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Glucuronic acid and the ethanol metabolite ethyl-glucuronide cause Toll-like receptor 4 activation and enhanced pain

Susannah S. Lewis^a, Mark R. Hutchinson^b, Yingning Zhang^a, Dana K. Hund^a, Steven F. Maier^a, Kenner C. Rice^c, and Linda R. Watkins^{a,*}

^aDepartment of Psychology & Neuroscience, University of Colorado at Boulder, Boulder, Colorado, USA

^bDiscipline of Pharmacology and Discipline of Physiology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia

^cChemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism National Institutes of Health, Rockville, Maryland, USA

Abstract

We have previously observed that the non-opioid morphine metabolite, morphine-3-glucuronide, enhances pain via a toll-like receptor 4 (TLR4) dependent mechanism. The present studies were undertaken to determine whether TLR4-dependent pain enhancement generalizes to other classes of glucuronide metabolites. *In silico* modeling predicted that glucuronic acid alone and ethyl glucuronide, a minor but long-lasting ethanol metabolite, would dock to the same MD-2 portion of the TLR4 receptor complex previously characterized as the docking site for morphine-3-glucuronide. Glucuronic acid, ethyl glucuronide and ethanol all caused an increase in TLR4-dependent reporter protein expression in a cell line transfected with TLR4 and associated co-signaling molecules. Glucuronic acid-, ethyl glucuronide-, and ethanol-induced increases in TLR4 signaling were blocked by the TLR4 antagonists LPS-RS and (+)-naloxone. Glucuronic acid and ethyl glucuronide both caused allodynia following intrathecal injection in rats, which was blocked by intrathecal co-administration of the TLR4 antagonist LPS-RS. The finding that ethyl glucuronide can cause TLR4-dependent pain could have implications for human conditions such as hangover headache and alcohol withdrawal hyperalgesia, as well as suggesting that other classes of glucuronide metabolites could have similar effects.

Keywords

glucuronic acid; ethanol; ethyl glucuronide; TLR4; (+)-naloxone; glucuronidation; metabolite

1. Introduction

Many drug metabolites are considered inert byproducts, but increasing evidence suggests certain metabolites can have more complex effects. For instance, morphine-3-glucuronide, a morphine metabolite, causes pro-inflammatory responses in the CNS (Komatsu et al., 2009,

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*Corresponding author: Linda R. Watkins, Department of Psychology, Campus Box 345, University of Colorado at Boulder, Boulder, Colorado, USA 80309-0345, linda.watkins@colorado.edu, Fax: (303) 492-2967, Phone: (303) 492-7034.

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Lewis et al., 2010). This response induces pain enhancement blocked by broad-spectrum immune inhibitors (e.g. minocycline) as well as the toll-like receptor 4 (TLR4) signaling inhibitor, (+)-naloxone (Lewis et al., 2010). These data raise the question of whether this TLR4-dependent pain-sensitizing effect is unique to morphine-3-glucuronide, or found more broadly in glucuronidated metabolites.

The pattern recognition receptor TLR4 and its co-receptor MD2 are present on many immune cells, including at least microglia in the CNS (Bsibsi et al., 2002). TLR4/MD2 activation initiates a proinflammatory cascade leading to proinflammatory cytokine release (Lehnardt et al., 2003). TLR4 is linked to enhanced pain, including neuropathic pain (Hutchinson et al., 2008), and decreased morphine analgesia (Hutchinson et al., 2010b), in addition to pain induced by morphine-3-glucuronide (Lewis et al., 2010). TLR4/MD2 signaling can be blocked non-stereoselectively by (+)- and (–)-naloxone as well as by lipopolysaccharide made by *Rhodobacter sphaeroides* (LPS-RS), a selective TLR4 antagonist (Hutchinson et al., 2008, Hutchinson et al., 2010b).

Several classes of commonly abused drugs have also been linked to increased TLR4 activity. Morphine has been demonstrated to competitively bind MD2, thereby inducing TLR4 signaling (Wang et al., 2012). TLR4 inhibition reduces the rewarding affects of morphine and cocaine (Galer et al., 2012, Hutchinson et al., 2012) and reduces sedative and ataxic effects of ethanol (EtOH) (Wu et al.). Additionally, TLR4 knockout mice display reduced ethanol-induced neuroinflammatory damage following chronic alcohol feeding (Pascual et al., 2011) and cultured microglia showed TLR4-dependent increases in pro-inflammatory cytokines in response to EtOH (Fernandez-Lizarbe et al., 2009). Intriguingly, none of these studies considered the metabolism of the drugs, leaving the potential that glucuronide or other metabolites could have contributed.

EtOH is primarily metabolized by conversion to acetaldehyde in the liver. Ethyl-β-D-glucuronide (EtG) is a minor ethanol metabolite with about 0.04% of an EtOH dose excreted as EtG (Goll et al., 2002). However, EtG has a much longer half-life than EtOH, and can be detected for up to 4 days after the elimination of EtOH from the body (Wurst et al., 2003). EtG has also been measured in the brain tissue and cerebrospinal fluid in a human who died due to acute alcohol intoxication (Wurst et al., 1999). Notably, a standard alcoholic drink has about 13 g EtOH, so even minor metabolites such as EtG can be present at physiologically relevant concentrations following heavy drinking. The inflammatory potential of EtG has not been previously investigated. However, the glucuronide component of the molecule, glucuronic acid (GA) alone can cause pain when injected intrathecally, which is blocked by the TLR4 antagonist (+)-naloxone (Lewis et al., 2010). EtG is structurally quite similar to GA, leading to the prediction that it may have similar TLR4 agonist effects.

We hypothesize that the TLR4 mediated nociceptive response to morphine-3-glucuronide described in Lewis et al. (2010) will generalize to other glucuronide molecules, namely GA and EtG. Further, we propose that EtOH will also have TLR4 agonist potential, as suggested by prior studies.

2. Methods

2.1. Drugs

Ethyl glucuronide (EtG) was purchased from Aurora Analytics (Baltimore, MD) and ethyl alcohol (EtOH) from Pharmco (Brookfield, CT). Sodium glucuronic acid (GA) was purchased from Sigma (St. Louis, MO). LPS-RS was purchased from Invivogen (Thousand Oaks, CA) and (+)-naloxone was obtained from the National Institute on Drug Abuse.

Endotoxin-free sterile water (Hospira, Lake Forest, IL) was used as a vehicle for all drugs *in vitro* (Experiment 2). Endotoxin-free, sterile 0.9% saline (Hospira, Lake Forest, IL) was used as a vehicle for all drugs in behavioral studies (Experiment 3). GA, EtG, EtOH, (+)-naloxone, saline and water were confirmed to be endotoxin-free by the limulus amoebocyte lysate assay (Lonza, Walkersville, MD) conducted per manufacturer's instructions. Where appropriate, doses are reported as a free base concentration.

2.2. In silico docking simulations

In silico docking simulation methods were identical to those previously described in detail (Hutchinson et al., 2010b). These methods were employed to examine the docking of gluconic acid (GA), ethyl glucuronide (EtG), and ethyl alcohol (EtOH) to the TLR4/MD-2 complex. The *in silico* docking analysis conducted in Experiment 1 used the recently published high-resolution crystalline structure of the dimer of human TLR4 and MD-2 (Park et al., 2009) and the *in silico* docking software suite AutoDock 4. Briefly, the complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). Modified pdb files were inputted into AutoDock 4.0 (<http://autodock.scripps.edu>), hydrogens added, and resaved in pdbqt format. GA and EtG structures were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator (<http://cactus.nci.nih.gov/services/translate/>). All dockings were executed with Lamarckian genetic algorithms.

2.3. In vitro assay for TLR4 signaling

A human embryonic kidney-293 (HEK 293) cell line was used in Experiment 2. This cell line was stably transfected by Invivogen (San Diego, CA) to over-express human TLR4 and coreceptor molecules (MD-2, CD14) (293-htr4a-md2cd14; referred to here as HEK-TLR4). In addition, these cells stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors, such as NF κ B and AP-1, activated as part of the TLR4 signaling cascade. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

HEK-TLR4 cells were grown at 37°C (5% CO₂; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selection [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5×10^3 cells/well) with the same media. After 48 h, supernatants were removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 μ l) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 μ l test samples were diluted in 45 μ l of 1x dilution buffer, transferred to 96-well plates (Thermo, Waltham, MA, USA), and heated at 65°C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 μ l/well) was added and, 5 min later, reaction buffer (50 μ l/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

2.4. In vivo behavioral assessments

2.4.1. Animals—Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used for all *in vivo* experiments. Rats (325–375 g at time of experiments) were housed in temperature (23 ± 3 °C) and light (12 h:12 h light:dark) controlled rooms with water and food given *ad libitum*. All habituation and behavioral testing procedures were performed during the light phase of the daily cycle. All procedures were approved by the University of Colorado-Boulder Institutional Animal Care and Use Committee. Each experimental group contains 6–8 rats.

2.4.2. Intrathecal catheter implantation and injections—All drugs administered *in vivo* in Experiment 3 were given intrathecally over lumbosacral spinal cord with acute intrathecal injections. Catheter placements via the L5/L6 intervertebral approach and drug microinjections were performed based on Milligan, et al. (1999). Rats were briefly anaesthetized under isoflurane anesthesia. An 18-gauge needle was placed between L5 and L6 into the intrathecal space and served as a guide. Polyethylene-10 tubing was threaded rostrally through the guide so to terminate over the lumbosacral enlargement. The catheters were pre-loaded with drugs at the intrathecal end and the remainder filled with sterile saline. Upon insertion, the drug was injected with a 8.5 μ l flush to ensure complete drug delivery, and the needle and catheter then removed. The rats were anaesthetized for, on average, 5 minutes and had no skin incision or implantations upon awakening.

GA was given at a dose of 0.32 μ g, consistent with previously published data showing that dose was capable of inducing pain following intrathecal injection (Lewis et al., 2010). EtG was given at a dose of 0.38 μ g, equimolar to the GA dose and also equimolar to a previously published dose of morphine-3-glucuronide that induced pain behaviors (Lewis et al., 2010). EtOH was also given equimolar to GA and EtG at 0.074 μ g. LPS-RS was given at an intrathecal dose of 40 μ g, consistent with previous literature showing this dose was capable of reversing neuropathic pain (Hutchinson et al., 2008).

2.4.3. von Frey test for tactile allodynia—All testing was conducted by an experimenter blind to group assignment. Rats received at least four 1 h habituations to the appropriate testing apparatus and environment prior to baseline testing, as in our previous studies (Lewis et al., 2010).

The response threshold to light touch on both plantar hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as described in Milligan et al. (2001). Briefly, a logarithmic range of hairs from 0.406–15.136 g force were used, allowing both analgesia and allodynia to be measured, following the standard up-down procedures previously described (Chaplan et al., 1994). Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described in Milligan et al. (2001).

2.5. Statistics

GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all statistical analyses. One-way ANOVAs with appropriate Bonferroni post hoc tests were used to compare experimental groups on *in vitro* experiments and to confirm that there were no baseline differences on behavioral measures. Two-way repeated-measures ANOVAs with Bonferroni posthoc tests when appropriate were used to determine statistical significance between behavioral measures. For all analyses, $p < 0.05$ was considered significant.

2.6. Experimental Procedures

2.6.1. Experiment 1: Does *in silico* docking predict TLR4-MD2 binding of GA, EtG and/or EtOH?—Initially, the *in silico* docking of GA and EtG to the entire TLR4 MD-2 dimer complex was conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). Additionally, all the ligands were docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized and the residues on MD-2 that interacted with that conformation determined for each of the experimental drugs.

2.6.2. Experiment 1B: For drugs predicted to dock to the TLR4/MD-2 complex in Experiment 1A, would previously docked (+)-naloxone reduce the likelihood of their *in silico* docking?—The (+)-naloxone conformation interacting with MD-2 from a previous study (Lewis et al., 2010) was saved as a combined MD-2/(+)-naloxone pdb file and the *in silico* docking was repeated on the combined MD-2/(+)-naloxone complex to determine the change in docking due to the presence of already docked (+)-naloxone.

2.6.3. Experiment 2A: Does *in vitro* GA, EtG or EtOH cause an increase in TLR4-dependent SEAP expression in HEK-TLR4 cells?—The experimental drugs, GA, EtG and EtOH, were each applied to HEK-TLR4 cells at concentrations of 0, 1, 10 or 100 μM as described above. Cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4-induced NF κ B activity.

2.6.4. Experiment 2B: Is the *in vitro* increase in TLR4-dependent SEAP expression seen in Experiment 2A blocked by the TLR4 antagonists LPS-RS and/or (+)-naloxone?—For drugs which increase HEK-TLR4 SEAP production in Experiment 2A, the most effective dose (100 μM for each) was applied to HEK-TLR4 cells as described above. Additionally, one of two TLR4 antagonists was coadministered. One, lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS, Invivogen, San Diego, CA), lacks functional TLR4 activity and acts as a competitive TLR4 antagonist. LPS-RS is the antagonist recommended by Invivogen for tests of TLR4 specificity (Invivogen technical support). LPS-RS was applied to the HEK-TLR4 cells at the same time as the GA, EtG or EtOH at concentrations of 0, 0.1, 1 or 10 ng/ml. The other, (+)-naloxone, is the opioid-inactive isomer of naloxone and has been shown to inhibit LPS-induced increases in HEK-TLR4 signaling (Hutchinson et al., 2010b). We have previously reported that (+)-naloxone does not suppress non-TLR4 signaling (Hutchinson et al., 2010b) and acts as a competitive inhibitor at MD2, the key coreceptor of TLR4 (Northcutt et al., manuscript in preparation). (+)-Naloxone was applied to the HEK-TLR4 cells at the same time as the GA, EtG or EtOH at concentrations of 0, 1, 10 or 100 μM . For all assays, cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4 activity.

2.6.5. Experiment 3: Does intrathecal GA, EtG and/or EtOH cause tactile allodynia? Is that allodynia TLR4 dependent?—Rats received either 0.32 μg GA, 0.38 μg EtG or 0.074 μg EtOH or equivolume (1 μl) saline vehicle via an acute intrathecal injection, as described above. Both injections had a saline flush for a total injection volume of 9.5 μl . Rats were tested for tactile allodynia 1 d prior to injection (baseline; BL) and again 1 and 3 h post-injection.

The drugs that produced significant allodynia (in this case, GA and EtG) were coadministered with the TLR4 antagonist LPS-RS. Rats received either 0.32 μ g GA or 0.38 μ g EtG or equivolume (1 μ l) saline with 40 μ g LPS-RS via an acute intrathecal injection.

3. Results

3.1 Experiment 1A: *In silico* modeling predicts that GA and EtG will bind to MD-2

An *in silico* docking study was done to determine if GA, EtG and/or EtOH would be predicted to dock to the TLR4/MD-2 complex and whether they interacted at the same residues as the TLR4 antagonist (+)-naloxone. This *in silico* technique has been successfully used previously to model the docking of compounds, including opioids, morphine-3-glucuronide, and tricyclic antidepressants and to predict whether the compounds would act as TLR4 agonists *in vitro* and *in vivo* (Hutchinson et al., 2010a, Hutchinson et al., 2010b, Lewis et al., 2010).

The EtOH molecule was too small to be effectively modeled in this paradigm. The *in silico* docking of GA or EtG to the entire human TLR4 MD-2 dimer complex was initially conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). These data demonstrated that the two ligands docked with human MD-2 independent of human TLR4 interactions. Therefore, the ligands were then docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized for GA and EtG and the residues that interacted with each compound determined (Table 1). The residues at which lipid A chains interacted in previously published crystallography studies (Park et al., 2009) are also included for comparison on Table 1.

Both GA and EtG were predicted to dock to MD-2, as demonstrated by negative predicted binding energies. EtG interacted at 7 residues, 6 of which were residues also known to interact with the lipid A moiety of lipopolysaccharide. GA interacted at 8 residues on MD-2, only 1 of which was in common with lipid A interactions. GA largely interacted with MD-2 at residues distant from the pockets where lipid A interacts.

3.2 Experiment 1B: (+)-naloxone alters the predicted MD-2 docking conformations of EtG and GA *in silico*

Given prior evidence that (+)-naloxone was able to block GA-induced pain (Lewis et al., 2010), we hypothesized that GA and the structurally similar EtG would dock such that when (+)-naloxone was docked to MD-2, there would be a lower probability of GA or EtG docking. The *in silico* docking analysis performed in Experiment 1A was repeated using a saved combined MD-2/(+)-naloxone pdb file to determine the change in docking due to the presence of already docked (+)-naloxone. The residues at which the lowest binding energy conformation of EtG docked to the MD-2 were altered when (+)-naloxone was bound (Table 1). In contrast, the residues at which the lowest binding energy conformation of GA docked were not altered by (+)-naloxone. Given prior evidence that (+)-naloxone was able to reverse GA-induced allodynia (Lewis et al., 2010), the *in silico* data here does not predict, in any obvious manner, the physiological effect observed. This discrepancy may be explained by a second conformation, other than the lowest binding energy conformation, being responsible for the physiological effect, an allosteric effect on MD-2/TLR4 interactions, differences in human and rat MD-2/TLR4 structure, or inverse agonist actions of (+)-naloxone at MD-2/TLR4, but such a distinction is beyond the capabilities of this *in silico* analysis.

3.2. Experiment 2A: In vitro TLR4-dependent SEAP expression in HEK-TLR4 cells is increased by GA, EtG and EtOH

Experiment 1 provided initial evidence suggesting that EtG and GA may have effects on TLR4. In order to determine if these effects were present in a cellular system, HEK-TLR4 cells were utilized. As detailed in the methods, HEK-TLR4 cells have been transfected with TLR4 and a NF κ B-dependent SEAP reporter gene. Thus, an increase in TLR4 signaling will lead to an increase in NF κ B transcription and subsequent increase in SEAP levels measured. Previous studies have confirmed that HEK cells with the SEAP reporter gene but without TLR4 overexpression did not increase SEAP expression due to the classic TLR4 agonist LPS (Hutchinson et al., 2010b). A dose-response function of TLR4 signaling changes for GA, EtG and EtOH was performed.

GA caused a significant increase in SEAP production in the HEK-TLR4 cells ($F=19.16$, $p<0.05$). Post-hoc tests showed that GA increased SEAP expression relative to vehicle at the 10 μ M and 100 μ M concentrations, but was not significantly different from vehicle at lower concentrations (Figure 1A). Similarly, EtG caused a significant increase in SEAP expression in HEK-TLR4 cells ($F=19.44$, $p<0.05$). Post-hoc tests showed that EtG increased SEAP expression relative to vehicle at the 10 and 100 μ M concentrations, but caused no significant changes at the lower concentrations (Figure 1B). Finally, EtOH also caused an increase in SEAP production in the HEK-TLR4 cells ($F=22.18$, $p<0.05$). However, Post-hoc tests showed that only the 100 μ M concentration of EtOH was significantly increased relative to vehicle, while the 0.01, 0.1, 1 and 10 μ M concentrations were not significantly different from the vehicle.

3.3. Experiment 2B: In vitro GA, EtG and EtOH induced increases in SEAP expression are blocked by the TLR4 antagonists LPS-RS and (+)-naloxone

Experiment 2A provides evidence that GA, EtG and EtOH were able to increase NF κ B-dependent SEAP expression in HEK-TLR4 cells. Here, the specificity of that response was tested by blocking the GA, EtG and EtOH-induced SEAP expression with each of two TLR4 antagonists. The first, LPS-RS, is structurally similar to LPS but fails to produce a TLR4-dependent proinflammatory response. It acts as a competitive antagonist of the LPS effects on TLR4 complex and has been shown to reduce the increase in SEAP expression caused by another glucuronidated metabolite, morphine-3-glucuronide (Lewis et al., 2010). The second, (+)-naloxone, has also been shown to inhibit the LPS response *in vitro* (Hutchinson et al., 2008, Hutchinson et al., 2010b). Further, (+)-naloxone was able to block the increase in HEK-TLR4 SEAP expression caused by morphine-3-glucuronide (Hutchinson et al., 2010b) as well as the *in vivo* pain responses caused by both morphine-3-glucuronide and GA (Lewis et al., 2010). (+)-Naloxone is a selective TLR4 antagonist as it has been screened across 68 different central nervous system receptors, second messengers, enzymes, and neurotransmitter transporters with no non-TLR4 effects observed (Hutchinson et al., 2012). The *in silico* results of Experiment 1 further predict that (+)-naloxone would be able to block EtG-induced HEK-TLR4 cell SEAP expression.

LPS-RS effectively blocked the increase in HEK-TLR4 SEAP expression caused by GA ($F=7.51$, $p<0.05$, Figure 2A), EtG ($F=7.99$, $p<0.05$, Figure 2C), and EtOH ($F=30.0$, $p<0.05$, Figure 2E). Post-hoc tests showed that when 100 μ M GA was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to 100 μ M GA alone. Similarly, when 100 μ M EtG was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to 100 μ M EtG alone. Finally, when 100 μ M EtOH was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to 100 μ M EtOH alone.

(+)-Naloxone was also able to block the increase in HEK-TLR4 SEAP expression caused by 100 μ M GA ($F=5.36$, $p<0.05$, Figure 2B), EtG ($F=4.92$, $p<0.05$, Figure 2D) or EtOH ($F=8.36$, $p<0.05$, Figure 2F). Post-hoc tests showed that when 100 μ M GA was incubated with 1, 10 and 100 μ M (+)-naloxone, SEAP expression was significantly decreased compared to 100 μ M GA alone. Similarly, when 100 μ M EtG was incubated with 1 or 10 μ M (+)-naloxone, SEAP expression was significantly decreased compared to 100 μ M EtG alone. Finally, when 100 μ M EtOH was incubated with 1 and 10 μ M (+)-naloxone, SEAP expression was significantly decreased compared to 100 μ M EtOH alone.

3.4. Experiment 3: Intrathecal GA and EtG cause tactile allodynia

The *in silico* and *in vitro* studies, above, strongly suggest that GA, EtG and EtOH are capable of activating TLR4. Previous studies have shown that other glucuronide metabolites that can act as TLR4 agonists are able to produce pain *in vivo* (Lewis et al., 2010). Additionally, previous studies have shown that GA was able to cause pain *in vivo* through TLR4-dependent mechanisms (Lewis et al., 2010). To confirm the nociceptive capability of GA and to determine if EtG and/or EtOH are able to cause allodynia, GA, EtG, EtOH or vehicle were injected intrathecally at equimolar doses and allodynia tested 1 and 3 h following injection. To test whether this TLR4 signaling activation was necessary for the development of GA and EtG-induced pain, LPS-RS was coadministered intrathecally with those drugs that caused a significant allodynia to develop. Intrathecal LPS-RS has been shown to block neuropathic pain for a minimum of 3 h after an intrathecal injection (Hutchinson et al., 2008). Experiment 2B further indicated that LPS-RS was able to block the TLR4 signaling caused by both GA and EtG *in vitro*.

In Experiment 3, no differences between right and left hind paws were detected. Thus, results are reported as the average of the response thresholds recorded for both paws. There were also no baseline differences between any groups ($F=0.458$, $p>0.05$).

Following intrathecal injection, GA caused significant allodynia ($F=17.1$, $p<0.05$, Figure 3A). Post-hoc test showed that 0.32 μ g GA i.t. produced a robust decrease in tactile threshold relative to vehicle 1 and 3 h following injection, replicating the results of Lewis et al. (2010). The TLR4 antagonist LPS-RS was able to block the development of allodynia at the 3 h time point.

EtG (0.38 μ g, i.t.) also produced a robust decrease in tactile threshold ($F=16.1$, $p<0.05$, Figure 3B). Post-hoc tests showed significant allodynia compared to vehicle at both 1 and 3 h following intrathecal injection. This allodynia was blocked by LPS-RS at 3 h post injection. EtOH (0.07 μ g, i.t.) failed to produce a significant change in tactile threshold relative to vehicle ($F=0.204$, $p>0.05$, Figure 3C).

4. Discussion

The present series of studies extend our prior observation of TLR4-dependent pain enhancement by the morphine metabolite, morphine-3-glucuronide (Lewis et al., 2010), by investigating whether such TLR4-dependent effects generalize to glucuronic acid (GA) itself or to other glucuronidated metabolites. These studies present the first evidence that GA and the long-lived ethanol metabolite, Ethyl- β -D-glucuronide (EtG) each activate TLR4 signaling *in vitro* and produce a TLR4-activity dependent allodynia *in vivo*. In addition, our *in silico* modeling based on the recently published crystal structure of TLR4 bound to its co-receptor MD-2 (Park et al., 2009) predicted that EtG would bind to MD-2 at a site in the same region as the recently documented TLR4 antagonist, (+)-naloxone (Hutchinson et al., 2010b). Further, the *in silico* docking of EtG to MD-2 was in a manner that was displaced by the prior docking of (+)-naloxone. These *in silico* results were substantiated *in vitro* using a

HEK-TLR4 cell line that was stably transfected to produce a reporter protein in response to TLR4 activation. Studies of these cells revealed that GA, EtG and EtOH induced increases in HEK-TLR4 reporter protein expression were blocked by both (+)-naloxone and the classical TLR4 antagonist, LPS-RS. Lastly, while EtOH was able to increase HEK-TLR4 SEAP expression, it did not enhance pain following an intrathecal injection at a dose equimolar to EtG and GA.

The seemingly contradictory finding that EtOH increased HEK-TLR4 cell reporter protein expression *in vitro*, but failed to cause allodynia when administered intrathecally, may arise from differences in *in vitro* versus *in vivo* conditions. First, EtOH was injected intrathecally at a dose equimolar to allodynia-inducing doses of GA and EtG. Thus EtOH was delivered at a dose of 0.074 μ g. It is possible that with such a small dose, there was not sufficient diffusion into spinal tissues from cerebrospinal fluid (CSF) and/or that EtOH so rapidly diffuses out of the central nervous system that there was insufficient exposure of lumbar dorsal horn to create a detectable change in pain threshold. While a higher dose may potentially cause significant allodynia, based on the HEK-TLR4 results, a higher intrathecal dose of EtOH was not tested here both because the strategy was to compare equimolar doses and because allodynia by higher doses had the potential to result from confounding toxicity of alcohol on central nervous system tissues. A second possible explanation for the apparent contradictory findings for EtOH is that EtOH also enhances the inhibitory function of GABA_A receptors (Lobo and Harris, 2008) which could counteract a TLR4-induced increase in neuronal excitability.

The rapid onset of pain behaviors seen with intrathecal injection is consistent with both the documented existence of pre-existing pools of proinflammatory cytokine protein in spinal cord as measured by ELISA (for example, (Yao et al., 2011, Christianson et al., 2012, Jin et al., 2012, Sun et al., 2012)) and with previous studies of intrathecally administered LPS and other TLR4 agonists. LPS has been shown to induce allodynia from 20 min–3 hours following intrathecal injection in rats (Saito et al., 2010, Loram et al., 2011), and 1–6 hours following intrathecal injection in male mice (Sorge et al., 2011). The glucuronidated metabolite morphine-3-glucuronide, a TLR4 agonist, can induce thermal hyperalgesia within 15 min and mechanical allodynia within 3 hours (Lewis et al., 2010). Current data support that these rapid effects are mediated via the release of proinflammatory cytokines. Previous studies report increased levels of several proinflammatory cytokines, including IL-1 β and IL-6 protein in the dorsal spinal cord at 1 and 4 hours following intrathecal LPS (Loram et al., 2012) and increases in IL-1 β in the CSF (indicative of IL-1 β release) at 1 hour after intrathecal morphine-3-glucuronide (Lewis et al., 2010). Intrathecal LPS also elevates tumor necrosis factor-alpha (TNF) in CSF within 2 hr (earliest timepoint tested) (Saito et al., 2010). That these rapid changes in proinflammatory cytokines can mediate pain enhancement is supported by the finding that interleukin-1 receptor antagonist (IL-1ra) and entanercept (TNF antagonist) prevent TLR4-dependent pain behaviors when co-administered with intrathecal LPS (Saito et al., 2010), and IL-1ra can reverse or prevent pain behaviors following intrathecal morphine-3-glucuronide (Lewis et al., 2010). Given that co-administration of IL-1ra blocks expression of pain enhancement as early as 15 minutes after administration of the TLR4 agonist (Lewis et al., 2010) provides further evidence for the rapid involvement of proinflammatory cytokines in TLR4-dependent pain behaviors. The cell type(s) involved in this rapid (1–3 h) response also remains unclear. Many cell types in the CNS have been reported to respond to LPS or other TLR4 agonists, including but not limited to immune cells in the meninges (Wiesler et al., 2006), CNS endothelial cells (Ramos et al., 2011), microglia (Loram et al., 2012), astrocytes (Chung and Benveniste, 1990), and neurons (Maroso et al., 2010, Due et al., 2012, Leow-Dyke et al., 2012), any of which could be involved in the effects observed here. Clearly, had systemic administration

been employed, instead of intrathecal, additional cell types may have responded to such TLR4 agonists.

The multiple mechanisms by which TLR4 stimulation may potentially lead to enhanced nociception is a topic of ongoing investigation. In addition to the known role of proinflammatory cytokines, another potential pathway is direct neuronal stimulation. TLR4 activation of dorsal root ganglion sensory neurons by LPS or morphine-3-glucuronide has been shown to lead to an increase in voltage-gated sodium currents, intracellular calcium and frequency of action potentials, all findings that could contribute to the enhanced nociception seen in the present study (Due et al., 2012). Whether that would occur following intrathecal administration of TLR4 agonists, as in the present case is unknown, but could well be a contributor to pain enhancement were such TLR4 agonists delivered systemically. LPS stimulation of TLR4 on nociceptive neurons has also been shown to sensitize TRPV1 responses to capsaicin, including increased intra-cellular calcium and calcitonin-related gene peptide (CGRP) (Diogenes et al., 2011). The exact mechanism by which LPS binding on neuronal TLR4 receptors leads to rapid (seconds-to-minutes) ion current changes is not yet known, as recognized by authors in the field (Due et al., 2012). Ethanol has also been linked to enhanced TRPV1-induced cation currents in response to capsaicin on peripheral sensory nerves, and subsequent release of substance P in the dorsal spinal cord, skin, esophagus (Trevisani et al., 2002), bronchi (Trevisani et al., 2004) suggesting a potential role for TRPV1 in initiating inflammatory responses and enhanced nociception following ethanol administration. Ethanol activation of TRPV1 channels on peripheral neurons in the stomach has also been linked to increased substance P and CGRP release and radical oxygen species generation (Gazzieri et al., 2007), and ethanol has been shown to activate TRPV1 in coronary arteries leading to the release of CGRP and coronary artery dilation (Gazzieri et al., 2006). The ethanol metabolite acetaldehyde has been linked to TRPA1 channel activation in peripheral sensory neurons and an increase in nociceptive behaviors following injection into the paw (Bang et al., 2007), and TRPA1 activation has been shown to lead to CGRP release in peripheral meningeal neurons and enhanced meningeal blood flow (Kunkler et al., 2011). TRPA1 agonist application to the dura also leads to enhanced pain behaviors (Edelmayer et al., 2012). Whether these ethanol-linked effects would occur with glucuronidated metabolites of alcohol is unknown.

The disparity between the length of time to develop a SEAP signal in vitro and the length of time to the onset of pain behaviors in vivo is not necessarily surprising given the very different environments and end points. There are several potential explanations for this disparity. First, the threshold for physiologic effect may be quite different than the threshold for detection in the SEAP assay. Second, numerous studies have shown rapid release of cytokines from cells in the CNS (Milligan et al., 2001, Saito et al., 2010, Loram et al., 2012), suggesting that these molecules are not synthesized de novo following stimulation in vivo, while the HEK-TLR4 SEAP assay requires a change in gene expression. Finally, the cellular environment and cell type (HEK cell vs. CNS cells in situ), including costimulatory molecule expression, cell surface molecules and second signals, are remarkably different in vivo than in vitro, and thus the response of the cells to TLR4 signaling is likely to also be quite different.

One important question is whether the TLR4-dependent effects of EtG might impact the known symptoms of EtOH use. In a model based on 75 g of EtOH ingestion, serum EtOH peaks 1–2 hours following the end of drinking, while serum EtG peaks approximately 6 hours after the end of drinking (Droenner et al., 2002). Little data are available on CSF levels of EtG, although EtG was detected at 0.31 µg/ml in a human who died due to acute alcohol intoxication (Wurst et al., 1999). Notably, several pain-related effects of EtOH in humans, such as alcohol withdrawal hyperalgesia (Jochum et al., 2010) and headache after

both chronic (Martinotti et al., 2010) and acute (e.g. hangover, (Howland et al., 2008)) EtOH ingestion, are associated not with high levels of EtOH, but with declining levels of EtOH. Additionally, withdrawal from chronic alcohol exposure in rats has been linked to an increase in microglial activation markers and allodynia (Narita et al., 2007). EtG concentrations peak while EtOH is declining, meaning that EtG levels are highest when these negative side effects are observed. Many alternative hypotheses for these pain-producing side effects have been postulated; however, the exact cause is unknown. While further work is needed to determine if EtG contributes to the negative side effects of EtOH, the potential exists and warrants additional study.

While EtOH is largely an exogenous drug, with only very small amounts made endogenously, GA is principally found endogenously throughout the body. However, it is usually either conjugated to form uridine 5'-diphosphoglucuronic acid, the substrate for the glucuronyltransferases that form glucuronidated metabolites, or polymerized with N-acetyl-D-glucosamine to form the extracellular matrix component hyaluronan. Hyaluronan polymers have been shown to have anti-inflammatory properties (Huang et al., 2011). However, smaller, low molecular weight hyaluronan polymers have been shown to activate TLR4 (Li et al., 2010, Leu et al., 2011). This finding is of particular significance in several pathological conditions, including pulmonary arterial hypertension (Ormiston et al., 2010), atherosclerosis (Cuff et al., 2001), and rheumatoid arthritis (Naor and Nedvetzki, 2003), where low molecular weight hyaluronan and its adhesion receptor CD44 have been associated with increased inflammation and disease progression. The studies here suggest that the glucuronide component of hyaluronan may contribute to the inflammatory effects of hyaluronan, and also suggest that TLR4 be considered as an additional candidate receptor for these inflammatory effects.

EtG and GA are structurally very similar. A logical question is whether the modification of GA by adding an ethyl group alters the *in silico*, *in vitro* and *in vivo* effects observed. *In silico*, EtG and GA appear to interact with different residues on MD-2, suggesting that there may be differences. However, *in vitro* and *in vivo* the results for the two drugs were not significantly different and in fact quite similar. It appears the addition of the ethyl group to glucuronic acid did not dramatically alter the effects observed, although it may have altered the most likely predicted docking site *in silico*.

These studies present evidence that the glucuronide molecules GA and EtG may produce pain through TLR4-dependent mechanisms. EtOH also had potential TLR4 activity, but did not cause pain in this particular *in vivo* model. The finding that GA and EtG can cause TLR4-dependent pain in and of themselves has potentially wide-ranging implications for conditions such as hangover, chronic alcohol withdrawal headache and several disorders, such as rheumatoid arthritis and atherosclerosis, linked to low molecular weight hyaluronan. Additionally, the TLR4-dependent pain effect of morphine-3-glucuronide has now generalized to additional classes of molecules, including glucuronic acid alone and ethyl glucuronide. This finding suggests that other classes of molecules that are glucuronidated, such as steroid hormones and non-steroidal anti-inflammatories, could also have TLR4-dependent pain producing effects.

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References

- Bang S, Kim KY, Yoo S, Kim YG, Hwang SW. Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. *The European journal of neuroscience*. 2007; 26:2516–2523. [PubMed: 17970723]
- Bsibsi M, Ravid R, Gveric D, van Noort JM. Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol*. 2002; 61:1013–1021. [PubMed: 12430718]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994; 53:55–63. [PubMed: 7990513]
- Christianson CA, Fitzsimmons BL, Shim JH, Agrawal A, Cohen SM, Hua XY, Yaksh TL. Spinal matrix metalloproteinase 3 mediates inflammatory hyperalgesia via a tumor necrosis factor-dependent mechanism. *Neuroscience*. 2012; 200:199–210. [PubMed: 22056600]
- Chung IY, Benveniste EN. Tumor necrosis factor- α production by astrocytes. Induction by lipopolysaccharide, IFN- γ , and IL-1 β . *Journal of immunology*. 1990; 144:2999–3007.
- Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Pure E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J Clin Invest*. 2001; 108:1031–1040. [PubMed: 11581304]
- Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *Journal of dental research*. 2011; 90:759–764. [PubMed: 21393555]
- Droenner P, Schmitt G, Aderjan R, Zimmer H. A kinetic model describing the pharmacokinetics of ethyl glucuronide in humans. *Forensic Sci Int*. 2002; 126:24–29. [PubMed: 11955827]
- Due MR, Piekarz AD, Wilson N, Feldman P, Ripsch MS, Chavez S, Yin H, Khanna R, White FA. Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *Journal of neuroinflammation*. 2012; 9:200. [PubMed: 22898544]
- Edelmayer RM, Le LN, Yan J, Wei X, Nassini R, Materazzi S, Preti D, Appendino G, Geppetti P, Dodick DW, Vanderah TW, Porreca F, Dussor G. Activation of TRPA1 on dural afferents: a potential mechanism of headache pain. *Pain*. 2012; 153:1949–1958. [PubMed: 22809691]
- Fernandez-Lizarbe S, Pascual M, Guerri C. Critical role of TLR4 response in the activation of microglia induced by ethanol. *J Immunol*. 2009; 183:4733–4744. [PubMed: 19752239]
- Galer EL, Northcutt AL, Cochran TA, Hutchinson MR, Miles NE, Rozeske RR, Amat J, Maier SF, Rice KC, Watkins LR. A role for TLR4 and proinflammatory signaling in neurochemical measures of cocaine reward. *Proc Society for Neurosciences*. 2012
- Gazzieri D, Trevisani M, Springer J, Harrison S, Cottrell GS, Andre E, Nicoletti P, Massi D, Zecchi S, Nosi D, Santucci M, Gerard NP, Lucattelli M, Lungarella G, Fischer A, Grady EF, Bunnett NW, Geppetti P. Substance P released by TRPV1-expressing neurons produces reactive oxygen species that mediate ethanol-induced gastric injury. *Free radical biology & medicine*. 2007; 43:581–589. [PubMed: 17640568]
- Gazzieri D, Trevisani M, Tarantini F, Bechi P, Masotti G, Gensini GF, Castellani S, Marchionni N, Geppetti P, Harrison S. Ethanol dilates coronary arteries and increases coronary flow via transient receptor potential vanilloid 1 and calcitonin gene-related peptide. *Cardiovascular research*. 2006; 70:589–599. [PubMed: 16579978]
- Goll M, Schmitt G, Ganssmann B, Aderjan RE. Excretion profiles of ethyl glucuronide in human urine after internal dilution. *J Anal Toxicol*. 2002; 26:262–266. [PubMed: 12166812]
- Howland J, Rohsenow DJ, Allensworth-Davies D, Greece J, Almeida A, Minsky SJ, Arnedt JT, Hermos J. The incidence and severity of hangover the morning after moderate alcohol intoxication. *Addiction*. 2008; 103:758–765. [PubMed: 18412754]
- Huang TL, Hsu HC, Yang KC, Lin FH. Hyaluronan up-regulates IL-10 expression in fibroblast-like synoviocytes from patients with tibia plateau fracture. *J Orthop Res*. 2011; 29:495–500. [PubMed: 20957732]
- Hutchinson MR, Loram LC, Zhang Y, Shridhar M, Rezvani N, Berkelhammer D, Phipps S, Foster PS, Landgraf K, Falke JJ, Rice KC, Maier SF, Yin H, Watkins LR. Evidence that tricyclic small molecules may possess toll-like receptor and myeloid differentiation protein 2 activity. *Neuroscience*. 2010a; 168:551–563. [PubMed: 20381591]

- Hutchinson MR, Northcutt AL, Hiranita T, Wang X, Lewis SS, Thomas J, van Steeg K, Kopajtic TA, Loram LC, Sfregola C, Galer E, Miles NE, Bland ST, Amat J, Rozeske RR, Maslanik T, Chapman TR, Strand KA, Fleshner M, Bachtell RK, Somogyi AA, Yin H, Katz JL, Rice KC, Maier SF, Watkins LR. Opioid Activation of Toll-Like Receptor 4 Contributes to Drug Reinforcement. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2012; 32:11187–11200. [PubMed: 22895704]
- Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, Sholar PW, Patel SJ, Crysdale NY, Harrison JA, Maier SF, Rice KC, Watkins LR. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci*. 2008; 28:20–29. [PubMed: 18662331]
- Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR. Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav Immun*. 2010b; 24:83–95. [PubMed: 19679181]
- Jin H, Li YH, Xu JS, Guo GQ, Chen DL, Bo Y. Lipoxin A4 analog attenuates morphine antinociceptive tolerance, withdrawal-induced hyperalgesia, and glial reaction and cytokine expression in the spinal cord of rat. *Neuroscience*. 2012; 208:1–10. [PubMed: 22366510]
- Jochum T, Boettger MK, Burkhardt C, Juckel G, Bar KJ. Increased pain sensitivity in alcohol withdrawal syndrome. *Eur J Pain*. 2010; 14:713–718. [PubMed: 20018536]
- Komatsu T, Sakurada S, Kohno K, Shiohira H, Katsuyama S, Sakurada C, Tsuzuki M, Sakurada T. Spinal ERK activation via NO-cGMP pathway contributes to nociceptive behavior induced by morphine-3-glucuronide. *Biochem Pharmacol*. 2009; 78:1026–1034. [PubMed: 19589334]
- Kunkler PE, Ballard CJ, Oxford GS, Hurley JH. TRPA1 receptors mediate environmental irritant-induced meningeal vasodilatation. *Pain*. 2011; 152:38–44. [PubMed: 21075522]
- Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A*. 2003; 100:8514–8519. [PubMed: 12824464]
- Leow-Dyke S, Allen C, Denes A, Nilsson O, Maysami S, Bowie AG, Rothwell NJ, Pinteaux E. Neuronal toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration in vitro. *Journal of neuroinflammation*. 2012; 9:230. [PubMed: 23034047]
- Leu SW, Shi L, Xu C, Zhao Y, Liu B, Li Y, Shiedlin A, Xiang C, Shen H, Quinn DA, Hales CA, Zhao H. TLR4 through IFN-beta promotes low molecular mass hyaluronan-induced neutrophil apoptosis. *J Immunol*. 2011; 186:556–562. [PubMed: 21098223]
- Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Zhang Y, Maier SF, Rice KC, Watkins LR. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. *Neuroscience*. 2010; 165:569–583. [PubMed: 19833175]
- Li Z, Potts EN, Piantadosi CA, Foster WM, Hollingsworth JW. Hyaluronan fragments contribute to the ozone-primed immune response to lipopolysaccharide. *J Immunol*. 2010; 185:6891–6898. [PubMed: 21037098]
- Lobo IA, Harris RA. GABA(A) receptors and alcohol. *Pharmacol Biochem Behav*. 2008; 90:90–94. [PubMed: 18423561]
- Loram LC, Sholar PW, Taylor FR, Wiesler JL, Babb JA, Strand KA, Berkelhammer D, Day HE, Maier SF, Watkins LR. Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats. *Psychoneuroendocrinology*. 2012; 37:1688–1699. [PubMed: 22497984]
- Loram LC, Taylor FR, Strand KA, Frank MG, Sholar P, Harrison JA, Maier SF, Watkins LR. Prior exposure to glucocorticoids potentiates lipopolysaccharide induced mechanical allodynia and spinal neuroinflammation. *Brain, behavior, and immunity*. 2011; 25:1408–1415.
- Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. *Nature medicine*. 2010; 16:413–419.
- Martinotti G, di Nicola M, Frustaci A, Romanelli R, Tedeschi D, Guglielmo R, Guerriero L, Bruschi A, De Filippis R, Pozzi G, Di Giannantonio M, Bria P, Janiri L. Pregabalin, tiapride and

- lorazepam in alcohol withdrawal syndrome: a multicentre, randomized, single-blind comparison trial. *Addiction*. 2010; 105:288–299. [PubMed: 20078487]
- Milligan ED, Hinde JL, Mehmert KK, Maier SF, Watkins LR. A method for increasing the viability of the external portion of lumbar catheters placed in the spinal subarachnoid space of rats. *J Neurosci Methods*. 1999; 90:81–86. [PubMed: 10517276]
- Milligan ED, O'Connor KA, Nguyen KT, Armstrong CB, Twining C, Gaykema RP, Holguin A, Martin D, Maier SF, Watkins LR. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *J Neurosci*. 2001; 21:2808–2819. [PubMed: 11306633]
- Naor D, Nedvetzki S. CD44 in rheumatoid arthritis. *Arthritis Res Ther*. 2003; 5:105–115. [PubMed: 12723975]
- Narita M, Miyoshi K, Suzuki T. Involvement of microglia in the ethanol-induced neuropathic pain-like state in the rat. *Neurosci Lett*. 2007; 414:21–25. [PubMed: 17284346]
- Ormiston ML, Slaughter GR, Deng Y, Stewart DJ, Courtman DW. The enzymatic degradation of hyaluronan is associated with disease progression in experimental pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2010; 298:L148–157. [PubMed: 19915162]
- Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*. 2009; 458:1191–1195. [PubMed: 19252480]
- Pascual M, Balino P, Alfonso-Loeches S, Aragon CM, Guerri C. Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. *Brain Behav Immun*. 2011
- Ramos K, Morgan KN, Lewis MT, Giganti PJ, Huey MG, Maier SF, Watkins LR. CNS endothelial cells are a critical cell type in mediating opioid-induced CNS inflammation: interaction with glia and peripheral immune cells. *Proc Society for Neurosciences*. 2011
- Saito O, Svensson CI, Buczynski MW, Wegner K, Hua XY, Codeluppi S, Schaloske RH, Deems RA, Dennis EA, Yaksh TL. Spinal glial TLR4-mediated nociception and production of prostaglandin E(2) and TNF. *Br J Pharmacol*. 2010; 160:1754–1764. [PubMed: 20649577]
- Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS. Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2011; 31:15450–15454. [PubMed: 22031891]
- Sun S, Yin Y, Yin X, Cao F, Luo D, Zhang T, Li Y, Ni L. Anti-nociceptive effects of Tanshinone IIA (THIA) in a rat model of complete Freund's adjuvant (CFA)-induced inflammatory pain. *Brain research bulletin*. 2012; 88:581–588. [PubMed: 22705002]
- Trevisani M, Gazzieri D, Benvenuti F, Campi B, Dinh QT, Groneberg DA, Rigoni M, Emonds-Alt X, Creminon C, Fischer A, Geppetti P, Harrison S. Ethanol causes inflammation in the airways by a neurogenic and TRPV1-dependent mechanism. *The Journal of pharmacology and experimental therapeutics*. 2004; 309:1167–1173. [PubMed: 14762103]
- Trevisani M, Smart D, Gunthorpe MJ, Tognetto M, Barbieri M, Campi B, Amadesi S, Gray J, Jerman JC, Brough SJ, Owen D, Smith GD, Randall AD, Harrison S, Bianchi A, Davis JB, Geppetti P. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nature neuroscience*. 2002; 5:546–551.
- Wang X, Loram LC, Ramos K, de Jesus AJ, Thomas J, Cheng K, Reddy A, Somogyi AA, Hutchinson MR, Watkins LR, Yin H. Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:6325–6330. [PubMed: 22474354]
- Wiesler JL, Jekich B, Mahoney J, Lewis SS, Coats B, Maier SF, Watkins LR. Considering the role of spinal cord meninges in exaggerated pain states. *Proc Society for Neurosciences*. 2006
- Wu Y, Lousberg EL, Moldenhauer LM, Hayball JD, Collier JK, Rice KC, Watkins LR, Somogyi AA, Hutchinson MR. Inhibiting the TLR4-MyD88 signalling cascade by genetic or pharmacological strategies reduces acute alcohol-induced sedation and motor impairment in mice. *British journal of pharmacology*. 2012; 165:1319–1329. [PubMed: 21955045]

- Wurst FM, Schuttler R, Kempter C, Seidl S, Gilg T, Jachau K, Alt A. Can ethyl glucuronide be determined in post-mortem body fluids and tissues? *Alcohol Alcohol*. 1999; 34:262–263. [PubMed: 10344787]
- Wurst FM, Skipper GE, Weinmann W. Ethyl glucuronide--the direct ethanol metabolite on the threshold from science to routine use. *Addiction*. 2003; 98(Suppl 2):51–61. [PubMed: 14984242]
- Yao M, Chang XY, Chu YX, Yang JP, Wang LN, Cao HQ, Liu MJ, Xu QN. Antiallodynic effects of propentofylline Elicited by interrupting spinal glial function in a rat model of bone cancer pain. *Journal of neuroscience research*. 2011; 89:1877–1886. [PubMed: 21812015]

Research Highlight

Drugs that form glucuronidated metabolites, including opioids and alcohol, can become agonists at toll-like receptor 4 (TLR4).

Figure 1.

GA, EtG and EtOH all cause a significant increase HEK-TLR4 cell SEAP reporter expression. GA caused a significant increase relative to vehicle at 10 and 100 μM concentrations ($F=19.16$, $p<0.05$, A). EtG caused a significant increase relative to the vehicle at 10 and 100 μM concentrations ($F=19.44$, $p<0.05$, B). EtOH caused a significant increase relative to vehicle at the 100 μM concentration ($F=22.18$, $p<0.05$).

Figure 2.

The TLR4 antagonist LPS-RS significantly attenuated the HEK-TLR4 cell SEAP increases seen with GA ($F=7.51$, $p<0.05$, A), EtG ($F=7.99$, $p<0.05$, C) and EtOH ($F=30.0$, $p<0.05$, E). Similarly, the TLR4 antagonist (+)-naloxone significantly attenuated the HEK-TLR4 cell SEAP increases seen with GA ($F=5.36$, $p<0.05$, B), EtG ($F=4.92$, $p<0.05$, D) and EtOH ($F=8.36$, $p<0.05$, F).

Figure 3.

GA (0.34 μ g, i.t.) caused a significant allodynia 3 hours post injection relative to vehicle injected rats, which was blocked by coadministered LPS-RS (40 μ g, i.t.). EtG (0.38 μ g, i.t.) caused significant allodynia 1 and 3 hours post injection compared to vehicle injected rats at both time points which was blocked by coadministered LPS-RS (40 μ g, i.t.). Finally, EtOH (0.07 μ g, i.t.) failed to cause allodynia within 3 hours of injection. All doses were equimolar. There were no baseline differences between any of the groups ($F=0.133$, $p>0.05$).

Table 1

In silico docking studies predict that GA and EtG will bind to the MD2 portion of the TLR4-MD2 complex. The residues on MD-2 at which GA and EtG interact with MD-2 are denoted with an X. Residues that interacted with lipid A chains of lipopolysaccharide in the crystal structure determined by Park et al., (2009) are included for reference. When the docking simulations were repeated with (+)-naloxone ((+)-Nal) already bound to the MD2 molecule, the residues at which EtG interacted were altered, but the residues that GA interacted with did not change. The residues that lost interaction with EtG when (+)-naloxone was already docked are denoted with a **X**. Residues that gained interaction when (+)-naloxone was already docked are denoted with a **G**.

residue	lipid A	(+)-Naloxone	GA	EtG
24	X	X		X
29			X	
32	X			
44				G
46	X	X		X
48	X	X		
52	X	X		
54	X	X	X	
55			X	
61	X			X
63	X			X
76	X			G
94	X			G
125			X	
126	X		X	
133				
135	X			X
147	X			G
149				X
151	X	X		X
153	X	X		
155			X	
157			X	
158			X	