Analgesic action of nicotine on tibial nerve transection (TNT)-induced mechanical allodynia through enhancement of the glycinergic inhibitory system in spinal cord

Md. Joynal Abdin[1], Norimitsu Morioka[1], Katsuya Morita[1], Tomoya Kitayama[1], Shigeo Kitayama[2], Toshikatsu Nakashima[3] and Toshihiro Dohi[1]

[1)] Department of Dental Pharmacology, Division of Integrated Medical Science, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

[2)] Department of Dental Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikada, Okayama, 700-8525, Japan

[3)] Department of Physical Therapy, Kio University, 4-2-2 Umaminaka, Koryo-cho, Kitakatsuragi-gun, Nara, 635-0832, Japan

Corresponding author : Toshihiro Dohi,
Address: 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
Tel: +81-82-257-5640, Fax: +81-82-257-5644,
e-mail: todohi@hiroshima-u.ac.jp

Abstract

The activation of cholinergic pathways by nicotine elicits various physiological and pharmacological effects in mammals. For example, the stimulation of nicotinic acetylcholine receptors (nAChRs) leads to an antinociceptive effect. However, it remains to be elucidated which subtypes of nAChR are involved in the antinociceptive effect of nicotine on nerve injury-induced allodynia and the underlying cascades of the nAChR-mediated antiallodynic effect. In this study, we attempted to characterize the actions of nicotine at the spinal level against mechanical allodynia in an animal model of neuropathic pain, tibial nerve transection (TNT) in rats. It was found that the intrathecal injection of nicotine, RJR-2403, a selective $\alpha4\beta2$ nAChR agonist, and choline, a selective $\alpha7$ nAChR agonist, produced an antinociceptive effect on the TNT-induced allodynia. The actions of nicotine were almost completely suppressed by pretreatment with mecamylamine, a non-selective nicotinic antagonist, or dihydro-β-erythroidine, a selective $\alpha4\beta2$ nAChR antagonist, and partially reversed by pretreatment with methyllycaconitine, a selective $\alpha7$ nAChR antagonist. Furthermore, pretreatment with strychnine, a glycine receptor antagonist, blocked the antinociception induced by nicotine, RJR-2403, and choline. On the other hand, the $GABA_A$

antagonist bicuculline did not reverse the antiallodynic effect of nicotine. Together, these results indicate that the α4β2 and α7 nAChR system, by enhancing the activities of glycinergic neurons at the spinal level, exerts a suppressive effect on the nociceptive transduction in neuropathic pain.

Keywords; nicotine, nicotinic ACh receptor, mechanical allodynia, tibial nerve transection, glycinergic system

Introduction

Damage to peripheral nerves triggered by surgery, an infection, or diabetes has been suggested to induce a tactile allodynia, which is a state of pain produced by innocuous stimuli. This painful sensation is often persistent, and very difficult to ameliorate because it is refractory to conventional treatments such as applications of anti-inflammatory drugs or opioids. Therefore, it is important to establish a therapeutic treatment for neuropathic pain. Nicotine has been shown to have various physiological and pharmacological effects such as the modulation of neurotransmission, neuroprotection, or differentiation through the activation of nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated ion channels composed of combinations of α (α2-α10) and β (β2-β4) subunits (Girod et al., 2000; Ryan et al., 2001; Arredondo et al., 2002). It has been pointed out that nasal applications of nicotine are effective at inhibiting pain following surgery (Flood and Daniel, 2004). In fact, it has been indicated that nicotine exerts antinociceptive effects in animal models of pain by interacting with one or more of the subtypes of nAChR which are present in the central or peripheral nerve system. For example, it has been suggested that the stimulation of peripheral nAChRs led to an antinociceptive effect on

formalin-induced pain (Gilbert et al., 2001). Furthermore, the intracerebroventricular administration of nicotine or nicotinic agonists produced a potent antinociceptive effect in models of acute pain (Rao et al., 1996; Wang et al., 2005). The intrathecal administration of nicotine has been indicated to have an analgesic effect on nerve injury-evoked thermal and mechanical hyperalgesia via the stimulation of $\alpha 4\beta 2$ nAChRs (Rashid and Ueda, 2002). Although several studies have suggested antinociceptive actions of nicotine or nAChR agonists, it remains unclear whether stimulation of nAChRs at the spinal level has an antinociceptive effect on nerve injury-induced allodynia, and which subtypes of the receptor might be involved in the analgesic action of nicotine. Therefore, in the present study, we attempted to characterize the effects of nicotine or nicotinic agonists at the spinal level on mechanical allodynia in a model of neuropathic pain.

Several animal models of chronic neuropathic pain in which sciatic or spinal nerves are partially ligated have been established for research into pain therapeutics. It has been suggested that the differences in the time-course of the induction and maintenance of allodynia after surgery and the degree of pain triggered by thermal or mechanical stimuli might be in part dependent on the

intensity or position of the ligation. For simplicity, here we selected a model in which the tibial branch of the sciatic nerve is transected; the tibial nerve transection (TNT) model (Lee et al., 2000; Hofmann et al., 2003).

In this study, it was found that the intrathecal administration of nicotine had an antinociceptive effect on TNT-induced allodynia, which might involve stimulation of the $\alpha 4\beta 2$ and $\alpha 7$ subtypes of nAChR at the spinal level. We further revealed that the glycinergic inhibitory system, but not the GABAergic inhibitory system, might contribute to the antinociceptive effect of nicotine on nerve injury-induced allodynia.

Methods

Surgery to transect the tibial nerve and drug administration

Male Wistar rats (200-300 g) were used in all experiments. The rats were housed at 22±2℃. All procedures and handling of the animals were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the guidelines of Hiroshima University, Hiroshima, Japan. The TNT was performed as described previously (Lee et al., 2000; Hofmann et al., 2003). Under anesthesia with sodium pentobarbital (50 mg/kg i.p.), the skin of the lateral surface of the right thigh was incised and the sciatic nerve and its three branches (the sural, common peroneal, and tibial nerves) were exposed. Then, the tibial nerve was transected just distal to the trifurcation of the sciatic nerve and furthermore, a 2-mm section of the transected nerve was cut to prevent rejoining. The other two nerves remained intact. The wound was closed by suturing first the muscle and then the skin. A sham-operation was also performed in which the tibial nerve was exposed but not transected. For intrathecal injections, rats were implanted with catheters according to the methods described by Yaksh et al. (1980). A polyethylene tube (PE-10) was inserted into the subarachnoid space through a

small incision, and the tip of the catheter was implanted close to the L4-L5 spinal segment and externalized through the skin. The volume of drug injected in all cases was 10 μl and a further 10 μl of saline was used to flush the catheter.

Measurement of mechanical allodynia

All behavioral tests to assess mechanical withdrawal threshold were conducted using a Dynamic planter aesthesiometer (Ugo Basile, Italy). Rats were placed inside acrylic cages on a wire mesh grid floor and allowed to acclimatize for a period of 30 min. The probe for the mechanical stimulus was then attached to the middle of the plantar surface of the right or left hindpaw. The pressure applied was automatically increased until the paw was withdrawn. Therefore, maximal accepted force was determined. The cut-off pressure was set at 30 g.

Drugs

Nicotine was purchased from Nacalai Tesque (Kyoto, Japan). Mecamylamine hydrochloride, dihydro-β-erythroidine hydrobromide (DHβE), choline chloride, strychnine hydrochloride, atropine sulfate and bicuculline methiodide were obtained from Sigma Chemical Co. (St. Louis, MO). Methyllycaconitine citrate

(MLA) and (*E*)-*N*-methyl-4-(3-pyridinyl)-3-buten-1-amine (RJR 2403) fumarate were from Tocris Cookson (Bristol, UK). Nicotine, mecamylamine, DHβE, choline, bicuculline, atropine and MLA were dissolved in physiological saline. Strychnine and RJR 2403 were first dissolved in distilled H_2O, and then diluted with saline.

Statistical analysis

Data were expressed as the mean±S.E. Differences between groups were determined using a one-way analysis of variance with a pairwise comparison by the Bonferroni method. The criterion of significance was a P value less than 0.05.

Results

Intrathecal nicotine suppressed the TNT-induced allodynia in rats.

In an initial series of experiments, we found that the rats significantly developed mechanical allodynia on the ipsilateral side about 3 weeks after TNT, and this lasted for about 5 weeks (Fig. 1a). There tended to be less allodynia on the contralateral side, but these responses were not significant. Sham-operated rats showed no apparent response to mechanical stimuli at any of the time points of measurement.

In the mechanical withdrawal threshold test, the effects of nicotine and several drugs on the mechanical allodynia were examined on days 20-35 post-surgery (Fig. 1b). Although nicotine had no significant effect on the TNT-induced allodynia at 0.1 nmol, it produced significant antinociception from 10 to 40 minutes post-injection at 1 nmol. This antinociceptive effect gradually declined, with a return to the pre-injection level at 60 min. Similarly, 10 nmol of nicotine also elicited a significant antinociceptive effect, but it disappeared earlier than did the effect at 1 nmol. Therefore, we selected 1 nmol as the dose of nicotine for subsequent experiments.

Effects of several nAChR antagonists on the antiallodynic effect of nicotine.

To characterize which subtypes of nAChR might be involved in the antinociceptive effect of nicotine in the TNT model, we investigated the actions of various nAChR antagonists. In this study, all antagonists were administered intrathecally 10 minutes before the injection of nicotine. Saline was injected as the vehicle of each antagonist. Pretreatment with mecamylamine (1 nmol), a non-selective and non-competitive nicotinic antagonist, completely inhibited the antinociceptive effect of nicotine on TNT-induced mechanical allodynia (Fig. 2a). Similarly, DHβE, a selective α4β2 nAChR antagonist (10 nmol), also blocked the antiallodynic effect (Fig. 2b). Furthermore, MLA, a selective α7 nAChR antagonist (10 nmol), had a less, but significant inhibitory effect on the antinociception induced by nicotine (Fig. 2c). We confirmed that the injection of these antagonists alone had no effect on the mechanical allodynia triggered by TNT in the rats (data not shown).

Effects of RJR-2403, a selective α4β2 agonist, or choline, a selective α7 agonist, on the TNT-induced allodynia.

We next examined the effect of nAChR agonists on the TNT-induced mechanical allodynia. As shown in Fig. 3a, the intrathecal injection of 10 or 100 nmol of RJR-2403, which is relatively selective of $\alpha 4\beta 2$ nAChRs (Papke et al., 2000), significantly reversed the mechanical allodynia. It was found that 10 or 100 nmol of choline, which is known to act as a selective $\alpha 7$ agonist (Damaj et al., 2000; Wang et al., 2005), also had an antiallodynic effect on the TNT-induced allodynia (Fig. 3b). Furthermore, we observed that pretreatment with MLA (Fig. 4a), but not atropine (Fig. 4b) or DHβE (data not shown), significantly inhibited the antinociception induced by choline.

Involvement of the glycinergic inhibitory system in the nicotine-induced antiallodynic action in the TNT model.

To elucidate the underlying systems of the nAChR-mediated antiallodynic effect in the rats, we first examined the interaction of the glycinergic system in the nicotine-induced antinociception using a glycine receptor antagonist, strychnine (Fig. 5a). In both the sham-operated rats and the TNT model, the intrathecal injection of 10 nmol of strychnine alone had no significant influence on behavior compared with before the injection. Pretreatment with 10 nmol of strychnine

completely blocked the nicotine-induced antiallodynic action in the transected rats. Furthermore, strychnine also significantly suppressed the antiallodynic effect of RJR-2403 or choline (Fig. 5b). Next, we investigated the involvement of the GABAergic system in the antinociceptive effect of nicotine. However, intrathecal pretreatment with 10 nmol of bicuculline, which is a selective antagonist for the $GABA_A$ receptor, did not inhibit the nicotine-induced antinociception (Fig. 6).

Discussion

We observed antinociceptive effects of nicotine or nicotinic agonists on mechanical allodynia using the TNT pain model. It has been recently demonstrated that the TNT model is a novel and surgically uncomplicated animal model for the study of neuropathic pain. Hofmann et al. (2003) have reported that this model shares several behavioral, molecular and pharmacological features with ligation models. For example, TNT resulted in an upregulation of the production of galanin and vasointestinal peptide, and downregulation of that of substance P and calcitonin gene-related peptide in ipsilateral L5 dorsal root ganglia. These alterations are the same as those observed in other models of neuropathic pain (Nahin et al., 1994; Honore et al., 2000). In this study, we found that on the side ipsilateral to the TNT, mechanical withdrawal threshold was markedly decreased compared with that in sham-operated rats. This sensation was induced about 3 weeks after the transection, and maintained for about 5 weeks. This observation is consistent with the finding that the mechanical allodynia in the TNT model was more robust and long-lasting than that in other models of peripheral nerve injury (Dowdall et al., 2005).

It has been demonstrated that nAChR agonists have an analgesic effect on persistent formalin-induced pain, inflammation-induced mechanical and thermal hyperalgesia, or acute thermal pain such as hot plate and tail-flick pain (Lawand et al., 1999; Damaj et al., 1999, 2000; Wang et al., 2005). In models of neuropathic pain, the intrathecal administration of nicotine has been indicated to have analgesic effects on both mechanical and thermal hyperalgesia via the stimulation of $\alpha 4\beta 2$ nAChRs (Marubio et al., 1999; Rashid and Ueda, 2002). Furthermore, Bannon et al. (1998) have shown that the systemic administration of ABT-594, a potent $\alpha 4\beta 2$ nAChR agonist, produced an analgesic effect in nerve injury-induced allodynia. However, it is not clear whether the intrathecal application of nicotine has antinociceptive effects on not only acute, inflammatory pain or hyperalgesia but also nerve injury-induced mechanical allodynia. As it is possible that the modulating systems of nociceptive transduction differ between the types of pain, it is important to investigate the effect of nicotine at the spinal level on mechanical allodynia in TNT models. In this study, we revealed that the intrathecal injection of nicotine significantly reversed TNT-induced mechanical allodynia with a maximal effect at 1 nmol. Nicotine had no greater, and in fact a shorter, antinociceptive effect at 10 nmol

than 1 nmol. It has been suggested that nicotine enhanced both inhibitory and excitatory transmission in the spinal dorsal horn (Genzen and McGehee, 2003; Cordero-Erausquin et al., 2004). Therefore, the excitatory action triggered by a high dose of nicotine might reduce the inhibitory effect of nicotine in the spinal cord. Another possibility for the shorter effect of nicotine at 10 nmol could be due to the desensitization to the nicotine effect, because it is characteristic of nicotine to develop the stimulatory and the subsequent inhibitory action, especially at high doses.

Together, our results strongly support the involvement of both $\alpha4\beta2$ and $\alpha7$ nAChRs at the spinal level in the antinociceptive effects of nicotine on TNT-induced mechanical allodynia. It has been demonstrated that the $\alpha4\beta2$ nAChR in the central nervous system is the most important receptor mediating nociceptive transduction (Marubio, et al., 1999; Rashid and Ueda, 2002). The finding in the present study that the intrathecal delivery of RJR-2403, a selective agonist for the $\alpha4\beta2$ nAChR, produced an antiallodynic effect similar to nicotine, and DHβE, a selective antagonist for the $\alpha4\beta2$ nAChR, suppressed the nicotine-induced antiallodynic effect, also supports an important role for the $\alpha4\beta2$ nAChR-mediated regulation of allodynia in nerve injury. DHβE also has

antagonistic effects on the other types of nAChR, especially those including β2 subunit. However, DHβE acts about 100-times more sensitive to α4β2 than α7 nAChR (Chavez-Noriega et al., 1997). On the other hand, the α7 nAChR has been indicated to be distributed extensively in the central nervous system and at peripheral sites and contribute to several functions such as the regulation of neuronal growth and differentiation or neuroprotection. However, the relationship between the α7 nAChR and nociception is largely unclear. Only recently, the administration of choline, a selective agonist for the α7 nAChR, was shown to elicit an antinociceptive effect in an acute pain model (Damaj et al., 2000) and in a formalin-induced inflammatory model (Wang et al., 2005). On the other hand, some studies have suggested that the selective α7 antagonist MLA was unable to block nicotinic agonist-induced analgesia (Khan et al., 1998; Rashid and Ueda, 2002). The present study demonstrated that the selective agonist for α7 nAChRs choline and antagonist for α7 nAChRs MLA, produced an antiallodynic effect and antagonized the antinociceptive effect of nicotine, respectively, although these effects were somewhat less potent than those of the agonist and antagonist for the α4β2 nAChR. Although choline is a full agonist of α7 nAChR, this drug may interact with most heteromeric nAChRs as a partial agonist or

indirectly through ACh synthesized from choline. However, this possibility could be excluded because the antiallodynic effect of choline was reversed by pretreatment with MLA, but not DHβE or atropine. Taken together, it is suggested from the results of the specific drugs for nAChRs that the involvement of both α4β2 and α7 nAChRs at the spinal level in the antinociceptive effects of nicotine on TNT-induced mechanical allodynia. Shytle et al. (2004) recently demonstrated that pretreatment with nicotine suppressed the endotoxin-induced release of tumor necrosis factor α (TNF-α) in glial cells via the activation of α7 nAChRs. Moreover, the expression of TNF-α increases in DRG after nerve injury (Schäfers et al., 2002, 2003). Since the upregulation of TNF-α production in both DRG and spinal cord is known to enhance the sensitivity of nociceptive perception, inhibition of the production or release of this cytokine through the stimulation of α7 nAChRs might lead to a decrease in the facilitation of pain. The possibility that these phenomena contribute to the antiallodynic action of nicotine via the activation of α7 nAChRs is of particular interest and should be investigated further.

Next, we attempted to identify the underlying cascades of the nAChR-mediated antiallodynic effect in the TNT model. The transduction cascades for the

antinociceptive action elicited by the activation of nAChRs at the spinal level should be the stimulation of an inhibitory system such as the glycinergic or GABAergic pathway. In fact, it has been demonstrated that the activation of nAChRs facilitated the release of glycine in rat spinal cord through the stimulation of $\alpha 4\beta 2$ or $\alpha 7$ nAChRs (Kiyosawa et al., 2001; Bradaia and Trouslard, 2002). Furthermore, Huang and Simpson (2000) have indicated that the continuous intrathecal administration of glycine suppressed neuropathic pain evoked by a loose unilateral ligation of the sciatic nerve in rats. In this study, pretreatment with strychnine, which is a glycine receptor antagonist, remarkably blocked the antinociception induced by not only nicotine but also nicotinic agonists for the $\alpha 4\beta 2$ or $\alpha 7$ nAChR in the TNT model. Therefore, we speculate that the endogenous glycine produced following the activation of $\alpha 4\beta 2$ or $\alpha 7$ nAChRs attenuates the hypersensitivity of nociceptive perception in the TNT model, although we could not determine the precise sites where nicotine or nicotinic agonists act in the spinal cord. It has been reported that the intrathecal administration of strychnine induced tactile allodynia in rats (Khandwala and Loomis, 1998; Milne et al., 2001; Loomis et al., 2001). In those studies, however, the dose of strychnine required to produce the allodynia was higher (about

10-fold) than that used in our study. Moreover, we confirmed that neither sham-treated nor transected rats treated with strychnine alone developed mechanical allodynia or motor dysfunction. Some studies showed that strychnine acts as a competitive antagonist of α7 nAChR (Seguela et al., 1993). Although the possibility can not be entirely ruled out that the antagonistic effect of strychnine on nicotinic agonists-induced antiallodynic effect might be due to the association of strychnine with α7 nAChR, we speculate that this possibility does not seem to hold true in this study for the following reasons. The affinity of strychnine to α7 nAChR is much lower than that to glycine receptors, for examples, Ki for strychnine as an inhibitor of the α7 nAChR and glycine receptors were found to be approximately 680 nM and 32 nM, respectively (Anand et al., 1993; Saitoh et al., 1994). Moreover, the IC_{50} for strychnine to block α4β2 nAChR-stimulated currents in hippocampal neurons was about 118 μM (Matsubayashi et al., 1998). Thus, the affinities of strychnine for these receptors are considerably different. In our preliminary experiment, an injection of 30 nmol of strychnine produced the convulsions. Considering about 20-fold low affinity of strychnine to α7 nAChR than glycine receptor, more than convulsive dose of strychnine would be required to block α7 nAChR. Whereas,

low dose of strychnine less than convusive dose almost completely blocked the effect of 100 nmol of choline. Together, the antagonistic action of strychnine in this study might be mainly due to the blockade of glycine receptors. On the other hand, we found that pretreatment with an intrathecal injection of bicuculline, a $GABA_A$ receptor antagonist, had no inhibitory effect on nicotine-induced antinociception in our model. In previous studies, nicotinic agonists produced analgesic effects on the hyperalgesia evoked by nerve injury though the activation of a spinal GABA-mediated mechanism (Rashid and Ueda, 2002). A disruption of GABAergic inhibitory tone in nociceptive transduction has been shown to lead to neuropathic states in several models (Drew et al., 2004; Coull et al., 2005). One possible explanation for the discrepancy between our results and previous reports may be the different models of nerve damage employed. The other possibility is that GABA released originally from GABAergic neurons might not be elicited by the stimulation of nAChRs on those neurons in the TNT model because of the damage to the spinal GABAergic system caused by TNT. Moore et al. (2002) have demonstrated a decrease in the level of the GABA-synthesizing enzyme glutamic acid decarboxylase and the selective loss of $GABA_A$-mediated inhibitory currents in the dorsal horn of rats following both

tibial and peroneal nerve injury. They suggested that the excitotoxicity due to an excessive release of glutamate elicited by nerve injury contributes to the death of GABAergic neurons. Therefore, enhancement of the glycinergic control of nociception through an unknown nAChR-stimulated pathway may indeed be evaluated using this model.

In conclusion, our results demonstrated that the stimulation of nAChRs, especially the $\alpha 4\beta 2$ or $\alpha 7$ subtype, elicited an antinociceptive effect on nerve injury-induced mechanical allodynia by enhancing the glycinergic system at the spinal level. Considering the non-responsive nature of various conventional drugs for neuropathic pain, the antiallodynic actions of nicotinic agonists are of real interest. Recently, studies have suggested that prostaglandins, ATP, TNF-α, lysophosphatidic acid, and platelet-activating factor contribute to the initiation of neuropathic pain, and furthermore, specific antagonists for each receptor or inhibitors of the biosynthesis of these molecules would be of potential use in the treatment of chronic pain (Zhao et al., 2000; Schäfers et al., 2003; Tsuda et al., 2003; Inoue et al., 2004; Fukuhara et al., 2004; Morita et al., 2004). However, once neuropathic pain has developed, such agents are unlikely to be of use in therapeutic treatment. Compared with the mechanisms of initiation or

development, the mechanisms for the maintenance of neuropathic pain are not very well understood. Therefore, our finding that the stimulation of nAChRs might help to ameliorate the mechanical allodynia evoked by nerve damage should be of particular interest.

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Legends

Fig. 1 Tibial nerve transection (TNT)-induced mechanical allodynia in rats was suppressed by pretreatment with an intrathecal injection of nicotine. a) The time-course of the development of mechanical allodynia triggered after TNT in rats. Each point represents the paw withdrawal threshold (g) in response to mechanical stimuli determined automatically by a Dynamic planter aesthsiometer (n=10-12). The following experiments were performed on days 20-35 post-surgery when neuropathic pain responses were constantly achieved. $^{**}p<0.01$ compared with the values of sham-ipsilateral for the corresponding post-operated days. b) Effects of nicotine on the mechanical allodynia. After the intrathecal administration of nicotine (0.1, 1 or 10 nmol) or saline, paw withdrawal threshold in response to mechanical stimuli was measured at different time points after the injection. Pre indicates the values before the injection of saline or nicotine. Each point represents the mean±S.E.M of 7-10 rats. $^{*}p<0.05$, $^{**}p<0.01$ compared with the values for the groups of rats injected with saline.

Fig. 2 Influence of nicotinic antagonists on the antinociceptive effect of

nicotine in the TNT model. a) Effects of mecamylamine, a non-selective nicotinic antagonist, on the nicotine-induced antinociception. After pretreatment with an intrathecal injection of mecamylamine (1 nmol; ■) or saline (▲) for 10 min, 1 nmol of nicotine was injected intrathecally. The rats injected with saline instead of the test drugs were used as a control (●). b) Effects of dihydro-β-erythroidine (DHβE), a selective α4β2 nAChR antagonist, on the nicotine-induced antinociception. After pretreatment with DHβE (10 nmol; ■) or saline (▲) for 10 min, 1 nmol of nicotine was injected intrathecally. The rats injected with saline instead of the test drugs served as a control (●). c) Effects of methyllycaconitine (MLA), a selective α7 nAChR antagonist, on the nicotine-induced antinociception. After pretreatment with MLA (10 nmol; ■) or saline (▲) for 10 min, 1 nmol of nicotine was injected intrathecally. The rats injected with saline instead of the test drugs were used as a control (●). The measurement of paw withdrawal threshold was performed at different time points after the injection of nicotine. Pre indicates values before the injection of drugs. Each point represents the mean ± S.E.M of 5-10 rats. $^{*}p<0.05$, $^{**}p<0.01$ compared with the values for the groups of rats injected with nicotine alone.

Fig. 3 Effects of nicotinic agonists on TNT-induced mechanical allodynia in rats. a), b) Effects of intrathecal RJR-2403, a selective $\alpha4\beta2$ nAChR agonist, or choline, a selective $\alpha7$ nAChR agonist, on the mechanical allodynia. After the intrathecal injection of RJR-2403 (1, 10 or 100 nmol; (a)), choline (10 or 100 nmol; (b)) or saline, paw withdrawal threshold in response to mechanical stimuli was measured at different time points. Pre indicates values before the injection of saline or agonists. Each point represents the mean±S.E.M of 4-8 rats. $^{*}p<0.05$, $^{**}p<0.01$ compared with the values for the groups of rats injected with saline.

Fig. 4 Influence of MLA, or atropine on the antinociceptive effect of choline in the TNT model. Effects of MLA (a), or atropine (b) on the choline-induced antinociception. After pretreatment with the intrathecal injection of MLA (10 nmol; (a)), or atropine (40 nmol; (b)) for 10 min, 100 nmol of choline was injected intrathecally. The rats injected with saline instead of the test drugs were used as a control. The measurement of paw withdrawal threshold was performed at different time points after the injection of choline. Pre indicates values before the injection of drugs. Each point represents the mean±S.E.M of 5-7 rats. $^{*}p<0.05$,

$^{**}p<0.01$ compared with the values for the groups of rats injected with choline alone.

Fig. 5 Involvement of the glycinergic system in nicotine or nicotinic agonist-induced antinociception in the TNT model. a) Effects of strychnine, a glycine receptor antagonist, on the nicotine-induced antinociception. After pretreatment with an intrathecal injection of strychnine (10 nmol; □) or saline (▲) for 10 min, 1 nmol of nicotine was injected intrathecally. The data for the rats injected with saline as a substitute for both strychnine and nicotine, and for nicotine alone, are indicated by ● and ▽, respectively. The data for sham-operated rats injected with strychnine alone are indicated by ◇. b) Effects of strychnine on the nicotinic agonist-induced antinociception. After pretreatment with strychnine or saline for 10 min, RJR-2403 or choline were injected intrathecally. The measurement of paw withdrawal threshold was performed at different time points after the injection of nicotinic agonists. Pre indicates values before the injection of drugs. Each point represents the mean±S.E.M of 5-7 rats. $^{*}p<0.05$, $^{**}p<0.01$ compared with the values for the groups of rats injected with nicotine (a) or RJR-2403 (b) alone. $^{+}p<0.05$, $^{++}p<0.01$ compared with the

values for the animals injected with choline (b) alone.

Fig. 6 Involvement of the GABAergic system in nicotine-induced antinociception in the TNT model. Effects of bicuculline, which is a $GABA_A$ receptor antagonist, on the nicotine-induced antinociception. After pretreatment with an intrathecal injection of bicuculline (10 nmol; □) or saline (▲) for 10 min, 1 nmol of nicotine was injected intrathecally. The data for the rats injected with saline as a substitute for both strychnine and nicotine are indicated by ●. The data for the sham-operated rats injected with bicuculline alone are indicated by ◇. The measurement of paw withdrawal threshold was performed at different time points after the injection of nicotine. Pre indicates values before the injection of drugs. Each point represents the mean±S.E.M of 5 rats.

Figure 1

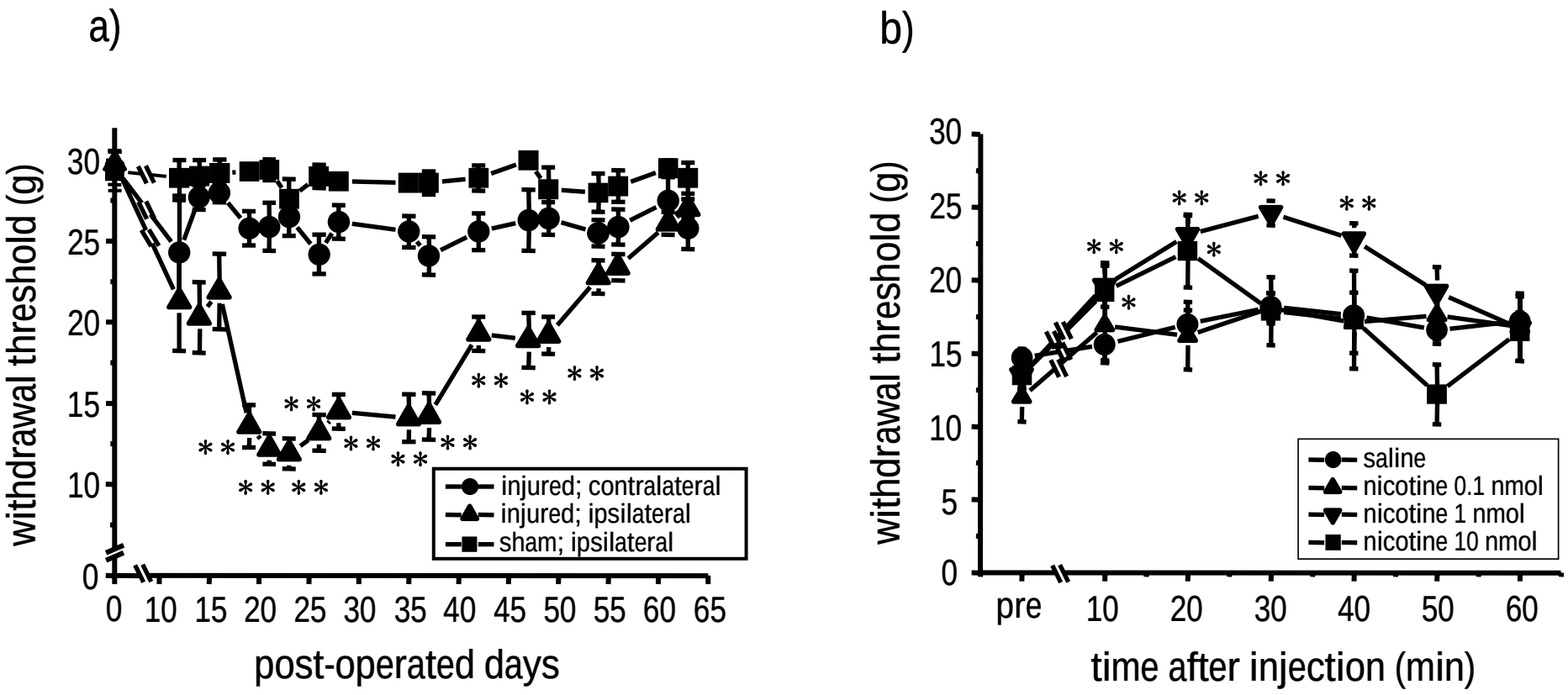

a)
withdrawal threshold (g)
30
25
20
15
10
0
0 10 15 20 25 30 35 40 45 50 55 60 65
post-operated days
injured; contralateral
injured; ipsilateral
sham; ipsilateral
b)
withdrawal threshold (g)
30
25
20
15
10
5
0
pre 10 20 30 40 50 60
time after injection (min)
saline
nicotine 0.1 nmol
nicotine 1 nmol
nicotine 10 nmol

Figure 2

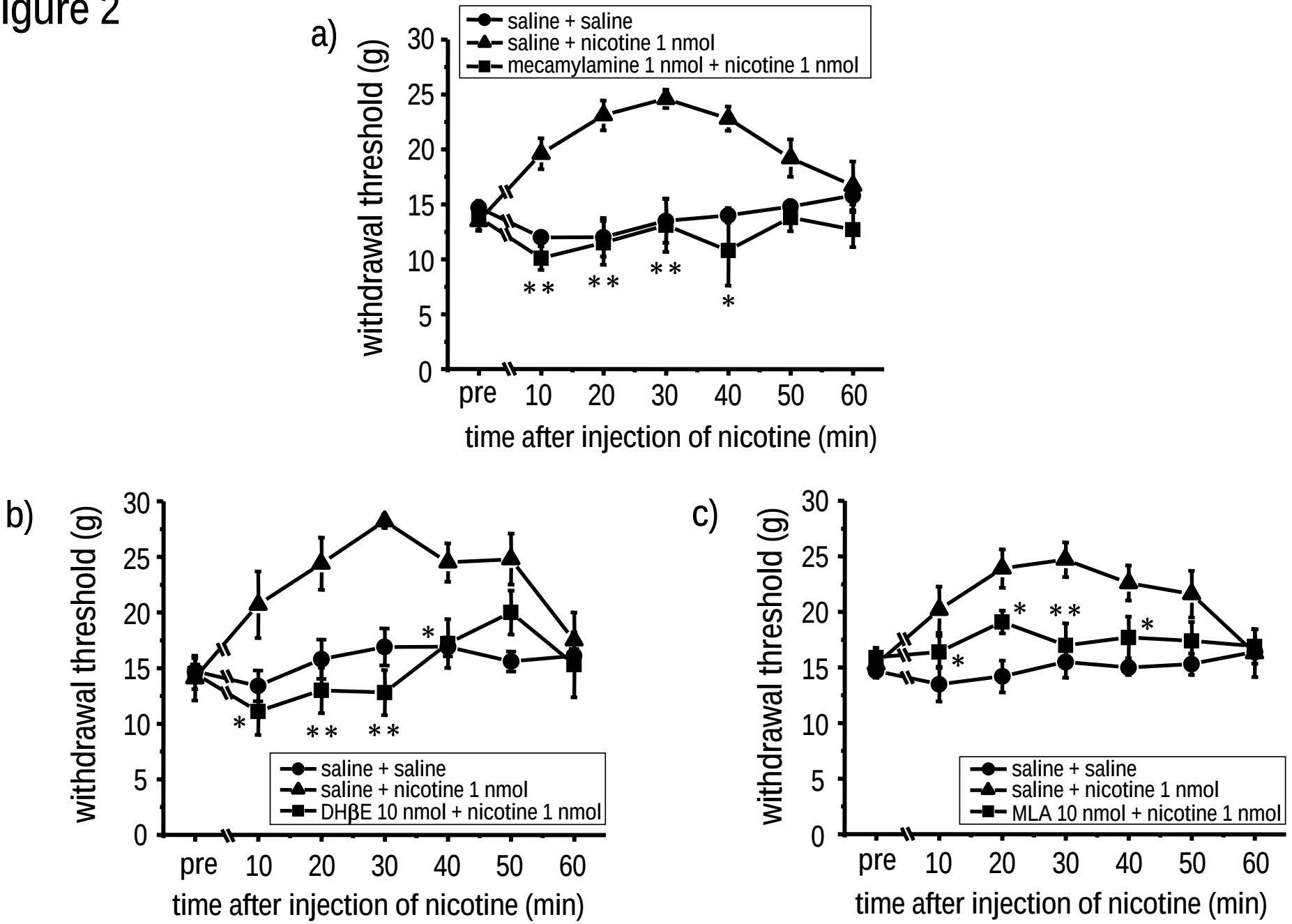

a)
saline + saline
saline + nicotine 1 nmol
mecamylamine 1 nmol + nicotine 1 nmol
withdrawal threshold (g)
0
5
10
15
20
25
30
pre
10
20
30
40
50
60
time after injection of nicotine (min)
b)
withdrawal threshold (g)
saline + saline
saline + nicotine 1 nmol
DHβE 10 nmol + nicotine 1 nmol
time after injection of nicotine (min)
c)
withdrawal threshold (g)
saline + saline
saline + nicotine 1 nmol
MLA 10 nmol + nicotine 1 nmol
time after injection of nicotine (min)

Figure 3

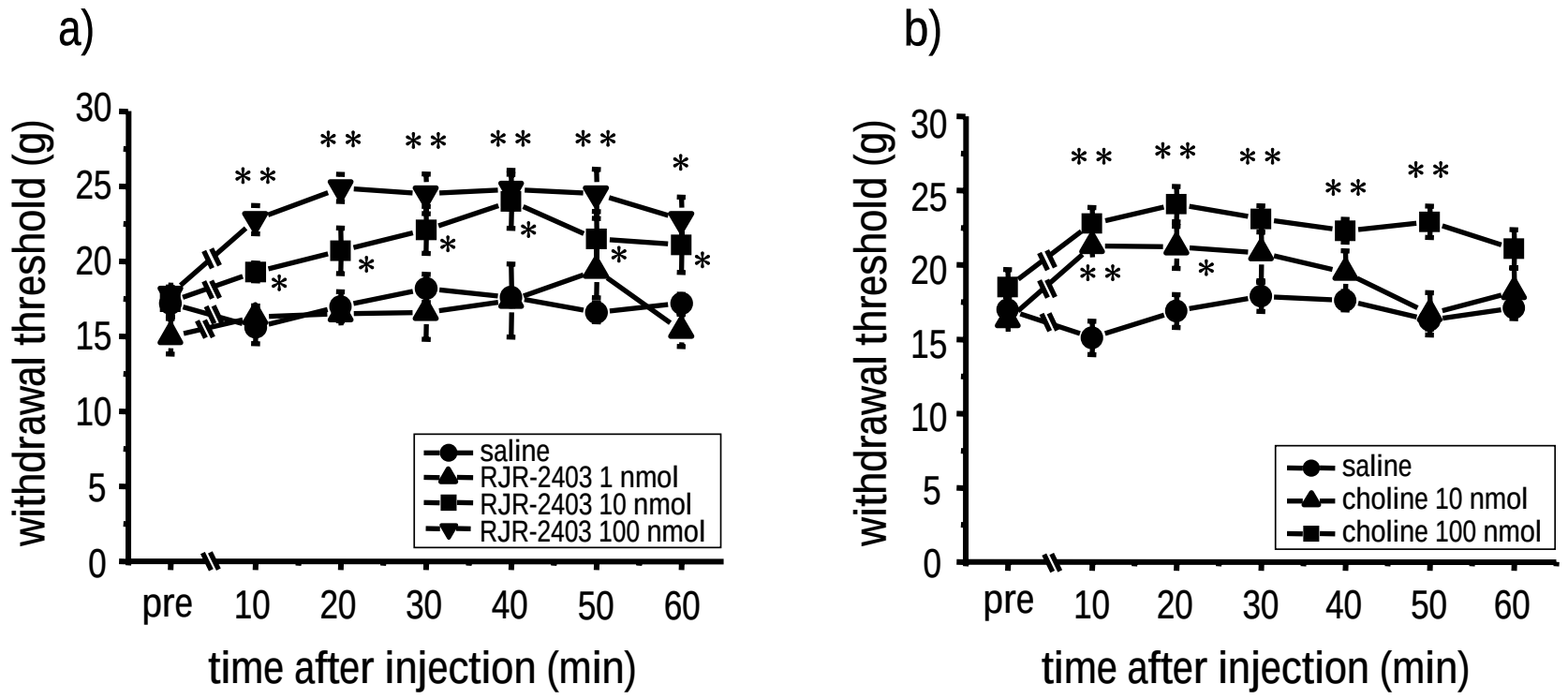

a)
withdrawal threshold (g)
30
25
20
15
10
5
0
pre
10
20
30
40
50
60
time after injection (min)
saline
RJR-2403 1 nmol
RJR-2403 10 nmol
RJR-2403 100 nmol
b)
withdrawal threshold (g)
time after injection (min)
saline
choline 10 nmol
choline 100 nmol

Figure 4

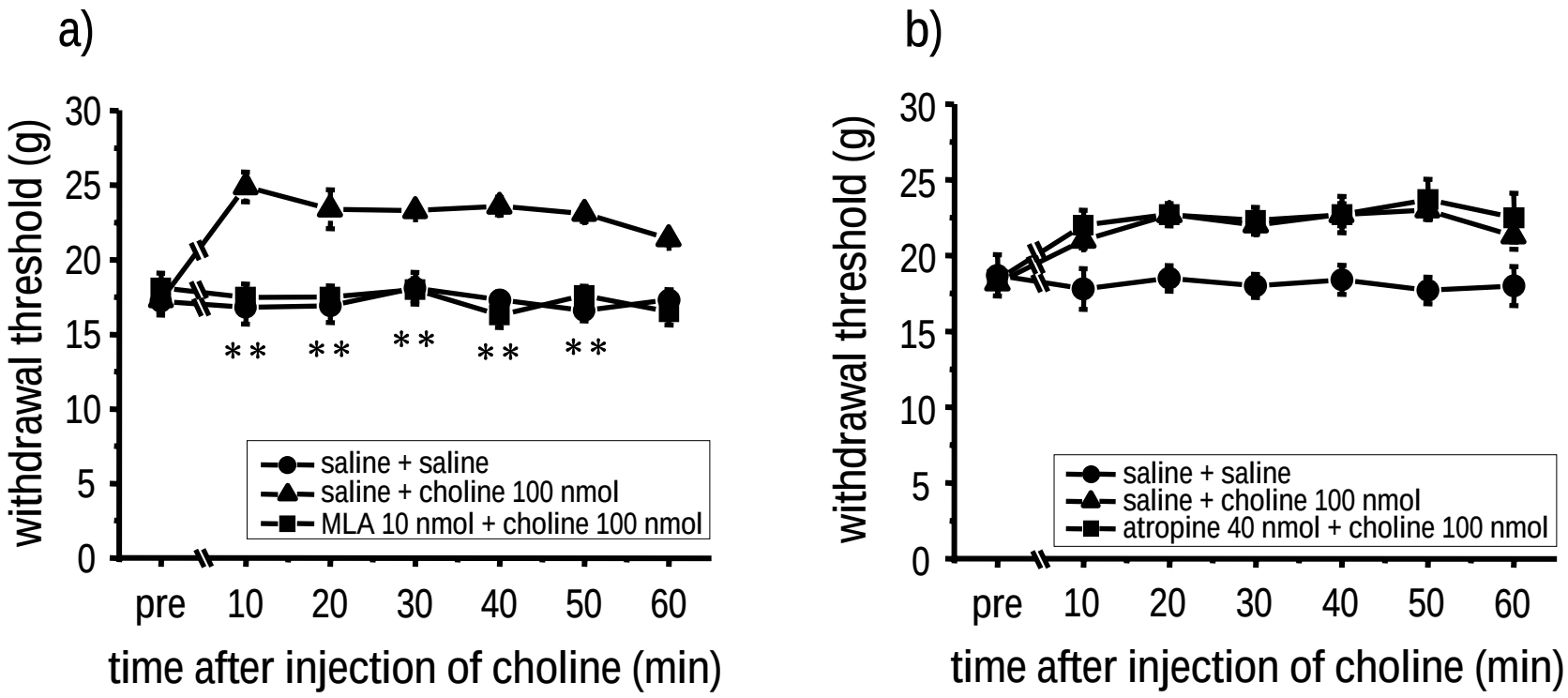

a)
withdrawal threshold (g)
30
25
20
15
10
5
0
pre
10
20
30
40
50
60
time after injection of choline (min)
saline + saline
saline + choline 100 nmol
MLA 10 nmol + choline 100 nmol
**
**
**
**
**
b)
withdrawal threshold (g)
time after injection of choline (min)
saline + saline
saline + choline 100 nmol
atropine 40 nmol + choline 100 nmol

Figure 5

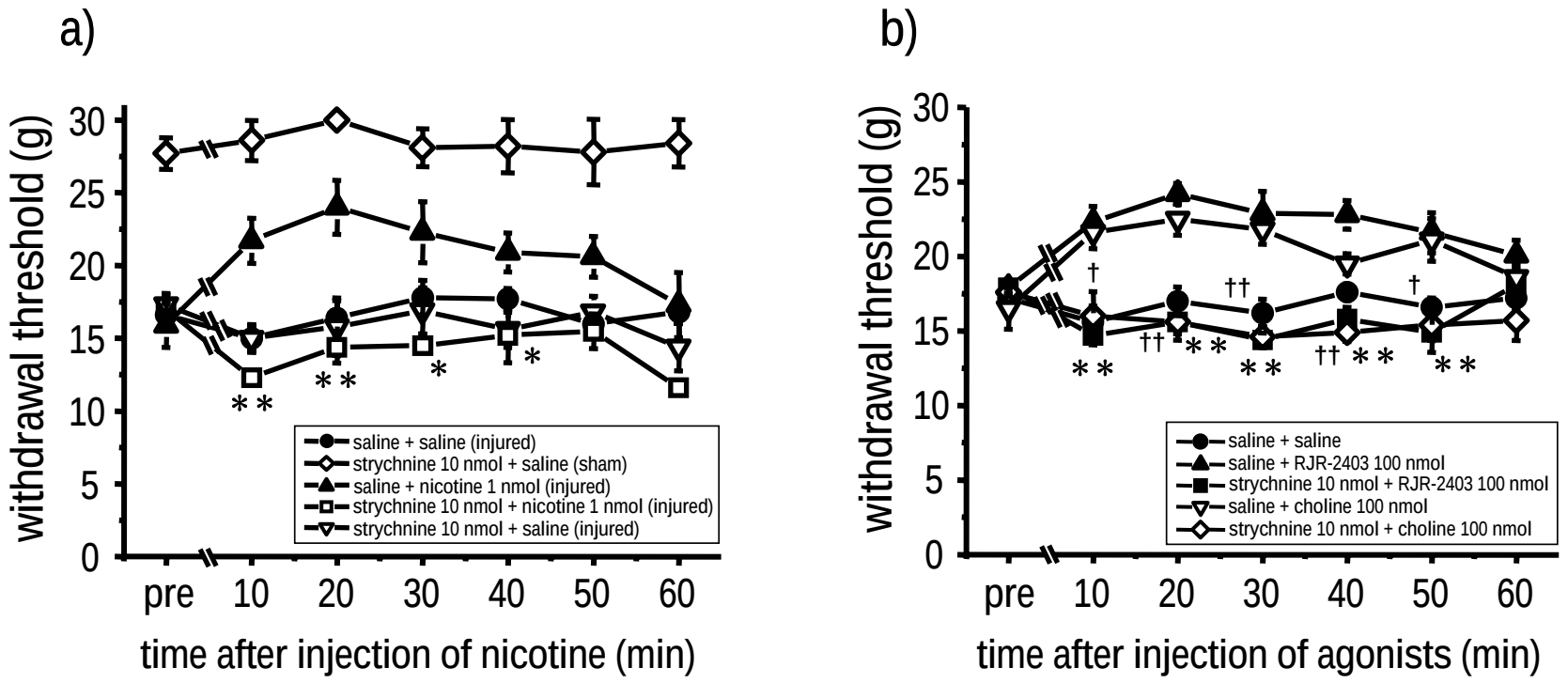
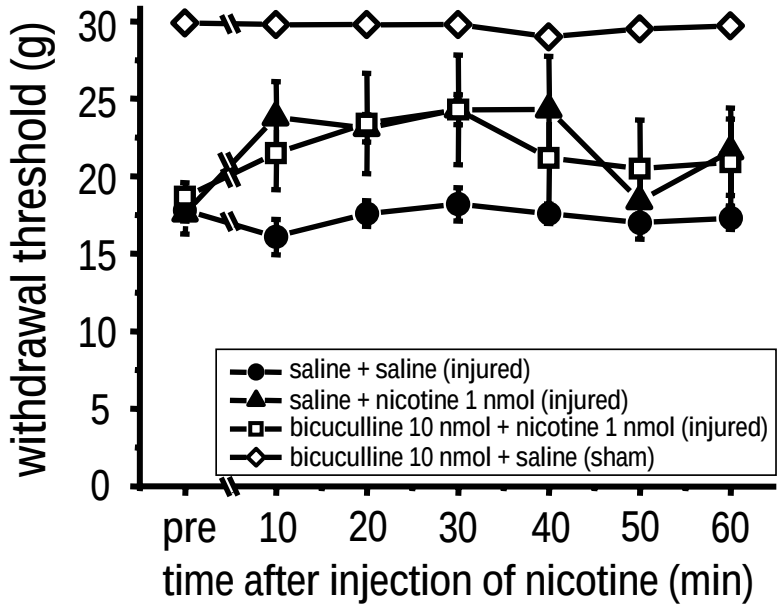

a)
withdrawal threshold (g)
30
25
20
15
10
5
0
pre
10
20
30
40
50
60
time after injection of nicotine (min)
saline + saline (injured)
strychnine 10 nmol + saline (sham)
saline + nicotine 1 nmol (injured)
strychnine 10 nmol + nicotine 1 nmol (injured)
strychnine 10 nmol + saline (injured)
b)
withdrawal threshold (g)
time after injection of agonists (min)
saline + saline
saline + RJR-2403 100 nmol
strychnine 10 nmol + RJR-2403 100 nmol
saline + choline 100 nmol
strychnine 10 nmol + choline 100 nmol

Figure 6


withdrawal threshold (g)
30
25
20
15
10
5
0
pre
10
20
30
40
50
60
time after injection of nicotine (min)
saline + saline (injured)
saline + nicotine 1 nmol (injured)
bicuculline 10 nmol + nicotine 1 nmol (injured)
bicuculline 10 nmol + saline (sham)