Therapeutic Effect of Nicotine in a Mouse Model of Intracerebral Hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) resulting from the leakage of blood into the brain parenchyma triggers severe tissue damage involving neurodegeneration and inflammation. Increasing lines of evidence indicate that the stimulation of central nicotinic acetylcholine receptors affords neuroprotection against various insults and also suppresses the proinflammatory activation of microglia. Therefore, the present study aimed to determine whether the administration of nicotine modifies the pathological consequences of ICH, using a mouse model of ICH induced by intrastriatal injection of collagenase. Daily intraperitoneal administration of nicotine (2 mg/kg), starting from 3 h after the induction of ICH, inhibited apoptosis and decreased the number of remaining striatal neurons at 3 days after the insult. We also found that nicotine administration increased the relative expression level of the antiapoptotic protein B cell lymphoma-2 versus that of the proapoptotic protein Bax in the brain. In addition, nicotine administration attenuated the activation of microglia/macrophages, infiltration of neutrophils, and increases in oxidative stress associated with ICH, without affecting hematoma expansion and brain edema. It is noteworthy that mice treated with nicotine exhibited improved sensorimotor performance and a marked increase in survival rate after ICH. These results indicate that nicotinic acetylcholine receptors may serve as a novel target for emergency therapy for ICH.

Introduction

Intracerebral hemorrhage (ICH) results from ruptures of blood vessels within the brain and leakage of blood constituents into the brain parenchyma. Brain edema and tissue damage, which frequently lead to enduring brain dysfunction and poor prognosis, are principal characteristics of ICH pathology (Qureshi et al., 2009). Although several therapeutic interventions including the regulation of osmotic pressure are in clinical practice, neuroprotective drug therapies have not been established to date (Katsuki, 2010).

Brain tissue damage associated with ICH involves substantial neuron loss in the region invaded by the hematoma. In addition to the direct cytotoxicity of blood constituents on the neurons, inflammatory reactions are considered to make an important contribution to the induction of neuron death (Wang and Dore, 2007b; Wasserman and Schlichter, 2007). Inflammatory reactions in the brain are represented by the activation of microglia/macrophages, which are found prominently in the perihematomal region (Gong et al., 2000). In this context, our recent study has shown that retinoic acid receptor stimulation can prevent several pathological events associated with ICH, including neuron loss, activation of microglia/macrophages, and expansion of oxidative stress (Matsushita et al., 2011). Thus, drugs with neuroprotective and anti-inflammatory properties are promising candidates for ICH therapy.

Nicotinic acetylcholine receptors (nAChRs) are expressed widely in the central nervous system and mediate fast synaptic transmission in the brain's cholinergic system (Taly et al., 2009). nAChRs are ionotropic receptors composed of five subunits, and their major subtypes in the central nervous system are homomeric α7 receptors and β2-containing heteromeric receptors (Hogg et al., 2003). Substantial lines of evidence indicate that the stimulation of nAChRs protects neurons from insults associated with several neurodegenerative disorders (Mudo et al., 2007). For example, in animal models of Parkinson disease, nicotine protects dopaminergic neurons from nigrostriatal damage induced by dopaminergic neurotoxins (Quik et al., 2009). Nicotine also protects cortical neurons in culture from Alzheimer disease-associated amy-
lloid β protein (Kihara et al., 2001). In the latter case, the protective effect of nicotine may be mediated by up-regulation of B cell lymphoma-2 (Bcl-2), an antiapoptotic protein (Tait and Green, 2010), via the phosphatidylinositol 3-kinase/Akt pathway (Kihara et al., 2001).

nAChRs are expressed not only in neurons but also in microglia/macrophages, and stimulation of nAChRs suppresses the inflammatory activation of these cells (Wang et al., 2003; Shytle et al., 2004; Lee et al., 2009). Indeed, nicotine has been shown to protect dopaminergic neurons via anti-inflammatory action in inflammation models of neurodegeneration in vitro and in vivo (Park et al., 2007). Overall, these findings indicate that nicotine may act as both a neuroprotectant and an anti-inflammatory agent.

Previously, we examined the effect of nicotine on thrombin-induced tissue injury in organotypic cortico-striatal cultures and found that long-term treatment with nicotine prevented microglial activation and afforded neuroprotection (Ohnishi et al., 2009). In this study, we investigated the potential therapeutic effect of nicotine in a mouse model of ICH in vivo.

Materials and Methods

Induction of ICH and Administration of Nicotine. All of the procedures were approved by our institutional ethical committee concerning animal experiments, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health (Institute of Laboratory Animal Resources, 1996) regarding the care and use of animals for experimental procedures. Male C57BL/6J mice at 8 to 10 weeks of age weighing 22 to 25 g were used to produce the collagenase-induced model of ICH, as described previously (Matsushita et al., 2011). Animals were maintained at constant ambient temperature (22 ± 1°C) under a 12-h light/dark cycle (lights on between 8:00 AM and 8:00 PM) with food and water available ad libitum. When surgery was performed, mice were placed in a stereotaxic frame after anesthesia with intraperitoneal injection and then daily at a 24-h interval. Control animals received intraperitoneal administration of the same volume of saline.

Nick-End Labeling. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was carried out with a commercial kit (Takara, Kyoto, Japan). Twenty-four hours after the induction of ICH, mice were decapitated under deep anesthesia, brains were isolated and frozen, and coronal brain sections of 30 μm thickness, and four sections around the injection site were collected every 120 μm. The number of NeuN-positive cells per 230 × 340 μm² was counted in the central region of the hematoma. The number of isoleictin B4 binding-positive cells per 230 × 340 μm² was counted in the peripheral region of the hematoma. Concerning isolecin B4 binding-positive cells, only cells exhibiting the morphology of activated microglia/macrophages, such as amoeboid appearance with short and thick processes, were incorporated in cell counting. For nitrotyrosine immunoactivity, threshold-based quantification of the immunopositive area was conducted with ImageJ (National Institutes of Health, Bethesda, MD) in a section containing the track of the cannula. The peripheral region of the hematoma is the region adjacent to the collagenase injection site defined by the track of the cannula.

Sham-operated mice received injection of the same volume of physiological saline. Body temperature was maintained at 37°C during surgery.

Nicotine tartrate dihydrate (Nacalai Tesque, Kyoto, Japan) was dissolved in 0.9% saline at a concentration of 0.1 or 0.2 mg/ml (as nicotine free base) and intraperitoneally administered to mice at a dose of 1 or 2 mg/kg once per day. Administration of nicotine first was performed 3 h after the induction of ICH by intrastriatal collagenase injection and then daily at a 24-h interval. Control animals received intraperitoneal administration of the same volume of saline.

Immunohistochemistry. Three days (72 h) after ICH, mice were anesthetized again with pentobarbital and perfused transcardially with 30 ml of ice-cold phosphate-buffered saline (PBS) followed by 30 ml of 4% paraformaldehyde. Brains were isolated and fixed in 4% paraformaldehyde overnight and then soaked in 15% sucrose overnight at 4°C. After being frozen, they were cut into sections of 30 μm in thickness, and four sections around the injection site were collected every 120 μm and mounted onto slides. Antigen retrieval was achieved by soaking specimens in 10 mM citrate buffer (pH 8.0 – 8.5) for 30 min at 80°C followed by incubation for 1 h at 22–25°C. After being rinsed with PBS containing 0.3% Triton X-100, specimens were treated with PBS containing Triton X-100 and blocking serum for 1 h at 22–25°C and then incubated with primary antibodies overnight at 4°C. Primary antibodies were mouse anti-NeuN (1:500; Millipore Corporation, Billerica, MA), rabbit anti-nitrotyrosine (1:500; Millipore Corporation), and rabbit anti-myeloperoxidase (MPO; 1:500; Dako Denmark A/S, Glostrup, Denmark). After being rinsed with PBS containing Triton X-100, specimens were incubated with the corresponding secondary antibodies for 2 h at 22–25°C. Biotinylated goat anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) were used as secondary antibodies. Microglia/macrophages were labeled by overnight incubation with biotinylated Orfina simplicifolia isolecint B4 (1:100; Vector Laboratories; Lee et al., 2009). After the incubation with biotinylated conjugates, specimens were treated with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories), and then peroxidase was visualized by diaminobenzidine and H2O2. The number of NeuN-positive cells per 230 × 340 μm² was counted at the central and the peripheral regions of the hematoma as described (Ohnishi et al., 2007; Matsushita et al., 2011). In this study, the central region of the hematoma refers to the region adjacent to the collagenase injection site defined by the track of the cannula. The peripheral region of the hematoma is the region adjacent to the edge of the hematoma defined by low NeuN immunoreactivity. Four coronal sections collected every 120 μm around the injection site in each mouse were examined for cell counting, and the averaged number of cells from these sections was taken as the value for each mouse. The number of MPO-positive cells per 230 × 340 μm² was counted in the central region of the hematoma. The number of isolecint B4 binding-positive cells per 230 × 340 μm² was counted in the peripheral region of the hematoma. Concerning isolecin B4 binding-positive cells, only cells exhibiting the morphology of activated microglia/macrophages, such as amoeboid appearance with short and thick processes, were incorporated in cell counting. For nitrotyrosine immunoactivity, threshold-based quantification of the immunopositive area was conducted with ImageJ (National Institutes of Health, Bethesda, MD) in a section containing the track of the cannula (Matsushita et al., 2011). The nitrotyrosine-positive area was presented as the total value obtained from four fields of 0.25 mm².

Double immunofluorescence histochemistry was performed for the combination of NeuN with Bax. Rabbit anti-Bax (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a primary antibody along with mouse anti-NeuN (1:500). Alexa Fluor 594-conjugated donkey anti-rabbit IgG(H + L) (1:500; Invitrogen, Carlsbad, CA) and Alexa Fluor 488-conjugated donkey anti-mouse IgG(H + L) (1:500; Invitrogen) were used to detect localization. Confocal images were obtained with the Fluoview FV300 system (Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was carried out with a commercial kit (Takara, Kyoto, Japan). Twenty-four hours after the induction of ICH, mice were decapitated under deep anesthesia, brains were isolated and frozen, and coronal brain sections of 30 μm in thickness were prepared and mounted onto slides. Specimens then were fixed with 4% paraformaldehyde for 15 min at 22–25°C. Epitopes were retrieved by treatment with 20 μg/ml proteinase K (Sigma-Aldrich) for 20 min at 22–25°C. Intrinsic peroxidase was inactivated with 3% H2O2 applied for 10 min, and after permeabilization, specimens were incubated with constructed labeling reaction solution containing terminal deoxynucleotidyl transferase (1:10) for 2 h at 37°C. After being rinsed with PBS, specimens were incubated with anti-fluorescein isothiocyanate horseradish peroxidase conjugate overnight at 37°C and then with biotinylated anti-rabbit IgG (1:200) for 2 h at 22–25°C. After being rinsed with PBS, specimens were treated with avidin-biotinylated horseradish peroxidase complex. Peroxidase was visualized by diaminobenzidine and H2O2.

Western Blot Analysis. Mice were deeply anesthetized and perfused transcardially with ice-cold PBS. The whole brain tissue except the cerebellum and the olfactory bulb was homogenized in ice-cold lysis buffer consisting of 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5
mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholate. After the incubation at 4°C for 30 min, lysates were centrifuged at 14,000g at 4°C for 30 min, and the protein concentration in each sample was determined by the bicinchoninate method. With added sample buffer containing 0.5 M Tris-HCl (pH 6.8), 10% SDS, 2-mercaptoethanol, glycerol and 1% bromphenol blue, each sample was heated at 99°C for 5 min. SDS polyacrylamide gel electrophoresis was performed on a 5.4% stacking gel with a 12% separating gel. After gel electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes. The blots were washed with Tris-buffered saline containing 0.1% Tween 20 and blocked with Blocking One (Nacalai Tesque) at 22–25°C for 1 h. The membrane was incubated with rabbit anti-Bcl-2 antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-Bax antibody (1:1000; Santa Cruz Biotechnology), and mouse anti-β-actin antibody (1:1000; Sigma-Aldrich) overnight at 4°C. After the incubation with horseradish peroxidase-conjugated secondary antibodies at 22–25°C for 1 h, bands were detected with the ECL Advance Western blotting detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) on a lumino-imaging analyzer (LAS-3000mini; Fuji Film, Tokyo, Japan).

Estimation of Lesion Size. Lesion volume, which reflected the extent of hematoma expansion, was estimated by Nissl staining with Cresyl violet (Wang and Dore, 2007a; Matsushita et al., 2011) of 30-µm coronal frozen brain sections obtained every 210 µm. Injured hemorrhagic areas in sections spanning the entire hematoma were measured by ImageJ software. Lesion volume (in mm³) was determined by the integration of the injured area in each section over the section depth.

Measurement of Brain Water Content. Three days after ICH, mice were decapitated under deep anesthesia with pentobarbital. Their brains were removed from their skulls and divided into ipsilateral and contralateral hemispheres along the midline, and then the olfactory bulbs and the cerebellums were removed. After the wet weight of the tissues was obtained, tissues were desiccated at 75°C for 12 h to give the dry weight. The water content was calculated by the following formula: [(wet weight – dry weight)/ wet weight] × 100 (%).

Behavioral Tests. Sensorimotor functions of mice were evaluated by means of the beam-walking test (Matsushita et al., 2011), modified limb-placing test (Jeong et al., 2003; Song et al., 2003), and adhesive removal test (Bouet et al., 2007; Beray-Berthat et al., 2010) at 1, 3, 7, and 14 days after the surgery. These tests were conducted by an experimenter blinded to the treatments. Mice were trained once daily for 3 days before the surgery.

In the beam-walking test, the mouse was placed on a beam (1.2 m in length, 1.5 cm in width, and 50 cm in height), and usage of the hind limb during beam crossing was analyzed on the basis of an eight-point scale as well as a fault rate. A score of 0 was given when the mouse could not balance on the beam (>5 s); 1 was given when the mouse remained on the beam for >5 s but could not cross the beam; 2 was given when the mouse could balance on the beam but not traverse it; 3 was given when the mouse traversed the beam with the affected limb extended and not reaching the surface of the beam or when the mouse made a turn on the beam; 4 was given when the mouse traversed the beam with 100% foot slips; 5 was given when the mouse traversed the beam with >50% but <100% foot slips; 6 was given when the mouse traversed the beam with <50% foot slips; 7 was given when mouse traversed the beam with two or fewer footslips. Performance on each day was expressed as a summated score of three trials. The fault rate was presented as an average from three trials.

The modified limb-placing test consisted of two limb-placing tasks that assess the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimuli.

![A](image1)

![B](image2)

![C](image3)

![D](image4)

![E](image5)

![F](image6)

![G](image7)

**Fig. 1.** Effect of nicotine on ICH-induced neuronal damage. A, a representative image of a coronal section immunostained with anti-NeuN antibody. The central region and the peripheral region for cell counts were denoted by rectangles with and without an asterisk, respectively. Scale bar, 1 mm. B–E, representative images of the central region of the hematoma in NeuN-immunostained coronal sections obtained 3 days after the induction of ICH. Mice received intrastriatal injection of saline (B) or collagenase (C–E). Administration of vehicle (saline; B and C) or nicotine at the indicated doses (D and E) was performed once daily for 3 days, starting from 3 h after the induction of ICH. Scale bar, 50 µm. F and G, quantitative results on the number of NeuN-positive cells in the central (F) and peripheral (G) regions of the hematoma. n = 6–8 mice for each condition. ***p < 0.001 versus sham group; †††, p < 0.001 versus vehicle group.
First, the mouse was suspended 10 cm over a table, and the stretch of the forelimbs toward the table was observed and evaluated: normal stretch, 0 points; abnormal flexion, 1 point. Next, the mouse was positioned along the edge of the table with its forelimbs suspended over the edge and allowed to move freely. Each limb (forelimb and hind limb) was pulled down gently, and retrieval and placement were checked. Finally, the mouse was placed toward the table edge to check for lateral placement of the forelimb. A total of 5 points means maximal neurological deficits, and a total of 0 points means normal performance.

In the adhesive removal test, the mouse was placed into a transparent box (345 mm/H11003×403 mm/H11003×177 mm) for 1 min to acclimate to the environment. Thereafter, adhesive tape (3 mm/H11003×4 mm) was applied on the palm of the impaired forepaw. The time when the mouse first touched the tape (contact time) and the time when the mouse removed the tape (removal time) were recorded, with a cutoff of 120 s for the removal time. The performance was expressed as the removal time after subtracting the contact time.

**Statistical Analysis.** All of the data are presented as mean ± S.E.M. Data were analyzed statistically by unpaired t test for two-group comparisons (data in Fig. 2). When data sets included more than two groups (data in Figs. 1, 3, 4, and 5), one-way analyses of variance followed by post hoc comparisons by Tukey-Kramer multiple comparisons test were used. Behavioral data (Fig. 6) were analyzed by two-way analysis of variance with repeated measures followed by post hoc comparisons with the Bonferroni method. Survival rate (Fig. 7) was analyzed by the log-rank test. Two-tailed probability values <0.05 were considered significant.

**Results**

**Nicotine Inhibits ICH-Induced Neuron Loss.** Neuro-pathological changes within the hematoma were assessed by immunohistochemistry against the neuronal marker NeuN at 3 days after the injection of collagenase into the striatum of the right hemispheres of the mice. At the macroscopic level, NeuN-positive signals were much lower in the hematoma region than those in the surrounding intact areas of immunostained sections (Fig. 1A). In a close-up view, the number of NeuN-positive cells in the center of the hematoma (central region) and in the region adjacent to the intact area (peripheral region) was found to decrease substantially (Fig. 1, B, C, F, and G), which was consistent with our previous findings (Matsushita et al., 2011). Daily administration of nicotine (1 and 2 mg/kg), starting from 3 h after collagenase injection, prevented the decrease in the number of NeuN-positive cells in the central region (Fig. 1, D–F). The effect of nicotine was dose-dependent and reached statistical significance at a dose of 2 mg/kg. In the peripheral region, the neuroprotective effect of nicotine was modest and did not reach statistical significance (Fig. 1G).

To confirm the protective effect of 2 mg/kg nicotine in the
central region of the hematoma, we next performed TUNEL staining. TUNEL detects apoptotic cell death associated with DNA fragmentation. We found that a substantial number of TUNEL-positive cells appeared in the central region of the hematoma at 1 day after the induction of ICH (Fig. 2A). Treatment with 2 mg/kg nicotine partially but significantly decreased the number of TUNEL-positive cells (Fig. 2, B and C).

**Nicotine Reduces Expression of the Proapoptotic Protein Bax.** Results above suggested that nicotine interfered with apoptotic neuron death. In this context, nicotine has been reported to up-regulate the expression of Bcl-2 via the stimulation of α7 nAChRs (Kihara et al., 2001; Akaike et al., 2010). Bcl-2 is an antiapoptotic protein localized in the mitochondria that interrupts the mitochondria-dependent apoptosis pathway by preventing cytochrome c release (Tsuji, 2003). Accordingly, we examined whether the same mechanisms could be involved in the protective effect of nicotine in vivo. When we examined the level of Bcl-2 protein expression in mice without ICH by Western blot analysis, we found that Bcl-2 in the brain tissue tended to increase at 3 h after intraperitoneal administration of 2 mg/kg nicotine. Concomitantly, the expression level of the proapoptotic protein Bax was decreased significantly by 2 mg/kg nicotine. Consequently, the expression ratio of Bcl-2/Bax was decreased significantly after nicotine treatment (Fig. 3, A and B). Moreover, double immunofluorescence histochemistry at 3 days after ICH revealed Bax immunoreactivity in NeuN-positive cells within the hematoma region of vehicle-treated mice (Fig. 3, C–E). Daily treatment with 2 mg/kg nicotine decreased neuronal Bax immunoreactivity (Fig. 3, F–H).

**Nicotine Reduces Activated Microglia/Macrophages, Neutrophils, and Areas Affected by Oxidative Stress.** Pathogenic events in ICH include the recruitment of activated microglia/macrophages and neutrophils, which are associated intimately with increased oxidative stress (Wang and Dore, 2007a,b). In the next set of experiments, we examined the effect of nicotine on these inflammatory processes, because nicotine has been reported to suppress microglial activation (Suzuki et al., 2006; Lee et al., 2009). To evaluate ICH-induced inflammation, activated microglia/macrophages, neutrophils, and oxidative stress were probed by isolectin B4 binding, MPO immunoreactivity, and nitrotyrosine immunoreactivity, respectively. Because isolectin B4 binding also detected endothelial cells and neutrophils, morphological criteria were applied to identify activated microglia/macrophages (Matsushita et al., 2011). At 3 days after the induction of ICH, activated microglia/macrophages accumulated mainly in the peripheral region of the hematoma. Daily administration of nicotine (1 and 2 mg/kg) resulted in a significant decrease in the number of activated microglia/macrophages in a dose-dependent manner (Fig. 4, A, D, and G). MPO-positive infiltrating neutrophils were distributed diffusely within the entire region of the hematoma. Nicotine at a dose of 2 mg/kg partially but significantly decreased the number of infiltrating neutrophils (Fig. 4, B, E, and H). Expansion of the area affected by oxidative stress as revealed by nitrotyrosine immunoreactivity also was suppressed significantly by daily treatment with 2 mg/kg nicotine (Fig. 4, C, F, and I). These results suggested that nicotine attenuated inflammatory reactions in response to ICH.

**Fig. 4.** Nicotine reduces the number of microglia/macrophages and neutrophils and the level of oxidative stress. A–F, representative images of cells positive for isolectin B4 binding in the peripheral region of the hematoma (A and D), MPO in the central region of the hematoma (B and E), and nitrotyrosine (C and F) at 3 days after the induction of ICH. Mice received daily intraperitoneal administration of vehicle (A–C) or 2 mg/kg nicotine (D–F). Scale bars, 50 μm (A and D), 20 μm (B and E), 500 μm (C and F). G–I, vehicle or nicotine at the indicated doses was administered intraperitoneally once daily for 3 days starting from 3 h after the induction of ICH by intrastriatal collagenase injection. Shown are quantitative results on the number of isolectin B4-binding-positive cells (G), the number of MPO-positive cells (H), and nitrotyrosine-positive area (I). n = 5–8 mice for each condition. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle group.
Nicotine Does Not Affect Hematoma Expansion or Brain Edema. We assessed lesion volume and brain water content to clarify whether nicotine suppressed hematoma expansion and brain edema. Lesion volume at 3 days after ICH induction was assessed by Nissl staining according to the procedures by Matsushita et al. (2011). Obtained values were not different between vehicle-treated mice and nicotine-treated mice (Fig. 5, A–C). Brain water content at 3 days after ICH induction was increased in the hemisphere ipsilateral to the hemorrhage compared with that of the sham-operated group, indicating edema formation (Fig. 5D). Treatment with nicotine (1 and 2 mg/kg) had no significant effect on the level of brain water content.

Nicotine Improves Neurological Deficits after ICH. We conducted several sets of behavioral experiments to verify whether nicotine treatment improved neurological deficits. Behavioral assessments were performed before and 1, 3, 7, and 14 days after the induction of ICH (Fig. 6A). After the induction of ICH, the foot fault rate in the beam-walking test was increased substantially. Mice treated with 2 mg/kg nicotine once daily for 3 days showed significant improvement in recovery from initial deficits (Fig. 6B). With regard to the performance score in the same test, nicotine treatment produced a tendency to improve recovery from the ICH-induced decrease, although the effect did not reach statistical significance (Fig. 6C).

In the modified limb-placing test, the neurological score was increased after the induction of ICH and remained elevated during 14 days of observations. Treatment with 2 mg/kg nicotine significantly lowered the score throughout the entire period (Fig. 6D). Nicotine was also effective in alleviating the deficit in performance in the adhesive removal test at 1 and 3 days after the induction of ICH (Fig. 6E). In these sets of behavioral experiments, sham-operated mice treated with 2 mg/kg nicotine showed performance indistinguishable from that of vehicle-treated sham-operated mice (data not shown).

Nicotine Increases the Survival Rate of Mice after ICH. Compared with sham-operated mice that showed 100% survival at 3 days after surgery, mice with ICH exhibited a decline in the survival rate, which reached 67.2% at 3 days after the induction of ICH. It is noteworthy that daily treatment with 2 mg/kg nicotine significantly improved the survival rate of mice after ICH to 87.5% (Fig. 7). Nicotine-treated sham-operated mice retained 100% survival (7 of 7 mice) at 3 days after surgery.

Discussion

The effect of nicotine on “ischemic” brain injury has been a subject of several studies. Acute pretreatment with nicotine attenuated ischemia-reperfusion injury in gerbil hippocampus (Nanri et al., 1998). Likewise, nicotine administered 5 min before occlusion prevented the delayed death of hip-
pocampal neurons in rats (Kagitani et al., 2000), but this effect was attributed to enhanced regional blood flow in the hippocampus by nicotine. In a chronic treatment regimen, two daily injections of nicotine for 12 days in rats, from 48 h after the unilateral devascularization of the motor cortex, facilitated the recovery of motor performance (Gonzalez et al., 2006). In contrast, continuous delivery of nicotine for 14 days by osmotic minipumps has been reported to exacerbate focal ischemia-induced injury (Wang et al., 1997). Therefore, depending on the treatment regimens, nicotine exerts divergent influences on the pathological consequences of ischemic brain injury. Despite a wealth of these findings on brain ischemia, there are no reports examining the effects of nicotine or nAChR agonists on hemorrhagic brain injury. In this study, we for the first time addressed this issue and found that nicotine exerted therapeutic effects in an ICH model in mice.

Intraperitoneal nicotine administration, starting from 3 h after the induction of ICH, significantly inhibited neuron loss. This effect was unexpected, because in an organotypic slice culture model of hemorrhagic injury long-term pretreatment with nicotine was required to produce significant neuroprotective effects (Ohnishi et al., 2009). In this context, our preliminary examinations in the ICH model in vivo revealed that pretreatment with nicotine from 1 or 7 days before the induction of ICH gave essentially similar results with those of the post-treatment regimen shown in the present study, although the degree of neuroprotection was somewhat greater in pretreatment regimens (data not shown). Reasons for the discrepancy of the results between in vitro and in vivo remain to be clarified, but the present results suggest that nAChRs are promising targets for emergency therapy for ICH.

The neuroprotective effect of nicotine was significant in the central region of the hematoma, where ICH-associated inflammatory reactions such as the accumulation of activated microglia/macrophages and increased oxidative stress (Matsushita et al., 2011) were less prominent than those in the

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**Fig. 6.** Nicotine alleviates neurological deficits after ICH. A, schematic representation of the experimental schedule. Vehicle or nicotine (2 mg/kg i.p.) was administered once daily for 3 days at a 24-h interval, starting from 3 h after the induction of ICH. Behavioral tests were performed at 1 day (24 h), 3 days (72 h), 7 days, and 14 days after surgery. That means that tests at 1 day were performed 21 h after the first injection of the vehicle or nicotine and that tests at 3 days were performed 21 h after the last injection of the vehicle or nicotine. B and C, performance of mice in the beam-walking test, evaluated by foot fault rate (B) and performance score (C). D and E, results of performance in the modified limb-placing test (D) and the adhesive removal test (E). $n = 14–19$ mice for each condition. *, $p < 0.05$; ***, $p < 0.001$ versus vehicle group.
consistent with this proposal.

investigations, but the effect of nicotine on the Bcl-2/Bax down-regulation of Bax should be explored in future and Deng, 2005). Precise molecular mechanisms leading to nicotine inactivates Bax by promoting its phosphorylation (Xin tine-containing nAChRs are involved in the coun-

7 nAChRs suppresses the activation of /H9251

alpha7 receptors in neuroprotection. (Ohnishi et al., 2009).

A finding that deserves consideration is the apparent discrepancy between the neuroprotective action and the anti-inflammatory action of nicotine. That is, the anti-inflammatory action of nicotine was observed mainly in the peripheral region where activated microglia/macrophages accumulated, but neuronal survival in the same region was not clearly promoted by nicotine. At present, we do not have clear explanations for this discrepancy, but in any case, these results propose the possibility that the neuroprotective action of nicotine has minimal relation with its action on microglia/ macrophages. However, the nicotine-induced attenuation of neutrophil infiltration, though its degree was small, may contribute to neuroprotection by nicotine, because infiltrated neutrophils were distributed in the central region of the hematoma. A recent study has demonstrated important roles of neutrophils in the pathogenesis of experimental ICH (Moxon-Emre and Schlichter, 2011).

Nicotine administration under the present experimental conditions produced no significant influences on either lesion volume (that reflected the extent of hematoma expansion) or edema formation. In contrast, a recent study on a brain ischemia model in vitro and in vivo has shown that nicotine exacerbates brain edema (Paulson et al., 2010). The difference between our study and that of Paulson et al. (2010) may be explained by different experimental conditions such as dosing regimens and also by different events involved in the formation of edema under hemorrhagic and ischemic conditions. In any case, the present results indicate that the neuroprotective effect of nicotine was independent of physical damage-related factors such as hematoma expansion and brain edema.

The most notable findings were that daily nicotine administration for 3 days enhanced the recovery of sensorimotor functions of mice and also improved the survival rate of mice after ICH. Although various receptors, enzymes, and signaling molecules have been proposed as potential targets for ICH therapy (Katsuki, 2010), to our knowledge, none of them has been shown to decrease the mortality rate of experimental animals. Overall, multiple beneficial effects of nicotine demonstrated in the present study may provide a basis for establishing novel therapeutic strategies for ICH.

**Authorship Contributions**

**Participated in research design:** Hijioka, Matsushita, Hisatsune, Isohama, and Katsuki.

**Conducted experiments:** Hijioka and Matsushita.

**Performed data analysis:** Hijioka, Hisatsune, Isohama, and Katsuki.

**Wrote or contributed to the writing of the manuscript:** Hijioka and Katsuki.

**Other:** Katsuki acquired funding for the research.

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