA-activated currents at different V_H . *, $p < 0.05$, paired t-test, compared to the value at -60 mV.

Figure 6. MB-induced inhibition in various GABA_A receptor subunit configurations

A, Representative traces showing whole-cell currents activated by EC₃₀ GABA recorded from HEK293 cells expressing varying GABA_A receptor subunit compositions (rat $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, $\alpha 5\beta 2\gamma 2$, $\alpha 6\beta 2\gamma 2$, $\alpha 1\beta 2$ or $\alpha 4\beta 2\delta$). MB (1–1000 μM) was co-applied with equipotent GABA (EC₃₀ of corresponding receptors) for 10 sec. **B**, A plot of the MB concentration-response relationship. All currents are normalized to the response to GABA without MB. The IC₅₀ values, Hill coefficients and efficacy of MB effect in various GABA_A receptor subunit configurations are shown in Table 2.

Figure 7. Mutation TM2 T6'F did not affect MB-induced inhibition of GABA response in rat $\alpha 1\beta 2\gamma 2$ receptors transiently expressed in HEK293 cells

A, Representative traces showing currents activated by EC_{30} GABA and by co-application of MB (1–300 μM) in wild type (WT) $\alpha 1\beta 2\gamma 2$ and mutant $\alpha 1\beta 2(\text{T6'F})\gamma 2$ receptors. **B**, Summary data for MB effect on WT and mutant receptors. Mutation T6'F at the $\beta 2$ subunit had little influence on MB-induced inhibition of GABA currents. The IC_{50} values, Hill coefficients and efficacy of MB effect are shown in Table 2.

Figure 8. Mutation of the residues in GABA binding pocket dramatically reduced MB-induced inhibition in human $\alpha 1\beta 2\gamma 2$ GABA_A receptors

A, Whole-cell currents activated by EC₃₀ GABA were recorded in the control and presence of MB (3–1000 μM) from the receptors containing $\beta 2(T205F)$ and/or $\alpha 1(F64A)$ mutation.

B, Summary data for the MB effect. All currents are normalized to the control (assigned as 100%). Compared to wild type, mutation of $\alpha 1(F64A)$ and/or $\beta 2(T205F)$ dramatically reduced both potency and efficacy of MB inhibitory effect. See Table 2 for IC₅₀ values, Hill coefficients and efficacy of MB effect.

Figure 9. MB inhibited GABA-activated currents recorded from mouse hippocampal CA1 pyramidal neurons

A, Representative whole-cell current traces showing MB (10–300 μM) caused a reversible and concentration-dependent inhibition of the currents activated by 10 μM GABA. **B**, Summary data for MB inhibition. All currents are normalized to control. $n=5-7$.

Figure 10. MB inhibited GABAergic miniature IPSCs (mIPSC) recorded from mouse hippocampal CA1 pyramidal neurons

A, mIPSCs were recorded in control, during 10 μ M MB perfusion and washout. GABAergic mIPSCs were isolated with 50 μ M AP-5, 10 μ M CNQX and 0.3 μ M TTX. **B**, Average of all IPSC events during control, MB treatment and washout. **C&D**, Summary data for the MB effect on mIPSC amplitude. **E**, Summary data for the MB effect on mIPSC kinetics. All values are normalized to control. $n=7$, *, $p<0.05$; **, $p<0.01$, paired t-test, compared to control.

Table 1GABA sensitivity of different GABA_A receptor configurations

Receptor configurations	EC ₅₀ (μM)	Hill coefficient	Sample size	EC ₃₀ (μM)
Human α1β2γ2	34 ± 4.9	1.1 ± 0.2	8	10
Human α1β2γ2 (MUS)	5.5 ± 0.5	1.2 ± 0.1	7	2.7
Rat α1β2γ2	8.8 ± 0.8	1.4 ± 0.1	4	5
Rat α3β2γ2 ¹	33 ± 2.1	1.1 ± 0.03	4	20
Rat α4β2δ ²	0.6 ± 0.1	1.0 ± 0.00	5	0.4
Human α5β2γ2	16 ± 1.0	1.3 ± 0.09	4	9
Rat α6β2γ2 ¹	1.6 ± 0.1	1.6 ± 0.1	4	0.5
Rat α1β2	1.6 ± 0.2	1.3 ± 0.1	4	0.8
Rat α1(T6'F)β2γ2	9.4 ± 0.4	1.2 ± 0.03	4	3
Human α1β2(Y205F)γ2 ³	655 ± 144	1.1 ± 0.10	6	300
Human α1(F64A)β2γ2 ³	16,000 ± 1,500	1.5 ± 0.21	3	5,000
Human α1(F64A)β2(Y205F)γ2 ³	60,000 ± 8,900	1.5 ± 0.08	5	25,000

1, 2 & 3 indicate the values reported in previous publications:

¹ (Bell-Horner et al., 2000);² (Feng et al., 2006);³ (Huang et al., 2004).

Table 2 Comparison of the potency and efficacy of MB at different GABA_A receptor configurations

Receptor configurations	IC ₅₀ (μM)	Hill coefficient	Efficacy ⁺ (% of control)	Sample size
Human α1β2γ2 (at 5 μM GABA)	19 ± 2.2	1.1 ± 0.1	3.2 ± 1.5	5
Human α1β2γ2 (at 10 μM GABA)	31 ± 1.8	1.3 ± 0.1	9.7 ± 1.9	6
Human α1β2γ2 (at 30 μM GABA)	218 ± 28 *	0.8 ± 0.05	47 ± 4.8	8
Human α1β2γ2 (at 2.5 μM MUS)	29 ± 2.8	1.3 ± 0.1	10 ± 2.8	5
Rat α1β2γ2	49 ± 10	1.0 ± 0.2	29 ± 5.9 *	7
Rat α3β2γ2	19 ± 1.9	1.3 ± 0.1	7.7 ± 2.8	5
Rat α4β2δ	35 ± 3.3	1.4 ± 0.2	28 ± 4.6 *	5
Human α5β2γ2	9.3 ± 1.1 *	1.4 ± 0.2	0 *	7
Rat α6β2γ2	60 ± 10 *	1.2 ± 0.2	26 ± 6.1 *	7
Rat α1β2	29 ± 2.4	1.2 ± 0.1	3.6 ± 1.9	4
Rat α1(T6'F)β2γ2	35 ± 6.0	1.5 ± 0.4	23 ± 6.8	6
Human α1β2(Y205F)γ2	93 ± 8.6 *	1.3 ± 0.1	34 ± 5.1 *	6
Human α1(F64A)β2γ2	n.d.	n.d.	85 ± 5.3 *	4
Human α1(F64A)β2(Y205F)γ2	n.d.	n.d.	91 ± 4.5 *	6

⁺Residual current is end-application current (% of control) in the presence of 300 μM MB.

n.d., not determined.

* p<0.05, one-way ANOVA followed by Holm-Sidak *post hoc* test.