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Metabolomics implicates altered sphingolipids in chronic pain of neuropathic origin

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Neuropathic pain is a debilitating condition for which the development of effective treatments has been limited by an incomplete understanding of its chemical basis. We show by using untargeted metabolomics that sphingomyelin-ceramide metabolism is altered in the dorsal horn of rats with neuropathic pain and that the upregulated, endogenous metabolite *N,N*-dimethylsphingosine induces mechanical hypersensitivity *in vivo*. These results demonstrate the utility of metabolomics to implicate unexplored biochemical pathways in disease.

Millions of individuals suffer from neuropathic pain[1,2], a disabling condition that develops after damage to the nervous system[3]. Treatment options for neuropathic pain are limited, associated with undesirable side effects and rarely provide complete therapeutic relief. Alterations in gene transcription, protein expression, ion-channel organization and trophic factors are all associated with the development of neuropathic pain symptoms[4,5], but the molecular etiology of the condition remains unclear and has limited the development of effective treatments.

To investigate the chemical basis of neuropathic pain, we performed MS-based metabolomics on samples collected from Sprague-Dawley rats suffering from tibial nerve transection (TNT). Transection of the tibial branch of the sciatic nerve is a well-established model of neuropathic pain that induces allodynia, a condition in which normally innocuous stimuli elicit a pain response. In the TNT model, allodynia persists for at least nine weeks after surgery[6], long after resolution of the initial peripheral injury (**Supplementary Fig. 1**). To focus on the chronic phase of neuropathic pain, the most clinically problematic symptom, we analyzed tissues from rats 21 d after TNT and compared them to control rats receiving a sham surgery. We profiled metabolites from rat dorsal horn, dorsal root ganglia (DRG), tibial nerve and plasma (**Supplementary Fig. 2**).

We separately compared each of the following in TNT rats to sham-operated rats 21 d after surgery: (i) the lumbar enlargement of the ipsilateral dorsal horn (L3–L5, where the sciatic nerve projects), (ii) the same region of the contralateral dorsal horn, (iii) the ipsilateral DRG, (iv) 1-mm segments of the tibial nerve proximal to the site of transection and (v) plasma. Out of the total number (733) of metabolic features that were detected as significantly ($P < 0.01$, fold change > 2) dysregulated between TNT and sham rats 21 d after injury, 94% were in the ipsilateral dorsal horn (**Fig. 1a**). Metabolic features with fold changes greater than 3 were detected only in the ipsilateral dorsal horn and not in the DRG, tibial nerve or plasma after TNT. Therefore, metabolic alterations in the spinal cord during the later stages of neuropathic pain seem to have an important role in perpetuating pain sensitivity and were the focus of our investigation.

We characterized dysregulated metabolites in the ipsilateral dorsal horn associated with the pain phenotype by using an untargeted workflow (**Supplementary Methods**). With this approach, we identified multiple alterations in sphingomyelin-ceramide metabolism 21 d after TNT injury (**Fig. 1b–d**). Sphingomyelin-ceramide metabolism plays an important part in many cellular processes including myelin formation, apoptosis and cell signaling[7]. We therefore hypothesized that dysregulated metabolites in this pathway may be linked to the physiological changes underlying neuropathic pain and represent possible new targets for therapeutic intervention.

The first step in the degradation of sphingomyelin is the formation of ceramide through activation of sphingomyelinase[8]. This biochemical reaction is coupled with the conversion of diacylgylcerols to phosphatidylcholines (**Fig. 1b**)[8]. We detected that both ceramide (d18:1/16:0) and several phosphatidylcholines are upregulated in the ipsilateral dorsal horn 21 d after TNT by more than three-fold (**Supplementary Figs. 3** and **4** and **Supplementary Table 1**). Consistent with the increased degradation of sphingomyelin, we found that several diacylglycerols are downregulated by a factor of 2–3 in the dorsal horn of TNT rats (**Supplementary Fig. 5** and **Supplementary Table 1**). Furthermore, several of the upregulated phosphatidylcholines and the downregulated diacylglycerols have the same fatty acid side chains. In the dorsal horn of TNT rats, we also detected a 3.8-fold increase of platelet-activating factor, a metabolite that has been reported to activate sphingomyelinase (**Supplementary Fig. 6**)[9,10]. Additionally, we found that monohexosylceramide (d18:1/24:1), sphinganine, sphingosine and *N,N*-dimethylsphingosine (DMS) were each significantly ($P < 0.007$) upregulated more than two-fold (**Fig. 1c,d** and **Supplementary Figs. 7–10**).

Reorganization of the central termination areas of primary sensory neurons is known to occur following peripheral-nerve injury[11–13], suggesting that cellular membrane degradation after TNT may contribute to alterations in sphingomyelin catabolism. In support of this, we found that the expression of acid ceramidase, the enzyme involved in ceramide catabolism, is significantly ($P < 0.0034$) upregulated by 2.3-fold in the ipsilateral DRG cell bodies 21 d after TNT injury (**Supplementary Fig. 11**). These data suggest that persistence of metabolite components of the cell membrane may contribute

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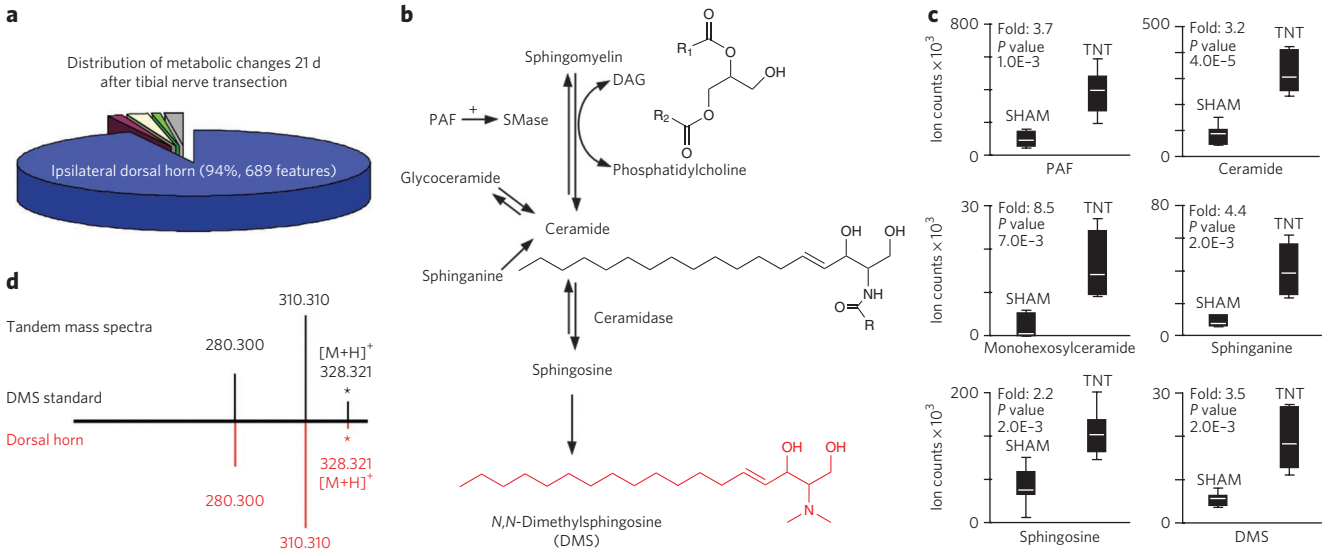

Figure 1 | Untargeted metabolomics identifies the dysregulation of sphingomyelin-ceramide metabolism in the ipsilateral dorsal horn during chronic neuropathic pain. (**a**) Distribution of the 733 statistically significant molecular features ($P < 0.01$) with changes greater than two-fold that are altered in TNT relative to sham rats 21 d after injury ($n = 7$ rats per group). The distribution of changes is 94% ipsilateral dorsal horn (blue), 1% contralateral dorsal horn (maroon), 2% ipsilateral dorsal root ganglia (white), 1% ipsilateral tibial nerve (green) and 2% plasma (gray). (**b**) The sphingomyelin-ceramide pathway, highlighting metabolites determined to be dysregulated. (**c**) Box-and-whisker plots of altered sphingomyelin-ceramide metabolites in the ipsilateral dorsal horn after TNT. The intensities represent ion counts from extracted ion chromatograms. Whiskers represent minimum and maximum values. (**d**) Tandem mass spectra from DMS standard and *m/z* 328.321 from the dorsal horn show the same fragments and relative intensities. SMase, sphingomyelinase.

to altered cell signaling and neuronal hypersensitivity during the development of neuropathic pain.

Among the dysregulated metabolites, endogenous DMS has not been previously investigated in the context of neuropathic pain. DMS is a catabolite of ceramide[14] that has been shown to increase the concentration of intracellular Ca^{2+} in astrocytes as well as inhibit glutamate uptake[15]. Deficient glutamate uptake by glial cells in spinal sensory synapses results in the excessive activation of *N*-methyl-D-aspartate receptors, which has been associated with neuropathic pain[16]. The role and concentration of endogenous DMS, however, are largely unknown, and a robust characterization of DMS as an endogenous metabolite has not been previously reported.

We characterized and quantified naturally occurring DMS in the ipsilateral dorsal horn of TNT rats 21 d after injury (**Supplementary Results**, **Supplementary Figs. 12** and **13**). By using selected reaction monitoring, we determined that there were 3.5 ± 1.2 fmol of DMS per mg of ipsilateral dorsal horn tissue 21 d after TNT. To determine whether DMS at these physiological concentrations is sufficient for the development of neuropathic pain behavior *in vivo*, we intrathecally injected DMS into healthy rats and measured the development of mechanical allodynia in the hind paw. Within 24 h of injections at concentrations of 0.25 micrograms per kilogram body weight (μg per kg body weight), rats developed mechanical allodynia that persisted for at least 3 d (**Supplementary Fig. 14**). In contrast, vehicle-treated rats showed no evidence of abnormal sensation to mechanical stimuli. We determined the concentration of DMS in the dorsal horn to be 4.1 ± 1.4 fmol per mg of tissue 2 h after injection with a concentration of 0.25 μg per kg body weight, an amount that approximates the amount of DMS detected in TNT rats after injury.

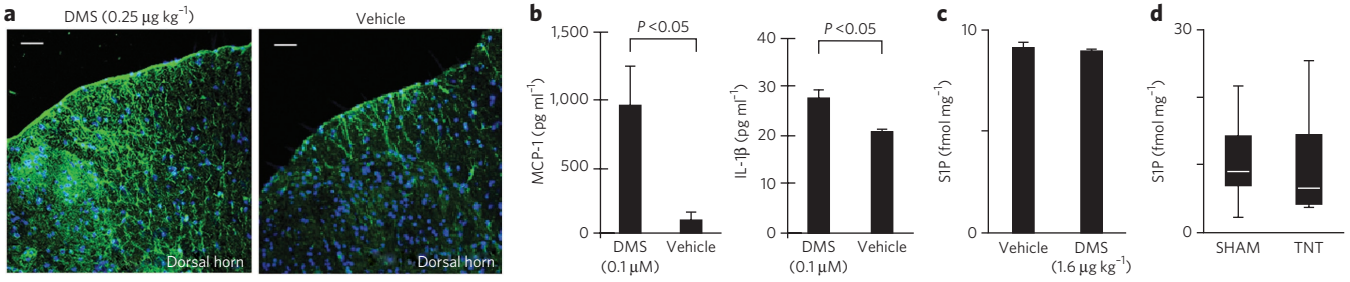

Figure 2 | DMS elicits neuropathic pain behavior and cytokine release. (**a**) Immunohistochemistry of the dorsal horn of rats intrathecally administered DMS at 0.25 μg per kg body weight (left) and vehicle (right) and stained with GFAP-specific antibody and an Alexa Fluor 488 anti-rabbit IgG-specific secondary antibody (green). Increased GFAP staining, a marker of astrocyte activation, is seen in the dorsal horn of DMS-treated rats relative to vehicle controls. Scale bars, 50 μm. (**b**) ELISAs of IL-1β and MCP-1 released into the supernatant of astrocyte cultures treated with DMS or vehicle. Cultures treated with 0.1 μM of DMS show a significant ($P < 0.05$) increase in MCP-1 and IL-1β release relative to vehicle controls. Data are expressed as mean ± s.e.m., with $n = 3$ cultures per group. (**c**) Comparison of S1P concentrations in the dorsal horn of rats that were intrathecally administered DMS at 1.6 μg per kg body weight and vehicle control. S1P was measured by selective reaction monitoring triple quadrupole MS (QqQ). S1P concentrations between the groups are not statistically different ($n = 4$ rats per group). S1P concentrations are represented as fmol per mg of spinal cord tissue. (**d**) Comparison of S1P concentrations in the ipsilateral dorsal horn of sham control rats relative to that in rats suffering from TNT 21 d after injury. S1P concentrations between the groups are not statistically different ($n = 7$ rats per group). S1P concentrations are represented as fmol per mg of spinal cord tissue.

A number of pathological alterations are associated with the development of mechanical allodynia, including abnormal astrocyte responses triggered by tissue damage and cellular dysfunction in the central nervous system (CNS)[17]. Therefore, we used immunohistochemistry to examine spinal cord glial fibrillary acidic protein (GFAP) expression in rats treated with DMS relative to that in vehicle-treated rats (**Fig. 2a**). Increased GFAP staining was detected in the spinal cord of rats following intrathecal DMS administration relative to staining in vehicle-treated controls, indicating an increase in astrocyte activation induced by DMS. Activated astrocytes release a variety of substances such as proinflammatory cytokines that have been shown to modulate neuronal hypersensitivity syndromes[18]. The cytokine interleukin 1β (IL-1β) is of special interest because it is known to be elevated in the cerebrospinal fluid of individuals with chronic pain[19]. Deletion of IL-1 receptor type 1 and transgenic overexpression of the naturally occurring IL-1 receptor antagonist delays the onset and severity of pain following peripheral nerve injury[20]. To determine whether DMS triggers IL-1β release, we treated cultured astrocytes with 0.1 μM of DMS and measured release of IL-1β by ELISA 24 h later (**Fig. 2b**). We observed a significant ($P < 0.05$) increase in IL-1β release from cells treated with DMS relative to that from vehicle-treated cultures. On the basis of our quantification, we estimate that this concentration of DMS is within the range of physiological concentrations after TNT injury.

Another inflammatory mediator that has a role in nociceptive responses is the chemokine monocyte chemoattractant protein-1 (MCP-1). MCP-1 recruits inflammatory cells to sites of injury and is upregulated in spinal cord astrocytes following nerve injury[21]. To determine whether DMS increases MCP-1 production, we treated astrocyte cultures with 0.1 μM of DMS and measured release of MCP-1 by ELISA 24 h later (**Fig. 2b**). We observed a significant ($P < 0.05$) increase in MCP-1 release from cells treated with 0.1 μM DMS relative to that from vehicle-treated controls. Taken together, our results show that DMS induces pathological responses in the dorsal spinal cord that are associated with the development of pain behaviors and that the mechanism by which DMS mediates mechanical allodynia may occur via production of inflammatory mediators such as IL-1β or MCP-1 in the CNS.

Previous studies have implicated another ceramide derivative, sphingosine-1-phosphate (S1P), in nociceptive processing[22]. DMS is an inhibitor of the enzyme sphingosine kinase, and treatment of cells with DMS blocks production of S1P[23]. To investigate the possibility that DMS affects hypersensitivity by regulating S1P, we compared S1P concentrations in allodynic rats intrathecally treated with 1.6 μg per kg body weight of DMS to that in rats administered a vehicle control and found no statistically significant ($P < 0.05$) difference between groups (**Fig. 2c**). Moreover, we determined that S1P concentrations were similarly not altered between TNT and sham rats 21 d after injury (**Fig. 2d**) and that sphingosine concentrations were not altered in astrocyte cultures treated with DMS (**Supplementary Fig. 15**). These results are consistent with the physiological concentrations of DMS being substantially lower than the 2.3–6.8 μM inhibition constant (K_i) values at which DMS has been reported to inhibit sphingosine kinase[23] and support that DMS sensitizes neurons in the CNS via an alternative mechanism that remains to be elucidated.

In summary, our results have shown that the majority of metabolic perturbations characterizing the chronic phase of neuropathic pain are localized to the spinal cord. The data presented here show that DMS, an endogenous metabolite that has not been previously implicated in nociception, induces mechanical allodynia in rats *in vivo* and elicits cytokine release from astrocytes *in vitro*. Further investigation is needed to determine the specific enzymes responsible for DMS biosynthesis. The capacity of mammalian tissues to achieve N-methylation of sphingoid bases, however, has been demonstrated previously for the analog sphingolipid safingol[24], and it has been suggested that an *S*-adenosyl-L-methionine–dependent N-methyltransferase is active in mouse CNS tissue[25]. Our results therefore suggest that inhibition of endogenous DMS production, with a methyltransferase or ceramidase inhibitor, for example, may be an attractive therapeutic candidate to treat this debilitating condition.

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Author contributions

G.J.P. and O.Y. contributed equally to this work. G.J.P., L.P.S. and J.C. performed work on rats. G.J.P., O.Y. and L.P.S. performed analytical experiments. G.J.P., O.Y., L.P.S., J.-P.C., R.T., M.M. and G.S. contributed to experimental design, performed data analysis and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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