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Minocycline increases phosphorylation and membrane insertion of neuronal GluR1 receptors

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Abstract

The tetracycline antibiotic minocycline beneficially affects neuronal functioning and also inhibits the enzyme 5-lipoxygenase (5-LOX). We hypothesized that similar to 5-LOX inhibitors, minocycline may increase phosphorylation and membrane insertion of the glutamate receptor GluR1. The experiments were performed in primary cultures of mouse striatal neurons and in the prefrontal cortex and striatum of minocycline-treated mice. *In vitro*, low micromolar minocycline concentrations increased GluR1 phosphorylation at Ser845 and Ser831 and increased the surface content of GluR1. Minocycline also increased GluR1 phosphorylation *in vivo*. Increased GluR1 phosphorylation and minocycline treatment have been associated with antidepressant and memory-enhancing activities. Direct consequences of minocycline-increased GluR1 phosphorylation are yet to be established.

Keywords

Minocycline; glutamate; AMPA; antidepressant; addiction; 5-lipoxygenase

Minocycline is a tetracycline with broad-spectrum antimicrobial activity often used for acne therapy. The drug crosses the blood-brain barrier and produces numerous beneficial neuronal effects. Initial findings suggested a neuroprotective action of minocycline in brain ischemia [21] and in models of Parkinson's disease [7]. More recent reports suggest minocycline may improve memory and cognitive impairment in models of Alzheimer's disease [4] and addiction [14], and that it may be antidepressant [12,15].

In addition to its antibiotic activity, minocycline inhibits poly(ADP-ribose) polymerase-1 (PARP-1) [1] and 5-lipoxygenase (5-LOX) [5,19,20], mechanisms that may contribute to neurotoxicity/neuroprotection. Recent research suggests that 5-LOX inhibition increases the phosphorylation of the GluR1 (also known as GluR-A) subunits of ionotropic glutamate/AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionate) receptors at Ser831 and Ser845 [11]. The phosphorylation of the GluR1 subunit regulates the membrane (i.e., surface) insertion of AMPA receptors. Altered GluR1 phosphorylation plays a role in mechanisms of addiction [3,6] and in learning and memory [18]. Pharmacologically increased GluR1 phosphorylation and AMPA receptor potentiation may be therapeutic for a number of central nervous system (CNS) disorders including depression [17].

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In this work, we tested the hypothesis that similar to 5-LOX inhibitors, minocycline may increase phosphorylation and membrane insertion of the glutamate receptor GluR1.

We performed in-vivo studies with eight-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) housed in groups of three with free access to laboratory chow and water. For in-vitro studies, we prepared primary cultures as described elsewhere [11]. Briefly, after removing the cortices, striatal tissue was excised from embryonic 16-day-old ICR (Harlan, IN) mouse pups and suspended in a serum-free neurobasal medium (Invitrogen-Gibco, Carlsbad, CA) containing 50 µg/ml gentamicin and B27 supplement (Invitrogen-Gibco). The cells were seeded in poly-d-lysine (Sigma-Aldrich, Inc., St. Louis, MO; 20 µg/ml) pre-coated 3.5-cm-diameter culture dishes (2 million/2 ml/dish). Cultures were maintained at 37 °C and 5% CO₂ for 8–10 days. The experimental protocol was approved by the Institutional Animal Care Committee.

For in-vivo treatment, minocycline hydrochloride (Sigma) was dissolved in sterile saline (0.9% NaCl) supplemented with 1 M HCl (10%, v/v) as described earlier [15]. Immediately before intraperitoneal (i.p.) administration, the pH was adjusted to approximately 6–7 by adding NaOH. Controls received the same pH saline. The prefrontal cortex and striatal samples were collected 30 min later. For in-vitro treatment, minocycline was dissolved in dimethylsulfoxide (DMSO; Sigma) and applied directly into the culture medium for 30 min. Controls were treated with a corresponding concentration of DMSO (0.1% final).

For GluR1 phosphorylation assay, brain and neuronal culture samples were homogenized in a buffer containing 20 mM Tris, 137 mM NaCl, 48 mM NaF, 2 mM Na₂VO₃, 1% NP-40, and 10% glycerol, and protease inhibitors: pefabloc SP 4 mM, aprotinin 0.3 µM, pepstatin 1 µM, and leupeptin 1 µM. Samples were centrifuged (10 min, 1000×g, 4°C) and the supernatants were collected. Samples of 40 and 80 µg protein (measured by the BCA protein assay kit; Pierce, Rockford, IL) were analyzed by quantitative Western blotting (loading buffer containing 50 mM Tris–HCl [pH 6.8], 4% β-mercaptoethanol, 1% [w/v] SDS [sodium dodecylsulfate], and 40% [v/v] glycerol). Samples were boiled and run on polyacrylamide Tris–HCl gels (7.5% [w/v]; running solution 125 mM Tris–base, 0.95 M glycine, 0.5% [w/v] SDS) and transferred electrophoretically to a nitrocellulose membrane (Amersham Piscataway, NJ) using a Mini-Transblot electrophoresis system (BioRad, Hercules, CA; transfer buffer 25 mM Tris–base, 192 mM glycine, and 20% [v/v] methanol; constant current of 150 mA, overnight, 4°C). The non-specific binding was blocked by 5% (w/v) non-fat dry milk, 10% (v/v) 10 × TBST buffer (100 mM Tris–base, 1.5 M NaCl, 5 ml/l Tween 20), 0.002% (v/v) Igepal, and 0.02% (w/v) SDS (pH 8) for 30 min. The membranes were incubated overnight at 4°C individually with the antibodies for anti-phospho-GluR1 Ser831 (1:1000; Tocris Cookson Ltd., UK) and anti-phospho-GluR1 Ser845 (1:1000; Affinity Bioreagents, CO), subsequently washed three times, and incubated for 4 h with horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG, Amersham; 1:1000). They were rinsed, treated with an ECL Kit (Amersham), and exposed to Hyperfilm ECL (Amersham). To quantify the signals of phospho-GluR1, the membranes were stripped with a Western Blot Recycling Kit (Alpha Diagnostic International, San Antonio, TX) and re-probed with anti-GluR1 antibodies (1:4000; overnight at 4 °C, Tocris Cookson Ltd., UK) and horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG, Amersham; 1:3000). Band densities were quantified using the Loats Image Analysis System (Loats Associates, Inc., Westminster, MD) [11]. The optical density of phospho-GluR1 bands was corrected by the optical density of the corresponding total GluR1 bands.

The surface protein cross-linking assay was used as described elsewhere [2]. Briefly, after vehicle and minocycline treatment, the cultures were washed twice with warm phosphate buffer saline (PBS) and incubated with BS³ (Bis[sulfosuccinimidyl] suberate; Pierce Biotechnology,

Rockford, IL; 1 mg/ml, 10 min, 4°C). The cells were washed three times with ice-cold quenching buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA pH 7.4, 1 mM phenylmethylsulfonamide, 50 mM ethanolamine, and 10 mM NaF) and harvested in 100 µl/dish quenching buffer plus 1 µM pepstatin and 100 µM leupeptin. As a negative control, another set of cultures was collected in the same way but without BS³ exposure. Western blotting was used for protein separation (4–15% acrylamide gradient gels, transferred to a nitrocellulose membrane [Amersham]). The blots were incubated overnight with the anti-GluR1 antibody (Tocris; 1:4000; 4°C) and with the horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG 1:3000, Amersham). In the absence of BS³, anti-GluR1 antibodies detect only one immunoreactive band; in the presence of BS³ they detect two bands, the upper surface GluR1 and the lower intracellular GluR1 signals. Levels of surface GluR1 insertion were determined as a ratio between the surface (S) and intracellular (I) band densities. To verify that the neurons remained intact during the BS³ treatment, the immunoreactivity of intracellular β-actin protein was measured on the same blots (anti-β-actin antibody; 1:20000, Sigma). The BS³ treatment did not affect the β-actin signals.

Statistical analyses were performed using SPSS software (version 12.0, SPSS Inc., Chicago, IL). Quantitative Western blotting results were evaluated by one-way ANOVA followed by the Dunnett's multiple comparison test (in-vitro) or the t-test (in-vivo). Significance was accepted as $p < 0.05$.

In a pilot in-vitro evaluation of different minocycline concentrations, we measured GluR1 Ser845 phosphorylation after a 30 min treatment and found the following (expressed as a percentage of the vehicle-treated control): 1 µM = 125%, 5 µM = 150%, 10 µM = 180%, 50 µM = 120%, and 100 µM = 100%. For further detailed analyses, we used 10, 50, and 100 µM minocycline. Only a concentration of 10 µM significantly altered (i.e., increased) both Ser845 and Ser831 GluR1 phosphorylation (Fig. 1).

The evaluation of the BS³ assay confirmed that only the membrane proteins (e.g., GluR1) and not the intracellular proteins (e.g., β-actin) were affected by this membrane protein-crossing agent (Fig. 2A). Using this assay, the quantitative analysis of the surface-GluR1/intracellular-GluR1 ratios revealed that 10 µM but not 100 µM minocycline increased the membrane GluR1 insertion (Fig. 2B). Although the example blot may convey the notion that total GluR1 content was affected by 100 µM minocycline, we found no evidence for significant alterations in total GluR1 content by minocycline (data not shown).

For brain delivery of minocycline, the drug is typically administered systemically in doses of 40–100 mg/kg. It was established that the plasma levels of minocycline 30 min after 90 mg/kg i.p. are comparable to the levels obtained with intravenous administration of lower doses [9]. We measured GluR1 phosphorylation in brain samples 30 min after i.p. injection of 80 mg/kg minocycline and found increased phosphorylation at both Ser845 and Ser831 (Fig.3).

Minocycline is a generic and widely available drug. The fact that it produces multiple potentially useful effects in the CNS has opened the possibility of its new uses in neurology and psychiatry. Several CNS-relevant molecular pathways have been considered as possible mediators of minocycline's actions in the brain [1,5,13,19,20]. Our data has shown for the first time that in the doses and concentrations used in previous in-vivo and in-vitro studies, minocycline increases the phosphorylation and membrane insertion of neuronal GluR1 receptors.

Both Ser831 phosphorylation (by calcium/calmodulin-dependent protein kinase II) and Ser845 phosphorylation (by cAMP-dependent protein kinase) of GluR1 receptors increase AMPA channel activity, and in particular, Ser845 phosphorylation increases GluR1 surface insertion [16]. These changes are associated with physiologically relevant synaptic changes, e.g.,

priming of AMPA receptors for long-term potentiation [16]. In contrast, activation of mitogen-activated protein kinase p38 (p38MAPK) is associated with a reduction of GluR1 surface expression [3,8].

At this time, it is unclear which molecular mechanisms are responsible for minocycline-increased phosphorylation and surface expression of GluR1 receptors. Increased Ser831 and Ser845 phosphorylation of GluR1 is triggered by 5-LOX inhibitors [11] and minocycline is capable of inhibiting neuronal 5-LOX [5,19,20]. On the other hand, GluR1 surface insertion is increased by p38MAPK inhibition [8] and minocycline inhibits this protein kinase [13].

In our experimental conditions, GluR1 receptors were affected by 10 μ M but not by higher concentrations of minocycline. Since minocycline may engage multiple molecular mechanisms it is possible that mechanisms triggered by higher concentrations influence GluR1 receptors differently from lower concentrations of this drug. For example, in cerebrocortical nerve terminals 50 μ M and 100 μ M minocycline reduced glutamate release while 10 μ M did not alter glutamate release [10]. Similarly, only high concentrations of minocycline inhibited the voltage-dependent calcium channels [10].

Minocycline-increased GluR1 phosphorylation and membrane insertion could participate in previously observed CNS actions of this drug. For example, both minocycline [12,15] and GluR1/AMPA-potentiators [17] produce antidepressant effects, may facilitate/increase learning and memory [4,14,16], and both are involved in the mechanisms of addiction [3,6,14]. Our results suggest that effects of minocycline on CNS GluR1 receptors should be taken into consideration in future studies of the CNS actions of minocycline.

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Fig. 1.

Effects of minocycline on GluR1 phosphorylation at Ser845 and Ser831 in-vitro. Primary cultures of mouse striatal neurons were treated with vehicle (control; CTR) or minocycline for 30 min. The upper panels show representative Western immunoblots of phosphorylated GluR1 signals (pGluR1) at Ser845 (A) and Ser831 (B) (upper bands) and the corresponding total GluR1 signals (lower bands). For each experimental condition, samples were loaded at two concentrations of proteins, 80 and 40 μg (from left to right). The pGluR1 signals were normalized by their respective GluR1 signals measured in the same blot. The values of two ratios for each experimental sample that were assayed at two different protein concentrations were similar and the average of these two ratios were used as a single value for each individual experiment. The corresponding lower panels (A and B) show the respective quantitative data. Results (mean ± SEM; n = 3 different culture preparations) are presented as a percentage of the corresponding vehicle-treated control (open bar = 100 %) and were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test. Only the 10 μM minocycline treatment significantly altered GluR1 phosphorylation at both Ser845 (A) and Ser831 (B) (*p<0.01; ** p<0.001 vs. corresponding control).

Fig. 2.

Effects of minocycline on the surface insertion of GluR1 proteins in primary cultures of mouse striatal neurons. The cultures were treated for 30 min with: 1) vehicle (control; CTR), 2) minocycline 10 μ M, and 3) minocycline 100 μ M. Experiments were performed in the presence and absence of a membrane impermeable protein cross-linking agent, BS³ (1 mg/ml; 10 min). The extracted proteins were analyzed by Western immunoblotting. The blots were probed for GluR1 immunoreactivity and re-probed for β -actin immunoreactivity. Representative immunoblots are shown in A. The BS³-caused surface (S) retention of the immunoreactive proteins was observed with GluR1 only (upper bands). In the absence of BS³, GluR1 immunoreactivity was observed in the intracellular (I) compartment only (lower bands). β -Actin immunoreactivity was not affected by BS³. Panel B shows the quantification of the extent of the membrane insertion of GluR1, expressed as S/I ratios (greater ratio equals greater surface insertion). Results (mean $\pm$ SEM; n = 4 different culture preparations) are presented as a percentage of the corresponding vehicle-treated control (open bar) and were evaluated by one-

way ANOVA followed by Dunnett's multiple comparison test. Only the 10 μ M minocycline treatment increased the membrane insertion of GluR1 (* $p < 0.05$ vs. control).

Fig. 3.

The effect of in-vivo minocycline treatment on (A) cortical and (B) striatal GluR1 phosphorylation. Minocycline (80 mg/kg, i.p.) and vehicle were administered for 30 min. The brain samples were analyzed by a quantitative Western blot assay of phospho GluR1 (pGluR1) and GluR1 immunoreactivities as described in Fig 1. For the quantitative assay, pGluR1 signals were normalized by the corresponding GluR1 signals. The results are expressed as the pGluR1/GluR1 ratios (mean $\pm$ S.E.M.; $n = 5-7$). Minocycline increased GluR1 phosphorylation at both Ser854 and Ser831 (* $p < 0.05$, ** $p < 0.01$; compared to vehicle-treated control mice; t-test).