Pharmacological analysis of the mechanisms underlying seizure induction mediated by nicotinic acetylcholine receptors

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Abstract

Convulsive seizures are caused by transitory excitation of the brain, often manifesting clonic or tonic-clonic convulsions. Convulsive seizures can be divided into “symptomatic” seizures associated with a brain injury or a metabolic/toxic disturbance of brain function and “idiopathic” seizures with unknown cause. Nicotinic acetylcholine (nACh) receptors are pentameric ligand-gated cation channels that control neuronal excitability and neurotransmitters release in the brain. Consequently, neural nACh receptors are responsible for regulating various pathophysiological functions including psychomotor activity, cognition, autonomic functions, movement disorders and seizure induction. Specifically, nACh receptors are suggested to be involved in seizures induction in various idiopathic epileptic disorders (e.g., generalized epilepsies–epilepsy with generalized tonic-clonic seizures and partial epilepsies–autosomal dominant nocturnal frontal lobe epilepsy and generalized epilepsy) as well as in nicotine intoxication. However, the mechanisms underlying nACh receptor-mediated seizure induction are still unknown. Among many available methods to evaluate brain activity, expression analysis of Fos protein, a biological marker of neural excitation, has been shown to be useful for exploring brain regions related to brain disorders and drug actions. In the present study, therefore, behavioral and Fos-immunohistochemical techniques were combined to delineate the underlying mechanisms of how stimulation of nACh receptor by nicotine results in convulsive seizures (nicotine-induced seizures) in rodents. Treatment of animals with nicotine (1–4 mg/kg, i.p.) produced motor excitement in a dose-dependent manner resulting in convulsive seizures at 3 and 4 mg/kg. The nicotine-induced seizures were abolished by a subtype non-selective nACh antagonist, mecamylamine (MEC), an α7 nACh antagonist, methyllycaconitine,

also significantly inhibited nicotine-induced seizures, whereas an α4β2 nACh antagonist, dihydro-β-erythroidine, affected only nominally. Topographical analysis of Fos protein expression, a biological marker of neural excitation, revealed that a convulsive dose (4 mg/kg) of nicotine region-specifically activated neurons in the piriform cortex, amygdala, medial habenula, paratenial thalamus, anterior hypothalamus and solitary nucleus among 48 brain regions examined, and this was also suppressed by MEC. Furthermore, electric lesioning of the amygdala, but not the piriform cortex, medial habenula and thalamus, specifically inhibited nicotine-induced seizures. In addition, microinjection of nicotine (100 and 300 μg/side) into the amygdala elicited convulsive seizures in a dose-related manner. Our findings strongly suggest that nicotine elicits convulsive seizures by activating amygdala neurons mainly via α7 nACh receptors. In addition, we also succeeded in characterizing the drug action, the NMDA receptor stimulant D-cycloserine, in modulating extrapyramidal motor disorders, using the Fos expression analysis.

Keywords: nicotine, convulsive seizures, nicotinic acetylcholine receptors, amygdala, Fos protein expression.

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ABREVIATIONS AND ACRONYMS

AcC: core region of nucleus accumbens

AcS: shell region of nucleus accumbens

ADNFLE: autosomal dominant nocturnal frontal lobe epilepsy

AH: anterior hypothalamus

AIC: agranular insular cortex

AM: anteromedial thalamic nucleus

AMG: amygdala

Apir: amygdalopiriform transition area

AuC: auditory cortex

BLP: basolateral amygdaloid nucleus

BMP: basomedial amygdaloid nucleus

CA: Cornu Ammonis area of hippocampus

CgC: cingulated cortex

CM: centromedial thalamic nucleus

DCS: D-Cycloserine

DG: dentate gyrus of the hippocampus

DHβE: dihydro-beta-erythroidine

DLEnt: dorsolateral entorhinal cortex

dlST: dorsolateral striatum

DM: dorsomedial hypothalamic nucleus

dmST: dorsomedial striatum

EPS: Extrapyramidal side effects

GP: globus pallidus

HAL: Haloperidol

IHC: Immunohistochemical

IO: inferior olive

IR: Immunoreactivity

LHb: lateral habenular nucleus

L-NAME: L-NG-Nitro-L-arginine methyl ester

LS: lateral septum

MC: motor cortex

MEC: mecamylamine

MePD: medial posterodorsal amygdaloid nucleus

MePV: medial posteroventral amygdaloid nucleus

MHb: medial habenular nucleus

MLA: methyllycaconitine

mPFC: medial prefrontal cortex

nACh: nicotinic acetylcholine

NMDA: N-Methyl-D-aspartate

NOS: Nitric oxide synthase

PH: posterior hypothalamus

PirC: piriform cortex

PMCo: posteromedial cortical amygdaloid nucleus

PRh-Ect: perirhinal-ectorhinal cortex

PT: paratenial thalamic nucleus

PV: paraventricular thalamic nucleus

RPC: parvocellular part of the red nucleus

SC: sensory cortex

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

Sol: solitary tract

VM: ventromedial thalamic nucleus

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1. Introduction and objectives

1.1 Seizures

Seizures are a transitory alteration of an abnormal, excessive and synchronized cerebral activity that can result or not in motor symptoms[1)], a recent recommendation of the International League Against the Epilepsy suggest to classify seizures according to the seizure onset, basically in focal, generalized and unknown onset[1, 2)]. Convulsive seizures can also be divided into "symptomatic" seizures associated with a brain injury or a metabolic/toxic disturbance of brain function (fever, head injury, brain infection, proconvulsive drugs like baclofen, clozapine), and "idiopathic" seizures with unknown cause[2, 3)]. Although idiopathic seizures are the main cause of the epilepsy, symptomatic seizures do not necessarily mean epilepsy, still there is a risk of subsequent epilepsy after symptomatic seizures according to the damage extension (e.g., reversible disturbances $\leq 3\%$ and brain abscesses $\geq 10\%$)[4)].

1.2 Nicotinic acetylcholine receptors

The pentameric ligand-gated cation channel nicotinic acetylcholine (nACh) receptors are composed of varying combinations of α (α1–α10), β (β1–β4) and other (δ, γ, ε) subunits[5-7)], these subunits assemble with different stoichiometry to form nACh receptors with different properties, including distinct cationic permeability, agonist affinity and desensitization properties[6)] (Figure 1). Specifically, neural nACh receptor subtypes are constructed from combination of 9 α (α2– α10) and 3 β (β2–β4) subunits. Among them, homomeric α7 and

heteromeric α4β2 nACh receptors are the most characterized and expressed subtypes in the brain (Figure 2) whereas α3β4 nACh receptors mainly function as peripheral ganglionic nACh receptors[5-9]. In the brain, nACh receptors control neuronal excitability and neurotransmitters release (GABA, dopamine, glutamate)[10]. Consequently, nACh receptors are responsible for regulating cognitive performance, vigilance, locomotor activity, body temperature, respiration, cardiovascular and gastrointestinal tract function, electroencephalographic activity, cortical blood flow and pain perception (for detailed information on the function of neural nACh receptors consult Lloyd and Williams [11]).

1.2.1 Potential role of nACh receptors in epileptic seizures

Intoxication with nicotine, an exogenous nACh receptor agonist, evokes motor excitement including tremors and convulsive seizures in humans[12, 13] and experimental animal models[14, 15]. In addition, nACh receptors are the first ion channels dysfunction reported to be involved in genetic epilepsies[16, 17]. Indeed, genetic polymorphisms of α4, β2 and/or α7 subunits of nACh receptors are involved in various epileptic disorders, including idiopathic generalized epilepsy (e.g., epilepsy with generalized tonic-clonic seizures and juvenile myoclonic epilepsy)[18-22] and partial epilepsy (e.g., autosomal dominant nocturnal frontal lobe epilepsy and benign epilepsy of childhood with centrotemporal spikes)[17, 23-25]. Therefore, nACh receptors could be involved in both “symptomatic” and “idiopathic” seizures, however, the role and mechanisms of nACh receptors in seizure generation and epileptogenesis are still unknown.

1.3 Fos protein

With regard to obtaining new information about the mechanism by which nACh receptors cause convulsive seizures, we decided to investigate which brain regions nACh receptors would be activate to cause convulsive seizures. For this purpose Fos protein, an immediate early gene product, can be used as a biological marker of neural excitation[26]. Fos protein expression occurs region-specifically and reproducibly with various other stimuli; detection of Fos expression is simple; the immunohistochemical (IHC) staining of Fos can also be applicable to double staining with other proteins, tracers and neurotransmitters; and the function of Fos is established as a part of the transcription factor activator protein 1 (AP-1; Figure 3). Hence, IHC mapping analysis of Fos expression is a useful method to identify brain regions affected by pathologies (e.g., emotional disorders, epilepsy and pain) and by various drug treatments[26-30] without the necessity to measure others metabolites (e.g., glucose usage)[31]. As a matter of fact, Fos expression applicability in neuroscience has lead me to study and acquire expertise on the subject. The result of this experience was transliterated in a book chapter about Fos protein expressional mechanism and neuroscience application [26].

1.4 Objectives of the studies

The primary purpose of the present study is to delineate the mechanisms of how stimulation of nACh receptors by nicotine result in convulsive seizures (nicotine-induced seizures) in rodents. Specifically, IHC analysis of Fos expression were performed in combination with behavioral studies to determine the seizure foci of nicotine-induced

seizures. In addition, we also tried to characterize the drug action, the NMDA receptor stimulant D-cycloserine, in modulating extrapyramidal motor disorders by using the Fos expression analysis as the second part of the experiments.

2 Pharmacological analysis of the mechanisms underlying seizure induction mediated by nACh receptors

2.1 Introduction

Nicotine, an alkaloid derived from leaves of *Nicotinia species*, is the primary active compound of tobacco products [32]. Nicotine acts as an exogenous agonist of nACh receptors causing a series of pharmacological actions including antidepressant effects[33, 34], cognitive enhancement[35, 36], positive reinforcement (addictive effects)[35, 37] and motor excitement[15, 36, 38, 39]. Acute intoxication with nicotine shows two phases of symptoms; early phase symptoms including nausea, vomiting, headache, tremors and seizures[13-15, 40-44], and delayed phase symptoms including CNS depression and coma[44, 45]. Nicotine evoked symptoms are mediated by nACh receptors. In the nervous system the neural homomeric α7 and heteromeric α4β2, whereas α3β4 nACh receptor in the peripheral ganglia[5-9]. The two main neural nACh receptor subtypes α4β2 and α7 nACh receptors are widely expressed in the CNS (Table 1) at the synapse (both pre- and postsynaptically) and extrasynaptically[5, 9, 46-48], both subtypes diverge around a higher affinity of its nicotine binding site (α4β2 nACh receptors)[9] and a higher calcium permeability (α7 nACh receptors)[49]. Therefore, while α4β2 nACh receptors respond for most actions induced by nicotine (≈90%)[50], α7 nACh receptors modulate intracellular signaling and neurotransmitter release[49].

In particular, motor excitement symptoms evoked by nicotine include Straub tail, tremors and convulsive seizures[15, 36, 38, 51, 52], suggesting the involvement of nACh receptors in the pathogenesis of epileptic and movement disorders. Indeed, genetic polymorphisms of α4, β2 and/or α7 subunits of nACh receptors are involved in various epilepticus disorders, including idiopathic generalized epilepsy (e.g., epilepsy with generalized tonic-clonic seizures, childhood absence epilepsy, juvenile absence epilepsy and juvenile myoclonic epilepsy)[18-22] and partial epilepsy (e.g., autosomal dominant nocturnal frontal lobe epilepsy and benign epilepsy of childhood with centrotemporal spikes[17, 23-25]. On the other hand, especially after the relatively recent findings that not only loss-of-function mutations in nACh receptors[19, 53, 54] are involved in the seizures generation, but also gain-of-function[55-57], it has become clear that little is known about the mechanism of how neural nACh receptors modulate seizures and epileptogenesis.

The functional mechanism of neural nACh receptors is very complex, each nACh receptor subtype composition affects the nACh receptor channel permeability and kinetics, whereas the localization in the neural network determines the precise contribution of a given nACh receptor population in a spatial- and time-dependent manner[58]. Nicotine is useful as a potential tool for understanding of the underlying mechanisms of nACh receptors in inducing motor impairments, as recently demonstrated by our research group[59, 60]. Fos protein, an immediate early gene product, as a biological marker of neural activation is widely used for mapping brain regions related to disease conditions (e.g., pain, epilepsy, and emotional disorders) and biological interactions of various drug treatments[26, 27, 29, 30]. In the present study, hence, we applied behavioral and Fos-IHC studies to delineate the mechanism underlying nicotine-induced seizures in rodents.

2.2 Materials and methods

The experimental protocols hereby were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

2.2.1 Experimental animals

Male ddY mice (Japan SLC, Shizuoka, Japan) weighing 25–35 g and male SD rats (Japan SLC, Shizuoka, Japan) weighting 200-300 g were used. The animals were kept in air-conditioned rooms under a 12-h light/dark cycle (light on: 8:00 a.m.) and allowed *ad libitum* access to food and water. The housing conditions and the animal care methods complied with the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2.2 Behavioral evaluation

Animals were intraperitoneally (i.p.) injected with nicotine (1-4 mg/kg) or saline (vehicle) and individually placed in an observation box (25 × 42 × 20 cm). Nicotine-induced behavioral excitement was evaluated over 15 minutes after the nicotine injection using a modified six point-ranked score (0: no effect; 1: mild head tremor and Straub tail; 2: apparent tremors in extended regions, 3: severe tremors with wild running; 4: clonic seizures; 5: tonic or tonic-clonic seizures)[59, 61]. Incidence of convulsive seizures was judged as positive when scores were higher than 3 (Figure 4A). In the experiments using nACh receptor antagonists, a subtype non-selective nACh antagonist, MEC (1 mg/kg), a specific α7 nACh antagonist,

MLA (10 mg/kg), a specific α4β2 nACh antagonist, DHβE (5 mg/kg) or saline (vehicle) was i.p. administered 15 min before the nicotine treatment (Figure 4A). The dosage of nACh antagonists was set to a sufficient level to antagonize its respective nACh receptor in previous studies [MEC: Gomita, et al. [62], DHβE: Blondel, et al. [63], and MLA: Blondel, et al. [63], Liu [64] and Kim, et al. [65]].

2.2.3 Analysis of Fos protein expression

Staining for Fos-immunoreactivity (IR) followed previously published methods[26, 28, 30]. Briefly, ddY mice were treated with a convulsive dose (4 mg/kg, i.p.) of nicotine and brain samples were obtained 120 min after the nicotine injection under pentobarbital (80 mg/kg, i.p.) anesthesia. In some experiments, mice were pretreated with MEC (1 mg/kg) 15 min before the nicotine injection. All animals were transcardially perfused with ice-cold phosphate-buffered saline (PBS), which was followed by 4% formaldehyde perfusion, following brains were stored in fresh fixative for at least 24 h. Following, coronal brain sections of 30 μm thickness were cut using a Microslicer (DSK-3000, Dosaka, Kyoto, Japan). Slices were incubated for 2 hours in the presence of 2% normal rabbit serum solution, and with goat c-Fos antiserum for an additional 18–36 hours. After PBS washing, the sections were then incubated with biotinylated rabbit anti-goat IgG for 2 hours and with PBS containing 0.3% hydrogen peroxide for 30 min to inactivate the endogenous peroxidase. Thereafter, the sections were incubated with avidin–biotinylated horseradish peroxidase complex for 2 hours. Fos-IR was visualized by the diaminobenzidine–nickel staining method and quantified by counting the number of Fos-IR positive nuclei in 48 regions[66], (1) the cerebral cortices (19 regions), mPFC, CgC, MC (1-4), SC (1-4), AIC, PirC (1-4), Apir, AuC, PRh-Ect and DLEnt, (2) the limbic regions and basal ganglia (14 regions), AcC, AcS, BLP,

BMP, PMCo, MePV, MePD, CA1, CA3, DG, dlST, dmST, GP and LS, (3) the diencephalic and lower brainstem regions (15 regions), MHb, LHb, PT, PV, AM, CM, VM, AH, PH, DM, RPC, SNr, SNc, Sol and IO.

2.2.4 Electrical lesion study

Electrical lesion studies were performed using SD rats as reported previously[59, 67]. Briefly, animals were anesthetized with pentobarbital (60 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, SR-6, Tokyo, Japan). A bipolar concentric electrode was bilaterally inserted into the thalamus (Th; A: -1.5 mm; L: ±0.4 mm; H: +4.2 mm); PirC (A: +1.3 mm; L: ±4.3 mm; H: +7.2 mm), MHb (A: +0.4 mm; L: ±0.4 mm; H: +4 mm); or amygdala (AMG; A: -3.1 mm; L: ±4 mm; H: +7.9 mm)[68]; and a direct current of 1 mA was delivered to the respective region for 15 s. After a recovery period (2-4 days) from the surgery, animals were treated with nicotine (4 mg/kg) or vehicle, individually placed in the observation box and underwent behavioral evaluation as described previously. After the experiments, the animals were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and the brain was removed from the skull in order to confirm each electrical lesion placement (Figure 4B).

2.2.5 Microinjection study

Microinjection studies were performed using SD rats as reported previously[69-71]. After the animals were fixed in a stereotaxic instrument under pentobarbital (40 mg/kg, i.p.) anesthesia, a stainless steel guide cannula was bilaterally inserted 1 mm above the AMG (A: -3.1 mm; L: ±4 mm; H: +7.9 mm)[68] and fixed on the skull with dental cement. After a

recovery period (2–4 days), an injection cannula was inserted into the AMG through a guide cannula and nicotine (100 or 300 μg/μL per side) was injected at a flow rate of 0.25 μL/min (Microinfusion pump KDS220; KD Scientific Inc., USA) for 4 min under freely moving conditions. The control animals were given the same volume of saline (vehicle) alone. Nicotine-induced behavioral excitement was evaluated as previously described using a six point-ranked score. After the experiment animals were deeply anesthetized with pentobarbital (80 mg/kg, i.p.), and their brains removed for subsequent guide cannula insertion site verification (Figure 4C).

2.2.6 Drugs

For the preparation of the experimental doses of nicotine (1, 2, 3 and 4 mg/kg, i.p.) a stock solution was prepared using 5 mg/mL (-)–nicotine solved in normal saline solution. Stock solution was diluted in saline to obtain, respectively, 0.05, 0.1, 0.6 and 0.8 mg/mL solutions. Experimental dose of MEC (1 mg/kg) was made from diluting MEC stock solution of 5 mg of MEC in 1 mL of 1% lactated saline solution in saline so 0.2 mg/mL MEC solution was attained. MLA and DHβE was directly diluted in saline (vehicle solution).

Nicotine, MEC hydrochloride, MLA citrate and DAB substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and DHβE hydrobromide from Tocris (Bristol, UK). The primary antibody against c-Fos was purchased from Santa Cruz Biotechnology Inc. (sc-52-G-Santa Cruz, CA, USA), and the secondary biotinylated anti-goat IgG antibody and ABC kit from Vector Laboratories (Burlingame, CA, USA). Others common laboratory reagents were also obtained from commercial sources.

2.2.7 Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance of differences among multiple groups was determined by Kruskal—Wallis test followed by the Steel—Dwass post-hoc test (behavioral scores) or one-way ANOVA followed by the Tukey's post-hoc test (Fos expression). Comparisons between two groups were determined by parametric Student's t-test (Fos expression) or non-parametric Mann-Whitney's U test (electrical lesion). Comparisons of the seizure incidence rate were done by $\chi 2$ test. A *P*-value of less than 0.05 was considered statistically significant.

2.3 Results

2.3.1 Nicotine-induced convulsive seizures

Nicotine at doses from 1 to 4 mg/kg (i.p.) dose-dependently induced motor excitement in mice and rats, inducing Straub tail and tremor (score 1-3) at low doses (e.g., 1-2 mg/kg, i.p.) and convulsive seizures (score 4-5) at high doses (e.g., 3-4 mg/kg, i.p., Figure 5A, 5B). Nicotine-induced motor excitement including seizures was normally transient and subsided within 10 min. The percentages of animals which showed clonic or tonic-clonic seizures with nicotine (4 mg/kg, i.p.) were 82% and 62.5% in mice and rats, respectively (Figure 5A, 5B).

To clarify the role of nACh receptors subtypes in nicotine-induced seizures, we tested the actions of nACh antagonists in mice. Pretreatment of animals with the subtype non-selective MEC (1 mg/kg, i.p.) markedly reduced the seizure intensity and incidence rate due to nicotine (4 mg/kg, i.p.). The α7 nACh antagonist MLA (10 mg/kg, i.p.) significantly

inhibited nicotine-induced seizures whereas the α4β2 nACh antagonist DHβE (5 mg/kg, i.p.) only slightly reduced the seizure intensity and incidence (Figure 6). We also tried co-administration of DHβE and MLA in order to clarify how much of nicotine-induced seizures can be attributed to α7 and α4β2 nACh receptors, although, all animals died by what seems to be peripheral respiratory failure (non-published data).

2.3.2 Nicotine-induced Fos expression

Using topographical analysis of Fos protein we explored brain regions excited with nicotine-induced seizures, a biological marker of neural excitation, in mice. Treatment of animals with nicotine at convulsive dose (4 mg/kg, i.p.) caused region-specific elevation of Fos expression in 8 out 48 brain verified regions (Figure 7). In the 19 cortical regions, nicotine increased the Fos expression in the PirC2 [$t(7) = 2.385$, P = 0.050], PirC4 [$t(12) = 4.783$, P < 0.001] and APir [$t(10) = 3.470$, P = 0.013] (Figure 8, 11). In the 29 subcortical regions, nicotine significantly enhanced Fos expression in the AMG (Figure 9, 11), MHb [$t(5) = 3.982$, $P = 0.010$], PT [$t(6) = 2.882$, $P = 0.027$), AH [$t(10) = 2.397$, $P = 0.037$] and in Sol [$t(5) = 3.121$, P=0.025] (Figure 10, 11). In the AMG, all investigated regions presented considerably high Fos expression (about two to four times the control level) after nicotine treatment, while it reached statistical significance only in the MePD [$t(6) = 2.439$, $P = 0.048$] (Figure 9. 11). Along with the hippocampus, other brain regions like striatum, GP, substantia nigra, did not show any significant changes in Fos expression (Figure 7).

In further analysis, to confirm the involvement of nACh receptors, we assessed the effects of MEC on nicotine-induced Fos expression in the above seven brain regions (e.g., PirC2, PirC4, Apir, MePD, MHb, PT and Sol). We confirmed that nicotine (4 mg/kg, i.p.) significantly augmented Fos expression in the PirC2 [$F(2,21) = 15.880$, $P < 0.001$], PirC4

[$F(2,23) = 7.498$, $P < 0.003$], MePD [$F(2,20) = 7.771$, $P < 0.003$], MHb [$F(2,25) = 86.928$, $P < 0.001$], PT [$F(2,20) = 16.097$, $P < 0.001$] and Sol [$F(2,21) = 35{,}564$, $P < 0.001$]. The nicotine-induced Fos expression was mostly abolished by MEC [PirC2, $F(2,21) = 15.880$, $P = 0.004$, PirC4, $F(2,23) = 7.498$, $P = 0.027$; MePD, $F(2,20) = 7.771$, $P = 0.026$; MHb, $F(2.25) = 86.928$, $P < 0.001$; PT, $F(2,20) = 16.097$, $P = 0.001$; and Sol, $F(2,24) = 35.564$, $P < 0.001$] (Figure 12), indicating that nicotine-induced Fos expression is mediated mainly by nACh receptors in these brain regions.

2.3.3 Electrical lesion studies

To determine the brain regions responsible for the generation of nicotine seizures, we next conducted electrical lesion studies of the sites which showed high Fos expression with nicotine in rats. Animals received electrical lesioning at the bilateral PirC, Th, MHb and MePD and AMG 2-4 days before nicotine seizure induction test. Under these conditions, only the lesion of the AMG noticeably reduced the intensity [$U(8) = 3.000$, $P = 0.028$] and the incidence ($\chi^2 = 0.225$, $P = 0.009$) of nicotine-induced seizures (Figure 13). In contrast, neither lesioning of PirC (Figure 14), Th (Figure 15) nor MHb (Figure 16) affected the seizure induction, suggesting that the AMG is responsible for nicotine seizures generation.

2.3.4 Microinjection

To further confirm the causative role of the AMG in nicotine-induced seizures, we performed a microinjection study with nicotine into the AMG. Under freely moving conditions, 100 and 300 μg/side of nicotine were injected into the bilateral AMG. Nicotine

caused motor excitement (100 μg/side; χ^2 = 13.602, df = 2, *P* = 0.0136, 300 μg/side; χ^2 = 13.602, df = 2, *P* = 0.005) and seizure generation (300 μg/side; χ^2 = 5.76, *P* = 0.016) in a dose-related manner (Figure 17). Reinforcing the role of AMG nACh receptors in the causation of nicotine-induced seizures.

2.4 Discussion

Nicotine has proconvulsive actions when overdosed, reports showing the convulsive action of nicotine in both humans and experimental animals are not uncommon[12, 44, 72]. Likewise, we confirmed that nicotine dose-dependently caused convulsive seizures in rodents (ddY mice and SD rats). The dosage (3-4 mg/kg, i.p.) of nicotine that caused convulsions was similar to those in previous reports[15, 73] where various mouse strains were evaluated for nicotine-induced seizure sensitivity (more sensitive ST/bj mice ED_{50} = 2.34 mg/kg, i.p. and less sensitive DB mice ED_{50} = 6.16 mg/kg, i.p.).

Several studies suggest that the proconvulsive action of nicotine is mediated by α7 nACh receptors[74-76], although the involvement of nACh receptors subtypes in nicotine-induced seizures are still uncertain. Here, nicotine-induced seizures were completely blocked by MEC, illustrating nACh receptor mediation. In addition, MLA (α7 nACh antagonist) was considerably more potent than DHβE (α4β2 nACh antagonist) in inhibiting nicotine-induced seizures. These results are consistent with previous studies[74-76] and suggest that α7 nACh receptors play a major role in inducing nicotine seizures. We have previously reported that kinetic tremors induced by a low dose (1 mg/kg, i.p.) of nicotine are mediated by α7 nACh receptors, whereas α4β2 nACh receptors are negligibly involved in tremor induction[59]. Therefore, α7 nACh receptors are likely to play a key role in producing motor excitations (e.g., tremor and seizure generation) with nicotine. However, we cannot completely deny a

possibility that α4β2 nACh receptors are partly involved in nicotine-induced seizures since DHβE slightly reduced the seizure induction, which is consistent with the previous finding that i.c.v. injection of DHβE reduced nicotine seizures by about 15%[74]. Actually, in an attempt to verify the complementary role of α4β2 nACh receptors in nicotine-induced seizures we co-administrated MLA and DHβE before the nicotine treatment, unfortunately, all animals died of what seemed to be peripheral respiratory failure. Since, we used the same concentrations when treating with only MLA or DHβE, this mortality can only be explained by a potentiation in the toxic actions of the DHβE[77] and MLA[78].

Fos protein expression is widely used as a marker of neural activation to explore the brain regions linked to disease conditions (e.g., epilepsy, essential tremors) and drug responses[26-28, 30, 79-84]. We previously demonstrated that a low dose (1 mg/kg, i.p.) of nicotine, which reportedly induces cognitive enhancement[51], antidepressant effects[33, 85] and positive reinforcement[37], as well as kinetic tremor[59], region-specifically elevated Fos expression in four brain regions; the PirC, MHb, Sol and IO. In the present study, a convulsive dose (4 mg/kg, i.p.) of nicotine further increased Fos expression in extended regions, the AMG and parts of the diencephalon (thalamus and hypothalamus). Thus, these regions excited by nicotine seemed to be related to seizure induction. In addition, the electrical lesion study revealed that only the AMG lesion, but not the PirC, Th, or MHb lesions, suppressed nicotine-induced seizures, suggesting that the AMG is the causative site for the induction of nicotine seizures. This possibility was further supported by the fact that microinjected nicotine into the AMG elicited convulsive seizures. The AMG is well known to be involved in seizure generation and epileptogenesis[86-89]. In addition, previous *in situ* hybridization and autoradiography studies revealed that in the AMG α7 and α4β2 nACh receptors highly expressed[8] what could explain the participation of both α7 and α4β2 receptors in nicotine-induced seizures[8, 90-100] (Table 1), studies using mice and brain slices[92, 101] and neuronal cell cultures[101, 102], show the direct activation of nicotine and acetylcholine in the amygdala α7

nACh receptors resulting mostly in the facilitation of glutamatergic transmission (60-40%) [92, 101, 102], while a small proportion resulted in GABAergic (20%), and both glutamatergic and GABAergic (17%) [102]. Therefore, it is most likely that the AMG, especially the medial AMG (e.g., MePD), is the primary foci of seizure generation by nicotine. However, we cannot limit the causative site to the medial AMG since other amygdaloid nuclei (e.g., BLP and BMP) also showed considerably high Fos expression with nicotine and are known to receive dense cholinergic input from the basal forebrain[24, 103, 104].

Although genetic polymorphisms of the gene (*CHRNA7*) encoding the α7 nACh receptor subunit are known to be involved in various epileptic disorders in humans, including idiopathic generalized epilepsy, childhood absence epilepsy, juvenile myoclonic epilepsy and benign epilepsy of childhood with centrotemporal spikes[18-21], the functional role and mechanisms of α7 nACh receptors in modulating seizure generation and/or epileptogenesis are still unknown. A line of studies showed that microdeletion of chromosome 15q13.3 including *CHRNA7* causes severe mental retardation, seizures and facial and/or digital dysmorphisms. This evidence implies that α7 nACh receptors are involved in the pathogenesis of mental illness (e.g., autism and schizophrenia) and negatively regulate seizure generation[19, 53, 54]. Nonetheless, the present results suggest that excessive stimulation of α7 nACh receptors elicits convulsive seizures by activating the AMG neurons, which are implicated in seizure generation not only due to nicotine intoxication, but also that caused by epileptic diseases. Concomitantly, gain-of-function mutation and/or copy number polymorphism (e.g., duplication and triplication) of *CHRNA7* are associated with epileptic disorders. Indeed, patients with duplication and triplication of *CHRNA7* (15q13.3 gains) have been shown to exhibit neuropsychiatric phenotypes including epileptic seizures[56, 57, 105]. Further studies are required to delineate the role and clinical relevance of the α7 nACh receptor in the pathogenesis of epileptic disorders.

In conclusion, we performed behavioral and Fos-IHC studies in rodents to clarify the mechanisms underlying nicotine-induced seizures. Treatment of animals with nicotine produced motor excitement and elicited convulsive seizures at 3 and 4 mg/kg. MEC and an α7 nACh antagonist, MLA, effectively blocked the nicotine seizures, but a α4β2 nACh antagonist, DHβE, did so only nominally. In addition, Fos expression analysis revealed that a convulsive dose (4 mg/kg) of nicotine region-specifically activated neurons in the PirC, AMG, MHb, PT, AH and Sol, among which electric lesioning of the AMG specifically inhibited nicotine seizure generation. Furthermore, microinjections of nicotine into the AMG evoked convulsive seizures in a dose-related manner. The present results strongly suggest that nicotine elicits convulsive seizures by activating AMG neurons primarily via α7 nACh receptors (Figure 18).

3 Pharmacological analysis of the mechanisms underlying the glycine-binding site stimulant of NMDA receptor-induced alleviation of extrapyramidal disorders using Fos expression analysis

3.1 Introduction

Schizophrenia is a complex disorder with diverse psychotic symptoms including positive (e.g., hallucinations, delusion) and negative (e.g., apathy, alogia) symptoms, neurocognitive impairments, and mood disturbances[106-113]. A hyperactive meso-limbic dopaminergic system is well-known to be related to the pathogenesis of schizophrenia (dopamine hypothesis)[114], accordingly numerous first-generation antipsychotics, which commonly antagonize dopamine D_2 receptors, have been developed[113, 115, 116]. These agents effectively

improve positive symptoms (e.g., hallucination, delusion, and excitement) in patients with schizophrenia through dopamine D_2 receptor blockade in the limbic regions (e.g., nucleus accumbens)[116]. However, they frequently induce extrapyramidal side effects (EPS) by blocking dopamine D_2 receptors in the basal ganglia (e.g., striatum)[117-119]. EPS include acute dystonia (sustained abnormal postures and muscles spasms, especially of the head or neck), akathisia (restlessness and pacing), Parkinsonism (tremor, skeletal muscle rigidity, and/or bradykinesia) and tardive dyskinesia (involuntary, repetitive facial, torso and limb movements)[117, 120]. EPS are serious, sometimes debilitating and one of the main causes of poor adherence to antipsychotic treatment[121, 122].

The assembling of the GluN1 and GluN2 subunits containing D-serine/glycine- and glutamate-binding sites, respectively, constitute the heteromeric tetrameric receptor N-methyl-D-aspartate (NMDA)[123, 124]. Along with the D-serine/glycine- and glutamate-binding sites, they also possess several regulatory sites sensitive to polyamines, Zn^{2+}, protons, and glutathione[123, 125]. Dysfunctions of NMDA receptors are also suggested to be involved in the pathogenesis of schizophrenia (glutamate hypothesis)[126-128]. The glutamate hypothesis was derived from evidences showing that NMDA receptor blockers including phencyclidine (PCP) and dizocilpine (MK-801) cause schizophrenia-like psychosis in human[126, 129-131]. Consequently, the glutamate hypothesis in schizophrenia encourages the development of new medication for schizophrenia using several agents (e.g., D-cycloserine (DCS), D-serine, and sodium benzoate)[127, 128, 132, 133], though their actions regarding the induction and/or modulation of EPS still require clarification.

As described in the section 1.3, Fos expression analysis has been shown to be useful to explore the mechanism of many psychostimulants and psychotropic agents including morphine[134, 135], caffeine[136, 137] and antidepressants[138]. Specifically, since dopamine D_2 receptors are known to tonically suppress Fos expression in the ST (caudate nucleus and putamen) and the nucleus accumbens (AcC, AcS) ST[83, 139-144]. Consequently, blockade of

the dopamine D_2 receptors activities by first generation antipsychotic agents (e.g., haloperidol [HAL]) results in elevation of the Fos expression in these structures[83, 141, 143, 145, 146], however, second generation antipsychotics (e.g., blonanserin) increase in the Fos expression are more focused in the AcS than the ST[140, 142-146]. Therefore, the comparison of antipsychotic-induced Fos expression between the AcS and dorsal ST has been used to differentiate the second generation antipsychotics alleviation of motor side effects from the first generation ones. Thus, we employed behavioral and striatal Fos-IHC in order to evaluate the effects of D-cycloserine, a glycine-binding site stimulant of NMDA receptors, on antipsychotic-induced EPS (i.e., bradykinesia).

3.2 Materials and methods

3.2.1 Animals

Male ddY mice (Japan SLC, Shizuoka, Japan) at 8–10 weeks of age were used. Animals were kept in air-conditioned rooms (24 ± 2°C and 50 ± 10% relative humidity) under a 12-h light/dark cycle (light on: 8:00–20:00) and allowed free access to food and water. Animal care methods complied with the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, and experimental protocols were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences (#17, 30 March 2015).

3.2.2 Evaluation of Bradykinesia

The pole test was performed as described previously by Shimizu, et al. [69]. Mice were placed at the top (head-upward) of a pole (diameter: 8 mm and height: 45 cm). The time for the animal to rotate downward (T_{turn}) and descend to the floor (T_{total}) was then measured with a cut-off time of 90 s. Only mice that showed T_{turn} < 8 s and T_{total} < 18 s in the pre-test trial (typically performed 2 h before the test trial) were used.

The glycine-site stimulant of NMDA receptors DCS (3–30 mg/kg, i.p.) was administered to animals 15 min before the HAL or vehicle injection, and the pole test was performed 30 min later.

3.2.3 Analysis of Fos Protein Expression

Regarding Fos immunohistochemical staining, brain samples were obtained from mice 120 min after the HAL injection. Under pentobarbital (80 mg/kg, i.p.) anesthesia, all mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS), which was followed by 4% formaldehyde perfusion. Brains were removed from the skull and stored in fresh fixative for at least 24 h. Fos immunohistochemical staining was performed using previously reported methods[26, 84, 147]. Coronal sections (thickness: 30 μm) were cut from the brain using a Microslicer (DSK-3000, Dosaka, Kyoto, Japan). Slices were incubated for 2 h with 2% normal rabbit serum, and with goat c-Fos antiserum for an additional 18–36 h. Sections were then incubated with a biotinylated rabbit anti-goat IgG secondary antibody for 2 h. After a 30 min incubation with 0.3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase, sections were incubated for 2 h with an avidin–biotinylated horseradish peroxidase complex. Fos-IR was visualized using the diaminobenzidine–nickel

staining method and quantified by counting the number of Fos-IR positive nuclei in dlST and AcS.

3.2.4 Drugs

HAL and DCS were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibody against c-Fos were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), the Vectastain ABC kit, DAB substrate and the secondary biotinylated anti-goat IgG antibody were purchased from Vector Laboratories (Burlingame, CA, USA) and others common laboratory reagents were also obtained from commercial sources.

HAL was dissolved in 1% lactate solution and then diluted with physiological saline. Other agents were dissolved in physiological saline. All drugs were injected i.p. in a volume of 5 mL/kg into mice.

3.2.5 Statistical Analysis

Data are expressed as the mean ± S.E.M. The significance of differences among multiple groups was assessed by a one-way ANOVA followed by Tukey's test or Kruskal–Wallis test (nonparametric one-way ANOVA) followed by the Steel–Dwass post-hoc test. Comparisons between only two groups were performed by the non-parametric Mann-Whitney's U-test or parametric Student's t-test. A *P*-value of less than 0.05 was considered significant.

3.3 Results

We confirmed that HAL at 1 mg/kg, i.p. markedly increased T_{turn} and T_{total} values, which were significantly reversed by DCS (30 mg/kg, i.p., Figure 19). Brain samples were then obtained from these animals 2 h after the HAL injection and subjected to Fos-IHC. Under these conditions, control (Vehicle + Vehicle) and DCS (30 mg/kg, i.p.) animals showed negligible Fos expression in the dlST (Figure 20, 21) and AcS (Figure 20, 22). The number of Fos-IR-positive cells was markedly increased by HAL (vehicle + HAL, dlST: $F(3, 21) = 14.979$, $P < 0.001$, AcS: $F(3, 21) = 8.3832$, $P = 0.003$). However, HAL-induced Fos expression was significantly inhibited by DCS in the dlST ($P = 0.032$). Haloperidol (vehicle + Hal) treatment showed an increment in the number of Fos-IR-positive cells in the dlST to approximately 21 cells/grid; on the other hand, the treatment with DCS reduced HAL-induced Fos expression to approximately 16 cells/gird. Interestingly, DCS did not significantly affect HAL-induced Fos expression in the AcS (Figure 20, 21).

3.4 Discussion

The present study demonstrated that DCS, a glycine-binding site agonist of NMDA receptors, significantly alleviated HAL-induced bradykinesia. The antibradykinetic doses of DCS in the pole test were 3–30 mg/kg, i.p., a dose range similar to those producing efficacy in animal models of schizophrenia with phencyclidine, a NMDA antagonist, (10-30 mg/kg, s.c.)[148]. Therefore, the glycine-binding site stimulants of NMDA are expected to reduce EPS associated with antipsychotic treatments in clinical settings.

Antipsychotics elevate the regional expression of the Fos protein, a biological marker of neural activation, both in the nucleus accumbens and striatum by blocking dopamine D_2 receptors[83]. Furthermore, dopamine D_2 receptor-mediated Fos expression reflects in the nucleus accumbens the effectiveness of the antipsychotics and in the striatum the EPS liability of antipsychotics[142, 144, 149-151]. Hence, second-generation antipsychotics with fewer EPS commonly lead to reduced Fos expression in the striatum[144, 152-154]. In the present study, we showed that DCS significantly reduced Fos expression in the dlST. This evidence further supports DCS counteracts striatal dopamine D_2 receptor blockade by HAL to attenuate the induction of EPS. The effects of DCS on Fos expression were region-specific and did not significantly alter HAL-induced Fos expression in the AcS. These results suggest that a combination of the glycine-binding site stimulants of NMDA receptors with antipsychotics preferentially attenuates EPS (D_2 blocking action in the striatum) without interfering with the therapeutic action of antipsychotics.

These results suggest that DCS induces the activation of neurons in the striatum alleviating the HAL-induced EPS. Therefore, based on the glutamate hypothesis, the glycine-binding site stimulants of NMDA receptors are expected to become a new medication for schizophrenia[155]. Clinical studies showed that several agents including DCS improved negative symptoms and/or cognitive impairment in patients with schizophrenia[156-159]. In conclusion, the present results suggest that glycine-binding site agonists are beneficial not only for its efficacy, but also side-effect management in the treatment of schizophrenia. Not to mention that the presented evidence shows that Fos protein expression evaluation is an effective way to strengthen the behavioral evidence and identify brain regions related to pharmacological drug effects/side-effects.

4 ACKNOWLEDGEMENTS

As the acknowledgements section presents an opportunity to students express their gratitude to all people that played an essential role in their graduation, without the concerning of social awkwardness, I would like to thank those that went far above and beyond what was professionally required of them. In this section, I also want to express my deepest gratitude to each person that contributed to the completion of this thesis.

Initially, I would like to say thank you to my supervisor, Prof. Yukihiro Ohno of the Laboratory of Pharmacology at Osaka University of Pharmaceutical Sciences, for his patient guidance, enthusiastic encouragement and providing me all necessary assets to accomplish the present work and improve myself as scientist. Your support and dedication to your laboratory team has earned you much deserved admiration. I am also thankful to Prof. Masahiko Taniguchi for his instruction, supervision, constant support and motivation, his wiliness to give his time so generously has been very much appreciated. I must also to include that his class was one the most interesting in this PhD course, I often use the contents in talks with laboratory fellows or friends with scientific curiosity.

I am grateful to Dr. Yoshiko Kawai, Dr. Saki Shimizu and all PhD students, especially to Dr. Kentaro Tokudome and Mr. Naofume Kunisawa, for sharing essential theory and technical knowledge, and also for their direct assistance in many experiments included in this dissertation. For all laboratory members past and present whom provided me with their excellent technical assistance and kind support. Thank you all for providing me with such a great laboratory environment.

I want to extend my thanks, to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for providing the opportunity to study and research in Japan as an international exchange student, allowing me to increase my knowledge in neuroscience and

in Japan's history, culture and language that I am so found of. To the OUPS that provided me all assistance to have comfortable graduate school lifestyle in order to allow me to focus my efforts in my research and studies. I am most grateful for your support.

I also would like to thank my family, relatives and friends who celebrated each achievement and helped to keep my head up and moving forward despite failures or difficulties I have faced. Especially my deceased Japanese teacher, Ms. Marcia Naomi Miyazaki, who helped with the Japanese documentations before coming to Japan, I am glad that my last memory was a tender smile for knowing that one of your students was going to Japan. I wholeheartedly appreciate everything you all have done for me.

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Table 1: Local expression of nACh receptor subtypes in the brain of human and rodents.

Brain region	Human[5,99]	Rodents[5,8]
Cortex	α3, α4, α7, β2	α3, α4, α5, α7, β2, β4
Striatum	α5, α7, β2, β4	α4, α5, α6, β2, β3
Hippocampus	α4, α5, α7, β2	α3, α4, α5, α7, β2, β4
Medial habenula	—	α3, α4, α7, β2, β3, β4
Thalamus	β2, β4	α4, β2
Hypothalamus	—	α4, α7, β2,
Substantia nigra	α6	α3, α4, α5, α6, α7, β2, β3, β4
Amygdala	α4, α7, β2	α4, α7, β2

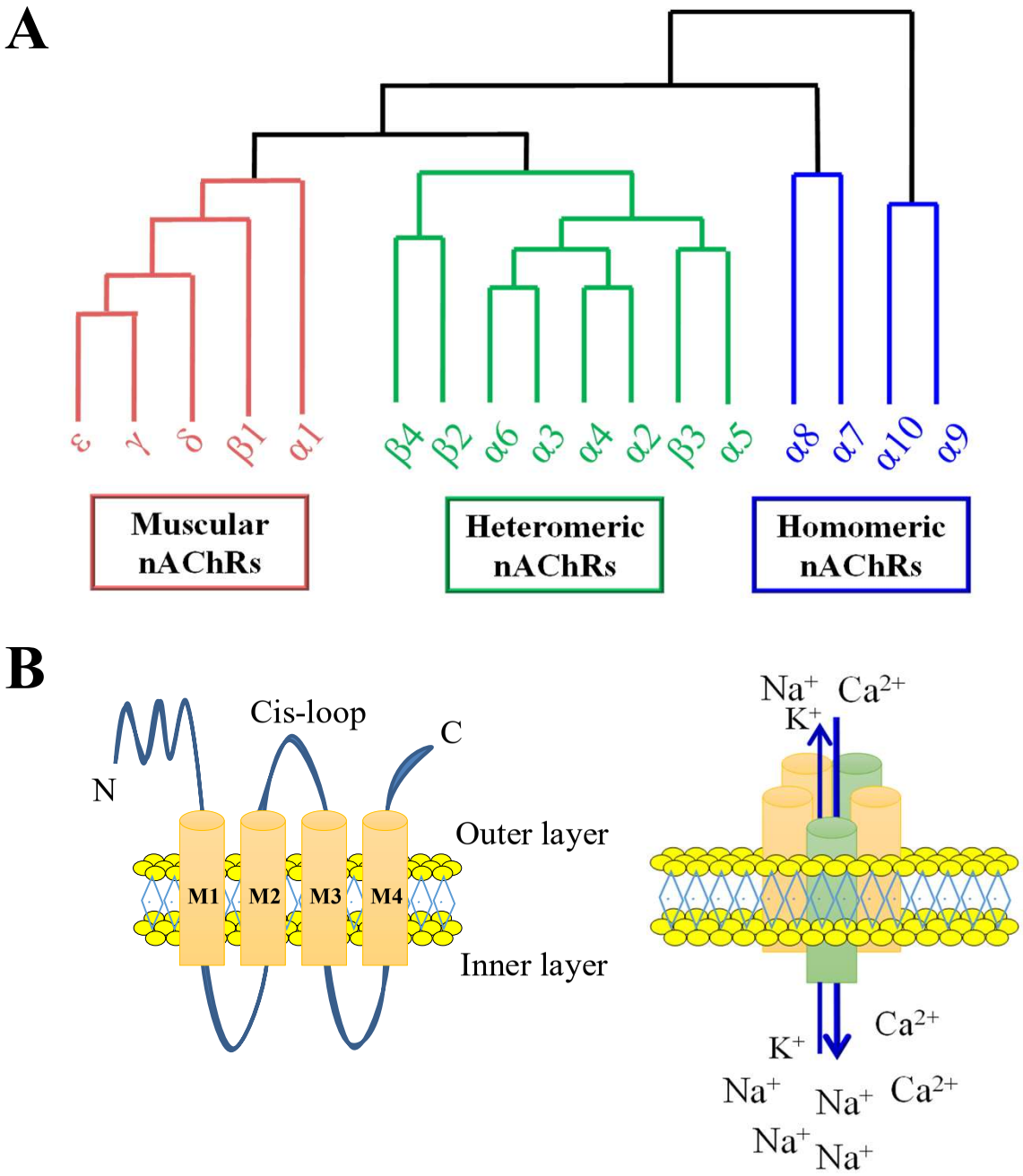

Figure 1: Nicotinic acetylcholine receptors resume. (A) Forms of nACh receptor subtypes pentameric assemblance. There are three basic patterns of nACh assembling α, β, δ and γ/ε (muscular forms), α and β (heteromeric forms) or only α subunits (homomeric forms). **(B)** Membrane topology of neuronal nACh receptor subunit, each nACh receptor subunit contains four transmembrane domains (M1-M4), an extracellular amino- and carboxy-terminus, and a prominent M3-M4 intracellular loop of variable length **(left)**, these five subunits coassemble to form a functional subunit **(right)**. Adapted from Hendrickson et al. (2013).

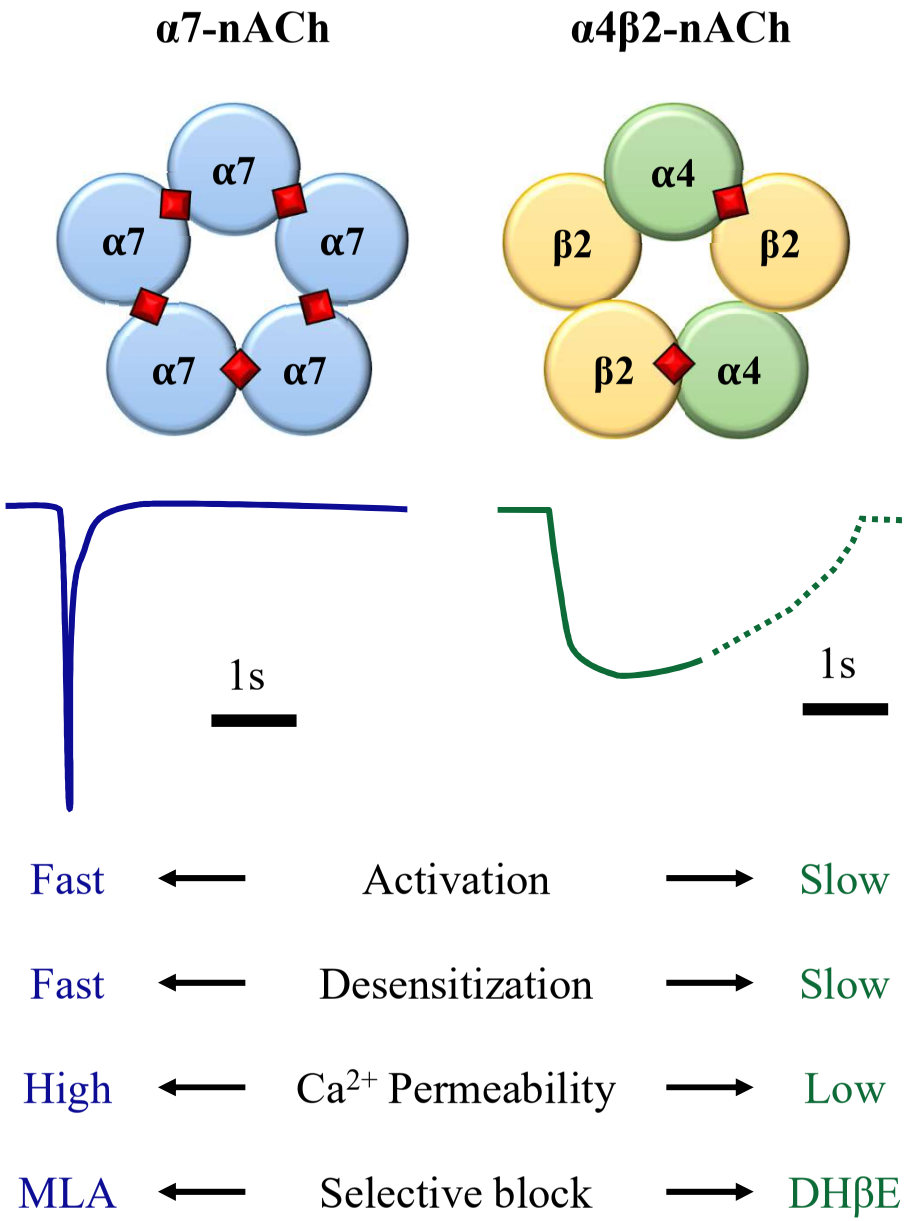

Figure 2: The two main neuronal nicotinic acetylcholine receptors subtypes. The two main neuronal nACh receptor subtypes consist of the α7 nACh receptors and the α4β2 nACh receptors. ACh binding sites are depicted as red diamonds. The basic functional and pharmacological properties of the α7 and α4β2 nACh receptor subtypes. Abbreviation: DHβE, dihydro-b-erythroidine; MLA, methyllycaconitine Adapted from Hendrickson et al.[160] and Dineley et al.[58]

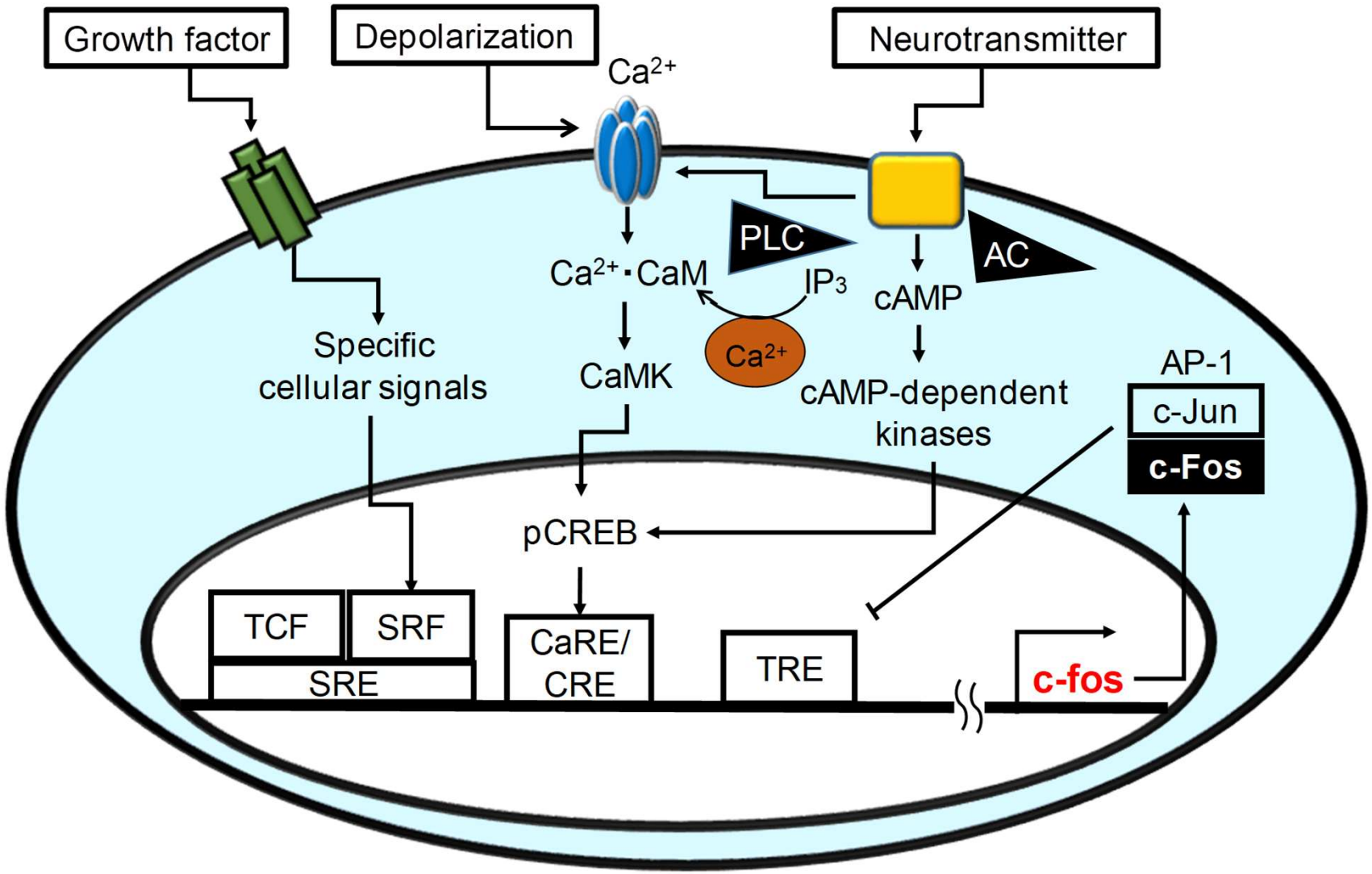

Figure 3: Illustration of main neuronal signal transduction pathways for Fos expression. Hormonal, ion channel and neutransmitters activation initiate cascade reactions that culminate in the transitory expression of c-fos transcript. c-fos transiently produce the Fos protein which may dimerize with c-Jun to form the AP-1 protein complex. AP-1 regulates both basal and inducible transcription of several genes including c-fos. Therefore, Fos protein is an optimal marker for transient neural signaling. Abbreviation: **AC** adenyl cyclase, **AP-1** activator protein 1, **CaM** calcium/calmoduline, **CaMK** CaM-dependent kinase, **CaRE** calcium responsive element, **CRE** cAMP responsive element, **pCREB** phosphorylated CREB, **PLC** phospholipase C, **SRE** serum response element, **SRF** serum response factor, **TCF** ternary complex factor, **TRE** TPA (12-O-tetradecanoylphorbol 13-acetate)-response element. Adapted from Iha et al.[26]

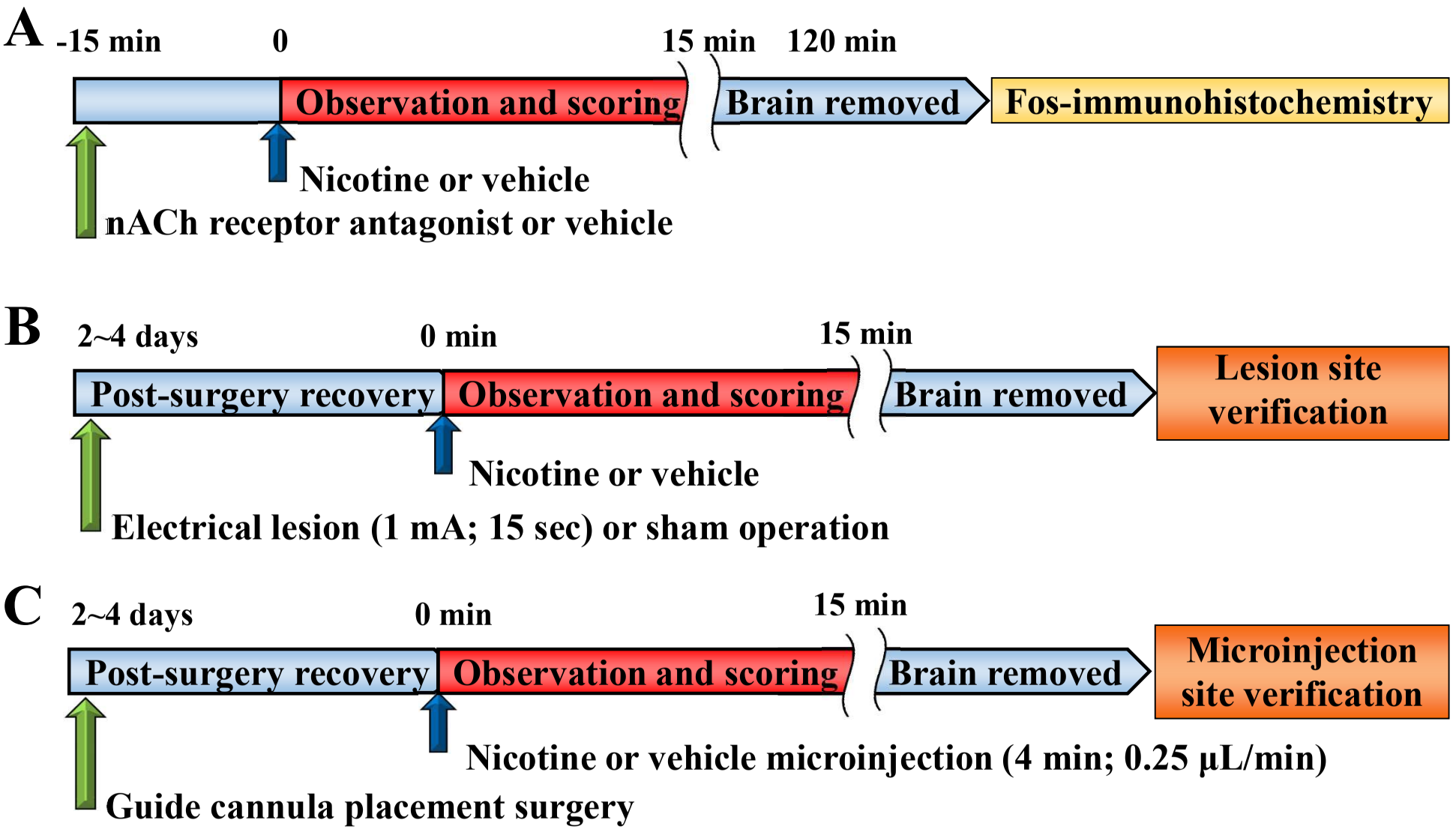

Figure 4: Experimental protocols for induction of convulsive seizures using nicotine. In all experiments animals were evaluated for 15 min immediately after the nicotine or saline (vehicle) injection for seizure incidence and intensity using a 6-point ranked score. The incidence of nicotine seizures was judged positive when animals displayed score 4 or higher. Behavior score 0: no effect; 1: mild head tremor and Straub tail; 2: tremors in extended regions; 3: severe tremors with wild running; 4: clonic seizures; and 5: tonic and tonic-clonic seizures. **(A)** Dose-response and nACh antagonists evaluation for nicotine-induced seizures; for the dose-response experiment animals were injected with saline or nicotine (1, 2, 3 or 4 mg/kg, i.p.) and for the nACh antagonists study animals were treated with MEC (1 mg/kg, i.p.), MLA (10 mg/kg, i.p.) or DHβE (5 mg/kg, i.p.) 15 min before nicotine (4 mg/kg, i.p.). Two hours after the nicotine injection the brains were removed for Fos-immunostaining. **(B)** Electrical lesion study, electrodes were bilaterally placed in the PirC, Th, MHb; and AMG before seizure induction with nicotine (4 mg/kg, i.p.). **(C)** Microinjection study were performed by bilaterally placing guide cannulas in the AMG region to inject saline or nicotine (100 or 300 µg/side). After the behavioral study brains were removed to verify lesion (B) or microinjection sites (C).

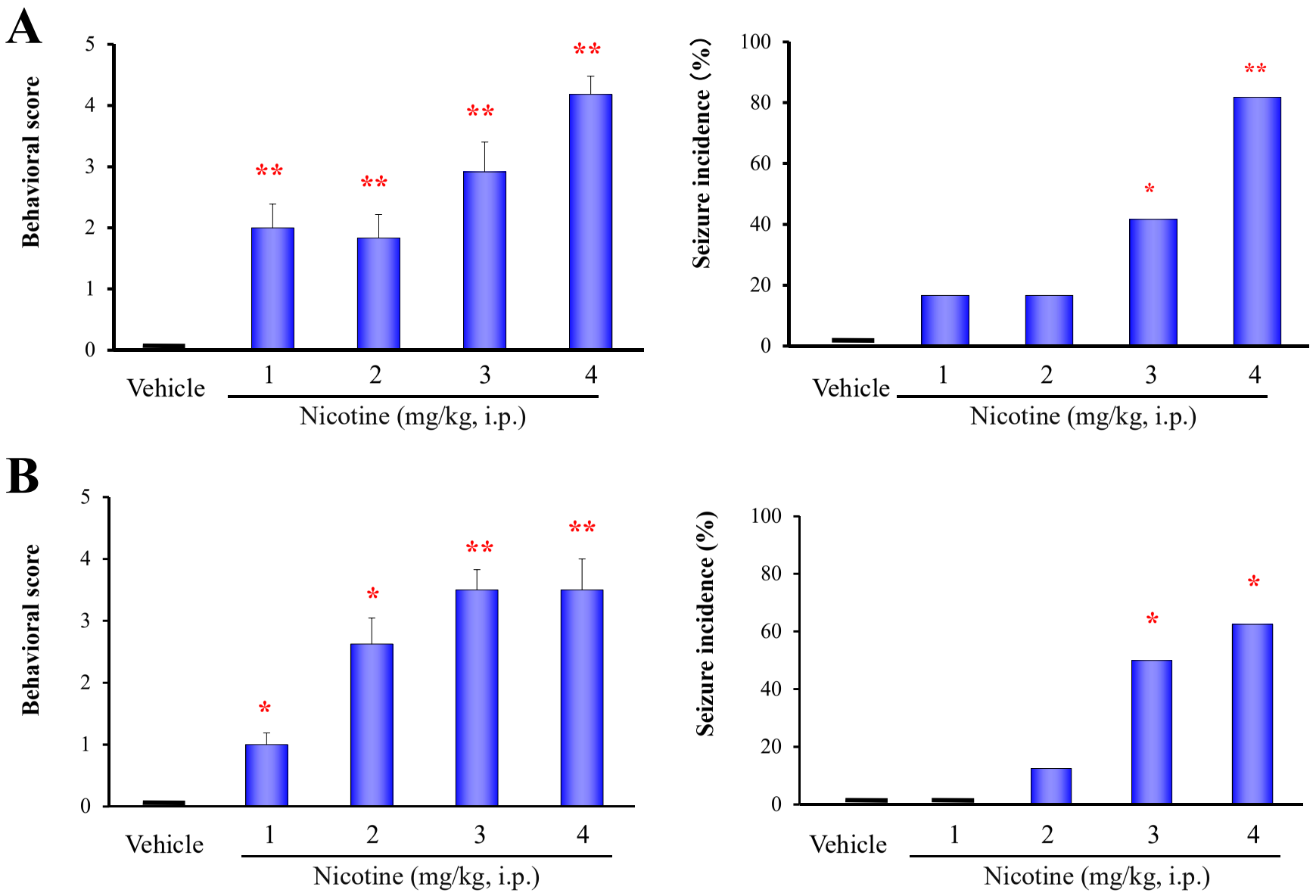

Figure 5: Dose-response effects of nicotine on convulsive seizure induction in rodents. Nicotine-induced convulsive seizures in mice **(A)** and rats **(B).** Behavioral scores are expressed as the mean of ± S.E.M. of 7-11 animals. Seizure incidence represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined. $^{*}P < 0.05$ P and $^{**}P < 0.01$; Significant different from the control animals treated with vehicle alone (Vehicle).

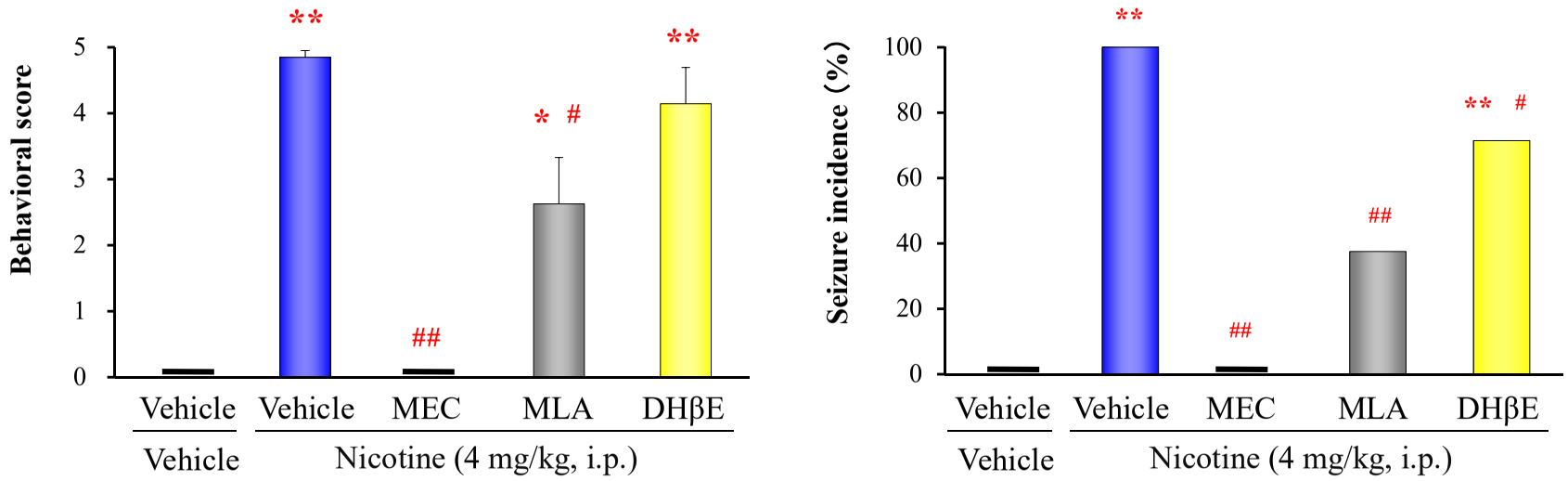

Figure 6: Effects of nACh antagonists in the nicotine-induced convulsive seizures in mice. Effects of nACh antagonists, MEC (non-selective; 1 mg/kg i.p.), MLA (α7 nACh antagonist; 10 mg/kg i.p.), and DHβE (α4β2 nACh antagonist; 5 mg/kg i.p.) on nicotine (4 mg/kg i.p.)-induced seizures in mice. nACh antagonists were injected 15 min before nicotine. Behavioral scores are expressed as the mean ± S.E.M. of 7-11 animals. Seizure incidence represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined. $^{*}P < 0.05$ and $^{**}P < 0.01$; Significant different from the control animals treated with vehicle alone (Vehicle+Vehicle). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$; Significant different from the value for nicotine group (Vehicle+Nicotine).

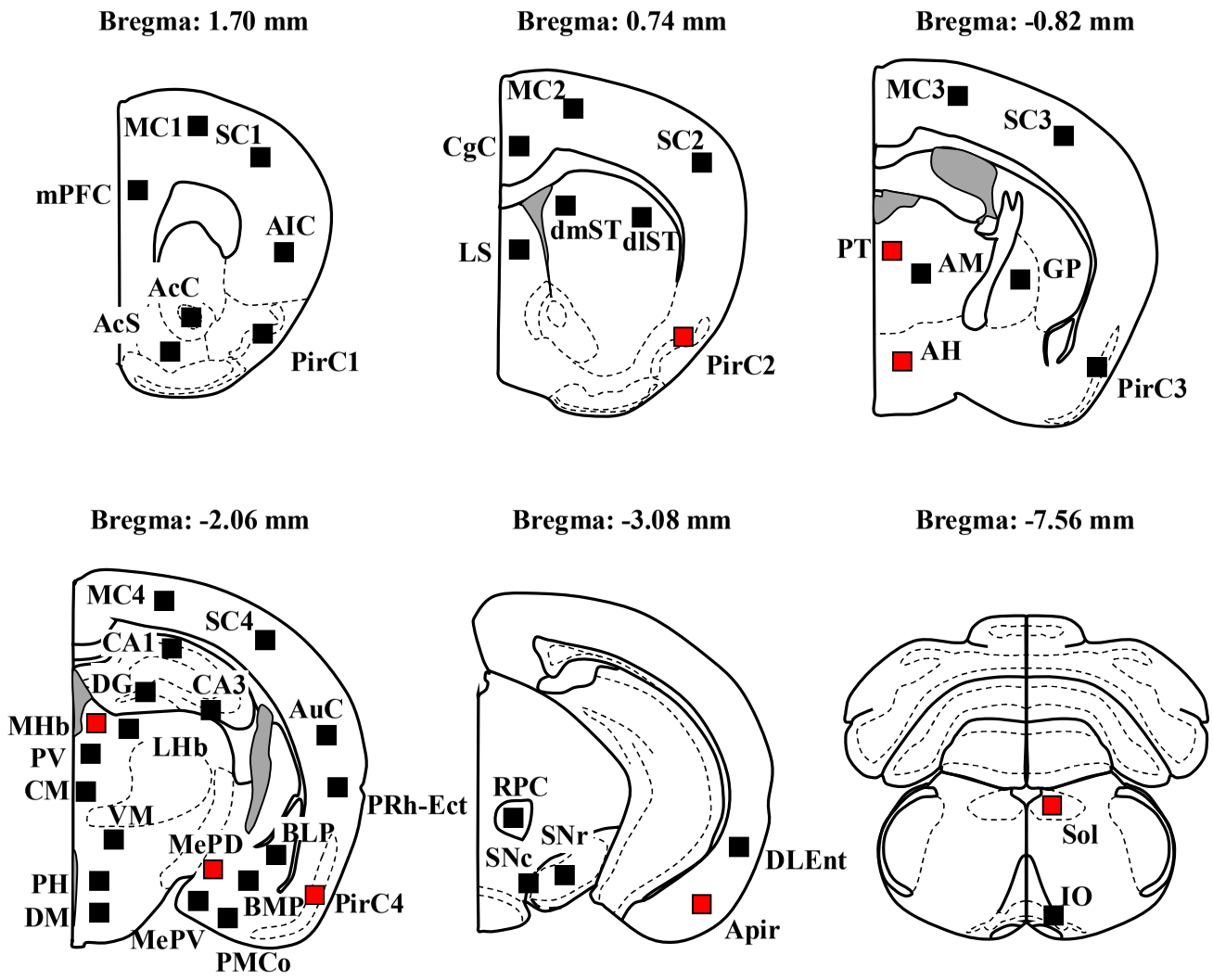

Figure 7: Schematic illustrations of the brain sections selected for quantitative analysis of Fos expression. Filled squares in each section indicate the sample areas analyzed and red filled squares represent the sites which showed significant increments in Fos expression by nicotine (4 mg/kg, i.p.). Anteroposteirior coordinate (distance from the Bregma) is shown above each section. Analysis of the MC, SC, and PirC were performed in four different levels from Bregma (Area 1 at +1.7 mm, Area 2 at +0.74 mm, Area 3 at –0.82 mm, Area 4 at –2.06 mm)

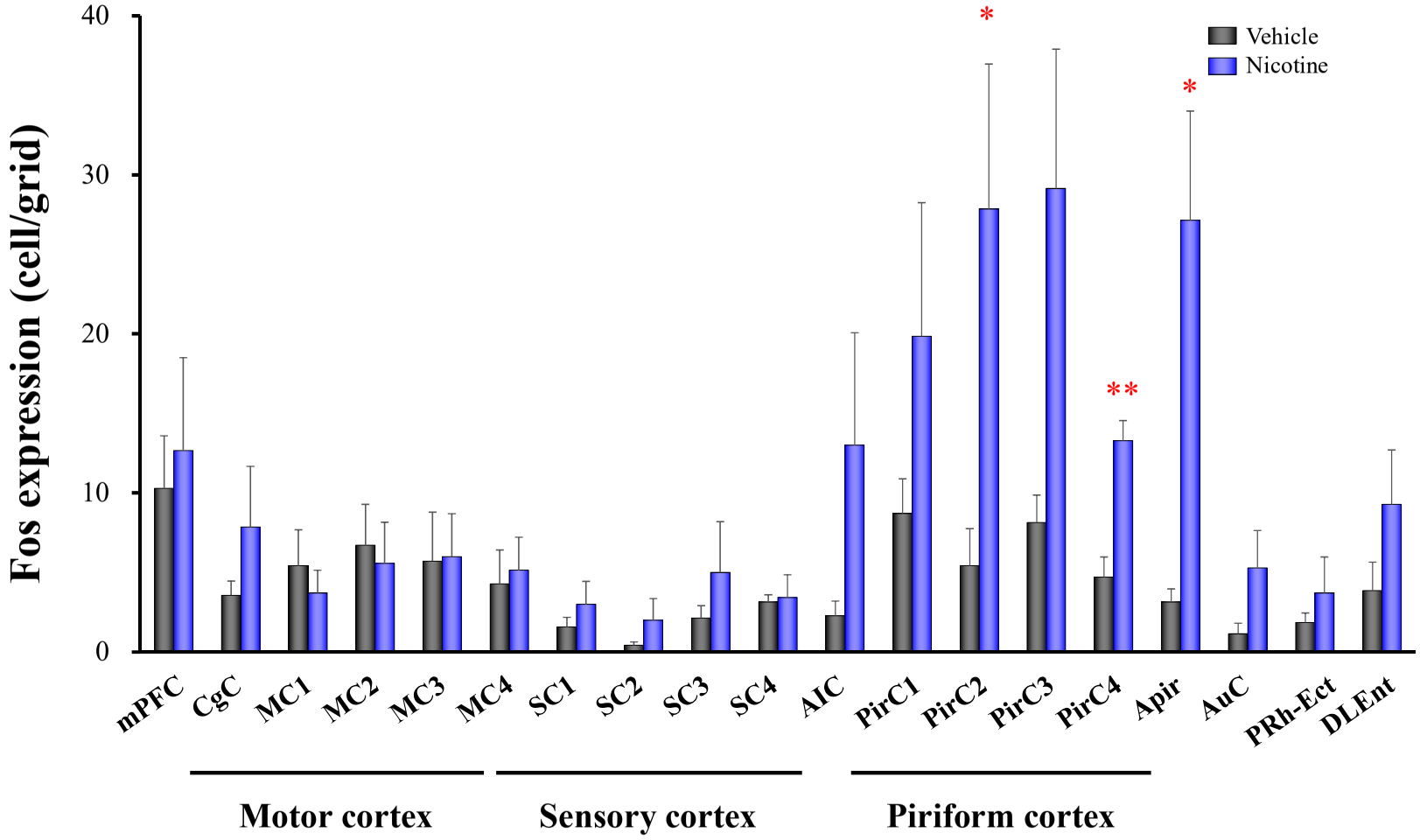

Figure 8: Effects of nicotine (4 mg/kg, i.p.) on Fos expression in cortical regions in mice. Brains were removed 2 h after the nicotine (4 mg/kg, i.p.) administration and subjected to the Fos-IHC staining. Representative photographs illustrating the Fos-IR-positive cells in the PirC4 are shown in the top. Each column represents the mean ± S.E.M. of 5-8 mice, $^{*}P < 0.05$ and $^{**}P < 0.01$; Significant different from the control animals treated with vehicle alone (Vehicle).

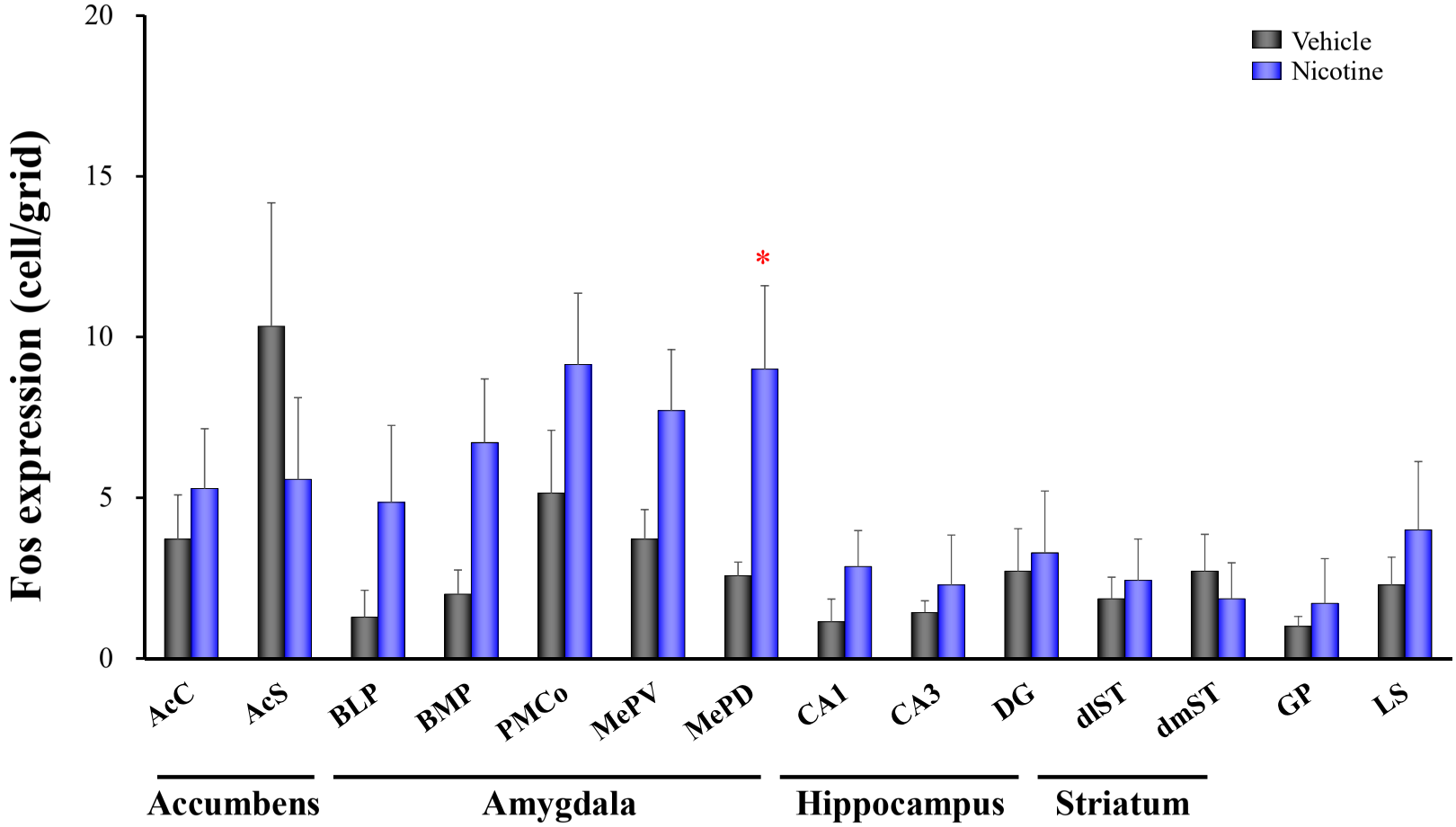

Figure 9: Effects of nicotine (4 mg/kg, i.p.) on Fos expression in limbic and basal ganglia regions in mice. Brains were removed 2 h after the nicotine (4 mg/kg, i.p.) administration and subjected to the Fos-IHC staining. Representative photographs illustrating the Fos-IR-positive cells in the MePD are shown in the top. Each column represents the mean ± S.E.M. of 5-8 mice, $^{*}P < 0.05$; Significant different from the control animals treated with vehicle alone (Vehicle).

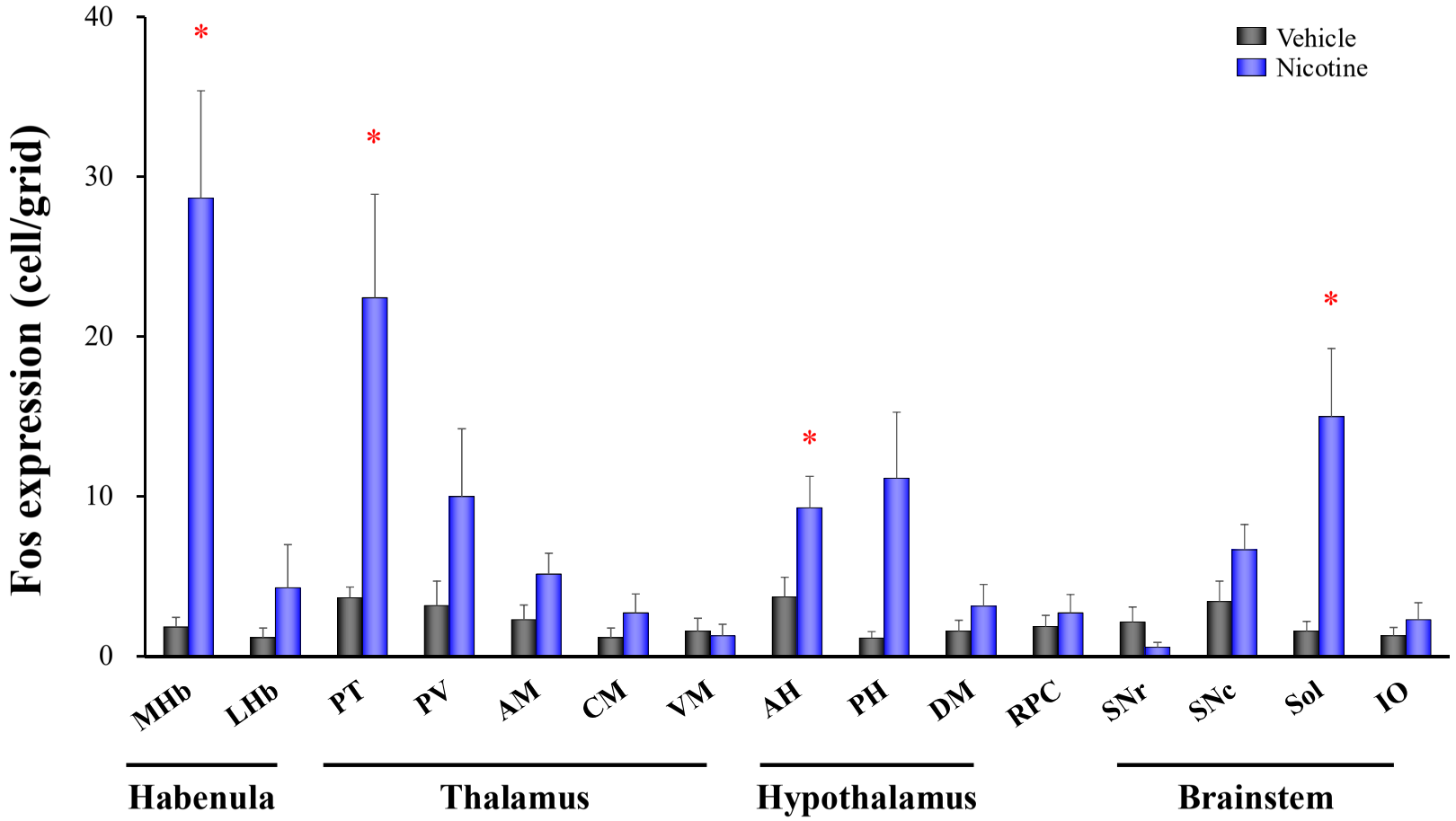

Figure 10: Effects of nicotine (4 mg/kg, i.p.) on Fos expression in diencephalic and brainstem regions in mice. Brains were removed 2 h after the nicotine (4 mg/kg, i.p.) administration and subjected to the Fos-IHC staining. Representative photographs illustrating the Fos-IR-positive cells in the PT are shown in the top. Each column represents the mean ± S.E.M. of 5-8 mice, $^{*}P < 0.05$; Significant different from the control animals treated with vehicle alone (Vehicle).

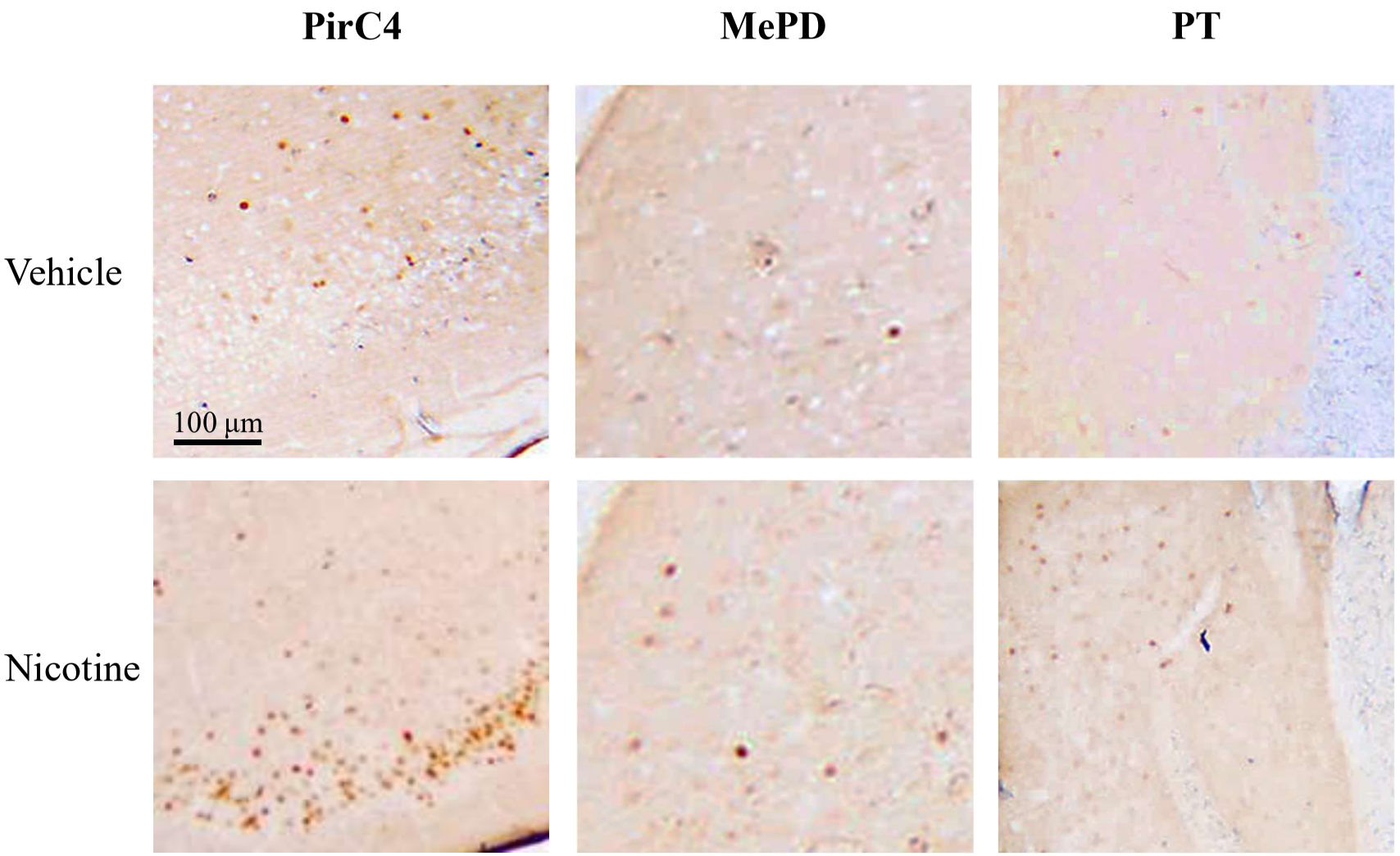

Figure 11: Immunohistochemical analysis of Fos protein immunoreactivity. Representative photographs illustrating the Fos protein expression in PirC (region 4, Bregma -2.06 mm), medial posterodorsal amygadaloid nucleus (MePD) and paratenial thalamus (PT) of mice treated with saline (Vehicle) and nicotine. Scale bar: 100µm.

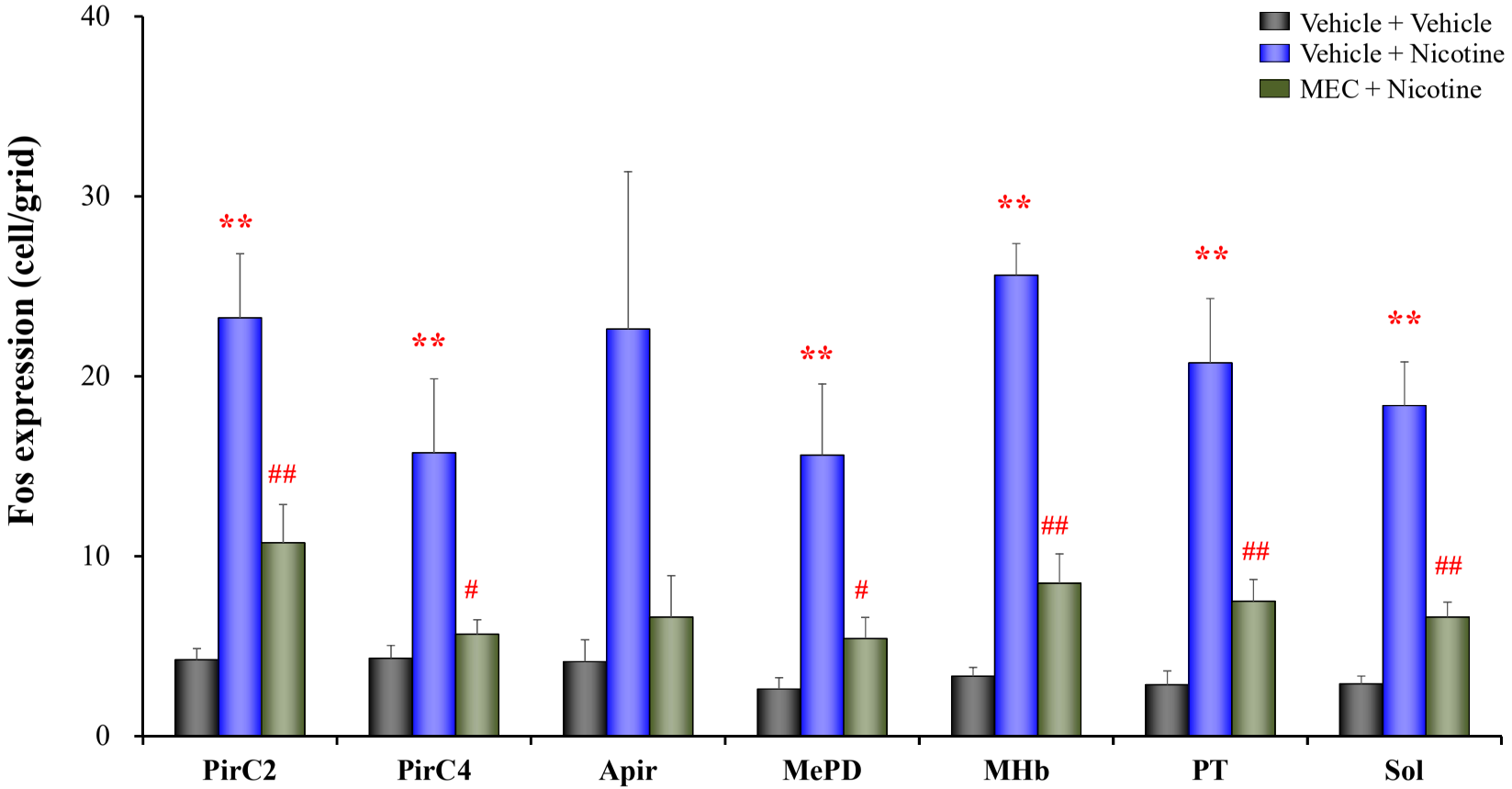

Figure 12: Effects of MEC on nicotine-induced Fos expression in mice. Animals were treated with MEC (1 mg/kg, i.p.) 15 min before the nicotine injection (4 mg/kg, i.p.). Each column represents the mean ± S.E.M. of 5-8 mice, $^{**}P < 0.01$; Significantly different from the control animals treated with vehicle alone (Vehicle + Vehicle). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$; Significantly different from the nicotine group (Vehicle + Nicotine).

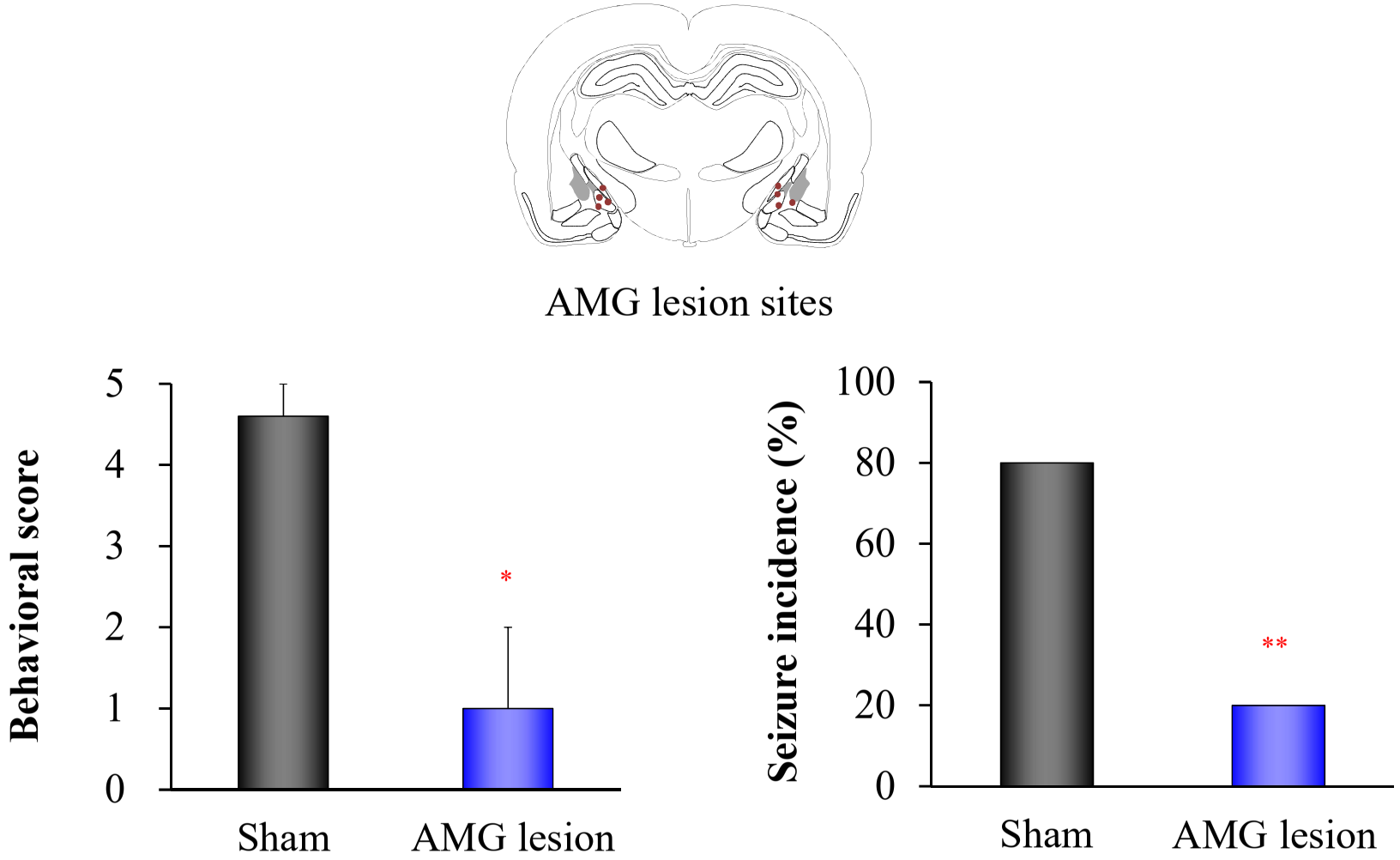

Figure 13: Effects of electrical lesionings of the amygdala (AMG) on nicotine-induced seizures in rats. Panels show the effects of nicotine (4 mg/kg, i.p.) on seizure generation after electrical lesionings at the AMG**.** Upper panel illustrate the electrical lesion sites in AMG. Behavioral scores (left graph) are expressed as the mean of ± S.E.M. of four or five animals. Seizure incidence (right graph) represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined. $^{*}P < 0.05$ and $^{**}P < 0.01$; Significant different from the Sham group.

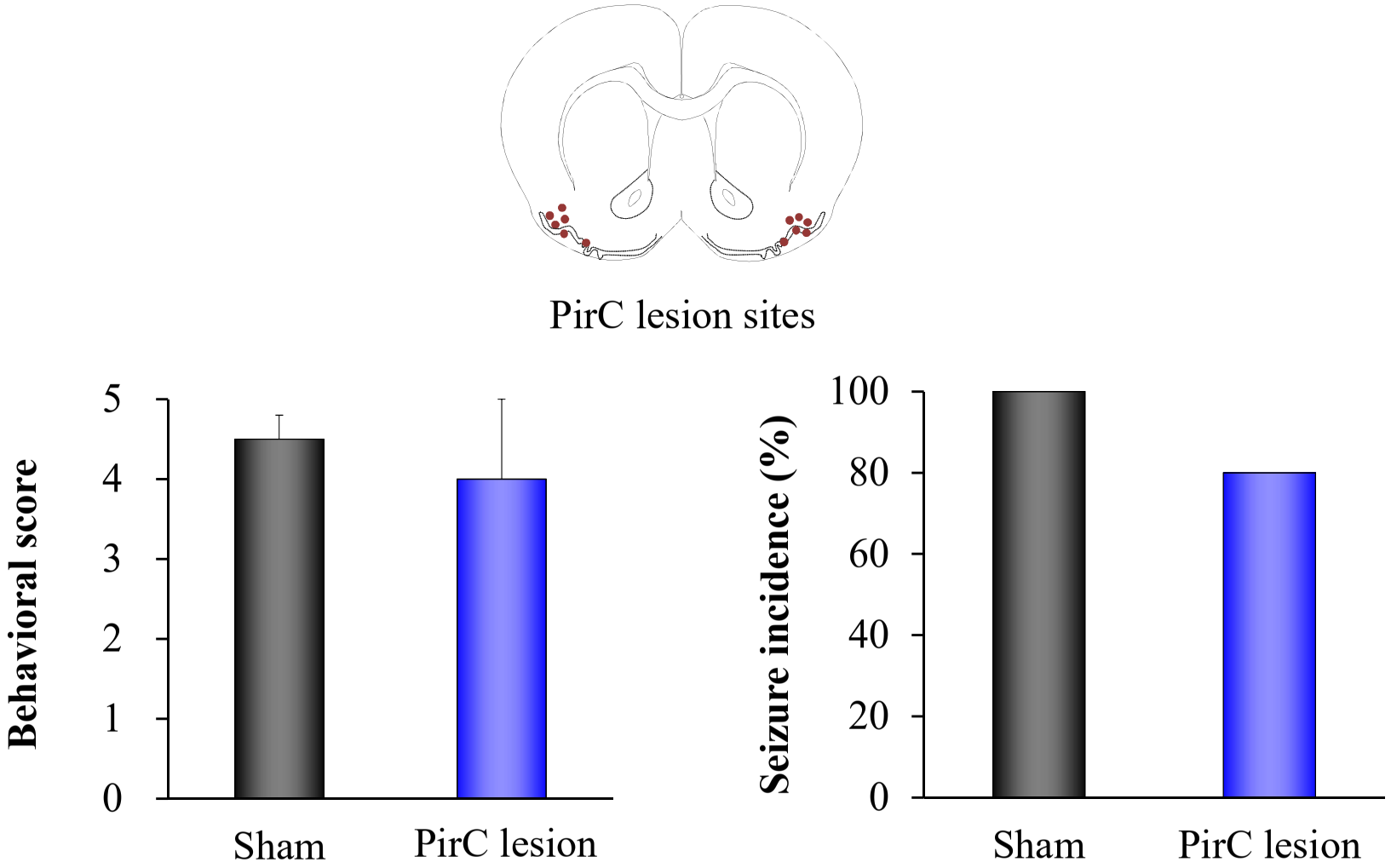

Figure 14: Effects of electrical lesionings of the piriform cortex (PirC) on nicotine-induced seizures in rats. Panels show the effects of nicotine (4 mg/kg, i.p.) on seizure generation after electrical lesionings at the PirC**.** Upper panel illustrate the electrical lesion sites in PirC. Behavioral scores (left graph) are expressed as the mean of ± S.E.M. of four or five animals. Seizure incidence (right graph) represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined.

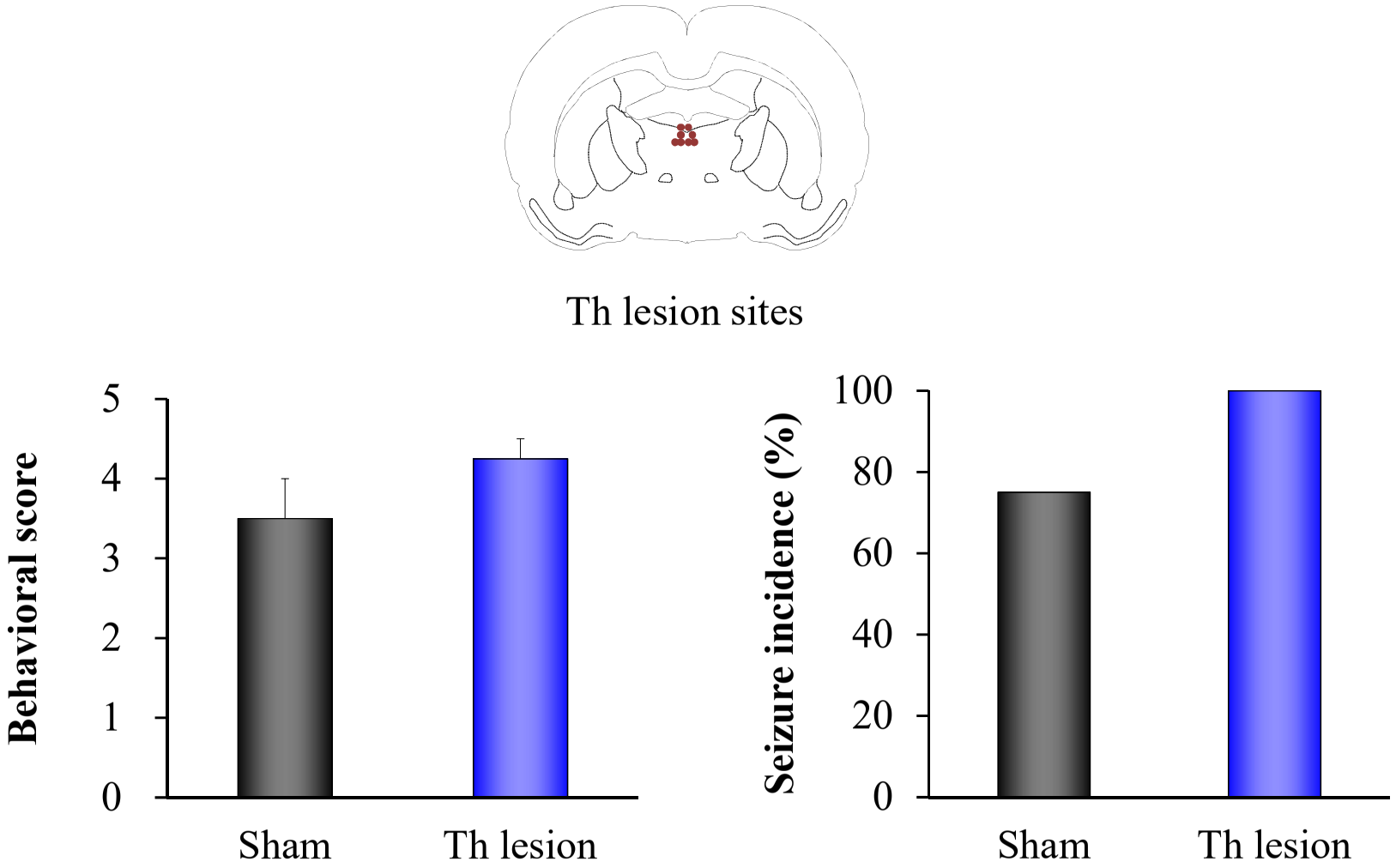

Figure 15: Effects of electrical lesionings of the thalamus (Th) on nicotine-induced seizures in rats. Panels show the effects of nicotine (4 mg/kg, i.p.) on seizure generation after electrical lesionings at the Th**.** Upper panel illustrate the electrical lesion sites in Th. Behavioral scores (left graph) are expressed as the mean of ± S.E.M. of four or five animals. Seizure incidence (right graph) represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined.

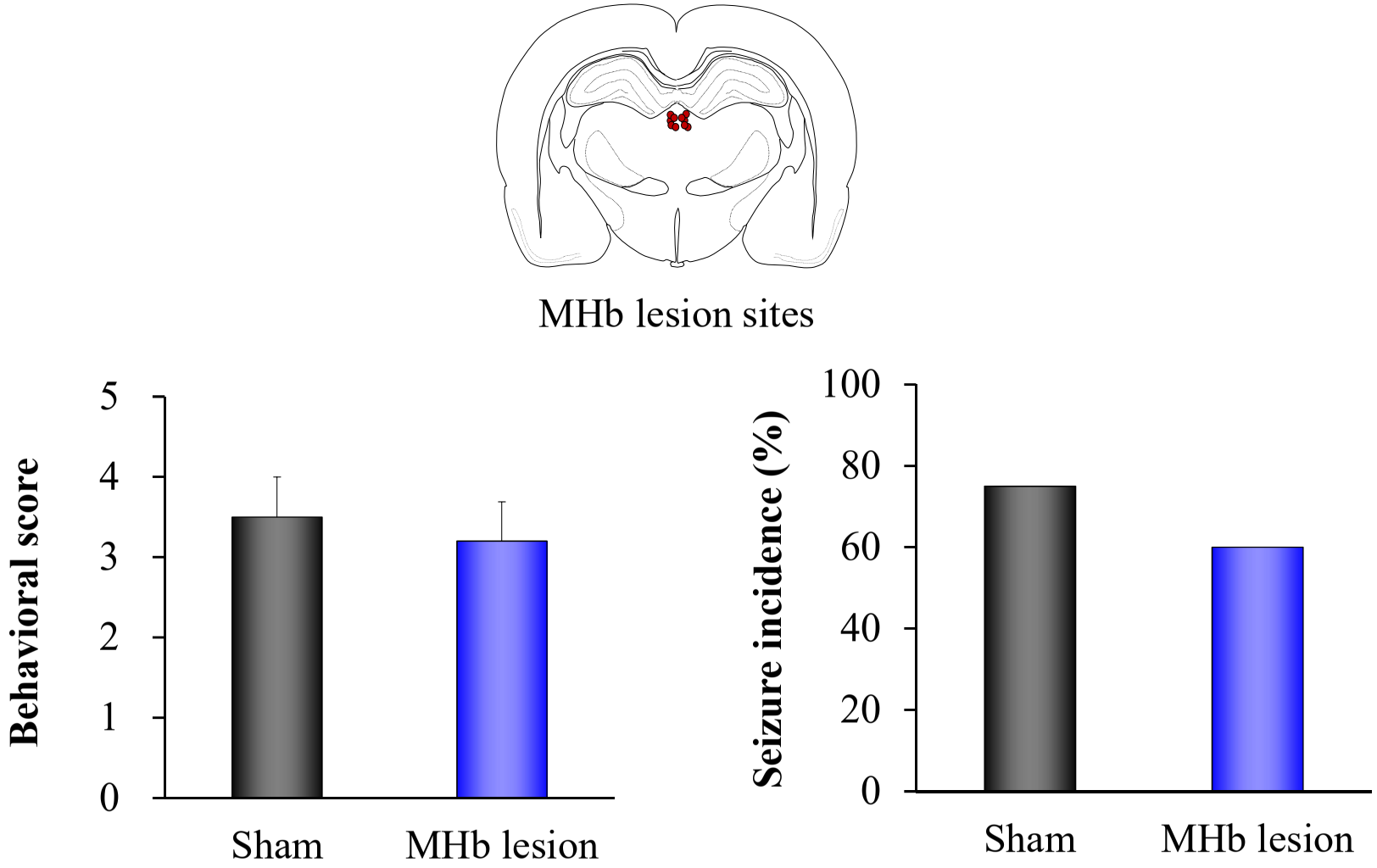

Figure 16: Effects of electrical lesionings of the medial habenula (MHb) on nicotine-induced seizures in rats. Panels show the effects of nicotine (4 mg/kg, i.p.) on seizure generation after electrical lesionings at the MHb**.** Upper panel illustrate the electrical lesion sites in MHb. Behavioral scores (left graph) are expressed as the mean of ± S.E.M. of four or five animals. Seizure incidence (right graph) represents the percentage of animals which showed convulsive seizures (scores 4 or 5) against total animals examined.

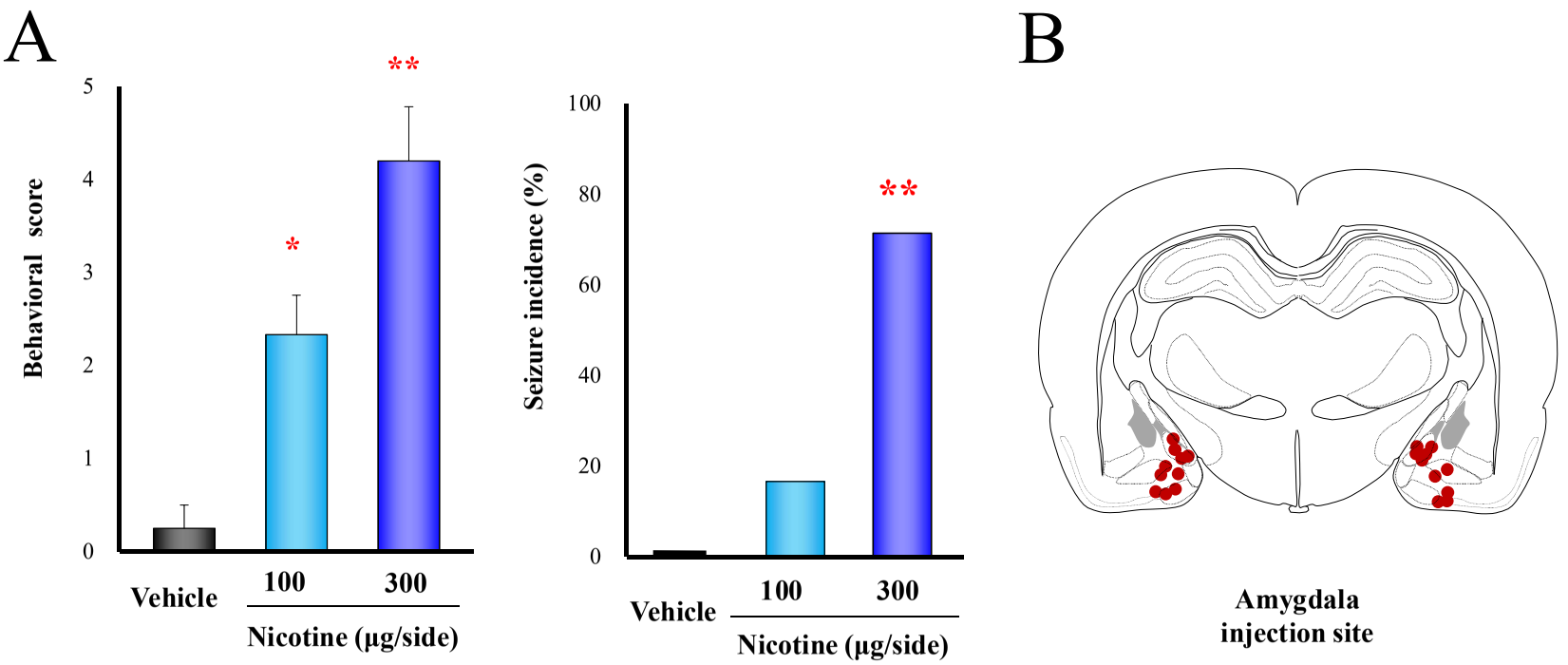

Figure 17: Effects of nicotine microinjected into the amygdala (AMG) on convulsive seizure induction in rats. Nicotine (100 or 300 μg/side) was locally injected into the bilateral AMG. **(A)** Behavioral scores (left graph) are expressed as the mean ± S.E.M. of 4–6 animals. Seizure incidence (right graph) represents the percentage of animals, which showed convulsive seizures (scores 4 or 5), against total animals examined. $^{*}P < 0.05$ and $^{**}P < 0.01$; Significant different from the control group injected with vehicle alone (Vehicle). **(B)** The injected sites of nicotine in the AMG.

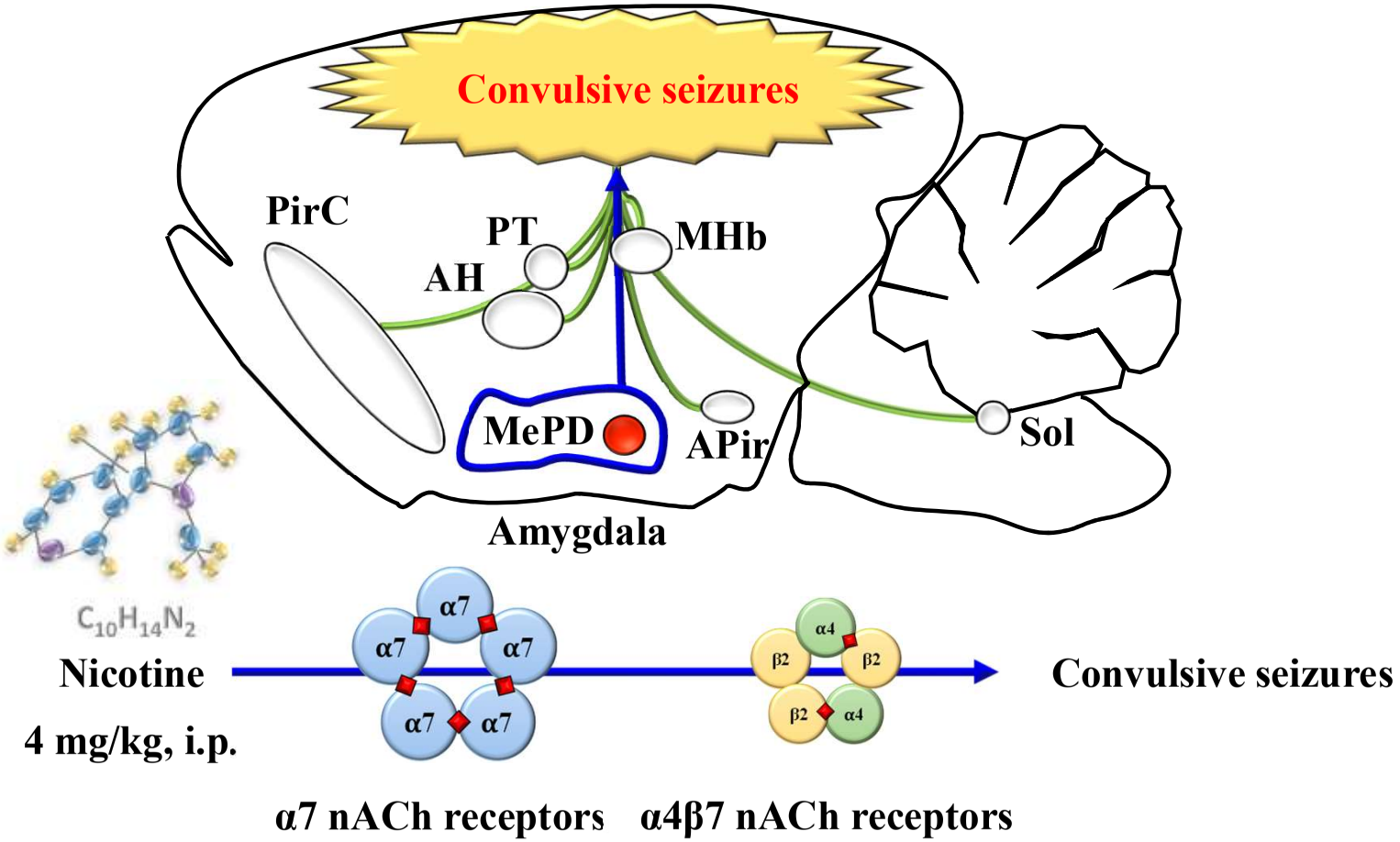

Figure 18: Schematic illustration of the mechanism of convulsive seizures induction by nicotine. According to our findings, nicotine at high doses (3-4 mg/kg, i.p.) elicited convulsive seizures primarily by activating neuronal α7 nACh receptors, with a nominal participation of α4β2 nACh receptors. Nicotine at convulsive doses caused the activation of neurons in the PirC, AH, PT, MePD, MHb, Apir and Sol (green lines). Finally, by using electrical lesion and the microinjection studies suggest not PirC, PT or MHb, but the amygdala are a causative site (blue arrow) of nicotine-induced seizures.

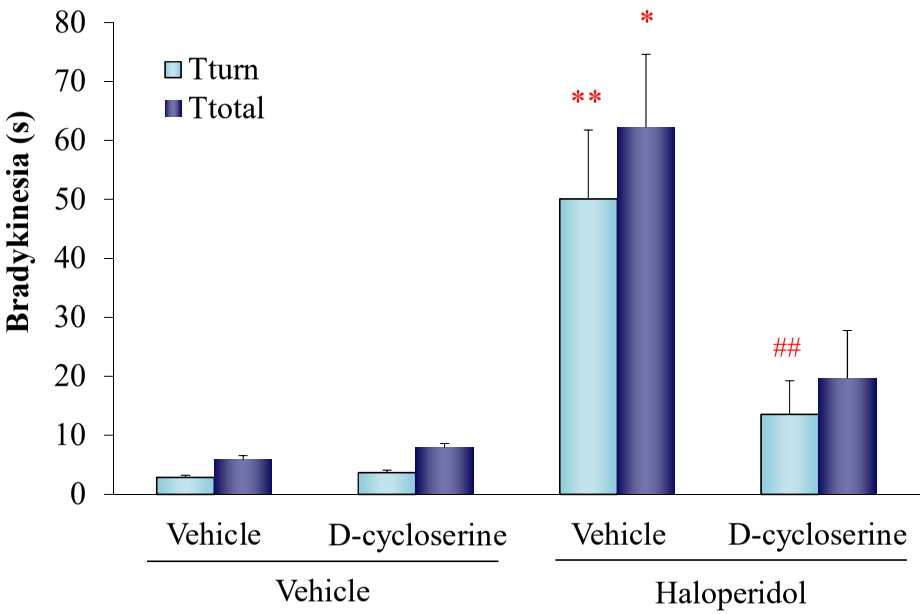

Figure 19: Effects of D-cycloserine (DCS) on haloperidol (HAL)-induced bradykinesia. DCS (30 mg/kg, i.p.) or vehicle was administered to animals 15 min before the HAL injection, which was followed by the pole test 30 min later. Each column represents the mean ± S.E.M. of 6–7 mice. These data were analyzed using the Kruskal−Wallis and Steel−Dwass tests. * $p < 0.05$, ** $p < 0.01$, significantly different from the value for Vehicle + Vehicle. ## $p < 0.01$, significantly different from the value for Vehicle + HAL.

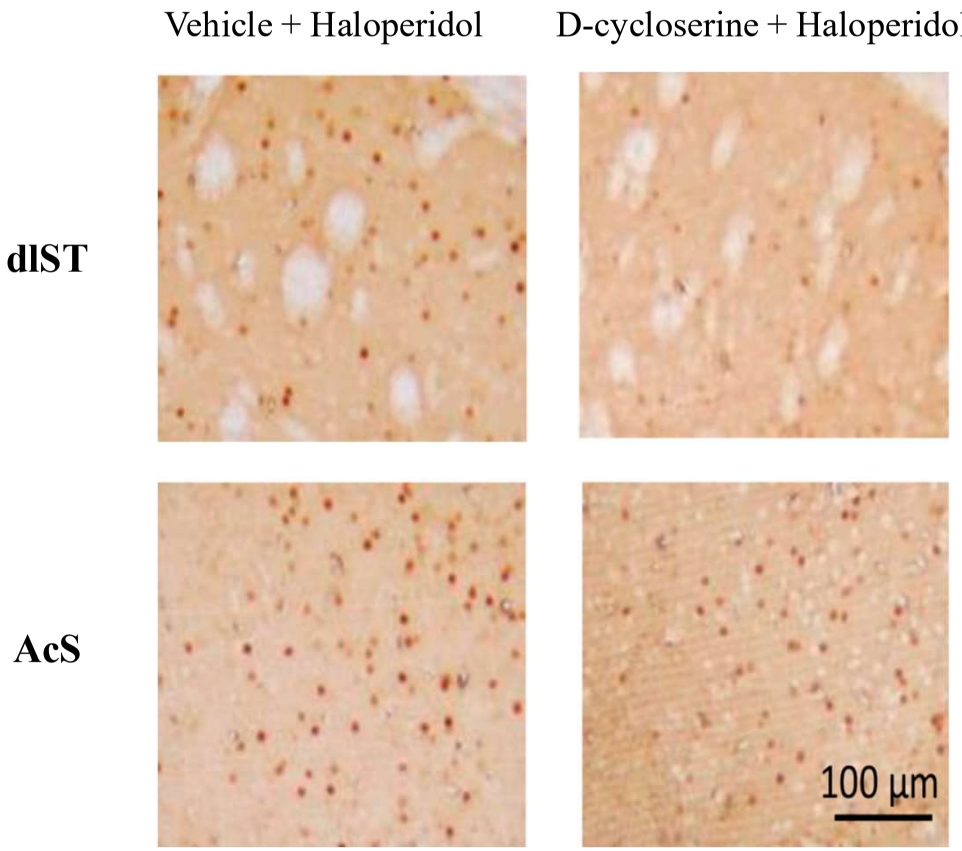

Figure 20: Immunohistochemical analysis of Fos protein immunoreactivity in the dorsolateral striatum (dlST) and accumbens shell (AcS). Representative photographs illustrating Fos protein expression in the dlST-positive cells in the dlST and AcS (left panel: Vehicle + HAL (1 mg/kg, i.p.)-treated mice, right panel: DCS (30 mg/kg, i.p.) + HAL (1 mg/kg, i.p.)-treated mice). Scale bar: 100 µm

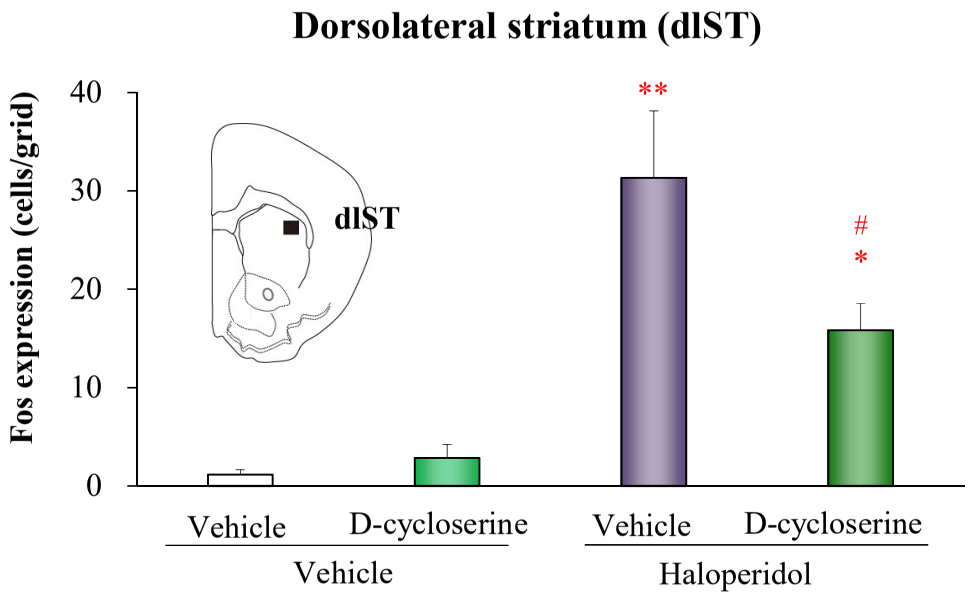

Figure 21: Effects of D-cycloserine (DCS) on haloperidol (HAL)-induced Fos expression in the dorsolateral striatum (dlST). DCS (30 mg/kg, i.p.) or vehicle was administered to animals 15 min before the HAL injection, brain was removed from animals 2 h after the HAL injection. Each column represents the mean ± S.E.M. of 6–7 mice. These data were analyzed using one-way ANOVA and Tukey's test. * $p < 0.05$, ** $p < 0.01$, significantly different from the value for Vehicle + Vehicle. # $p < 0.05$, significantly different from the value for Vehicle + HAL.

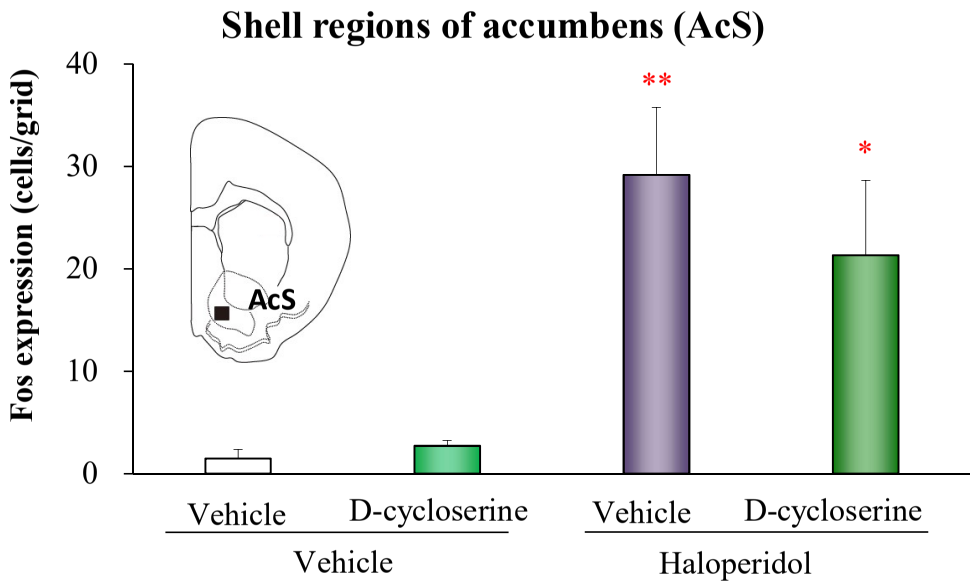

Figure 22: Effects of D-cycloserine (DCS) on haloperidol (HAL)-induced Fos expression in the accumbens shell (AcS). DCS (30 mg/kg, i.p.) or vehicle was administered to animals 15 min before the HAL injection, brain was removed from animals 2 h after the HAL injection. Each column represents the mean ± S.E.M. of 6–7 mice. These data were analyzed using one-way ANOVA and Tukey's test.
* $p < 0.05$, ** $p < 0.01$, significantly different from the value for Vehicle + Vehicle.