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Adverse Effects of Methylene Blue on the Central Nervous System

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Background: **An increasing number of clinical observations suggest adverse neurologic outcome after methylene blue (MB) infusion in the setting of parathyroid surgery. Hence, the aim of the current study was to investigate the potentially neurotoxic effects of MB using a combination of *in vivo* and *in vitro* experimental approaches.**

Methods: **Isoflurane-anesthetized adult rats were used to evaluate the impact of a single bolus intravascular administration of MB on systemic hemodynamic responses and on the minimum alveolar concentration (MAC) of isoflurane using the tail clamp test. *In vivo*, MB-induced cell death was evaluated 24 h after MB administration using Fluoro-Jade B staining and activated caspase-3 immunohistochemistry. *In vitro*, neurotoxic effects of MB were examined in hippocampal slice cultures by measuring excitatory field potentials as well as propidium iodide incorporation after MB exposure. The impact of MB on dendritic arbor was evaluated in differentiated single cell neuronal cultures.**

Results: **Bolus injections of MB significantly reduced isoflurane MAC and initiated widespread neuronal apoptosis. Electrophysiologic recordings in hippocampal slices revealed a rapid suppression of evoked excitatory field potentials by MB, and this was associated with a dose-dependent effect of this drug on cell death. Dose–response experiments in single cell neuronal cultures revealed that a 2-h-long exposure to MB at non–cell-death-inducing concentrations could still induce significant retraction of dendritic arbor.**

Conclusions: **These results suggest that MB exerts neurotoxic effects on the central nervous system and raise questions regarding the safety of using this drug at high doses during parathyroid gland surgery.**

METHYLENE blue (MB) is a cationic thiazine dye being used in a wide range of different fields, such as biology, chemistry, and medicine. In clinical practice, administration of this compound has been shown to be effective in the treatment of methemoglobinemia[1] as well as ifosfamide-induced encephalopathy[2] and was proposed to be a potentially helpful adjunct in the treatment of vasoplegia accompanying septic shock.[3,4] Because MB rapidly crosses cell membranes and temporally stains living cells, direct application of low concentrations of this compound onto mucosal membranes is commonly used in endoscopic diagnostic procedures to allow better visualization of tissue structures.[5–7]

When injected intravenously, MB selectively accumulates in parathyroid glands, thereby facilitating the identification of these structures during surgery.[8] Indeed, preoperative intravenous administration of MB is now a universally used practice in parathyroid gland surgery, and numerous investigators have suggested the safety of this technique, even at higher doses (7.5–10 mg/kg).[9–11] Pharmacokinetic studies in rodents revealed that intravascular administration of MB leads to a rapid and extensive accumulation of this drug in the central nervous system (CNS).[12] In line, during the past few years, an emerging number of clinical observations have suggested that MB infusion during parathyroidectomy (5–10 mg/kg) might induce prolonged disorientation in the postoperative period.[13–21] These recent results are in accord with early descriptions reporting that intravenous injection of MB in comparable doses to healthy volunteers induced dizziness, headache, tremors, and mental confusion[22] and raise questions about the safety of use of MB in the setting of parathyroid surgery.

In line with the aforementioned clinical observations, experimental data also suggest potential toxic effects of MB on the CNS. *In vitro*, exposure of organotypic slice cultures of the young rat cerebellum to this compound resulted in a progressive destruction of differentiating cells,[23] whereas epidural injections of MB in cats induced axonal swelling and inflammation of leptomeninges.[24] However, the question whether the observed CNS dysfunction after intravascular injection of MB can reflect histopathologic manifestations of neurotoxicity remains to be elucidated. In the current study, we explored this possibility using a combination of *in vivo* and *in vitro* models permitting us to assess, both functionally and morphologically, the dose-dependent toxic effects of MB on the CNS.

Materials and Methods

Animals and Hemodynamic Monitoring

The experimental protocol was reviewed and approved by the Ethics Committee for Animal Research of the University Medical Center of Geneva and by the

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Cantonal Veterinary Office, Geneva, Switzerland. Sprague-Dawley male rats (400–430 g) were purchased from Iffa Credo (Saint-Germain sur l'Arbresle, France). All procedures were performed according to the guidelines stated in the National Institutes of Health publication *Guide for the Care and Use of Laboratory Animals*. Animals were placed in an induction chamber to which 5% isoflurane (Abbott Laboratories, Chicago, IL) was directed at a continuous oxygen flow of 3 l/min (isoflurane vaporizer; Ohmeda, Steeton, West Yorkshire, United Kingdom). After 5 min, rats were withdrawn from the induction chamber and positioned in dorsal recumbency, intubated with a 16-gauge polyethylene catheter, and mechanically normoventilated (40% O_2 in air) with a constant volume-cycled rodent ventilator (model 683; Harvard Apparatus Corp., South Natick, MA; tidal volume, 7 ml/kg; positive end-expiratory pressure, 2.5 cm H_2O; respiratory rate, 70–80 breaths/min). Airway pressures were measured using a calibrated pressure transducer (Validyne DP 45; Northridge, CA). Respiratory gases (isoflurane, carbon dioxide, oxygen) were monitored with a daily calibrated infrared spectrometer (Ultima; Datex/Instrumentarium, Helsinki, Finland). The left femoral artery was cannulated for continuous arterial pressure monitoring and blood gas analysis (Radiometer, Acid Base Laboratory, model 505; Copenhagen, Denmark). Physiologic variables were stored at a sampling rate of 200 Hz *via* an analog–digital interface converter (Biopac, Santa Barbara, CA) on a microcomputer allowing the continuous and precise recording of hemodynamic and respiratory variables over time. Heart rate was derived from the pressure tracings. Rectal temperature was monitored and maintained between 37° and 38°C by means of a heating pad. After baseline measurements, either normal saline (n = 5) or two doses of MB (1%; Sigma-Aldrich, Inc., Buchs, Switzerland; 5 or 50 mg/kg, n = 5 each) diluted in 2 ml normal saline were injected intraarterially over 30 s, and variables were continuously recorded for 10 min.

Determination of Minimum Alveolar Concentration of Isoflurane

The minimum alveolar concentration of isoflurane (MAC_{iso}) was determined according to the classic method described by Eger *et al.*[25] A noxious stimulus was applied with a long hemostat clamped to the first ratchet lock on the tail for 20 s. The tail was always stimulated proximal to a previous test site. The response was considered positive when a gross purposeful movement of the head, body, extremities, or combination of these was observed. Isoflurane concentration was decreased every 10 min in steps of 0.2% from an initial concentration of 2.0% until a gross purposeful movement or paw withdrawal was noticed; the concentration of isoflurane at this point was defined as MAC_{iso}. After baseline determination of MAC_{iso}, the same procedure was repeated 10 min after either 2 ml normal saline intraarterial injection (control group, n = 7) or two different doses of MB (1%; Sigma-Aldrich, Inc.; 5 mg/kg, n = 10; or 50 mg/kg, n = 3, diluted with normal saline to a total volume of 2 ml).

Cultures and Reagents

Organotypic hippocampal slice cultures were prepared from 6-day-old neonate Sprague-Dawley rats and maintained in culture for 10–15 days at the interface on a porous and transparent membrane as previously described.[26] The culture medium was composed of 50% minimal essential medium, 25% horse serum, and 25% Hanks' solution buffered to pH 7.2 by addition of 5 mM Tris and 4 mM $NaHCO_3$, with penicillin and streptomycin added. Cultures were kept in an incubator with 5% CO_2 at 37°C for the first 4 days and then transferred at 33°C. Slice cultures were usually maintained for 10–15 days in culture before being tested. To detect dying cells, cultures were exposed to propidium iodide (PI; 5 μg/ml; Sigma, St. Louis, MO) for 30 min at 33°C before visualization.

Dissociated neuronal cultures from the subventricular zone were prepared from newborn rats (P0) as described previously.[27] Briefly, the subventricular zone was dissected from coronal slices, dissociated mechanically, trypsinized, and purified using Percoll gradient centrifugation. Cells were plated onto polyornithine (Sigma)–coated coverslips in 35-mm Petri dishes (Falcon, Plymouth, United Kingdom) at a plating density of 5,000 cells/cm^2 and allowed to grow in Neurobasal medium (Invitrogen, Paisley, United Kingdom) with 2% B27 supplement (Invitrogen), 2 mM L-glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma). MB was added to the culture medium at the sixth day *in vitro* for 2 h, as described in the Results section.

Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma; 200 μM) was used for pharmacologic blockade of nitric oxide synthase, whereas H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma; 10 μM) was used to specifically inhibit guanylyl cyclase.

Histology, Immunofluorescence, and Fluoro-Jade Staining

Animals were killed 24 h after MB injection by an overdose of pentobarbital (50 mg/kg) and were perfused transcardially with a 4% paraformaldehyde (pH 7.4) solution (4% PAF). Brains were then removed and fixed overnight in 4% PAF. After cryoprotection using a 30% sucrose solution, brains were frozen and cut using a cryostat (Leica, Wetzlar, Germany). Coronal slices of 20 μm were plated onto gelatin-coated slides and postfixed for 30 min with 4% PAF. For immunolabeling to detect caspase-3 activity, sections were rinsed three times in phosphate-buffered saline (PBS) and then incubated with an anti–caspase-3 antibody (Cell Signaling Technology, Danvers, MA; 1:2,000 dilution) in 0.5% bovine serum

albumin and 0.3% Triton X-100 in PBS overnight at 4°C. Slices were then rinsed and incubated for 2 h with a goat anti-rabbit biotinylated antibody (Vector Laboratories Inc., Burlingame, CA; 1:500 dilution). Subsequently, bound antibodies were revealed using the avidin–biotin peroxidase complex (Vectastain ABC Elite PK6100; Vector Laboratories, Burlingame, CA; 1 h), and finally with the chromogen 3,3′ diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). After staining, slices were dehydrated, cleared with xylene, and coverslipped.

Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR) staining was performed according to the protocol of Schmued and Hopkins.[28] Briefly, 12-μm-thick 4% PAF–perfused sections were cut, collected on gelatin-coated slides, and dried overnight. Potassium permanganate (0.06%) was applied for 10 min to ameliorate signal-to-noise ratio, and slides were then incubated with Fluoro-Jade B (0.0004%) for 20 min. Slides were rinsed through three changes of distilled water for 1 min per change and air dried. Sections were then costained with 4′,6′-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO), a fluorescent nuclear stain, and coverslipped with DPX nonfluorescent mounting media (Sigma-Aldrich).

Immunocytochemistry in organotypic and single cell cultures was performed as described previously.[27,29] Briefly, organotypic and single cell cultures were fixed with cold (4°C) 4% PAF. Then, they were rinsed three times in PBS and incubated overnight at 4°C with the primary antibody diluted in PBS containing bovine serum albumin (0.5%) and Triton X-100 (0.3%). The mouse monoclonal antibody directed against β-tubulin isotype III (Sigma; 1:400 dilution) was used to identify neurons. Bound antibodies were revealed with Alexa-conjugated secondary antibodies (1/1,000 dilution; Molecular Probes, Carlsbad, CA) diluted in PBS and were applied for 90 min at room temperature.

Image Acquisition and Data Analysis

In the *in vivo* experiments, the number of activated caspase 3–positive neurons in the somatosensory cortex was counted with a Nikon Eclipse TE2000-U microscope (Nikon Corp., Tokyo, Japan). For these counts, a 10× Nikon objective and a digital camera (Retiga EX; Qimaging, Burnaby, Canada) controlled by Microbrightfield Neurolucida software (Microbrightfield, Williston, VT) was used. Under each experimental condition, five sections per brain were used to calculate the density of apoptotic cells in three different brains.

Propidium iodide fluorescence intensity in organotypic cultures was recorded with a Nikon Eclipse TE2000-U fluorescence microscope. All the exposure parameters were kept constant under each session of measurements. Quantification of fluorescence intensity was performed with Image J software.†† Values are expressed as the percentage of increase in mean pixel intensity of the whole slice at each treatment and at each time point compared with the mean pixel intensity of that slice before treatment (time 0).

Immunostained cultures were examined with a Nikon Eclipse TE2000-U microscope. For cell counts, a 10× Nikon objective and a digital camera (Retiga EX) controlled by Openlab software (version 3.1.2; Improvision, Coventry, England) was used. On each coverslip, 30 samples were randomly taken, and then samples were pooled (*i.e.*, total surface measured per coverslip was approximately 5 mm^2). Cell counts were made using Image J software (data are expressed as the number of neurons per mm^2 ± SEM and reflect results obtained from at least three independent experiments). For quantitative analysis of dendritic arbors, β-tubulin isotype III–positive cells were photographed using a 40× Nikon objective linked through the digital camera to the Openlab software. Before analysis, brightness and contrast were optimized with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The following parameters of dendritic shape and extent were then determined: number of primary dendrites, length of dendrites, and number of dendritic branches. Total dendritic length was measured drawing all visible processes with Image J. The remaining parameters were manually scored on the image. Processes shorter than 5 μm were excluded from the analysis.

Electrophysiologic Recordings

Slices that were 11–15 days *in vitro* (n = 5) were maintained in a recording chamber at 33°C and superfused at 1 ml/min with artificial cerebrospinal fluid containing 124 mM NaCl, 1.2 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM glucose, and 2 mM ascorbic acid, saturated with 95% O_2 and 5% CO_2.

Excitatory postsynaptic potentials (EPSPs) evoked by stimulation of Schaffer collaterals at low frequency (0.05 Hz) were recorded, through an artificial cerebrospinal fluid–filled glass pipette placed in the stratum radiatum of the CA1 region and using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). All data were processed by a homemade software developed with Labview (National Instruments, Austin, TX), and maximum amplitude of EPSPs were reported as a function of time.

The effects of MB on evoked EPSP were tested by switching the perfusion after 30–50 min of stable recordings to artificial cerebrospinal fluid supplemented with 10 μM MB and maintained in perfusion for approximately 1 h. In some experiments, MB was washed out for up to 2 h 30 min to test the reversibility of the effect.

Statistical Analysis

Throughout the study, values were expressed as mean ± SEM and analyzed for statistical significance. Differences

†† Available at: http://rsb.info.nih.gov/ij. Accessed September 12, 2007.

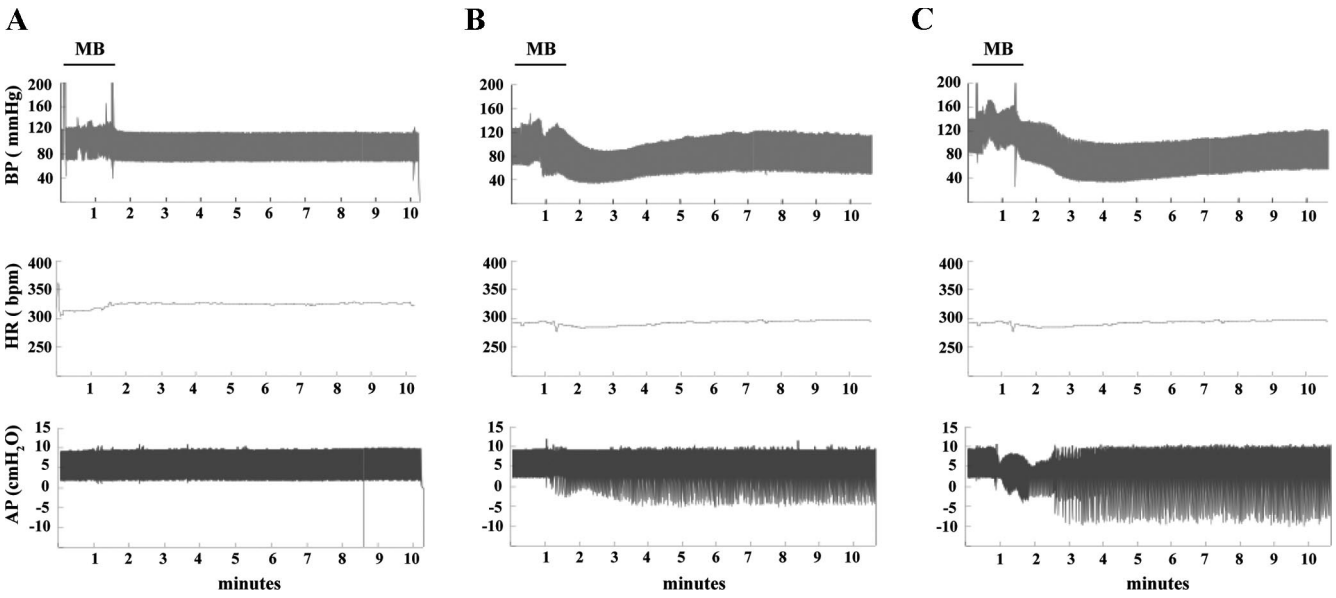

Fig. 1. Physiologic recordings from adult rats receiving intravascular bolus injections of saline as control (*A*), 5 mg/kg methylene blue (MB) (*B*), and 50 mg/kg MB (*C*). Data depict mean values from five animals per group (blood pressure [BP] and airway pressure [AP] tracings are averaged max [systolic or peak AP] and min [diastolic or minimal AP]). HR = heart rate.

between groups were first discriminated by one-way analysis of variance and then the unpaired *t* test was performed, where *t* was corrected for multiple comparisons against the untreated group using the Bonferroni test. $P < 0.05$ compared with the untreated control group. Statistical analyses were performed using Excel (Microsoft, Seattle, WA) and Prism (Graphpad Software, San Diego, CA) Software.

Results

Physiologic Response to Bolus Methylene Blue Injection

Because rapid intravascular injection of MB, through the inhibition of guanylyl cyclase in the vascular smooth muscle, could potentially alter systemic hemodynamics, we first checked the effects of single bolus dose administration of this agent at the systemic level. To this end, hemodynamic parameters were monitored in anesthetized and ventilated animals up to 10 min after the intravascular injection of a bolus dose of MB (5 and 50 mg/kg) over 30 s. Blood pressure and heart rate remained within the normal physiologic ranges after MB administration at any doses (fig. 1 and table 1). A small decrease in mean arterial blood pressure and heart rate was found in MB compared with saline-injected control animals during the first minutes after MB injections, but values returned to baseline levels by 10 min after bolus administration (fig. 1 and table 1). Also, no significant differences between blood gas parameters, hematocrit, and oxygen saturation levels could be detected between treatment groups at this time point (table 1). In contrast, we found a sustained increase in respiratory muscle activity after MB administration, manifested by a decrease in end-expiratory pressure during the imposed constant-volume mechanical ventilation, a pattern that was especially evident with the large MB dosage (fig. 1). This effect was not a compensatory consequence of the

Table 1. Physiologic Variables during Methylene Blue Administration

	Control		MB 5		MB 50	
	5 min	10 min	5 min	10 min	5 min	10 min
MAP, mm Hg	85.5 ± 3.5	82.5 ± 4.4	78.5 ± 7	70.4 ± 14.4	73.2 ± 7	81 ± 3.8
Heart rate, beats/min	323 ± 8	321 ± 8	321 ± 15	317 ± 33	323 ± 7	340 ± 11
pH	7.37 ± 0.01	7.37 ± 0.02	7.37 ± 0.02	7.35 ± 0.01	7.36 ± 0.02	7.35 ± 0.02
P_{O_2}, kPa	28 ± 1.3	27.5 ± 1.5	22.4 ± 1.4	25.4 ± 0.1	21 ± 6	20.5 ± 3.8
P_{CO_2}, kPa	5.6 ± 0.2	6 ± 0.3	5.7 ± 0.2	6.1 ± 0.1	5.8 ± 0.9	5.9 ± 1.2
Hematocrit, %	41.9 ± 0.3	43.5 ± 0.6	42.5 ± 0.1	42.2 ± 0.2	43.7 ± 0.3	44.3 ± 0.4

Data were collected during isoflurane anesthesia. Values are expressed as mean ± SEM; n = 3 animals per group.

MAP = mean arterial pressure; MB 5 = experimental group receiving single bolus methylene blue injection at a concentration of 5 mg/kg; MB 50 = experimental group receiving single bolus methylene blue injection at a concentration of 50 mg/kg; P_{CO_2} = partial pressure of carbon dioxide; P_{O_2} = partial pressure of oxygen.

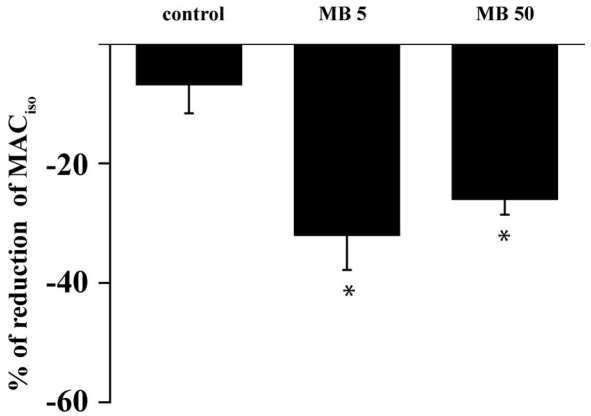

Fig. 2. Effect of methylene blue administration on the minimum alveolar concentration (MAC) of isoflurane. Results are expressed as mean ± SEM; n = 3–10 animals per group. * *P* < 0.05 compared with the untreated control group. MB 5 = experimental group receiving single bolus methylene blue injection at a concentration of 5 mg/kg; MB 50 = experimental group receiving single bolus methylene blue injection at a concentration of 50 mg/kg.

acid load accompanying MB injection, because injection of an equivalent volume of physiologic saline, titrated to the same pH as MB (pH 3.5), did not have any effect on respiratory muscle activity (fig. 1A).

Intravascular Injections of Methylene Blue Reduces the Minimum Alveolar Concentration of Isoflurane

Intracerebroventricular administration of MB has been previously shown to reduce the MAC of sevoflurane.[30] To determine whether systemic administration of MB could also reduce anesthetic requirements, in a series of experiments we evaluated the effects of intravascular MB injections on MAC_{iso} using the tail clamp test.[25] As seen in figure 2, bolus administration of MB at concentrations of both 5 and 50 mg/kg significantly reduced MAC_{iso}, indicating the potential effect of systemic MB administration on the CNS.

Single Bolus Injection of Methylene Blue Induces Apoptosis in the Brain

To investigate whether systemic administration of MB induces cell death in the CNS, brains of vehicle- as well as MB-treated animals were assessed 24 h after drug exposure. At this time point, Fluoro-Jade B staining revealed an important increase in the number of degenerating neurons in the brains from animals receiving bolus injections of both 5 and 50 mg/kg MB but not in control groups where saline injections were applied (figs. 3A–F). Activated caspase-3 immunohistochemistry confirmed these observations, showing apoptotic neurons in all brain regions of MB-treated rats (figs. 3G–I). Quantitative analysis of activated caspase 3–positive cells in the somatosensory cortex revealed a nearly fourfold increase in the number of apoptotic neurons after a single bolus injection of 5 mg/kg MB, whereas this effect was even

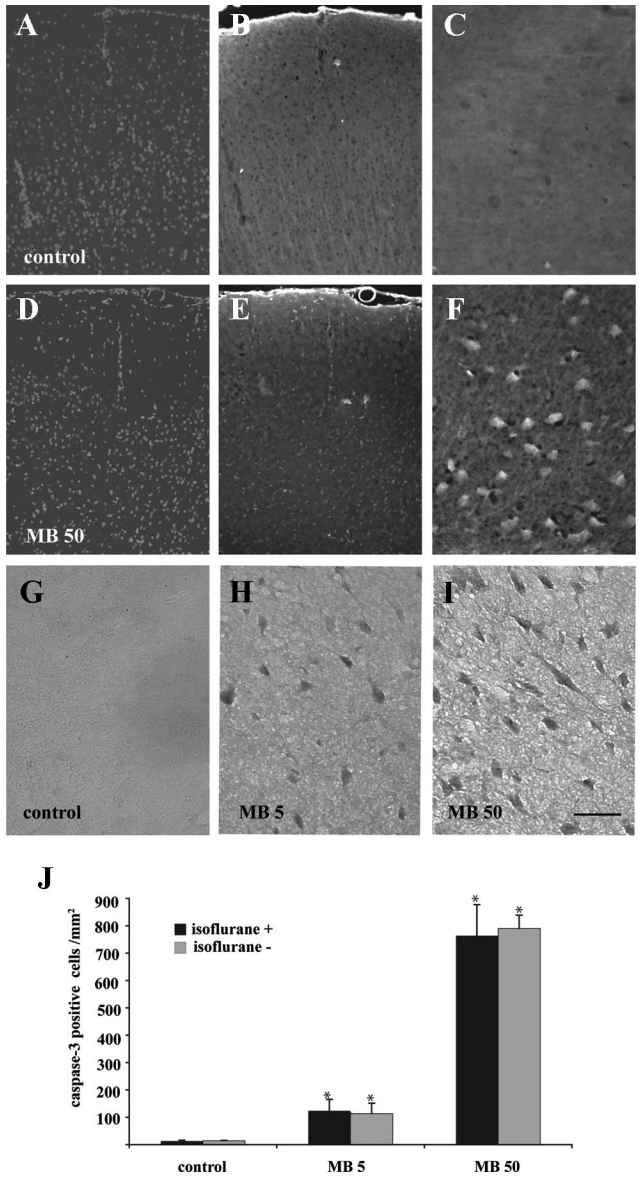

Fig. 3. Single bolus injection of methylene blue induces apoptosis in the brain. (*A*) Representative Hoechst counterstained photomicrograph of the cerebral cortex in the control group 24 h after single bolus saline injection. (*B* and *C*) Fluoro-Jade B staining of this same section demonstrates the absence of dying neurons in the control group. (*D*) Hoechst counterstaining of cortical sections from methylene blue–treated rats (50 mg/kg). In contrast to the control group, Fluoro-Jade B staining (*E* and *F*) reveals numerous dying neurons in the cerebral cortex. (*G–I*) Representative photomicrographs of cleaved caspase-3 immunohistochemistry from the control (*G*), 5 mg/kg methylene blue (MB 5; *H*), and 50 mg/kg methylene blue (MB 50; *I*) groups 24 h after bolus injections revealing the dose-dependent effect of methylene blue on neuronal apoptosis. (*J*) Quantitative analysis of methylene blue–induced apoptosis in the cerebral cortex in the concomitant presence (isoflurane+) or absence (isoflurane−) of anesthesia. Correction bar *A–B*, *D–E*: 1 mm; *C*, *F*: 35 μm; *G–I*: 30 μm. In *J*, results are presented as mean ± SEM; n = 3 animals for each treatment expressed. Values are expressed as the number of neurons/mm^2. * *P* < 0.05 compared with the untreated control group.

more pronounced when 50 mg/kg MB was administered, indicating the dose-dependent effect of MB on neuronal death (fig. 3J). Intraperitoneal administration of MB to nonanesthetized animals induced neuronal apoptosis in a comparable extent to experimental groups receiving this molecule during isoflurane anesthesia (fig. 3J). These results suggest that the apoptogenic effects of MB are neither attenuated nor potentiated by simultaneous exposure of the organism to isoflurane.

Time Course and Dose Response of Methylene Blue–induced Cell Death in Hippocampal Organotypic Cultures

Organotypic cultures retain a well-preserved morphofunctional organization of neural tissue and provide a valuable auxiliary tool to study drug effects on the CNS.[26] We therefore took advantage of hippocampal organotypic cultures and performed both functional and morphologic analysis to further explore the potentially toxic effects of MB on the CNS. Based on pharmacokinetic studies, evaluating plasma concentration and blood–brain partition of MB in rodents,[12] we exposed hippocampal slices to MB in a concentration range extending from 10 to 1,000 μM. As a first test, we measured the size of excitatory field potentials evoked in hippocampal slices before, during, and after exposure to MB (10 μM). As seen in figure 4A, perfusion of hippocampal slices with MB completely abolished evoked EPSPs within 1 h. The effect of MB on EPSPs was irreversible, because evoked synaptic activity could not be rescued by extensive washing of cultures (up to 2 h 30 min; data not shown). Pharmacologic blockade of nitric oxide synthase (L-NAME, 200 μM) as well as guanylyl synthase (ODQ, 10 μM) induced neither abolition nor decreased amplitude of EPSPs, indicating that the effects of MB on synaptic transmission are not mediated by the nitric oxide–cyclic guanosine monophosphate pathway (data not shown).

Morphologic analysis, using incorporation of PI, a fluorescent marker that stains the nuclei of permeabilized, dying cells, further confirmed MB-induced neurotoxicity in hippocampal organotypic cultures (fig. 4B for experimental protocol). Under control conditions, *i.e.*, before MB or saline exposure, organotypic slice cultures showed only scarce PI staining (fig. 4C). However, after a 2-h-long MB exposure, we detected a significant and dose-dependent increase in PI staining of slice cultures at each concentration tested. This was not observed in slices exposed to saline, and the peak fluorescent intensity occurred 24 h after MB exposure (figs. 4C–I). In contrast to MB, continuous exposure of hippocampal slice cultures to the nitric oxide synthase inhibitor L-NAME (200 μM) up to 48 h did not result in an increased PI incorporation (not shown), indicating that, at least in this experimental setting, the nitric oxide pathway is not implicated in MB-induced neuronal toxicity.

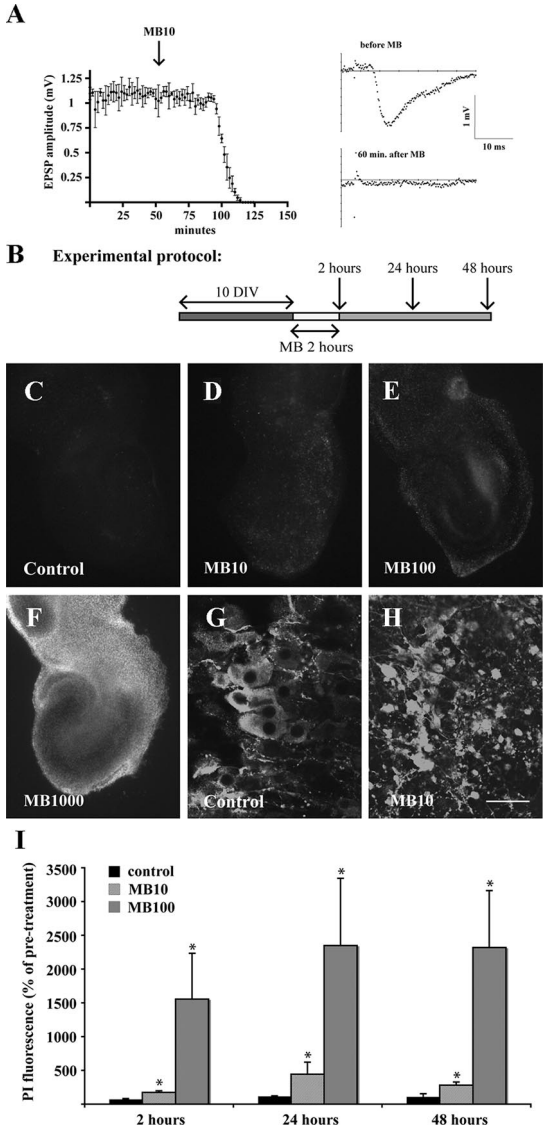

Fig. 4. Neurotoxic effects of methylene blue on hippocampal organotypic slice cultures. (*A*) *Left panel:* Changes in amplitude of evoked excitatory postsynaptic potentials (EPSPs) recorded in organotypic cultures before and after perfusion with methylene blue (10 μM; *arrow*). Normalized amplitudes of EPSPs are reported as a function of time, and *error bars* represent the SEM. *Right panels:* Illustration of the responses evoked by the same stimulation pulse and recorded in the pyramidal CA1 layer before (*upper panel*) and 60 min after methylene blue (10 μM) administration (*lower panel*). (*B*) Experimental setup to analyze methylene blue–induced cell death in organotypic cultures. (*C*) Illustration of propidium iodide (PI) staining in a healthy control hippocampal slice culture with only a few cells labeled. (*D–F*) PI incorporation of hippocampal slices 24 h after methylene blue treatment at concentrations of 10 μM (MB 10; *D*), 100 μM (MB 100; *E*), and 1,000 μM (MB 1,000; *F*). (*G*) Immunohistochemistry using the neuron-specific marker tubulin-β-III revealed healthy neurons in control cultures. (*H*) In contrast, immunostaining with this same antibody showed pyknotic neurons in methylene blue–exposed slices. (*I*) Quantitative dose–response and time-course analysis of PI fluorescence intensity in hippocampal organotypic cultures after methylene blue treatment. Correction bar *C–F*: 300 μm; *G–H*: 15 μm. In *I*, results are presented as mean ± SEM; n = 3 independent experiments (3 slice cultures per experiment) for each time point and each treatment expressed. * $P < 0.05$ compared with the placebo-treated control group. DIV = days *in vitro*.

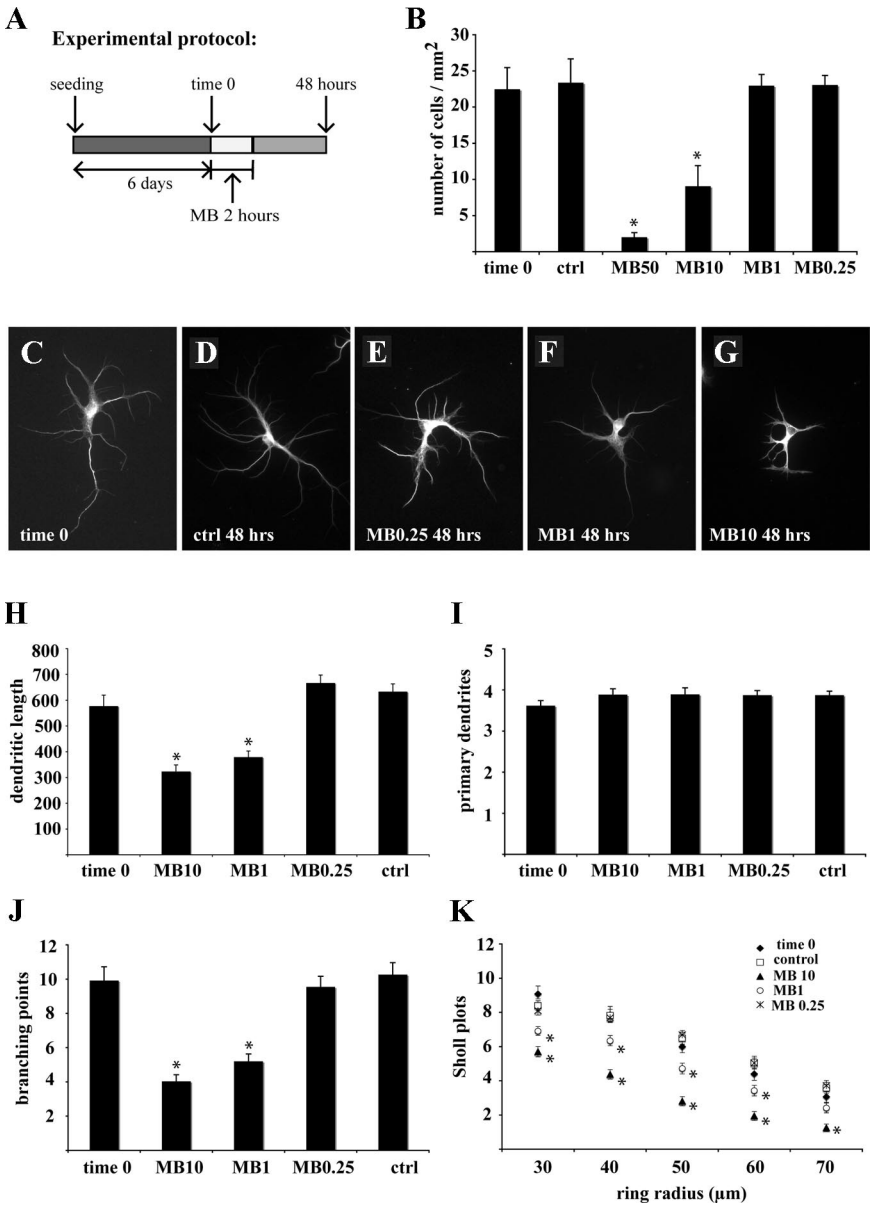

Fig. 5. Dose-dependent effects of methylene blue on the survival and dendritic architecture of isolated γ-aminobutyric acid–mediated neurons. (*A*) Experimental protocol. (*B*) Quantitative analysis of cell survival 48 h after a 2-h-long exposure to methylene blue (MB). (*C*) Representative epifluorescent micrograph of an isolated 6-day-old neuron before treatment. (*D*) In placebo-treated control (ctrl) cultures, neuronal arbor architecture remained stable up to 48 h after treatment. Similarly, a 2-h-long exposure to methylene blue at concentrations of 0.25 μM or less did not have any long-term effect on neuronal morphology (*E*). In contrast, methylene blue at concentrations as low as 1 μM induced significant dendritic pruning of differentiated neurons (*F*), and these effects were even more robust when the concentrations reached 10 μM (*G*). (*H–K*) Quantitative assessment of dendritic arbor architecture in terms of dendritic length, number of primary dendrites, branching points, and Sholl analysis. In photomicrographs *C–G*, cells were stained with the neuron-specific marker tubulin-β-III antibody. Correction bar *A–D*: 20 μm. Results are presented as mean ± SEM; n = 3 independent experiments (3 culture dishes per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. * $P < 0.05$ compared with the placebo-treated control group.

Non–cell-death-inducing Concentrations of Methylene Blue Initiate Dendritic Arbor Retraction In Vitro

To further extend our investigations on the potentially neurotoxic effects of MB, we investigated whether low concentrations of this drug that did not induce cell death could still impair important morphofunctional parameters of neurons, such as dendritic arbor architecture. For this purpose, we used our previously described single cell culture model where immature neuroblasts, isolated from the newborn rat subventricular zone, differentiate into γ-aminobutyric acid–mediated neurons.[27] We have recently shown that neurons in these cultures acquire mature phenotype by the sixth day *in vitro*.[31] Therefore, in a series of experiments, we exposed cultures at the sixth day *in vitro* to MB for 2 h and assessed the survival and dendritic arbor architecture of these cells 48 h after this treatment paradigm (for experimental protocol, see fig. 5A). At this time point, we found a significant loss of differentiated neurons after a 2-h-long exposure to MB at concentrations of 10 μM and greater (fig. 5B). Most importantly, analysis of neuronal dendritic architecture revealed a significant remodeling of dendritic arbor, including retraction of dendrites as well as elimination of branching points at MB concentrations of 1 μM and greater (figs. 5C–K). These *in vitro* results thus suggest that MB at non–cell-death-inducing concentrations can still induce persistent changes of dendritic arbor architecture.

Discussion

An increasing number of clinical observations report adverse neurologic outcome after single bolus MB injec-

tions in the setting of parathyroid gland surgery.[13–21] Hence, the current experimental study was aimed to investigate whether intravascular administration of MB can induce neurotoxic effects on the CNS. We show that, while single intravascular bolus injections of MB up to 50 mg/kg did not induce important hemodynamic effects, they significantly reduced MAC_{iso} in anesthetized adult rats and initiated widespread neuronal apoptosis in the brains of these animals. In accord with these observations, electrophysiologic recordings in hippocampal organotypic cultures revealed a rapid suppression of evoked excitatory field potentials by MB, and this was associated with a dose-dependent effect of this drug on cell death. Finally, dose–response experiments in single cell neuronal cultures revealed that a 2-h-long exposure to MB at non–cell-death-inducing concentrations could still induce significant retraction of the neuronal dendritic arbor. Altogether, these results strongly suggest that MB, at clinically relevant concentrations, can indeed exert neurotoxic effects on the CNS and raise questions regarding the safety of using this drug at high doses during parathyroid gland surgery.

Methylene blue is a potent inhibitor of the soluble guanylyl cyclase, the primary receptor for nitric oxide, and the nitric oxide–cyclic guanosine monophosphate signaling pathway has been suggested to play an important role in the mechanisms' actions of general anesthetics.[32–35] Our results, showing that systemic administration of MB significantly reduces MAC_{iso} without inducing hemodynamic compromise, are in accord with this hypothesis and corroborate previous observations where intracerebroventricular injection of this drug has been shown to decrease the MAC of sevoflurane in rats.[30] It is also important to note that MB may inhibit the action of nitric oxide synthase itself[36] and could also inactivate nitric oxide directly, possibly through the generation of superoxide anions.[37] In addition to interfering with the nitric oxide–cyclic guanosine monophosphate pathway, MB has also been shown to directly activate calcium-dependent potassium channels[38] and to enhance release of noradrenaline from intracellular stores.[39]

Using a combination of *in vivo* and *in vitro* experimental approaches, we found a dose-dependent neurotoxic effect of MB on the CNS. Although, to our knowledge, we provide here the first experimental demonstration that systemic administration of MB can induce neuronal death at clinically relevant concentrations, previous reports also suggest a potentially toxic effect of this drug on neural tissue.[23,24,40] In these studies, MB-induced cytotoxicity seems to be mediated through the generation of oxygen free radicals, as application of oxygen radical scavenging enzymes or a decrease in oxygen partial pressure effectively reduced cell death.[23,40]

In vitro models provide a useful complementary approach to *in vivo* animal experiments in the evaluation of potential drug-induced adverse effects on an organism.[41] Hence, we used two different *in vitro* systems to corroborate our *in vivo* observations. In organotypic cultures of the hippocampus, retaining a well-preserved three-dimensional architecture of neural network, electrophysiologic recordings revealed a fast, complete, and irreversible disappearance of evoked electrical activity in less than 1 h after MB exposure. In these cultures, dose-dependent MB-induced cell death was detected as early as 2 h after exposure, with a peak effect at 24 h. Importantly, pharmacologic blockade of the nitric oxide–cyclic guanosine monophosphate pathway affected neither synaptic transmission nor cell death in this model, indicating that other molecular mechanisms might be responsible for MB-induced adverse effects on neural tissue. Given the multiple sites of action of MB, further studies are needed to elucidate this question.

Neuronal apoptosis is not the only parameter to be considered in evaluating potential adverse effects of drug exposure on the nervous system. It is now well established that interference with the finely tuned molecular mechanisms orchestrating the maintenance of dendritic arbors in differentiated neurons can induce important reorganization of synaptic connections, leading to persistent changes in behavior and psychological functions.[42] In this context, an important finding of the current study was that a 2-h-long exposure of differentiated neurons in single cell neuronal cultures to low, non–cell-death-inducing concentrations of MB can still induce a significant atrophy of dendrites, including retraction of branches and elimination of branching points. Given that even minor changes in neuronal morphology have been shown to exert a significant impact on neuronal responses to stimuli,[43] these results could have important functional consequences, and an essential next step should be to determine whether low concentrations of MB initiate reorganization of neuronal arbor in a more complex and physiologic environment.

Extrapolation of our data to clinical practice requires caution. One should not underestimate the importance of interspecies differences,[44] and therefore, we cannot exclude an increased sensitivity to MB in rats compared with humans. However, several lines of clinical evidence suggest that MB can exert adverse effects on the human CNS. Intrathecal administration of MB, a current diagnostic procedure in the past to visualize cerebral ventricles and to detect rhinorrhea, was reported to be associated with the development of persistent cauda equina dysfunction or even higher paraplegia a few hours after injection.[45–47] More relevant to systemic administration, Nadler *et al.*[22] have shown that intravenous injection of 500 mg MB to healthy individuals induced mental confusion for several hours in the majority of the subjects. Descriptions made in this early report are similar to the emerging number of recent observations pointing to MB-

associated encephalopathy.[13–21] It is important to note that less than 10% of patients receiving high-dose MB bolus developed encephalopathy, and an association between the concomitant use of serotonin reuptake inhibitors and the development of encephalopathy was observed.[13,21] Also, no CNS dysfunction was reported in patients receiving lower doses of MB to treat, *e.g.*, methemoglobinemia or ifosfamide-induced encephalopathy. On the other hand, MB-associated alterations in cognitive functions were also reported in subjects without concomitant use of serotonin reuptake inhibitors,[22] and to our knowledge, the effect of MB administration on higher-order cognitive functions, using sophisticated neuropsychological tests, has not been studied. In light of results presented in our study, conducting such clinical trials would be of interest to initiate discussion about the risk–benefit evaluation of high-dose MB administration in the setting of parathyroid surgery.

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