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An adenosine kinase inhibitor, ABT-702, inhibits spinal nociceptive transmission by adenosine release via equilibrative nucleoside transporters in rat

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1. Introduction

Purinergic systems are well known to regulate neuronal activities in the central nervous system (CNS), including the spinal cord, via adenosine and P_2 (ATP) receptors (Abbracchio et al., 2009). Adenosine receptors are classified into four subtypes, the A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al., 2001). The activation of A_1 receptors inhibits neuronal activities (Haas and Selbach, 2000), and also contributes to neuroprotection by suppressing excessive excitation (de Mendonça et al., 2000; Wardas, 2002).

Distinct purine turnovers take place inside and outside cells, and transmembrane transport of purines greatly affects the actions of adenosine. Intracellularly, adenosine is degraded to inosine by adenosine deaminase (ADA) and/or is converted to AMP by adenosine kinase (AK). Extracellularly, ATP released from the cell is degraded rapidly to adenosine by a series of ecto-enzymes (Matsuoka and Okubo, 2004; Robson et al., 2006). Extracellular adenosine is then incorporated into the cells via nucleoside transporters.

Equilibrative nucleoside transporters (ENTs) transport adenosine with bidirectional facilitated diffusion across the membrane (King et al., 2006). Among four

ENT isoforms, ENT1 and ENT2 reportedly play major roles in adenosine transport across the cell membrane. ENTs are inhibited by S-(4-nitrobenzyl)-6-thioinosine (NBTI) and some coronary vasodilators, such as dipyridamole (DIP), dilazep and draflazine. ENTs usually function as uptake transporters for adenosine, because intracellular adenosine level is maintained at a lower level than extracellular adenosine level by the activities of AK and ADA. These purine turnover cycles control the extracellular level of adenosine, and thus, they are expected to directly influence CNS functions via adenosine receptors.

In the spinal cord, adenosine and its analogs produce analgesia, which is mediated by inhibiting neuronal activities via A_1 receptors in the superficial layers of the dorsal horn (Salter et al., 1993: Sawynok, 1998; Sawynok and Liu, 2003). In deep layers of the ventral horn, adenosine likewise inhibits excitatory synaptic transmission, potentially facilitating neuroprotection and/or motor impairment (Miyazaki et al., 2008; Carlsen and Perrier, 2014). Moreover, AK inhibitors release adenosine from the spinal cord (Golembiowska et al., 1995; 1996), and intrathecal administration of these inhibitors yields analgesia (Poon and Sawynok, 1995; McGaraughty et al., 2001; Zhu et al., 2001), although some nucleoside AK inhibitors, such as 5-iodotuberdicin and 5’-amino-5’-deoxyadenosine, have therapeutic limitations because of its adverse effects,

poor oral bioavailability or a short half-life *in vivo* (Ugarkar et al., 2000; McGaraughty et al., 2005).

Systematic administration of ABT-702 (4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl) pyrido[2,3,-d] pyrimidine), a potent and selective non-nucleoside AK inhibitor, also generates analgesic effects in animal models of pain (Jarvis et al., 2000; Kowaluk et al., 2000; Suzuki et al., 2001), suggesting that ABT-702 suppresses nociceptive neuronal pathways in the CNS including the spinal cord. However, there is little direct demonstration showing the effects of AK inhibitors on spinal synaptic transmission. We previously reported that ABT-702 increases extracellular adenosine levels in the isolated spinal cord of neonatal rats (Takahashi et al., 2010). Accordingly, ABT-702 may affect neuronal activities of nociceptive pathways in the spinal cord. In addition, the effects of ABT-702 are possibly influenced by the activities of ADA and ENTs during purine turnover, which can cause adverse effects.

In this study, we evaluated two types of spinal reflex potentials recorded from the isolated neonatal rat spinal cord: monosynaptic reflex potentials (MSRs) and slow ventral root potentials (sVRPs). MSRs are mainly mediated by non-NMDA receptors at monosynaptic neuronal pathways from primary afferent fibers to motoneurons, while

sVRPs are mediated by NMDA and various metabolic receptors (e.g., neurokinin and metabolic glutamate receptors) at polysynaptic pathways from primary afferent fibers to intrinsic neurons. sVRPs are thought to reflect spinal nociceptive transmission, because they are preferentially inhibited by analgesics such as opiates and α_2 agonists (Akagi and Yanagisawa, 1987; Nussbaumer et al., 1989; Woodley and Kendig, 1991; Faber et al., 1997; Otsuguro et al., 2005). In terms of analgesics, on the other hand, MSR inhibition seems to be implicated in adverse effects such as motor impairment. By using this preparation, therefore, these therapeutic and adverse effects could be evaluated. The purpose of the current investigation was to examine the influence of ABT-702 on these nociceptive and motor reflex pathways, and adenosine release with and without inhibitors for ADA and/or ENTs in the rat spinal cord.

2. Materials and methods

2.1. Animals and spinal cord preparation

All animal care and experimental protocols were approval by the Institutional Animal Care and Use Committee (IACUC) at Hokkaido University. Every effort was made to minimize animal suffering and to reduce the number of animals used. Wistar rats (0–4

days old) of either gender were killed by decapitation, and the spinal cords were isolated and used for experimentation as described below.

2.2. Recording of spinal reflex potentials

The hemisected spinal cord preparation with lumber spinal nerve roots was fashioned as previously described (Otsuguro et al., 2006; 2011), placed in a bath, and superfused (3 ml/min) with artificial cerebrospinal fluid (ACSF) at 27 ± 2°C. The composition of the ACSF was as follows (mM): NaCl 138, KCl 3.5, $CaCl_2$ 1.25, $MgCl_2$ 1.2, $NaHCO_3$ 21, NaH_2PO_4 0.6, and glucose 10. The ACSF was saturated with 95% O_2 and 5% CO_2, and the pH was maintained at ~7.3.

For spinal reflex potential recording, stimulating and recording suction electrodes were placed on the dorsal and ipsilateral segmental ventral roots (L3–L5), respectively. The preparation was equilibrated for 1 h before experimentation. MSRs and sVRPs were evoked by electrical stimulation (40V, 200 μs) every 2 min and evaluated by measuring the peak amplitude (mV) and area under the curve above the resting level (mV·s), respectively (Kawamoto et al., 2012). The reflex potential magnitude was expressed in each case as a percentage of the mean of the first five responses. The effects of ABT-702 or other agents on the reflex potentials were

evaluated as the mean of three responses around their maximal responses. Electrical responses were detected using a high gain amplifier (MEZ-7200, Nihon Kohden, Tokyo, Japan) equipped with a low-pass filter at 10 kHz. MSRs were recorded using a thermal arraycorder (WR7800, Graftec, Yokohama, Japan) with a sampling time of 80 μs. sVRPs were digitized by using an analog/digital converter (PowerLab, ADInstruments, Castle Hill, Australia) with a sampling time of 10 ms. Data were stored in a personal computer and analyzed with LabChart 6 software (ver. 6.0, ADInstruments).

2.3. Measurement of adenosine release

The amount of adenosine released from the isolated spinal cord was measured by using HPLC according to previously reported methods (Takahashi et al., 2010), with some modifications. Briefly, the isolated spinal cord was cut into four pieces and equilibrated in ACSF for 1 h before experimentation. For measurement of the adenosine concentration, ACSF (1 ml) was changed every 10 min, and sample aliquots (250 μl) were collected. All experiments were conducted at 35°C.

Collected aliquots were immediately chilled on ice, and 0.1 M citrate-phosphate buffer, pH 4.0 (90 μl), 2 μM α,β-methylene ADP (internal standard, 25 μl), and 45% chloroacet aldehyde (10 μl) were added. The mixtures were incubated at

80°C for 40 min to generate ethenoderivatives, which were separated by using an analytical column (Accucore aQ, 150 × 4.6 mm, particle size = 2.6 μm, Thermo Fisher Scientific, Waltham, MA, USA) at 40°C. The ethenoderivatives of ATP, ADP, AMP, and adenosine were detected using an HPLC system equipped with a fluorescence detector (FP-2020, Nihon-Koden, Tokyo, Japan). The wavelengths for excitation and emission were set at 270 and 420 mm, respectively. The mobile phase buffer consisted of 150 mM KH_2PO_4, 5 mM tetra-*n*-butylammonium bromide, and 2.0% CH_3CN adjusted to pH 3.3 with H_3PO_4. The flow rate was 0.8 ml/min. The amount of adenosine released over 10 min was expressed relative to the tissue wet weight (pmol/mg).

2.4. Immunohistochemistry

Isolated spinal cords were immediately fixed with 4% paraformaldehyde/0.1 M phosphate buffer overnight at 4°C, and cut into 3-μm-thick paraffin sections. The deparaffinized sections were autoclaved with 20 mM Tris-HCl buffer (pH 9.0) for 20 min at 105°C. They were then incubated in 3% hydrogen peroxide/methanol solution for 20 min. Sections blocked in normal goat serum were then incubated with a primary antibody rabbit anti-ADA antibody (1:500; Merck Millipore, Billerica, MA, USA) or rabbit anti-ADK antibody (1:500; Sigma-Aldrich, St. Louis, USA) at 4°C overnight. For

immunohistochemistry, the sections were incubated with goat anti-rabbit IgG conjugated with biotin (SABPO Kit, Nichirei Bioscience, Tokyo, Japan) and subsequently with streptavidin-peroxidase complex (SABPO Kit) each for 30 min at room temperature. The labeled sections were developed using 3,3′-diaminobenzidine-H_2O_2 solution and counterstained with hematoxylin.

For immunofluorescence, the deparaffinized sections were treated with 20 mM Tris-HCl buffer for 20 min at 105°C, treated with normal donkey serum, and incubated with mixture of either mouse anti-GFAP antibody (1:100; Immuno-Biological Laboratories, Fujioka, Japan) and rabbit anti-ADA antibody (1:500) or mouse anti-GFAP antibody (1:100) and rabbit anti-ADK antibody (1:500) at 4°C overnight. The sections were then incubated with mixture of Alexa Fluor 488-labeled donkey anti-mouse IgG, Alexa Fluor 546-labeled donkey anti-rabbit IgG secondary antibody (1:500; Life Technologies, Carlsbad, CA, USA), and Hoechst33342 (1:500; Dojindo, Kumamoto, Japan) for 30 min. The immunofluorescence signals were analyzed under a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

2.5. Data Analysis

Results were expressed as means ±SEM. The IC_{50} value was calculated with software

(Origin, ver 8.6J, OriginLab, Northampton, MA, USA). Statistical comparisons between two groups were performed by applying the paired- or unpaired Student's t-test. For multiple comparisons, ANOVA followed by Dunnett's test was used. In all cases, a *P* value of less than 0.05 was considered significant.

2.6. Drugs

ABT-702 dihydrochloride, *N*-(2-Methoxyphenyl)-*N'*-[2-(3-pyrindinyl)-4-quinazolinyl]-urea (VUF5574) and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) were purchased from Tocris (Bristol, UK). Adenosine, N^6-cyclohexyladenosine (CHA), 8-cyclopentyl-1,3-dipropylxanthine (8CPT), dipyridamole (DIP), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) hydrochloride, 5-iodotubericidin, α,β-methylene ADP sodium, S-(4-nitrobenzyl)-6-thioinosine (NBTI) and PSB 1115 potassium salt hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

3.1. ABT-702 inhibits spinal reflex potentials via adenosine A_1 receptors

Bath application of ABT-702 (3 μM) to the isolated neonatal rat spinal cord for 20 min gradually decreased sVRPs with a slight decline in MSRs, which were both rapidly recovered by 8CPT (3 μM), an adenosine A_1 receptor antagonist (Fig. 1A). On the other hand, both sVRP and MSR inhibitions by ABT-702 were not affected by potent and selective adenosine A_{2A}, A_{2B} and A_3 receptor antagonists, ZM241385 (0.1 μM), PSB1115 (0.1 μM) and VUF5574 (0.1 μM), respectively (Fig. 1C and D). Compared with ABT-702 alone, a mixture of ABT-702 (3 μM) and EHNA (5 μM), an adenosine deaminase inhibitor, more potently inhibited sVRPs (Fig. 1B and 2D), although EHNA even at 10 μM had little effect on the reflex potentials (MSRs: 96 ± 5% of control, n = 4; sVRPs: 92 ± 13% of control, n = 4; Fig. 2C). In addition, ABT-702/EHNA markedly inhibited MSRs, albeit with a delayed onset. 8CPT recovered both MSR (114 ± 7% of control, n = 4) and sVRP inhibition (125 ± 10% of control, n = 4) by ABT-702/EHNA.

ABT-702 (0.03–10 μM) inhibited sVRPs in a concentration-dependent manner with an IC_{50} value of 0.32 μM (Fig. 2A, B, and D). The agent caused a perceptible and maximal inhibition of sVRPs at 0.03 and 3 μM, respectively. On the other hand, ABT-702 caused only a slight inhibitory effect against MSRs, even at a high

concentration (10 μM). We also examined the effects of 5-iodotubercidin, a general nucleoside AK inhibitor, on reflex potentials (Fig. 3). 5-Iodotubercidin caused obvious 8CPT-sensitive inhibition of not only sVRPs (maximal inhibition ~ 70%, IC_{50} = 0.02 μM) but also MSRs (maximal inhibition ~ 40%, IC_{50} = 0.31 μM).

3.2. Nucleoside transporters contribute to ABT-702-evoked inhibition of reflex potentials

To examine the contribution of ENTs to the ABT-702-evoked inhibition of reflex potentials, a mixture of high concentrations of NBTI (5 μM) and DIP (10 μM) was applied to inhibit ENT1 and ENT2. As shown in Fig. 4A, NBTI/DIP gradually decreased sVRPs for 60 min (50 ± 8% of control, n = 5), but had little inhibitory impact on MSRs (92 ± 10% of control, n = 5). Both reflex potentials were recovered by 3 μM 8CPT (sVRPs: 84 ± 12% of control, n = 4; MSRs: 125 ± 8% of control, n = 4). In the presence of NBTI/DIP, ABT-702 (3 μM) failed to evoke additional inhibition of MSRs (NBTI/DIP: 116 ± 1% of control, n = 6; ABT-702: 115 ± 4% of control, n = 6) or sVRPs (NBTI/DIP: 68 ± 8% of control, n = 6; ABT-702: 61 ± 7% of control, n = 6; Fig. 4B). On the other hand, as shown in Fig. 4C, the inhibition of sVRPs by ABT-702 (71 ± 3% of control, n = 5) was attenuated by NBTI/DIP (88 ± 5% of control, n = 5, $P < 0.01$).

When ABT-702/EHNA was applied to simultaneously block AK and ADA, a marked inhibition of sVRPs occurred (39 ± 7% of control, n = 10; Fig. 4D). The further addition of NBTI/DIP partially reversed the sVRP inhibition (59 ± 10% of control, n = 10, $P < 0.05$). However, MSR inhibition by ABT-702/EHNA (80 ± 8% of control, n = 10) was not significantly recovered by NBTI/DIP (77 ± 10% of control, n = 10), while, in some cases (4 out of 10), a relatively small MSR inhibition was completely recovered.

As adenosine supposedly mediates the inhibitory actions of ABT-702, the effect of adenosine on reflex potentials was next examined. Bath application of adenosine, like ABT-702, also inhibited sVRPs more potently than MSRs, both of which recovered with the further application of 8CPT (Fig. 5A and B). Nevertheless, unlike ABT-702, adenosine rapidly inhibited the reflex potentials. Moreover, inhibitory effects of adenosine were enhanced by NBTI/DIP (Fig. 5C–E). Then we used a potent and stable A_1 receptor agonist, CHA, which is a poor substrate for ENTs (Marangos et al., 1982; Geiger et al., 1985). Although CHA caused a similar inhibition to adenosine, it was hardly affected by NBTI/DIP (CHA: 52 ± 6% of control for sVRPs, 97 ± 1% of control for MSRs, n = 5; NBTI/DIP: 51 ± 6 % of control for sVRPs, 107 ± 3 % of control for MSRs, n = 6; Fig. 6).

3.3. ABT-702 releases adenosine via nucleoside transporters

To evaluate the effect of ABT-702 on adenosine release from the spinal cord, the amount of adenosine effluxed into the ACSF was measured. Both ABT-702 (3 μM) and EHNA (10 μM) significantly elevated adenosine release, and ABT-702 tended to increase adenosine more rapidly than EHNA (Fig. 7A and B). The combination of ABT-702 and EHNA caused a substantial further release (Fig. 7C). NBTI (5 μM)/DIP (10 μM) also augmented adenosine release. In the presence of NBTI/DIP, the adenosine increase evoked by ABT-702 was almost completely abolished, while EHNA significantly enhanced adenosine release to the same extent as that observed in the absence of NBTI/DIP (Fig. 7D). In addition, ABT-702/EHNA caused a marked further increase in adenosine release. The increase in adenosine in response to ABT-702/EHNA was significantly reduced in the presence vs. the absence of NBTI/DIP. In all experiments, AMP levels were not significantly altered, and ADP and ATP levels were undetectable (data not shown).

3.4. AK and ADA express in spinal neurons and astrocytes

Immunoperoxidase staining revealed that both AK and ADA expressed in various types

of cells including dorsal and ventral horn neurons throughout the spinal sections (Fig. 8A and B). In neurons, AK expressed in nuclei more potently than cytosols, and there was no apparent difference in expression levels between dorsal and ventral neurons. The profound AK expression in nuclei was also reported in the brain (Studer et al., 2006). On the other hand, ADA uniformly expressed in nuclei and cytosols, and strong stains were found in large motoneurons in the ventral horn. To investigate astrocytic expression, we then performed double immunofluorescence staining. Similar to neurons, cells with astrocytic marker GFAP-positive processes strongly expressed AK, and the strongest stain was found in the nuclei. ADA also expressed in the astrocytes, while astrocytic ADA expression was found in not only the nuclei but also the cytosols and processes.

4. Discussion

Here, we demonstrated that ABT-702, an AK inhibitor, increased the extracellular levels of adenosine and inhibited sVRPs more potently than MSRs in the isolated neonatal rat spinal cord. The inhibition by ABT-702 was attenuated by the blockade of ENTs and A_1 receptors. Our findings indicate that ABT-702 releases adenosine through ENTs to

inhibit spinal nociceptive transmission via the activation of A_1 receptors (Fig. 9).

AK plays a key role in the regulation of the intracellular turnover of adenosine in the CNS (Lloyd and Fredholm, 1995; Wall et al., 2007; Etherington et al., 2009; Diógenes et al., 2014). In the present investigation, inhibition of AK activity by ABT-702 strongly inhibited sVRPs. It is likely that ABT-702 increases the intracellular content of adenosine, which is in turn released into the extracellular space. AK inhibitors reportedly release adenosine from the spinal cord (Golembiowska et al., 1995; 1996; Takahashi et al., 2010), and in agreement with these observations, the present study demonstrated an increase in extracellular adenosine levels by ABT-702. In this study, 5-iodotuberdicin showed more potent inhibitory effects on the reflex potentials than ABT-702. Although ABT-702 inhibits AK activity more potently than 5-iodotuberdicin in the cell-free conditions, the IC_{50} value of ABT-702 for AK is higher than that of 5-iodotuberdicin in intact cells (Jarvis et al., 2000). In tissue levels such as the isolated spinal cord, 5-iodotuberdicin may also show a more potent inhibitory effect on AK compared with ABT-702. In addition, 5-iodotubercidine reportedly inhibits Na^+-gradient dependent concentrative NTs with similar IC_{50} values for AK inhibition (Parkinson and Geiger, 1996). Therefore, 5-iodotubercidine may accumulate a large amount of adenosine in extracellular spaces, resulting in MSR inhibition.

Unlike AK inhibition, the inhibition of ADA activities by EHNA had little effect on spinal reflex potentials, although EHNA did increase extracellular adenosine levels. One explanation for this discrepancy is that the adenosine increase seen herein was not sufficient to inhibit reflex potentials. In this study, the time course of the adenosine increase by EHNA tended to be slower than that by AK. Alternatively, we measured adenosine released from the whole spinal cord preparation, and thus the elevation of adenosine levels by EHNA could occur not only at synaptic regions expressing A_1 receptors, but also in various parts of the spinal cord. Therefore, it is suspected that the elevation of adenosine levels by EHNA occurs at different regions in the spinal cord from that by AK. ADA on the cell surface (as ecto-ADA) was also reported in many types of cells (Franco et al., 1997; 1998). Although ecto-ADA is argued to play a minor role in adenosine metabolism compared with cytosolic ADA (Arch and Newsholme, 1978), EHNA may augment extracellular adenosine levels by blockade of ecto-ADA more rapidly than by blockade of intracellular ADA, resulting in the adenosine increase at different regions from that by ABT-702. In addition, AK was appeared to similarly express in both dorsal and ventral horn neurons, while the highest expression of ADA was found in motoneurons at the ventral horn, at where MSRs were mediated. MSRs were less sensitive to the activation of A_1 receptors than sVRPs, which

may also contribute to the different effect between EHNA and ABT-702. On the other hand, there was no apparent difference in astrocytic AK expression between dorsal and ventral horn. AK was scarcely expressed in astrocytic processes, which should surround synaptic regions, suggesting that the neuronal AK is more important for synaptic regulation than the astrocytic AK. In the mouse brain, it has been shown that AK expression shifts from neurons to astrocytes during postnatal development (Studer et al., 2006). The astrocytic AK may become more important in the spinal cord of mature animals. Further investigation is needed to determine its underlying mechanisms. On the other hand, EHNA substantially enhanced the ABT-702-mediated adenosine release and inhibition of reflex potentials. Within cells, ADA probably acts more efficiently under conditions in which intracellular adenosine levels are excessively increased, because the Km values for ADA are higher than those for AK in the rat brain (Phillips and Newsholme, 1979).

ENTs transport adenosine in a transmembrane adenosine gradient-dependent fashion (Baldwin et al., 2004; King et al., 2006). We showed that NBTI/DIP increased extracellular adenosine levels and inhibited sVRPs, suggesting that adenosine levels, at least in the vicinity of the cell membrane, are higher in the extracellular vs. the intracellular space. Thus, ENTs apparently function as uptake transporters under normal

conditions. To the contrary, NBTI/DIP decreased the adenosine increase in response to ABT-702. In the presence of ABT-702 and increasing intracellular adenosine levels due to AK inhibition, ENTs are thought to transport adenosine from the cell interior to the exterior through an inversed adenosine gradient across the membrane. Therefore, ABT-702 failed to inhibit sVRPs in the presence of NBTI/DIP. These results indicate that ENTs function as adenosine efflux pathways in response to ABT-702. On the other hand, NBTI/DIP did not significantly inhibit the adenosine increase by EHNA. This result also supports our speculation that the EHNA-evoked adenosine increase is mediated at least in part by ecto-ADA inhibition.

Like ABT-702, adenosine and its analogs inhibit spinal reflex potentials (Nakamura et al., 1997; Otsuguro et al., 2009). A_1 receptors seem to be responsible for these actions, because the rank order of the inhibitory potencies of these agonists is consistent with the rank order of their affinities for A_1 receptors. Furthermore, the inhibitory effects of adenosine were competitively antagonized by 8CPT, an A_1 receptor antagonist, as were those of CHA and ABT-702. However, unlike ABT-702, the inhibition by adenosine was enhanced by the ENT inhibitors. The effect of CHA, a stable A_1 receptor agonist, was not changed by them, indicating that the ENT inhibitors did not affect the downstream signaling of A_1 receptor activation. Extracellular

adenosine is controlled by a rapid uptake into cells (Arch and Newsholme, 1978). It is likely that exogenous adenosine is rapidly removed from local extracellular spaces near A_1 receptors by ENTs, and thus the ENT inhibitors instantly enhanced the adenosine effect. In clinical applications, ENT inhibitors might exert different effects in patients treated with AK inhibitors from A_1 receptor agonists. In addition, chronic activation of A_1 receptors reportedly influences the activities and/or expression levels of adenosine receptors and ENTs (Hettinger et al., 1998; Sheth et al., 2014; Hughes et al., 2015). Therefore, the long-term treatment of ABT-702 may affect the activities and expression of molecules in purinergic systems, and thus lead to unexpected effects.

sVRPs were more sensitive to ABT-702 than MSRs. This result agrees with previous reports that the activation of A_1 receptors more potently inhibits sVRPs than MSRs (Nakamura et al., 1997; Otsuguro et al., 2009). Importantly, sVRPs are thought to reflex C-fiber-evoked nociceptive transmission. Nociceptive signals mediate primary afferent C-fiber inputs to the superficial dorsal horn, where A_1 receptors are highly expressed, especially in intrinsic spinal neurons (Geiger et al., 1984; Choca et al., 1988). Furthermore, intrathecal application of adenosine analogs generates antinociceptive effects via the activation of A_1 receptors (Salter et al., 1993; Sawynok, 1998). MSR inhibition by high concentrations of adenosine and adenosine analogs may contribute to

motor impairment and other adverse events (Sosnowski et al., 1989; Karlsten et al., 1990). Even at high concentrations, ABT-702 showed little inhibition of MSRs. Although 5-iodotuberdicin inhibited sVRPs more potently than ABT-702, it caused a marked inhibition of MSRs, which may contribute to its adverse effects such as motor impairment (Davies et al., 1986). We suggest that a marked and rapid increase in intracellular adenosine levels by ABT-702 is normally prevented by ADA activity.

In conclusion, ABT-702 preferentially inhibited sVRPs compared with MSRs. The preferential inhibition of sVRPs is expected to lead to good analgesic effects of ABT-702, and thus, ABT-702 and other AK inhibitors are possible candidates for pain control. Because MSR inhibition by ABT-702 seems to occur in a time-dependent manner, long-term application of the drug may lead to adverse events. Furthermore, in the case of ADA dysfunction or deficiency, caution may be required for the use of ABT-702 and other AK inhibitors, given that these agents might release excessive amounts of adenosine.

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Conflicts of interest

There are no conflicts of interest, real or potential, associated with this work.

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Figure legends

Fig. 1. Effect of ABT-702 on spinal reflex potentials. (A) ABT-702 (3 μM) was applied to the spinal cord. 8CPT (3 μM) was then added in the presence of ABT-702. (B) ABT-702 (3 μM) was applied together with EHNA (5 μM). Each upper panel shows representative traces of MSRs and sVRPs evoked by electrical stimulation (arrowheads). (C, D) Summary of the effects of 8CPT (3 μM), ZM214385 (0.1 μM), PSB1115 (0.1 μM) and VUF5574 (0.1 μM) on MSRs (C) and sVRPs (D) in the presence of ABT-702 (3 μM). Data represent the means ± SEM (n = 4–6). $^{**}P < 0.01$ (paired Student's t-test).

Fig. 2. Concentration-response relationship of ABT-702 actions on spinal reflex potentials. (A, B) ABT-702 (0.3 μM, n = 4, A; 10 μM, n = 4, B) were applied to the spinal cord. 8CPT (3 μM) was then added in the presence of the drug. (C) EHNA (1–10 μM) was cumulatively applied to the spinal cord (n = 4). (D) Summary of the rate of MSR and sVRP inhibition by ABT-702 in the presence or absence of EHNA. Data represent means ± SEM (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 3 μM ABT-703 (unpaired Student's t-test).

Fig. 3. Concentration-response relationship of 5-iodotubercidin actions on spinal reflex potentials. (A, B) 5-Iodotubercidin (5-IOD, 0.03 μM, n = 4, A; 3 μM, n = 4, B) were applied to the spinal cord. 8CPT (3 μM) was then added in the presence of the drug. (C) Summary of the rate of MSR and sVRP inhibition by 5-iodotubercidin. Data represent means ± SEM (n = 4).

Fig. 4. Effect of ENT inhibitors on spinal reflex potentials. (A, B) A mixture of NBTI (5 μM) and DIP (10 μM) was applied to the spinal cord. 8CPT (3 μM, n = 4–5, A) or ABT-702 (3 μM, n = 6, B) was then added in the presence of NBTI/DIP. (C, D) ABT-702 alone (3 μM, n = 5, C) or together with EHNA (5 μM, n = 10, D) was applied to the spinal cord. NBTI (5 μM)/DIP (10 μM) was then added in the presence of ABT-702.

Fig. 5. Effect of adenosine on spinal reflex potentials. (A, B) Adenosine (Ado, 30 μM, n = 8, A; 100 μM, n = 6, B) was applied to the spinal cord. 8CPT (3 μM) was then added in the presence of adenosine. (C) NBTI (5 μM)/DIP (10 μM) was added to the spinal cord in the presence of adenosine (Ado, 30 μM, n = 8). The number for each

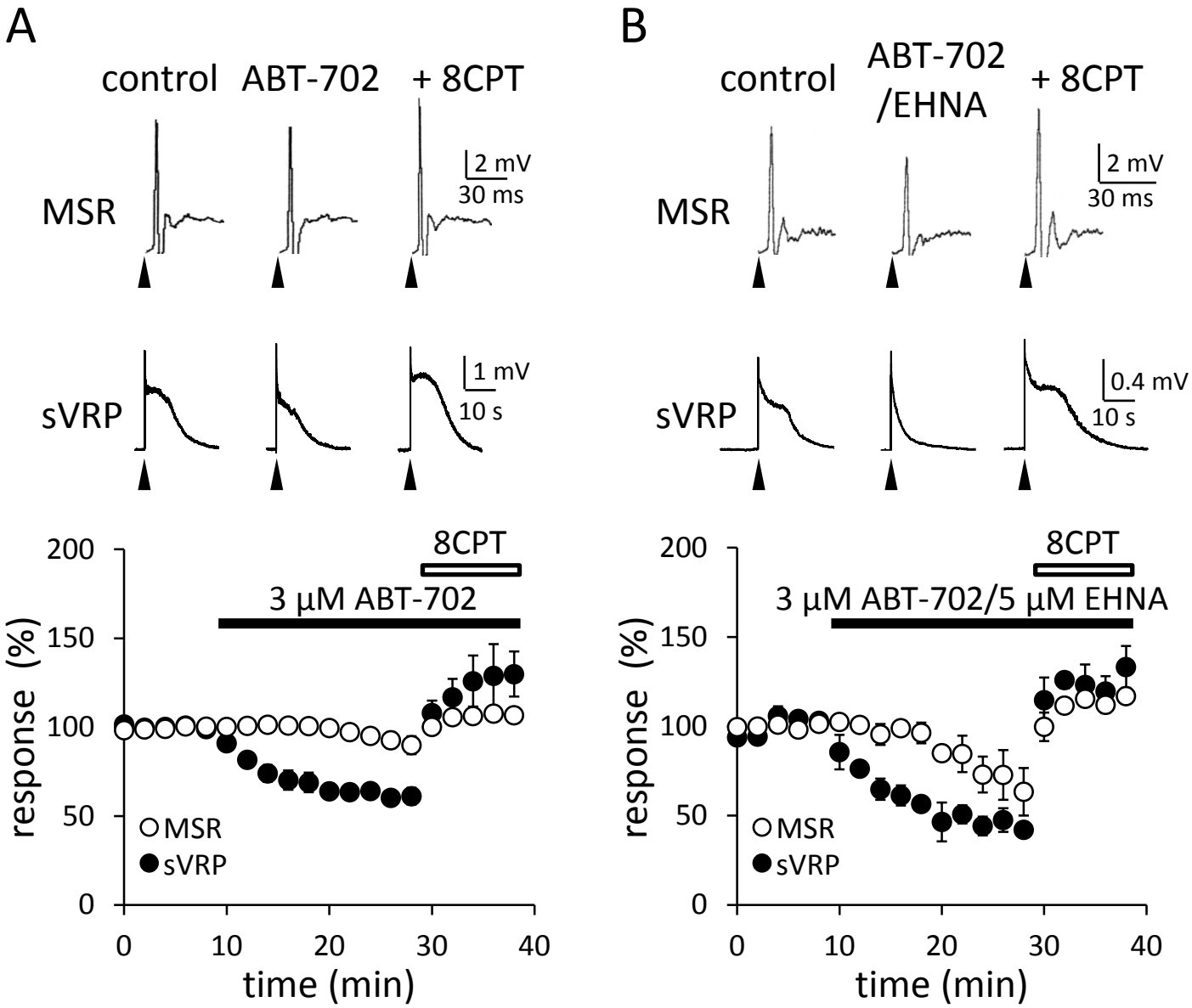
representative reflex potential trace corresponds to that in the accompanying graphs. (D, E) Summary of MSR inhibition rate (n = 6–8, D) and sVRP inhibition rate (n = 6–8, E) by adenosine in the presence and absence of NBTI/DIP, $*P < 0.05$, $**P < 0.01$ (unpaired Student's t-test).

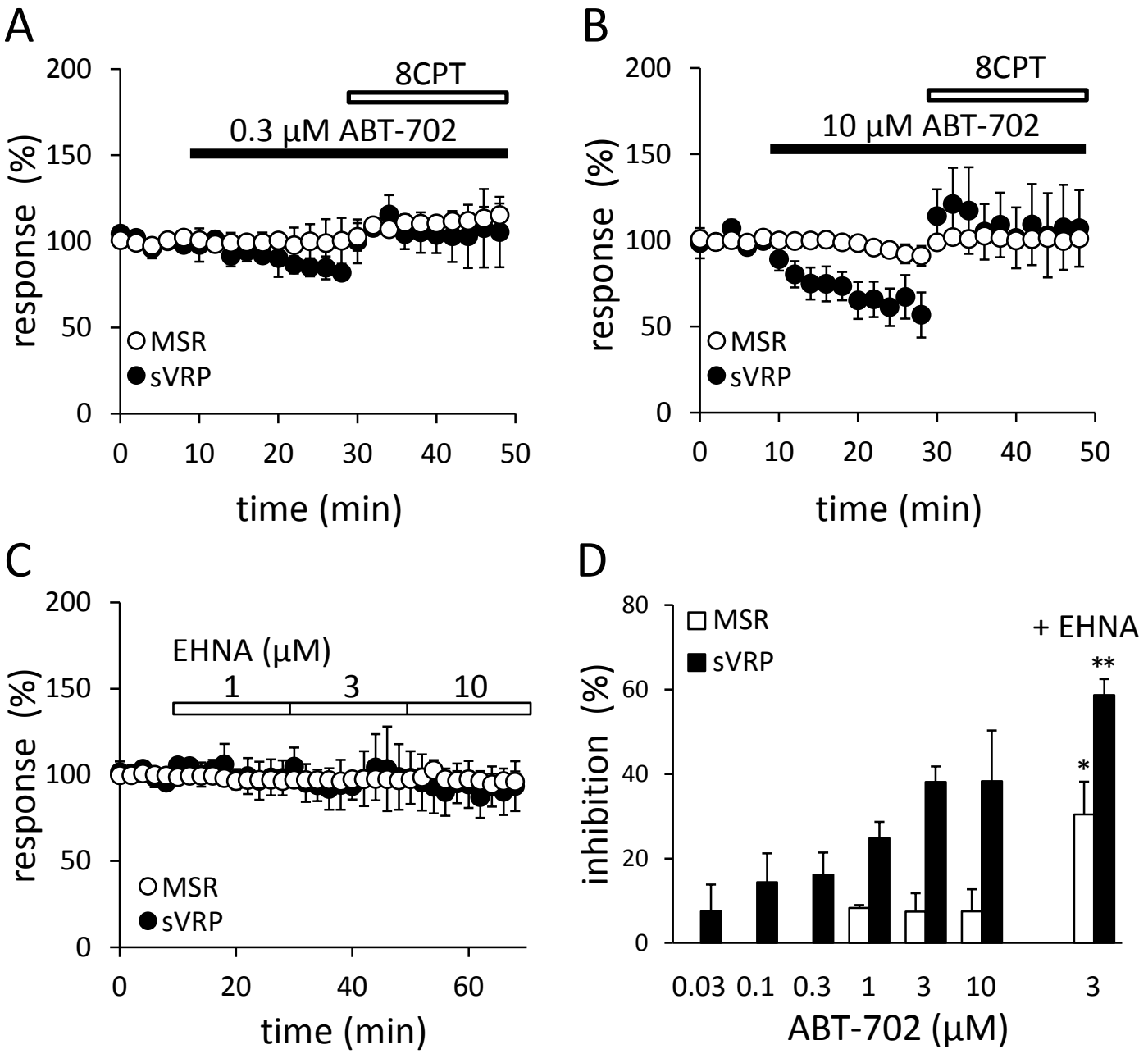
Fig. 6. Effect of CHA on spinal reflex potentials. CHA (30 nM) was applied to the spinal cord. (A, B) 8CPT (3 μM, n = 5, A) or NBTI (5 μM)/DIP (10 μM) (n = 6, B) was then added in the presence of CHA.

Fig. 7. Effect of ABT-702 on adenosine release from the spinal cord. (A, B) The time course of the adenosine increase by ABT-702 (3 μM, n = 6, A) and ENHA (10 μM, n = 7, B). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. open column (Dunnett's test). (C) ABT-702 (3 μM) and EHNA (10 μM) were applied to the spinal cord for 10 min in the presence and absence of NBTI (5 μM)/DIP (10 μM) (n = 6–8). $*P < 0.05$ vs. control (paired Student's t-test), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. hatched column (Dunnett's test). (D) The increment in adenosine by ABT-702, EHNA or ABT-702/EHNA is shown in the presence and absence of NBTI/DIP (n = 6–8). $*P < 0.05$, $**P < 0.01$ vs. control (unpaired Student's t-test).

Fig. 8. Immunohistochemical staining for AK and ADA in spinal cord. (A, B) Immunoperoxidase staining of AK (A) and ADA (B). Boxed regions at dorsal (upper part) and ventral horn (lower part) are enlarged in the right panels. Scale bars indicate 100 μm. (C, D) Immunofluorescent staining for AK, ADA and GFAP at ventral horn. (C) AK (red) expressed in GFAP (green)-positive astrocytes (arrow heads), especially in the nuclei (blue). (D) ADA (red) expressed in GFAP-positive astrocytes (arrow heads). ADA expression was found in astrocytic nuclei, cytosols (arrow heads) and processes (arrows). Scale bars indicate 20 μm.

Fig. 9. Schematic representation of inhibition of neuronal activity by ABT-702 in spinal cord. ABT-702, an AK inhibitor, increases intracellular adenosine, which is released via ENTs into extracellular spaces. Extracellular adenosine activates A_1 receptors, resulting in suppression of spinal neuronal activity.


A
control
ABT-702
+ 8CPT
MSR
2 mV
30 ms
sVRP
1 mV
10 s
8CPT
3 µM ABT-702
response (%)
200
150
100
50
0
MSR
sVRP
0
10
20
30
40
time (min)
B
control
ABT-702
/EHNA
+ 8CPT
MSR
2 mV
30 ms
sVRP
0.4 mV
10 s
8CPT
3 µM ABT-702/5 µM EHNA
response (%)
200
150
100
50
0
MSR
sVRP
0
10
20
30
40
time (min)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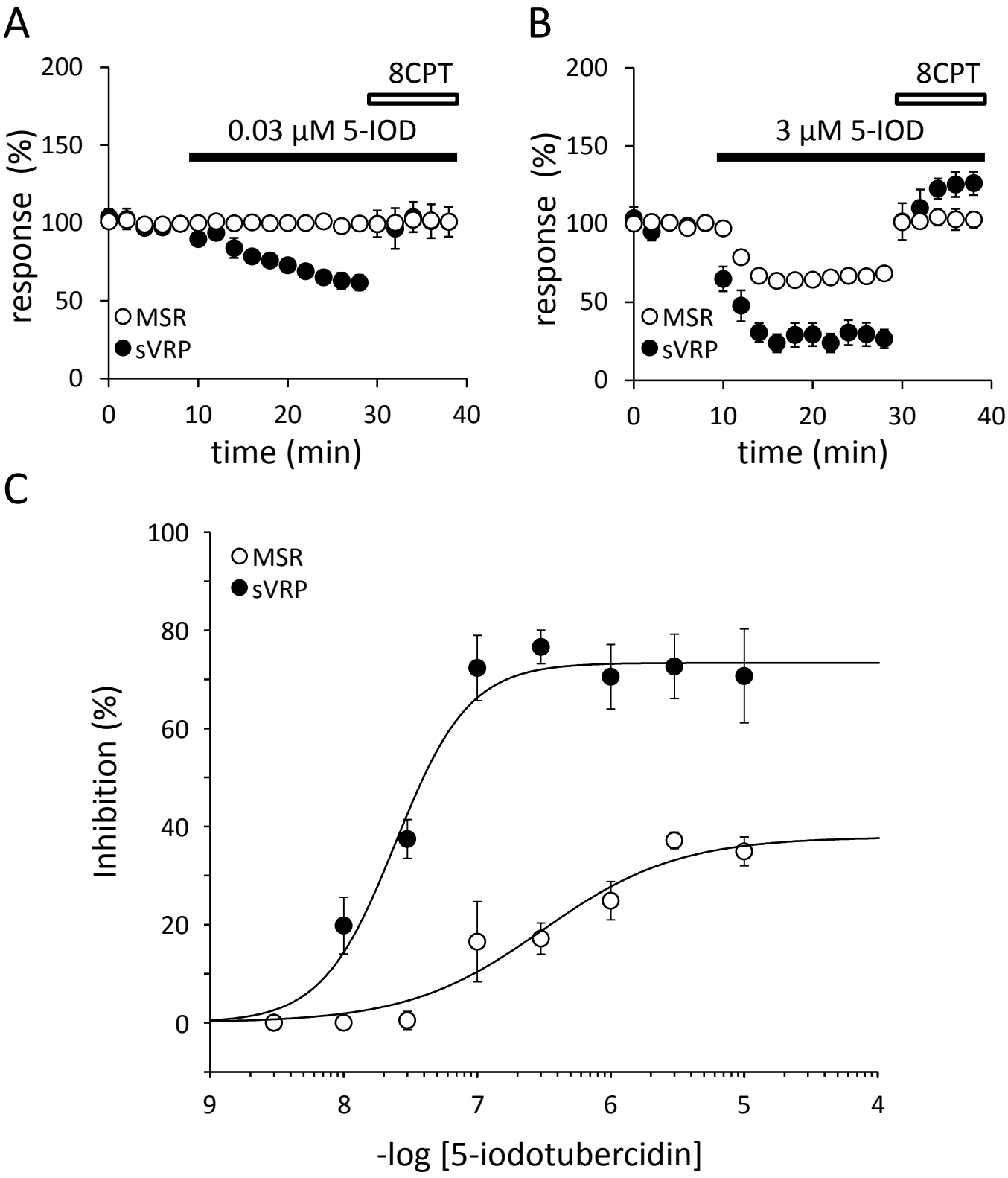
response (%)
MSR
sVRP
time (min)
B
8CPT
10 µM ABT-702
response (%)
MSR
sVRP
time (min)
C
EHNA (µM)
1
3
10
response (%)
MSR
sVRP
time (min)
D
MSR
sVRP
+ EHNA
inhibition (%)
0.03 0.1 0.3 1 3 10 3
ABT-702 (µM)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B
C
8CPT
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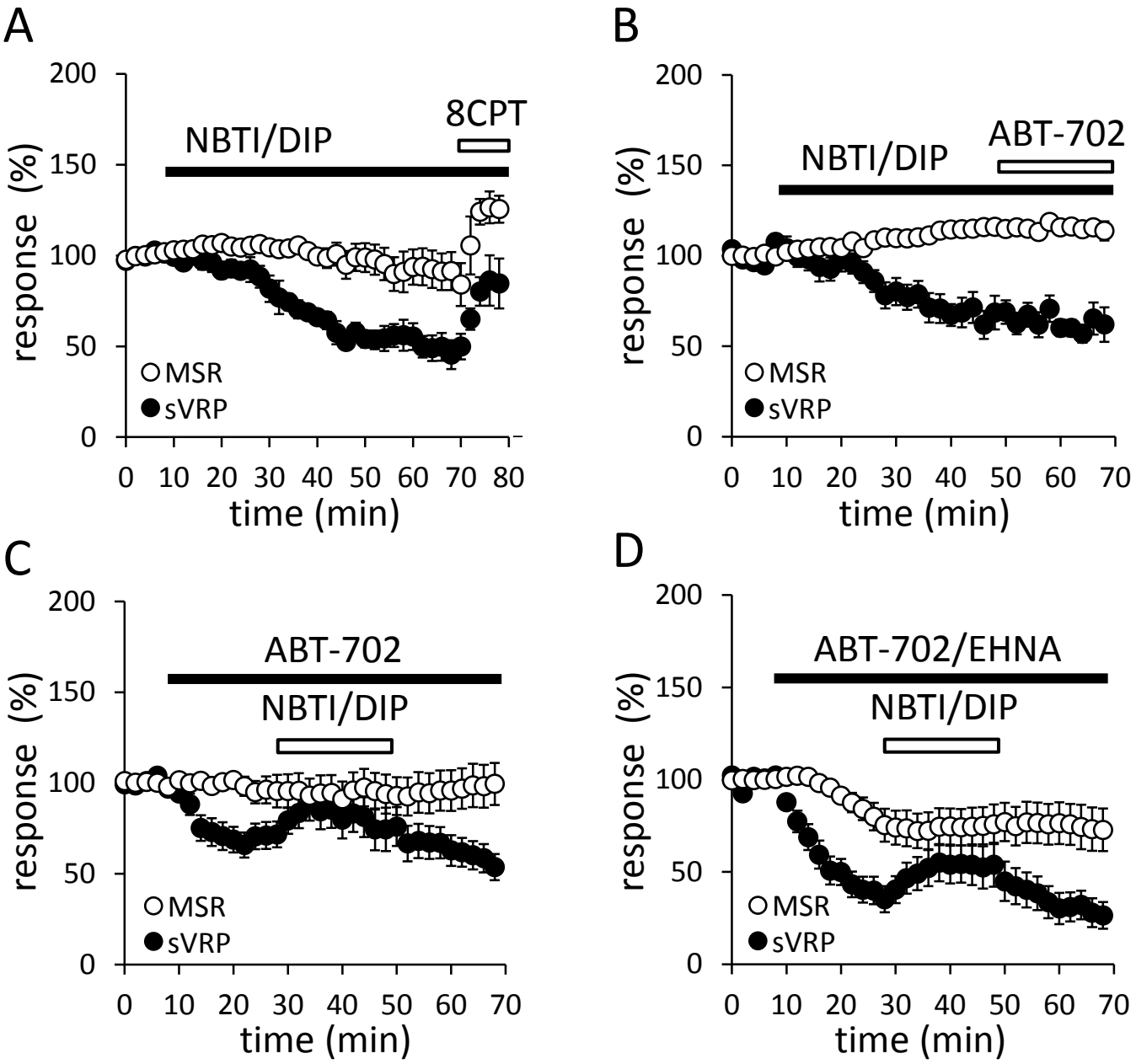
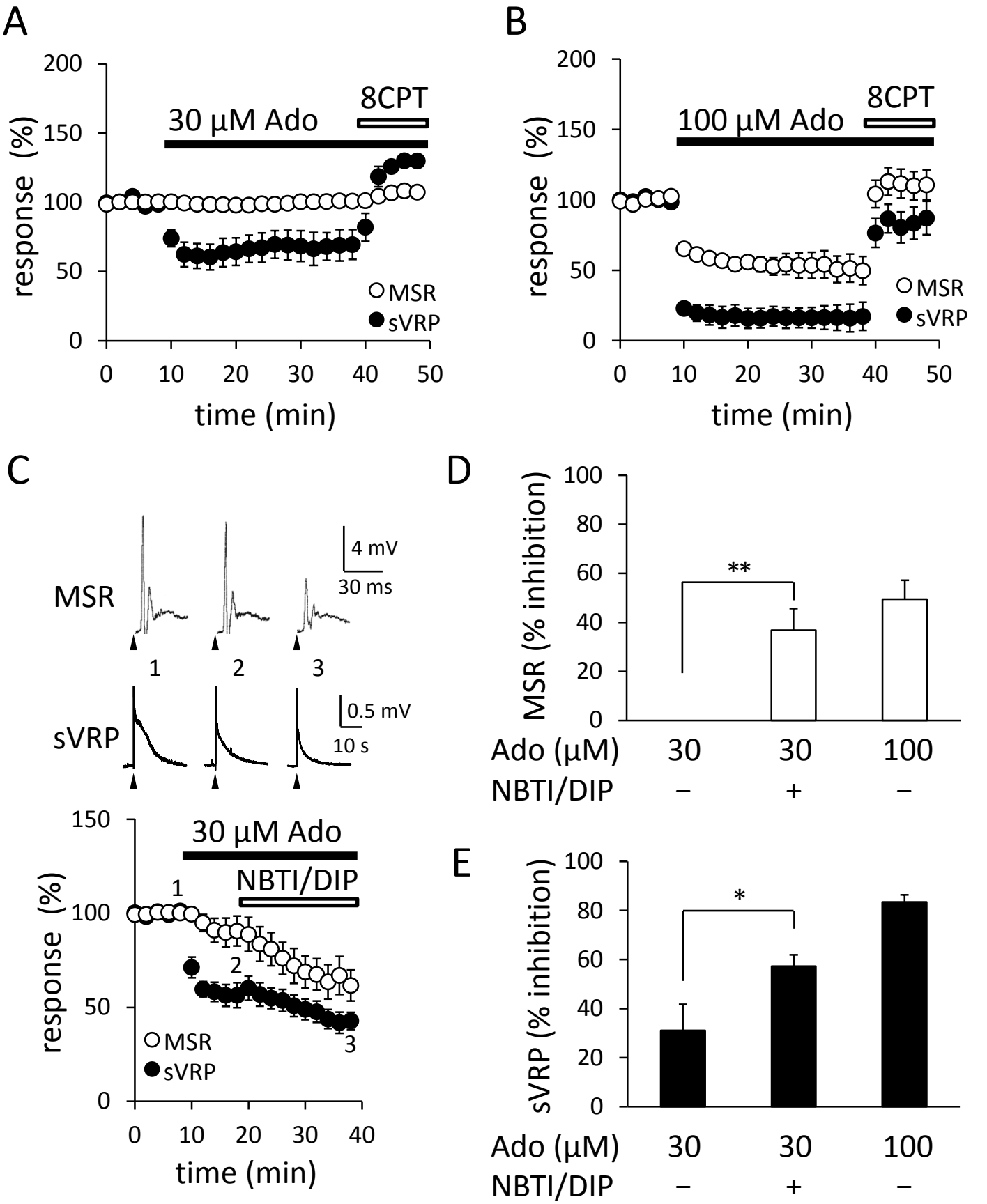
3 µM 5-IOD
response (%)
time (min)
MSR
sVRP
Inhibition (%)
-log [5-iodotubercidin]


A
B
C
D
NBTI/DIP
8CPT
ABT-702
ABT-702/EHNA
MSR
sVRP
response (%)
time (min)
0
50
100
150
200
0 10 20 30 40 50 60 70 80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150
100
50
0
response (%)
30 μM Ado
8CPT
MSR
sVRP
0
10
20
30
40
50
time (min)
B
200
150
100
50
0
response (%)
100 μM Ado
8CPT
MSR
sVRP
0
10
20
30
40
50
time (min)
C
MSR
4 mV
30 ms
1
2
3
sVRP
0.5 mV
10 s
150
100
50
0
response (%)
30 μM Ado
NBTI/DIP
1
2
3
MSR
sVRP
0
10
20
30
40
time (min)
D
MSR (% inhibition)
100
80
60
40
20
0
**
Ado (μM)
30
30
100
NBTI/DIP
–
+
–
E
sVRP (% inhibition)
100
80
60
40
20
0
*
Ado (μM)
30
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100
NBTI/DIP
–
+
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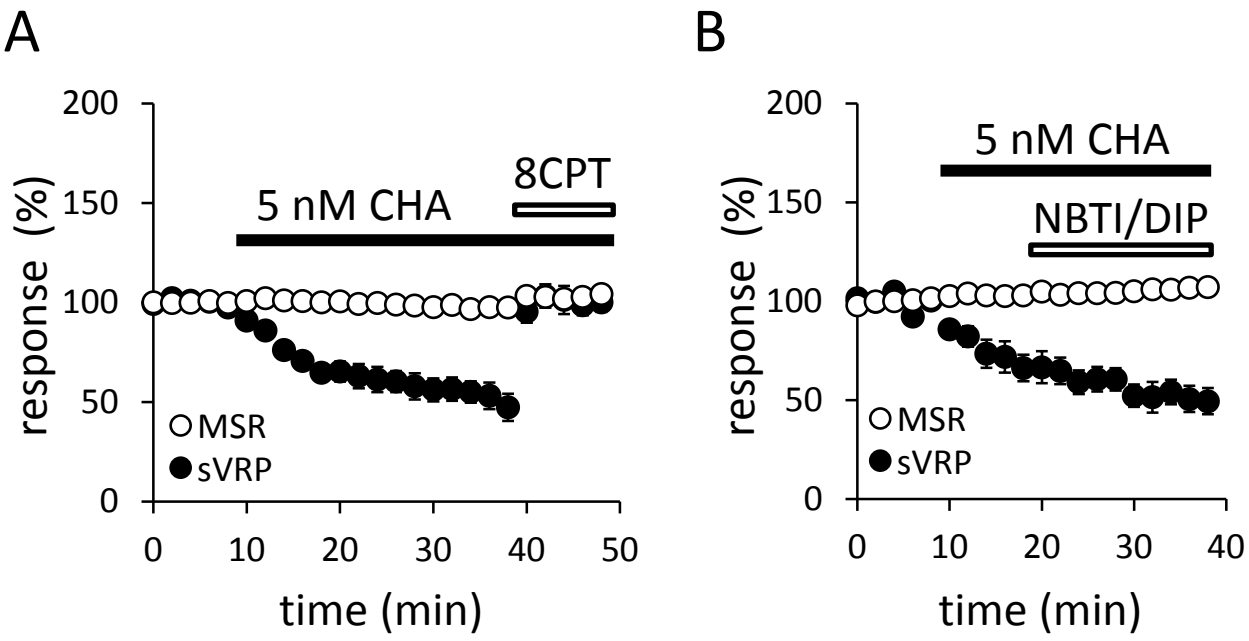
Fig. 6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MSR
sVRP
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time (min)
B
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response (%)
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MSR
sVRP
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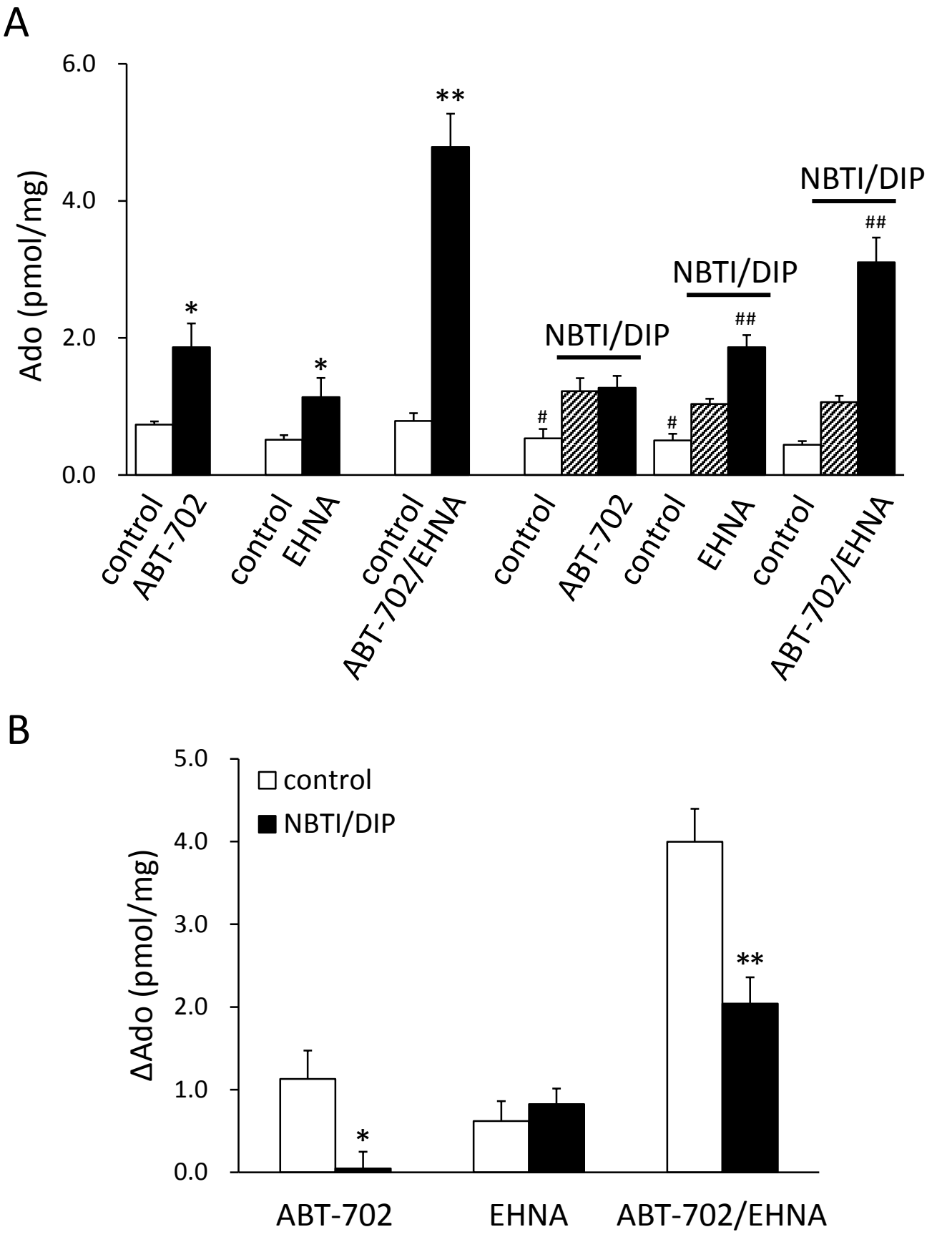
time (min)


A
6.0
4.0
2.0
0.0
Ado (pmol/mg)
*
*
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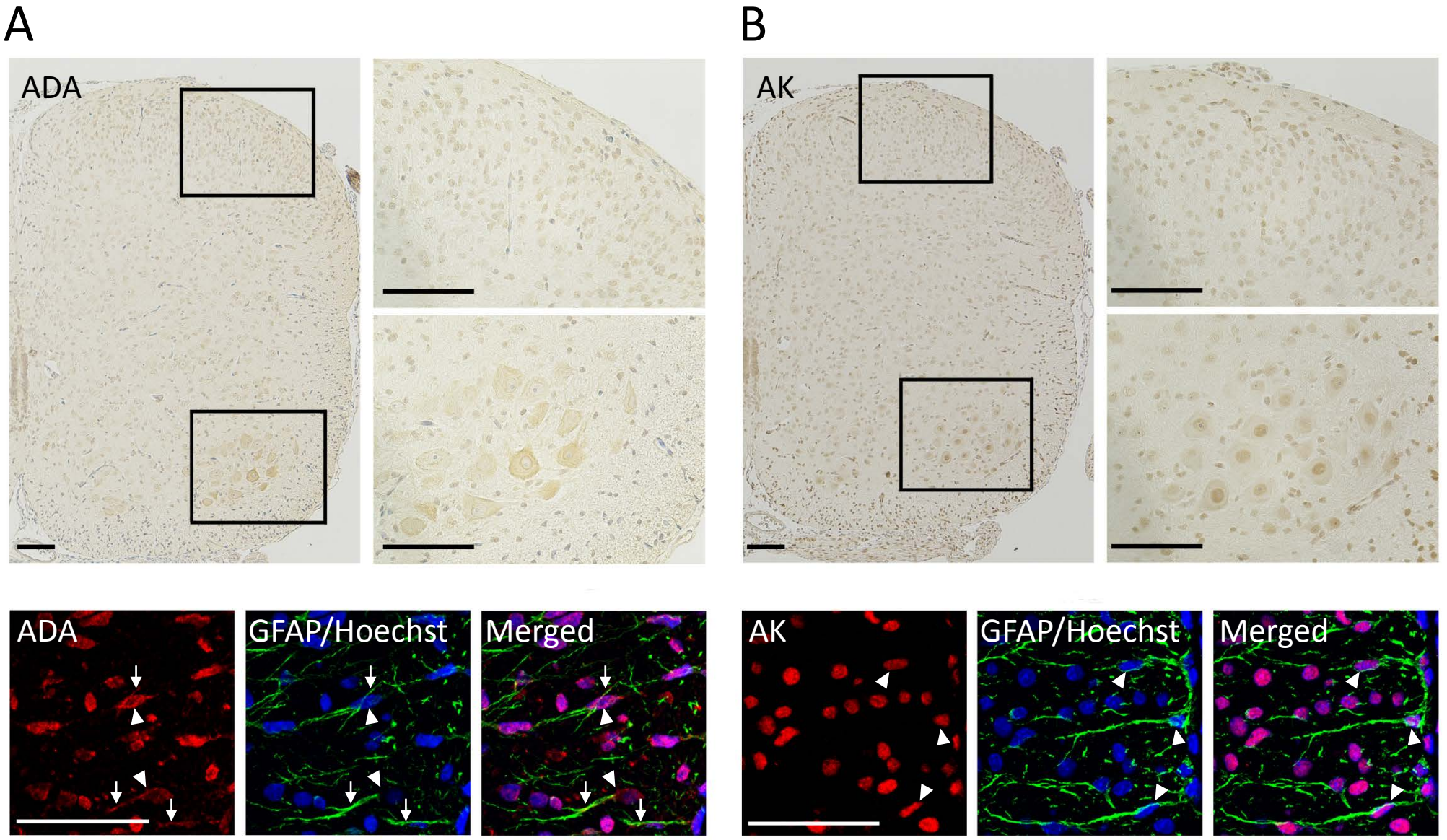
#
#
##
##
NBTI/DIP
NBTI/DIP
NBTI/DIP
control
ABT-702
control
EHNA
control
ABT-702/EHNA
control
ABT-702
control
EHNA
control
ABT-702/EHNA
B
5.0
4.0
3.0
2.0
1.0
0.0
ΔAdo (pmol/mg)
control
NBTI/DIP
*
**
ABT-702
EHNA
ABT-702/EHNA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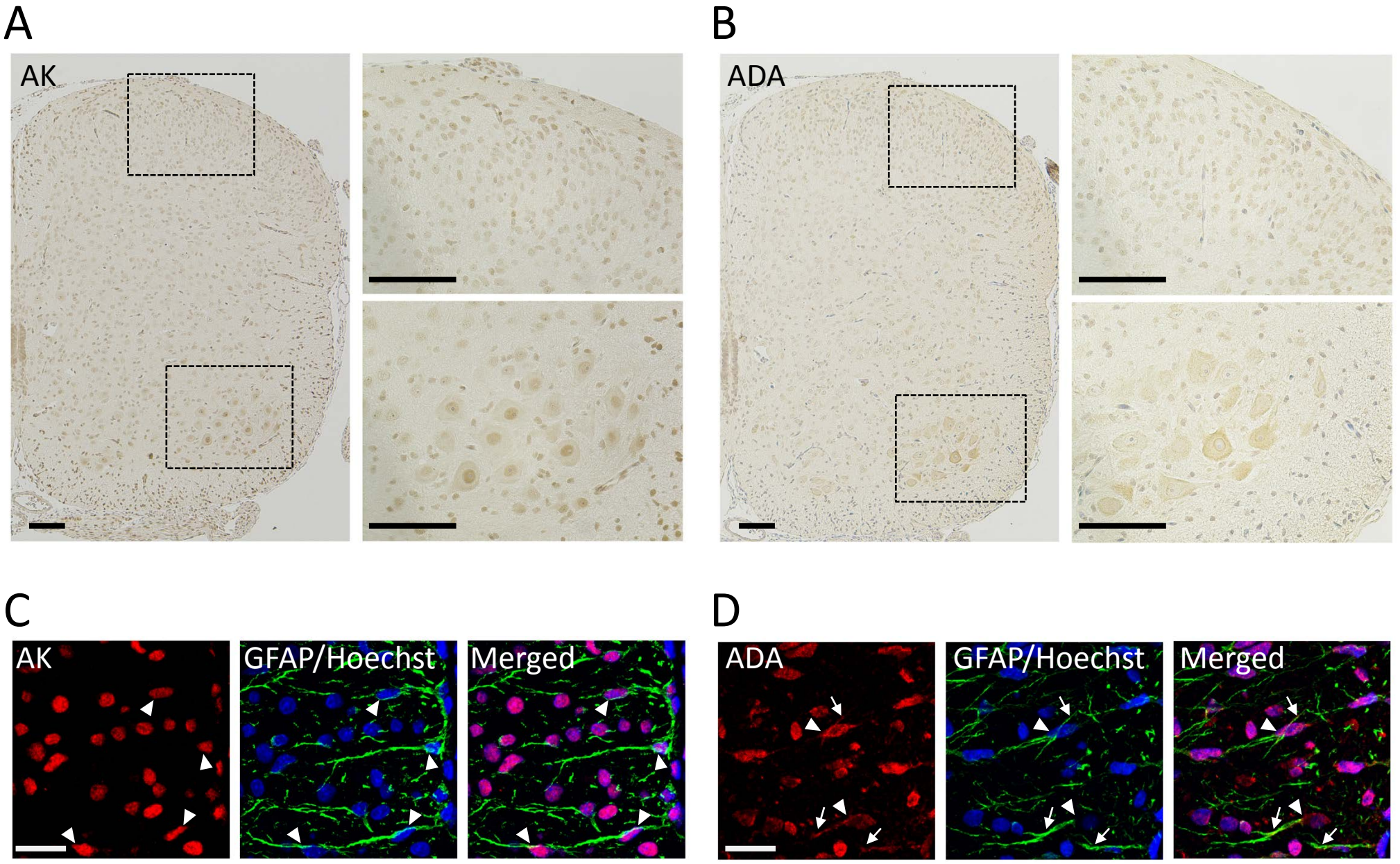
ADA
B
AK
ADA
GFAP/Hoechst
Merged
AK
GFAP/Hoechst
Merged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C
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GFAP/Hoechst
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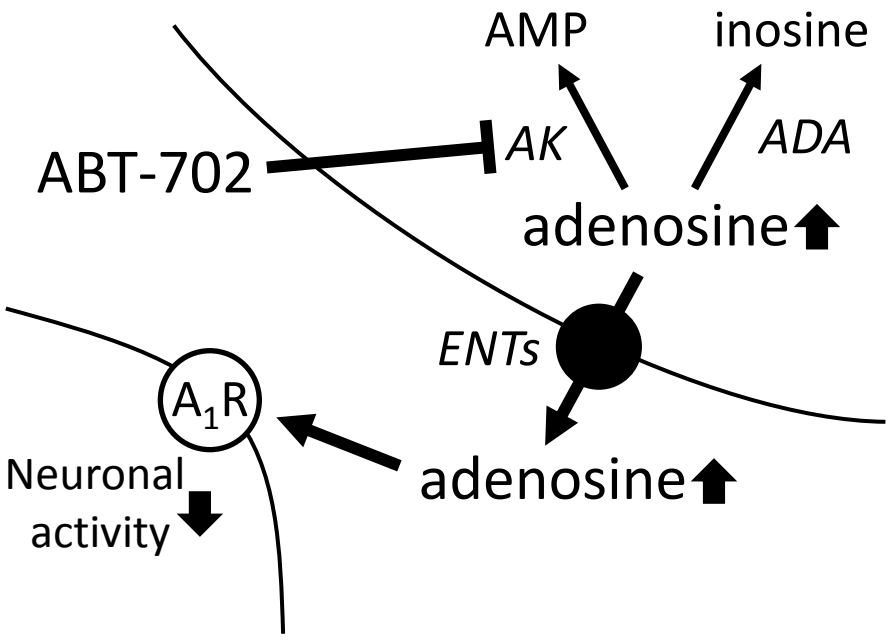
D
ADA
GFAP/Hoechst
Merged

Fig. 9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ABT-702
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ENTs
A1R
Neuronal
activity
adenosine