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Differential effect of glutamate transporter inhibition on EPSCs in the morphine naïve and morphine tolerant neonatal spinal cord slice

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Abstract

Opioid analgesic tolerance is a phenomenon defined as a need for increasingly higher doses of opiates to maintain suitable pain relief following repeated drug exposure. Research suggests that analgesic tolerance may result from heightened NMDA receptor (NMDAR) activity, but little is known regarding the mechanisms by which this elevated NMDAR activity develops. Recent evidence suggests that glutamate transporter down-regulation follows repeated opiate exposure and contributes to heightened pain sensitivity. Though glutamate transporter inhibition has been shown to increase activity of spinal cord neurons, it is unknown whether this increase contributes to the heightened NMDAR activity that underlies opiate tolerance. We directly tested this hypothesis by comparing the effects of glutamate transporter inhibition on excitatory post-synaptic currents (EPSCs) in the spinal cord dorsal horn of opiate naïve and opiate tolerant rats. We show that non-selective glutamate transporter inhibition increases the rate of spontaneous excitatory post-synaptic currents (sEPSCs) in the opiate naïve, but not opiate tolerant slice. This potentiation occurs in the presence of the sodium channel blocker tetrodotoxin (TTX) and is blocked by the NMDAR antagonist D-2-amino-5-phosphonovalerate (APV). The sEPSC rate is elevated at baseline in the opiate tolerant spinal cord slice compared to the opiate naïve slice, and glutamate transporter inhibition eliminates this difference. Taken together, we conclude that glutamate transporter inhibition directly contributes to heightened NMDAR activity. Furthermore, we propose that the increased neural activity observed in the opiate tolerant slice is due to a state of glutamate transporter down-regulation and resultant heightened NMDAR activity.

Keywords: Morphine tolerance; Glutamate transporters; Spinal cord; Whole cell voltage clamp

Opioid analgesic tolerance is a laboratory and clinical phenomenon defined as a need for increasingly higher levels of opiates to maintain suitable pain relief following repeated drug exposure [15]. Although much *in vitro* evidence demonstrates a desensitization of opiate receptors, *in vivo* studies have also suggested that the development of increased pain sensitivity, or hyperalgesia, mediates this phenomenon [12,15]. This hyperalgesic mechanism is thought to develop through heightened NMDA receptor (NMDAR) activity, as tolerance can be blocked by NMDAR antagonists [2,4,7,12,16,21]. Recent evidence suggests glutamate transporter down-regulation following repeated morphine exposure as a facilitator of this heightened NMDAR activity [10,11,13,14,23,24].

Glutamate transporters normally function to maintain homeostasis of extra-cellular glutamate levels [3]. The spinal cord glutamate transporters are: the predominately astroglial glutamate transporter, glutamate transporter 1 (GLT1); the glutamate/aspartate transporter (GLAST); and the neuronal glutamate transporter, excitatory amino acid carrier 1 (EAAC1) [3,5,6,8,17,18,22].

Chronic morphine administration in rats induces the down-regulation of these glutamate transporters [11,13,23]. Moreover, pharmacological inhibition and potentiation of spinal glutamate transporters attenuates and potentiates, respectively, the analgesic effect of morphine in morphine tolerant rats [13,14]. Additionally, application of a glutamate transporter inhibitor increases the rate of spontaneous and evoked action potentials of spinal cord neurons *in vitro* [24] and intrathecal administration of a glutamate transporter inhibitor increases evoked and induces spontaneous pain behaviors *in vivo* [10], pointing to the spinal cord as a potentially critical site for glutamate transporter modulation of both morphine analgesia and pain sensation.

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We have recently observed an NMDAR-mediated increase in spontaneous activity in Lamina I of the spinal cord dorsal horn of neonatal rats made tolerant to opiates (morphine), which correlates with an increase in primary afferent terminal NMDAR number. Such an increase in NMDAR number, however, does not rule out the possibility that an increase in extra-cellular glutamate [10,11,13,14,23,24] also contributes to opiate tolerance. In these studies we investigate the effects of glutamate transporter inhibition in spinal cord slices from opiate naïve and tolerant rats to directly test this hypothesis.

All experiments were conducted on 12–17-day-old Sprague-Dawley rats. All efforts were made to minimize distress to the rats throughout the experiment and all studies were in accordance with the guidelines of the U.S. Guide for the Care and Use of Laboratory Animals.

Opiate analgesic tolerance was induced with 6 twice daily (morning and evening) subcutaneous injections of morphine (15, 30 and 45 mg/kg on Days 1, 2, 3, respectively). We have previously shown this regimen to induce analgesic tolerance (approximately a 10-fold shift in the dose-response) on a radiant heat paw withdrawal test (Zeng et al., unpublished observations; see also [25]).

Electrophysiological recordings were made on either Day 4 or 5. No behavioral (e.g., jumping, tremors (c.f. [20,26]) or cellular (no naloxone-induced potentiation of excitatory post-synaptic currents (EPSCs)) signs of withdrawal were observed in any rats during this period (Eastman et al., unpublished observations). We have previously observed no differences in frequency or amplitude of spontaneous EPSCs in spinal cord slices from vehicle-injected rats and rats who have not received any injections (Zeng et al., unpublished observations), and thus, opiate naïve rats in these studies received no injections *in vivo*.

Whole cell voltage clamp was performed on spinal cord slices from 12- to 17-day-old Sprague-Dawley rats as previously described [19]. Briefly, a laminectomy was performed while rats were anesthetized with isoflurane. After euthanasia by decapitation, the spinal cord was quickly removed and placed in low melting point agar (2%, w/v). Using a Vibratome (Ted Pella Inc.), 300–400 μm transverse sections of the spinal cord were cut and held in O_2-bubbled modified artificial cerebrospinal fluid (aCSF) (113 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM bicuculline, 1 μM strychnine) for approximately 1 h prior to electrophysiological recordings. Spinal cord slices were then transferred to a Plexiglas recording chamber in which Lamina I cells, identified as cells within 10 μm from the dorsal edge of the slice, were visualized using a CCD camera (Dage-MTI). A glass recording electrode (125 mM $KMeSO_4$, 8 mM NaCl, 10 mM HEPES, 2 mM MgATP, 0.5 mM NaGTP, 5 mM EGTA)(15 MΩ resistance; pH 7.3; 285 mOsm) was advanced using a micro-manipulator (Sutter) until whole cell configuration was obtained. Whole cell voltage clamp was maintained at a resting potential of −70 mV using an Axopatch 200B amplifier (Axon Instruments) and Pulse software (HEKA). Initial series resistance and capacitance were noted and a change of greater than 20% was used as the upper limit for inclusion of data in analysis.

Mini-Analysis software (Synaptosoft) was used to analyze the frequency, maximal amplitude, and decay time (measured as time for response amplitude to decrement from 90% to 37% of maximum) of the sEPSCs. Spontaneous EPSCs were recorded in 5 min epochs, with the last of two pre-treatment epochs serving as baseline. Except where otherwise indicated, data were transformed [(post-drug/pre-drug) × 100] to illustrate the percent change from baseline response. Data were analyzed using One-Way or Repeated Measures ANOVAs as appropriate and *post hoc* comparisons were performed using Fisher's LSD test (GB-STAT for Macintosh). A statistically significant effect was based on a p-value of less than 0.05.

Bicuculline methiodide, strychnine hydrochloride, dihydrokainic acid (DHK) and D-2-amino-5-phosphonovalerate (APV) were purchased from Sigma–Aldrich. DL-threo-beta-benzyloxyaspartic acid (DL-TBOA) was purchased from Tocris Cookson Inc. Tetrodotoxin (TTX), *Fugu* sp. was purchased from CalBioChem. Except where otherwise indicated, all drugs were added to the perfusate in a 1:1000 dilution.

The non-selective glutamate transporter inhibitor, TBOA (100 μM in water, 1:100 dilution to aCSF) caused a significant increase in the rate of sEPSCs in spinal cord slices from opiate naïve, but not opiate tolerant rats ($F(1, 53) = 23.39$, $p < .0001$; Fig. 1A and B). No effects on amplitude or decay time by TBOA were observed in either group (data not shown). The sEPSC rate in the opiate tolerant slice showed a decrease over time, perhaps due to a gradual run-down (i.e., a non-specific decline in sEPSCs). However, potential group differences in slice viability do not appear to mediate the differential effect of TBOA on sEPSC rate, as the normalized (fold-change over baseline) rate of dorsal horn neurons in untreated spinal cord slices from opiate naïve and opiate tolerant rats were not different from each other (Fig. 1B) and we do not observe differences in anatomic (cell body diameter) or electrophysiological characteristics (e.g., input resistance, capacitance) of Lamina I cells in the opiate naïve and opiate tolerant spinal cord slice (Zeng et al., unpublished observations).

In addition to group differences in responding to TBOA, it is also possible that spinal cord slices from opiate naïve and opiate tolerant spinal cord slices have different baseline sEPSC rates. Indeed, analysis of sEPSC rates prior to TBOA application revealed a significantly elevated sEPSC rate in the opiate tolerant spinal cord slice that was no longer apparent following TBOA application ($F(2, 28) = 3.61$, $p < .05$; Fig. 1C). Therefore, not only are Lamina I cells potentiated by TBOA, but this potentiation appears to offset the higher baseline EPSC rate observed in the opiate tolerant spinal cord slice.

In summary, we propose that TBOA does not increase the rate of sEPSCs in spinal cord slices from opiate tolerant rats because the rate is already elevated, and that this elevation is the result of glutamate transporters already having been down-regulated during the development of opiate tolerance [13].

The intent of the next two experiments was to further characterize the TBOA- induced increase in sEPSC rate in dorsal horn neurons. Because TBOA did not increase sEPSC rate in the spinal cord slice from the opiate tolerant rat, the