

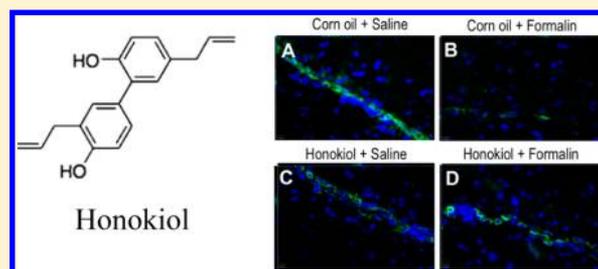
## Honokiol for the Treatment of Neonatal Pain and Prevention of Consequent Neurobehavioral Disorders

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**ABSTRACT:** This study examined the short- and long-term neuroprotective and analgesic activity of honokiol (a naturally occurring lignan isolated from *Magnolia*) on developing brains in neonates exposed to inflammatory pain, known to cause neuronal cell death. Postnatal day 4 (P4) neonatal rat pups were subjected to intraplantar formalin injection to four paws as a model of severe neonatal pain. Intraperitoneal honokiol (10 mg/kg) or corn oil vehicle control was administered 1 h prior to formalin insult, and animals were maintained on honokiol through postnatal day 21 (P21). Behavioral tests for stress and pain were performed after the painful insult, followed by morphological examinations of the brain sections at P7 and P21. Honokiol significantly attenuated acute pain responses 30 min following formalin insult and decreased chronic thermal hyperalgesia later in life. Honokiol-treated rats performed better on tests of exploratory behavior and performed significantly better in tests of memory. Honokiol treatment normalized hippocampal and thalamic c-Fos and hippocampal alveus substance P receptor expression relative to controls at P21. Together, these findings support that (1) neonatal pain experiences predispose rats to the development of chronic behavioral changes and (2) honokiol prevents and reduces both acute and chronic pathological pain-induced deteriorations in neonatal rats.



Early exposure to pain in neonates alters normal neuronal connections and causes anatomic, electrophysiological, and molecular changes that manifest as neurologic and behavioral deficits later in childhood, adolescence, and even adulthood.<sup>1–6</sup> In neonatal rats, prior studies have shown that inflammatory pain insults can cause selective neuronal cell death in the cortex, hippocampus, and hypothalamus with long-term decreases in thresholds for pain and stress;<sup>1,7</sup> repeated injections of formalin result in severe pain that leads to apoptosis and changes in the developing brain;<sup>8</sup> it has been speculated that a similar mechanism explains the development of neuropsychiatric sequelae (anxiety, depression, learning disabilities, and chronic pain) in humans born prematurely, since these infants are typically exposed to multiple needlesticks and other painful procedures including catheterization, circumcision, and chest tube placement.<sup>2,4</sup> Current pharmacologic options include opioids as the mainstay (morphine, fentanyl, methadone), ketamine, sucrose, local/topical anesthetics, nonsteroidal anti-inflammatory drugs, and midazolam; interventional procedures such as nerve blocks and neuraxial blockade may also be performed.<sup>9</sup>

Despite the search for nonopioid analgesics and adjuncts for pain management, current options for treatment of pain are often accompanied by a host of undesirable side effects.<sup>10</sup> Early, repeated exposure to opioids in children with prolonged hospital stays and repeated surgeries can increase their potential for adrenocortical suppression, endocrine abnormalities, apnea, addiction, and tolerance,<sup>11,12</sup> and neonatal exposure to opioids

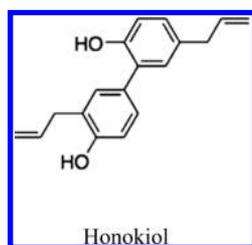
may alter developing brain structures.<sup>13</sup> A superior analgesic agent would be one that confers not only analgesia but also neuroprotection in order to prevent negative neurological/cognitive consequences.

A naturally occurring lignan, honokiol, was examined for its potential to reduce the neuropsychiatric sequelae related to early pain experiences. Honokiol has been shown to have neuroprotective and neurotrophic effects<sup>14–16</sup> as well as anti-inflammatory effects at multiple levels in the cytokine cascade, including downregulation of NF-κB activation through MEKK-1 inhibition.<sup>17–20</sup> Inflammatory pain involves the release of neurotransmitters (e.g., glutamate and serotonin), neuromodulators (e.g., substance P), and inflammatory mediators (e.g., bradykinin, histamine, and prostaglandin) from dorsal horn primary afferents following tissue damage.<sup>21–35</sup> Honokiol alleviates formalin-induced inflammatory pain without motor and cognitive side effects in adult rodents, providing evidence for its nonopioid analgesic activity.<sup>36</sup> The mechanisms by which honokiol decreases inflammatory pain may include (1) inhibiting NMDA-induced licking behavior and thermal hyperalgesia, (2) blocking glutamate-, substance P-, and PGE2-induced inflammatory pain, and (3) decreasing glutamate-induced c-Fos protein expression in superficial laminae of the L4–L5 dorsal horn.<sup>37</sup> On the basis of these findings, the present investigation tested the hypothesis that honokiol may

Received: March 23, 2015

Published: November 5, 2015

provide an alternative or adjunctive treatment of inflammatory pain in neonates.

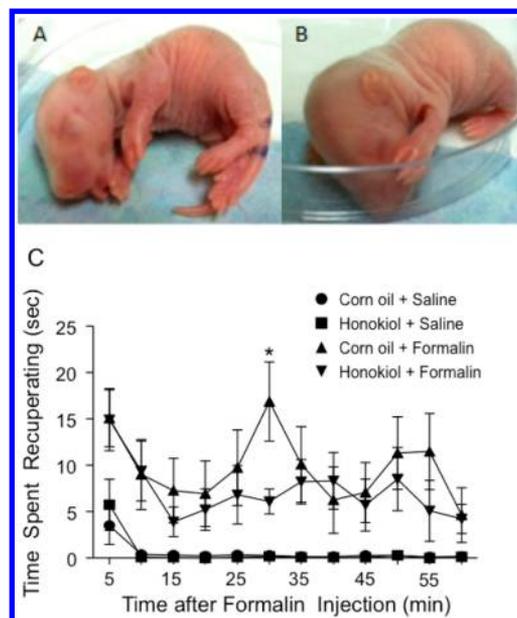


## RESULTS AND DISCUSSION

**Honokiol Attenuates Acute Nociceptive Alterations from Inflammatory Pain.** At P7, honokiol (10 mg/kg) dissolved in corn oil (treatment) or corn oil alone (control) was injected intraperitoneally (ip), and 1 h later animals were subjected to either formalin or saline subcutaneous (sc) injection into their left forepaw. During the next 60 min, rats were observed for 60 s at 5 min intervals for signs of pain-induced recuperation behaviors, including paw-licking, paw-lifting, flinching, and rolling. Since formalin injection is known to create a biphasic pain response, with the first phase (direct pain) occurring at 0–5 min and the second phase (inflammatory pain) occurring at 10–40 min,<sup>25</sup> data were collected and compared for a 60 min period that spanned these two phases. In rats receiving formalin-induced pain stimuli and honokiol treatment, significant reduction in recuperation time was seen at 30 min ( $n = 8$  per group,  $p < 0.05$ ) compared to rats receiving the painful insult and corn oil injection alone (Figure 1).

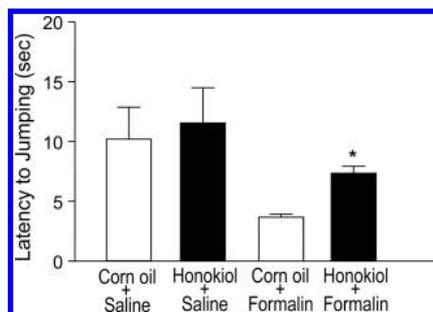
**Repeated Honokiol Treatment Decreases Chronic Thermal Hyperalgesia Resulting from Early Pain.** Using the severe inflammatory pain model, repeated honokiol treatment was tested to prevent long-term alterations in pain sensation. In this experiment, one group of rats was pretreated with either honokiol (10 mg/kg, ip) or vehicle control (corn oil, ip) prior to formalin injection daily from P4 to P6. This model of severe neonatal pain has been previously tested in a neonatal rat model.<sup>1,8</sup> After the 3-day pain insult, these rats were continuously treated with maintenance honokiol or vehicle control every other day until P17–P21. Animals in all groups (saline injection vehicle control, saline injection plus honokiol, formalin injection with vehicle control, and formalin plus honokiol) were then subjected to the hot plate test. The latency of responding to the hot surface ( $55 \pm 2$  °C) was noticeably shortened in rats of the formalin groups, suggesting the development of thermal hyperalgesia and chronic pain lasting many days after original pain stimuli. Meanwhile, honokiol treatment largely prevented the expression of thermal hyperalgesia compared to formalin-vehicle controls ( $n = 10$ –22 per group,  $p < 0.0001$ ) (Figure 2). The response latency of honokiol-treated rats showed no significant difference from that of saline-injected normal control rats.

**Honokiol Prevents Behavioral Abnormalities Resulting from Early Pain.** In P21 rats, the defensive withdrawal and novel object recognition tests were performed to evaluate delayed behavioral alterations 2 weeks after the neonatal inflammatory pain insult. In the defensive withdrawal test, rats in the honokiol–formalin group were more likely to leave shelter and showed improved exploratory behavior over those that were pretreated only with corn oil and exposed to formalin



**Figure 1.** Honokiol attenuates formalin-induced acute pain responses in neonatal rats. The response to inflammatory pain was tested in the “paw-licking test”, which included evaluation of all recuperation behaviors (licking, flinching, and rolling) in postnatal day 7 (P7) pups. Honokiol (10 mg/kg, ip) was dissolved in corn oil. Corn oil alone was tested as the vehicle control for honokiol treatment. Formalin (5%) sc injection is an established inflammatory pain model; saline injection was used as the control for the formalin insult. Honokiol or corn oil was administered 1 h before either formalin or saline injection into the left forepaw. (A) Formalin injection triggered recuperation behaviors including marked and prolonged licking activity of the injected paw at 5 min and again at 30 min after the formalin insult. (B) Saline injection did not cause significant increases in recuperation behaviors in either corn oil or honokiol pretreated rats. (C) Although there was no significant difference 5 min after the painful stimulus between rats in honokiol + formalin vs corn oil + formalin groups, honokiol treatment significantly reduced the licking duration at 30 min after formalin injection. Results are presented as the mean  $\pm$  SEM ( $n = 8$  animals per group). \* $p < 0.05$  between corn oil + formalin vs honokiol + formalin groups at 30 min.  $p < 0.05$  for corn oil + saline vs corn oil + formalin at 5, 30–35, and 55 min; honokiol + saline vs corn oil + formalin at 25–35 and 50–55 min; corn oil + saline vs honokiol + formalin at 5 min also. There was no statistically significant difference among the other groups and times analyzed.

insults ( $n = 6$ –14 per group,  $p = 0.1619$ ) (Figure 3). Rats treated with honokiol after formalin injury performed better on the novel object recognition test, indicating an improved memory capacity and possible attenuation of early pain-induced hippocampal injury. In novel object recognition, the discrimination ratio for honokiol-treated, formalin-injured rats was significantly greater than for corn-oil control, formalin-injured rats ( $n = 7$ –12 per group,  $p < 0.05$ ), indicating an improved ability to recognize a novel object (Figure 3). Of the pups that exhibited hyperalgesia and anxiety behaviors within days and weeks after the initial pain insults, it is important to note that honokiol treatment normalized pain sensitivity and mitigated behavioral anxiety. This impact on cognition is likely due to honokiol’s neuroprotective, anti-inflammatory, and analgesic effects, which may reduce pain-induced cell death in the hippocampus and cortex of neonatal rats during a critical period of development.<sup>1,38,39</sup>

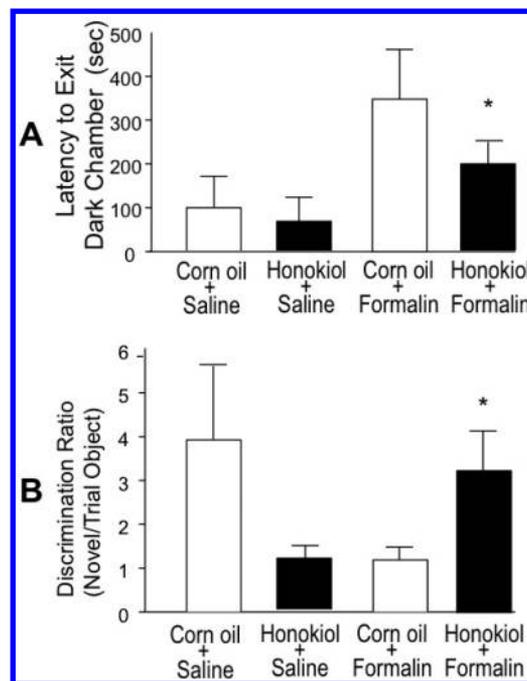


**Figure 2.** Inflammatory pain induces chronic enhancement of pain sensation, attenuated by honokiol. Hot plate testing for thermal hyperalgesia was performed in P16/P17 rats subjected to control or formalin injection from P4 to P6. Honokiol or corn oil vehicle control was administered prior to formalin injection daily during the 3-day insult. Saline injection was used as a noninflammatory pain control for formalin. Rats in honokiol and corn oil groups were continuously treated with honokiol and vehicle control, respectively, every other day until P21 for the hot plate test. In saline injection control rats, the response latency to jumping on the hot surface ( $55 \pm 2^\circ\text{C}$ ) was similar between corn oil and honokiol groups. The latency of response, however, was noticeably shortened in rats of the formalin/corn oil group. Honokiol treatment largely prevented the development of thermal hyperalgesia compared to corn oil controls. Average 3 trials per animal. Results are presented as the mean  $\pm$  SEM ( $n = 11, 10, 23, 22$  in corn oil + saline, honokiol + saline, corn oil + formalin, honokiol + formalin groups, respectively). \* $p < 0.0001$  for honokiol + formalin vs corn oil + formalin rats.  $p < 0.001$  for honokiol + saline vs corn oil + formalin rats. No other groups showed statistically significant differences.

Prior studies have investigated hippocampal theta activation relative to formalin nociception, showing that formalin hindpaw injections evoked biphasic increases in duration of dorsal CA1 theta independent of duration of nociception.<sup>40</sup> Honokiol may attenuate these effects of formalin injury on the hippocampus, but further electrophysiologic research with honokiol would need to be performed. Further studies should also be performed to determine whether honokiol attenuates cell death and the expression of inflammatory mediators, microglia, and neutrophils within the hippocampus, cortex, and thalamus of neonatal rats exposed to inflammatory pain.

Interestingly, honokiol decreased the discrimination ratio for novel object recognition in rats not exposed to formalin injection (Figure 3). This may be a result of honokiol's GABAergic and other inhibitory effects on the brain, which may lead to decreased cognition in an uninjured control rat when there is no painful insult to suppress.<sup>41–43</sup>

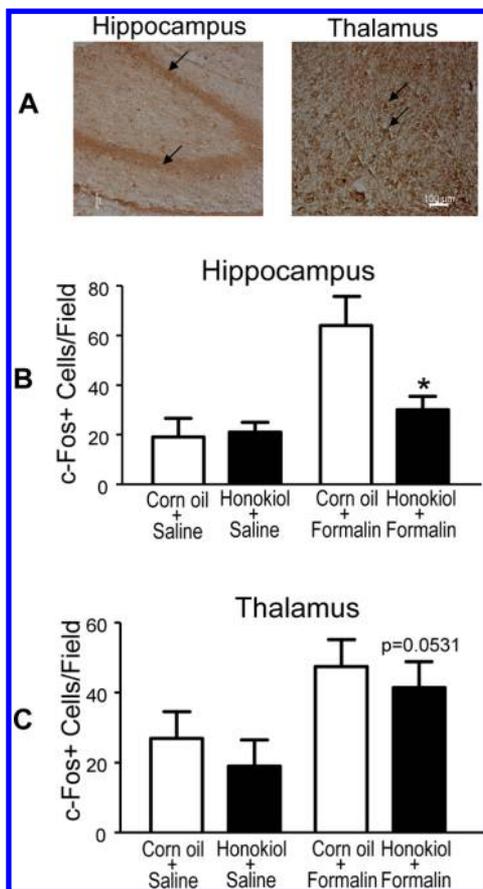
**Honokiol Does Not Significantly Affect Exploratory Behavior.** While honokiol has been shown to have anti-inflammatory analgesic effects without cognitive or motor impairment in adult mice following formalin hindpaw injection,<sup>36</sup> the compound had not been evaluated in a neonatal model of inflammatory pain until now, and chronic/long-term effects conferred by the drug have not been studied. At P21, open-field testing was performed in a  $50 \times 50 \times 50$  cm box during the dark cycle, 1 h following ip injection of either honokiol or corn oil. There was no significant difference in exploratory behavior in terms of distance traveled during the 5 min period between any of the groups. All groups traveled a mean distance of 4000–4500 mm ( $n = 3$  or 4 per group,  $p = 0.9162$ ), indicating no difference in exploratory behavior or anxiety between the groups.



**Figure 3.** Honokiol prevents behavioral abnormalities resulting from early pain experiences. Two weeks after the neonatal inflammatory pain insult or control procedure, P20–21 rats were tested for chronic changes in their behavior activities. (A) In the defensive withdrawal test, rats that received formalin inflammatory pain preferred to hide inside the dark shelter, while rats in the formalin and honokiol group were more likely to leave the shelter of darkness for exploration outside the shelter. Results are presented as the mean  $\pm$  SEM ( $n = 6, 7, 14,$  and  $14$  for corn oil + saline, honokiol + saline, corn oil + formalin, and honokiol + formalin groups, respectively). \* $p < 0.05$  for honokiol + formalin vs corn oil + formalin and for honokiol + saline vs corn oil + formalin.  $p = 0.1619$  for corn oil + saline vs honokiol + saline, indicating corn oil or honokiol alone did not change the defensive withdrawal behavior in normal saline control animals unexposed to formalin injury. (B) In novel object recognition testing, rats were exposed to a novel object after familiarization with the original object. The discrimination ratio (time spent with novel object vs time spent with original object) decreased in rats subjected to early inflammatory pain. Honokiol treatment prevented this functional deficit, and the discrimination ratio for honokiol + formalin treated rats was significantly increased compared to corn oil + formalin controls. Results are presented as the mean  $\pm$  SEM ( $n = 7, 7, 12,$  and  $12$  for corn oil + saline, honokiol + saline, corn oil + formalin, and honokiol + formalin groups, respectively). \* $p < 0.05$  for honokiol + formalin vs corn oil + formalin groups. No other groups showed statistically significant differences.

**Honokiol Attenuates Pain-Related Increase in c-Fos from Early Pain Experiences.** Upregulation of c-Fos in neurons is a marker of increased signaling in chronic pain. The upregulation of c-Fos in honokiol-treated and untreated formalin-injured animals was evaluated after the third day of injury. Honokiol treatment significantly attenuated c-Fos expression in the hippocampus ( $n = 6$ – $8$  per group,  $p = 0.0122$ ) and also appeared to attenuate c-Fos expression in the thalamus of injured rats ( $n = 6$ – $8$  per group,  $p = 0.0531$ ) (Figure 4).

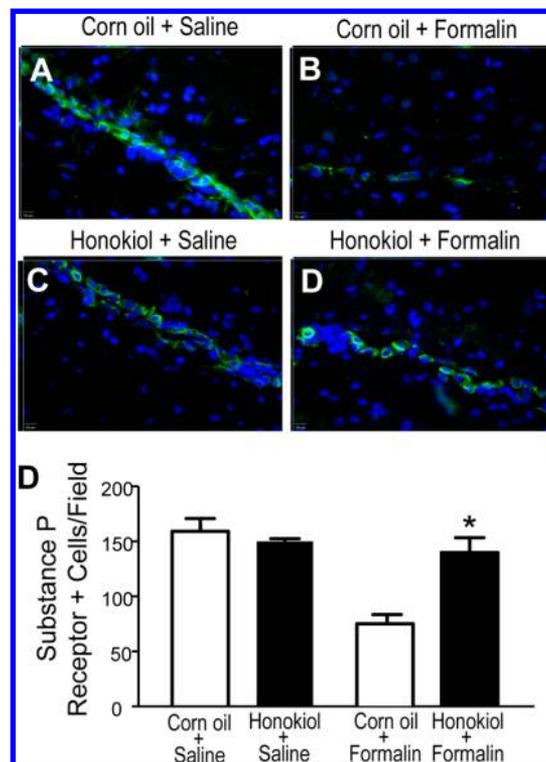
**Honokiol Attenuates Pain-Related Long-Term Down-regulation of Substance P Receptor.** In chronic pain, substance P receptor expression is typically reduced over time.<sup>44,45</sup> To evaluate the development of chronic pain at the molecular level, substance P receptor was stained in the alveus



**Figure 4.** Honokiol attenuates pain-related upregulation of c-Fos expression in the brain. (A) c-Fos expression as an early marker of chronic pain was measured using immunohistochemical staining (dark brown) in brain sections from P7 rats after the 3-day formalin or control treatment (20 $\times$  field). (B and C) Quantified data of c-Fos expression in the hippocampus (B) and thalamus (C). Results are presented as the mean  $\pm$  SEM ( $n = 6, 6, 7, 8$  animals per corn oil + saline, honokiol + saline, corn oil + formalin, and honokiol + formalin groups, respectively, for both hippocampal and thalamic sections). Formalin-induced inflammatory pain markedly increased c-Fos expression in the hippocampus. Honokiol largely prevented this increase of the chronic pain markers in formalin-injured animals ( $*p = 0.0122$  for honokiol + formalin vs corn oil + formalin;  $p < 0.05$  for corn oil + saline and honokiol + saline groups vs corn oil + formalin). The honokiol effect in the thalamus was not significant for honokiol + formalin vs corn oil + formalin groups ( $p = 0.0531$ ).  $p < 0.05$  for corn oil + formalin vs honokiol + saline groups. There was no difference in c-Fos expression within the saline groups or other groups in either the hippocampus or thalamus.

of honokiol-treated and control animals 14 days following formalin injury in P21 rats. Cells expressing substance P were counted in the alveus, along the border of the hippocampus. Honokiol treatment normalized the expression of substance P in the alveus of formalin-injured rats ( $n = 3-7$  per group,  $p = 0.0018$ ), although it did not completely restore the expression of the substance P receptor to uninjured control levels (Figure 5). The functional benefits of honokiol are likely mediated by the cellular and molecular mechanisms related to attenuation of pain, as indicated by a normalization of substance P receptor and c-Fos expression in the brain tissues of treated animals.

In summary, these findings support that (1) neonatal pain experiences predispose the rats to the development of chronic behavioral changes including the development of thermal



**Figure 5.** Inflammatory pain results in chronic downregulation of substance P receptor levels, attenuated by honokiol. In P21 rats, immunohistochemical staining with the substance P receptor antibody was used to detect the long-term effect of inflammatory pain on this pain-related receptor in the hippocampus. (A to C) The expression of substance P receptor in the brain was decreased 2 weeks after formalin insult. Honokiol treatment noticeably maintained the substance P receptor expression in the hippocampus. (D) Quantified data of the number of substance P receptor positive cells in the hippocampus. Saline or honokiol alone did not alter the expression level. There was a marked reduction in substance P receptor level 2 weeks after the 3-day inflammatory pain protocol. Honokiol treatment normalized the expression of substance P in the alveus of formalin-injured rats. Results are presented as the mean  $\pm$  SEM ( $n = 7, 3, 6, 7$  rats in corn oil + saline, honokiol + saline, corn oil + formalin, and honokiol + formalin groups, respectively).  $*p = 0.0018$  for honokiol + formalin vs corn oil + formalin rats.  $p < 0.01$  for corn oil + saline vs corn oil + formalin groups analyzed.

hyperalgesia, decreased exploratory behavior, and short-term memory derangements that can be reflected at the histopathological level as an increase in c-Fos and decrease in substance P receptor expression within the brain tissue and (2) honokiol prevents and reduces both acute and chronic pathological pain-induced deteriorations in neonatal rats, as seen in the respective studies.

## EXPERIMENTAL SECTION

**Test Compound and Reagents.** Honokiol was obtained from Calbiochem/EMD-Millipore (CAS 35354-74-6). The compound obtained was determined to be  $\geq 98\%$  pure by high-performance liquid chromatography (HPLC). Substance P receptor antibody was obtained from the same manufacturer (#AB5060; Millipore, Billerica, MA, USA), as was anti-c-Fos antibody (#AB1584; Millipore) for immunohistochemistry.

**General Experimental Procedures.** Intraplantar subcutaneous injection of formalin results in an early, acute pain response followed by a late, tonic phase that is manifested behaviorally as flinching and

licking of the paw. The late, tonic phase is thought to more accurately represent the inflammatory nociceptive response. In this study, formalin injection was used as a neonatal model of peripheral inflammatory pain, and intraperitoneal injections of either honokiol or corn oil vehicle were given as treatment and control, respectively. In the acute pain model, postnatal day 4 Wistar rat pups were gently restrained and injected with either 5% formalin (10  $\mu$ L, sc) or an equivalent amount of normal saline into the left forepaw. This procedure was performed once for the acute pain model. In the chronic severe pain model, however, each paw was sequentially injected at 1 h intervals over the course of 4 h (left forepaw, right forepaw, left hindpaw, right hindpaw), and this was repeated daily for 3 days. The chronic severe pain model has been previously validated and was justified in this experiment to reflect the pain experienced by premature infants in the neonatal intensive care unit on a daily basis.<sup>1,8,46–48</sup> Prior studies have shown that acute minor injury to only one forepaw will not create long-term neurological changes, whereas injury to all four paws on a daily basis does create long-term neurological changes including cell death.<sup>1</sup> Pups were returned to their mothers immediately after each injection, and the health condition of each pup was monitored each day. Animals with skin infection, excessive swelling, and pain, as monitored by a veterinarian, were sacrificed without further testing. At P7 and/or P21, rats were subjected to behavioral tests and then sacrificed for morphological assessments. Experimental groups include (1) saline sc injection with corn oil ip control (CS), (2) saline injection sc with honokiol treatment ip (HS), (3) formalin injection sc to model inflammatory pain with corn oil ip control (CF), and (4) formalin injection sc plus honokiol treatment ip (HF). Female and male rats were equally distributed across groups to account for gender differences. After sacrifice, brain tissues were collected for immunostaining. All procedures were approved by the Emory Institutional Animal Care and Use Committee (IACUC) under protocol DAR-2000868-110714BN. All behavioral tests were performed under blinded or double-blinded conditions.

**Paw-Lick Test for Acute Pain.** This test is performed to detect the response of animals to an inflammatory pain stimulus at the early stage of the pain model. P4 rat pups were given either normal saline (10  $\mu$ L) or 5% formalin (10  $\mu$ L) intraplantar with a 27G needle administered into the left forepaw. Honokiol (10 mg/kg, ip) or corn oil was administered 1 h before formalin injection in the honokiol-treated or control group, respectively. The duration of paw licking of the affected paw and other signs of recuperation were measured for 60 s at 5 min after formalin injection, designated as phase I response to acute and direct nociceptive pain, and observed for 60 s at 5 min intervals afterward for an additional 55 min as a phase II tonic inflammatory pain period (5–60 min after formalin injection).

**Open-Field Test and Defensive Withdrawal Test.** Two classic tests originally designed for anxiety were used to detect motor/behavioral changes after inflammatory pain and to evaluate the potential sedative effects of honokiol by examining exploratory behavior following ip injection. Rat pups received a 3-day course of formalin insults, and their anxiety and exploratory behaviors were tested at P21. In the open-field test, rats were allowed to freely move in an open-field container (50  $\times$  50  $\times$  50 cm box) during the dark cycle (between 19:00 and 07:00) and allowed to explore for 5 min under a video-camera attached to the TopScan program (CleverSys, Inc., Reston, VA, USA). The total traveled distance during the 5 min period was calculated and compared between groups. Animals were also tested for exploratory behavior using the defensive withdrawal test on P21. The animals were placed in a dark chamber placed along the wall of a large 50  $\times$  50  $\times$  50 cm box during the dark cycle. Animals were habituated to the open field prior to the test by allowing them to explore the open field for 10 min without access to the defensive withdrawal chamber. The latency was measured as the time it took each animal to place its four paws outside the small chamber.

**Hot Plate Test.** On P21, thermal hyperalgesia and pain sensitivity to heat was measured on a hot plate at  $55 \pm 2$  °C. Latency was measured as the time for the rat to jump with a maximum allowed time of 30 s. Jumping was used as the most apparent objective sign of heat

intolerance. Three distinct readings, separated by at least 15 min, were averaged for each animal.

**Novel Object Recognition Test.** As a test of memory at P21, animals were allowed 3 min in the training phase to acclimate to the arena and become familiar with two identical objects. To qualify for the trial phase, the animal must have spent at least 20 s exploring the objects during the familiarization/training period. After a 30 min delay, each qualifying animal was then again placed in the arena with one of the original objects and a novel object for the trial period (3 min). The amount of time spent with each object was then recorded and expressed as a discrimination ratio (time spent with novel object: time spent with original object). Behavioral experiments were performed in the dark, during the dark cycle, by two separate individuals blinded to the identity of the groups.

**Immunohistochemical Staining in Brain Sections.** Substance P receptor staining was performed to evaluate the extent of downregulation of the substance P receptor secondary to chronic pain. Brain sections were dried for 30 min and fixed in formalin for 10 min followed by cold methanol for 10 min. Sections were placed in Triton X-100 0.2% for less than 3 min to prevent degradation of the receptor and then blocked in 1% fish gel (Sigma-Aldrich, cat. no. G7765) for 1 h. Slides were incubated in substance P receptor antibody for 24 h, then stained with secondary antibody prior to mounting with Vectashield (Vector Laboratories Inc., cat. no. H-1000). Substance P receptor was found most commonly in the alveus of the hippocampus, an area of GABAergic neurons commonly associated with inhibition of pain and epileptiform activity.<sup>49–51</sup>

Staining for c-Fos was performed using the DAB protocol on P7 brain sections as an early indicator of chronic pain. Brain sections were dried for 20 min and fixed in formalin, then allowed to react with 0.3% H<sub>2</sub>O<sub>2</sub> at room temperature for 5 min, washed in PBS and Triton X-100, incubated in 1% fish gel for 60 min, and then washed with PBS prior to application of 1:500 anti-c-Fos antibody for 24 h, after which, sections were washed and incubated with biotinylated IgG at room temperature for 60 min. Then, DAB solution was applied and slides were dehydrated and mounted with Vectamount. All staining of positive cells was quantified using cell counting.

**Stereologic Cell Counting.** Cell count was performed following the principles of design-based stereology. Systematic random sampling was employed to ensure accurate and nonredundant cell counting. Every section under analysis was at least 90  $\mu$ m apart. A total of six 20  $\mu$ m thick sections spanning the regions of interest were randomly selected for cell counting from each animal. Counting was performed on six nonoverlapping randomly selected 20 $\times$  fields per section. Sections from different animals represented the same corresponding area in the anterior–posterior direction. For statistical analysis, each animal (six sections) represented one sample, and more than six animals were used for each group.

**Statistical Analysis.** All analyses were performed using GraphPad Prism 4.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed by Kruskal–Wallis testing using assumptions for nonparametric data. Single comparisons were performed using Mann–Whitney U testing, likewise for nonparametric data assumptions. Changes were considered significant at  $p < 0.05$ , and the appropriate Bonferroni correction was made for multiple comparisons. All results are reported as the mean  $\pm$  SEM.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported by a Foundation for Anesthesia Education and Research (FAER) Research Fellowship grant (A.W.), NIH grants NS075338 (L.W.), NS062097 (L.W.), and

NS0458710 (S.P.Y.), an AHA Established Investigator Award (L.W.), and an AHA Grant-in-Aid grant 12GRNT12060222 (S.P.Y.). We gratefully acknowledge helpful conversations about the pharmacology of honokiol and mechanisms of neonatal pain involving A. Jenkins, O. Mohamad, and J. Arbiser.

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