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BRIEF REPORT

Alterations in Spinal Cord Metabolism during Treatment of Neuropathic Pain

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Abstract Therapeutic options for neuropathic pain have improved over the last 20 years yet still only provide partial relief with numerous side effects. Recently, metabolomics revealed that the concentration of the endogenous metabolite *N,N*-dimethylsphingosine (DMS) is increased in the spinal cord in a model of neuropathic pain. Additionally, it was shown that introduction of DMS to the central nervous system (CNS) resulted in mechanical allodynia. Here, we have examined two compounds; pregabalin (Lyrica®), a drug used to treat neuropathic pain, and *N*-oleoylethanolamine (NOE), an endogenous endocannabinoid-like compound that is known to affect multiple lipid pathways. We found that the concentration of DMS in the spinal cord was not significantly altered upon pregabalin treatment of rats suffering from neuropathic pain. We further explored whether modulating lipid metabolism may impact neuropathic pain by testing NOE as a potential novel therapeutic.

Keywords Neuropathic pain · Antimetabolite · *N*-oleoylethanolamine · Pregabalin · *N,N*-dimethylsphingosine · Metabolomics

Introduction

Injury to the peripheral or central nervous system (CNS) can result in neuropathic pain (Calvo and Bennett 2012). While there have been many advances to managing this type of pain, most treatment options provide only partial relief and have side effects which result in premature treatment cessation (Dworkin et al. 2007). Metabolomics can reveal biomarkers and/or metabolic pathway perturbations in metabolic homeostasis (metabostasis), providing new therapeutic targets. Recently, metabolomics was carried out on a model for neuropathic pain; tibial nerve transected (TNT) rats, and revealed a correlation between *N,N*-dimethylsphingosine (DMS) (Metlin ID 34487) in the dorsal horn and neuropathic pain (Patti et al. 2012). DMS was further demonstrated to induce mechanical hypersensitivity (allodynia) after injection into the spinal cord (Patti et al. 2012).

More recently DMS was shown to be produced in human oligodendroctyes when challenged with agents that damage white matter (Chen et al. 2014). Furthermore, DMS-treated astrocytes produce pro-inflammatory cytokines such as IL-1 that can increase neuronal hypersensitivity. Therefore, DMS-induced pathological responses associated with neuropathic pain may be regulated through the production of pro-inflammatory mediators.

We hypothesize that DMS is a metabolite in the sphingomyelin-ceramide pathway. This pathway involves the

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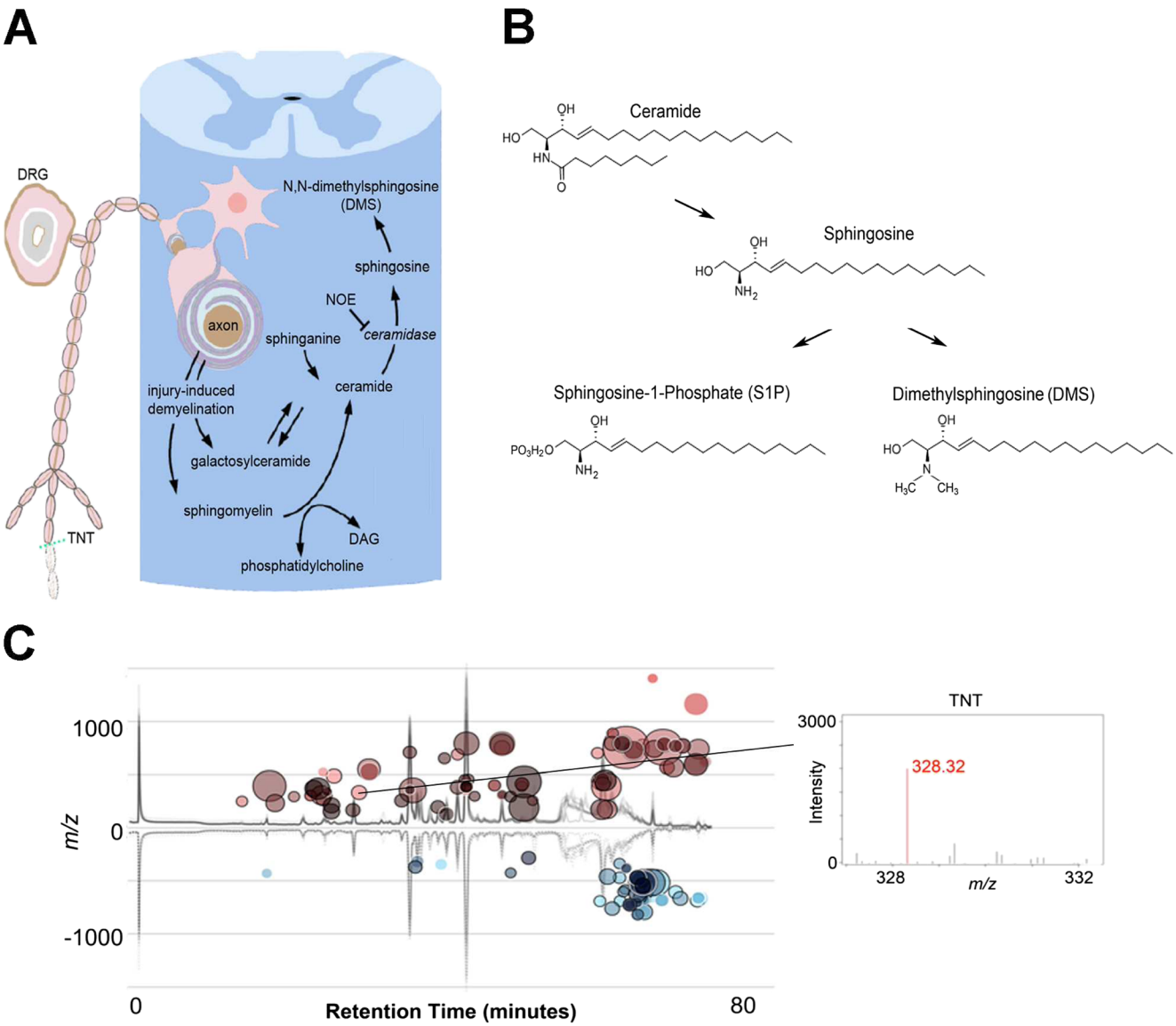

Fig. 1 **a** Proposed actions of *N*-oleylethanolamine (NOE) on *N,N*-dimethylsphingosine (DMS) production, **b** Sphingomyelin-ceramide metabolism. **c** Untargeted metabolomics of ipsilateral dorsal horn tissue from sham rats compared to ipsilateral dorsal horn tissue from tibial nerve transected (TNT) rats. Cloud plot generated by XCMS Online displaying dysregulated features between sham rats (*lower panel*) and TNT rats (*upper panel*), larger and brighter circles represent larger fold changes and higher p-values respectively. Mass spectrum of DMS from the ipsilateral dorsal horn of TNT rats

breakdown of ceramide to sphingosine by acid ceramidases (Fig. 1a, b). Sphingosine can then either be dimethylated by uncharacterized methyltransferase(s) to DMS or phosphorylated by sphingosine kinase to sphingosine 1-phosphate (S1P). The balance between these lipid mediators influences multiple cellular functions. DMS is an inhibitor of sphingosine kinase, and S1P production is tightly regulated. Increased levels of ceramide can induce cell death while elevated levels of S1P can lead to cell survival and proliferation (Draper et al. 2011). These results suggest that the sphingomyelin-ceramide pathway could be linked to the physiological changes that underlie neuropathic pain and be used as a potential therapeutic target.

Metabolic therapeutic targets can be investigated through the use of antimetabolites that target enzymes involved in related pathways, and gene knockdown/knockout strategies. Enzyme inhibitors or "antimetabolites" were first described in the 1940's and 50's by D.W. Woolley as "structural analogs antagonistic to metabolites"(Woolley and White 1943; Woolley 1952). Antimetabolites are endogenous compounds or compounds that are structurally similar to a particular metabolite designed to inhibit a specific metabolic process. Well known examples include antimetabolite cancer drugs such as fluorouracil, which is a pyrimidine analog that inhibits thymidylate synthase and thymidine production (Tattersall et al. 1975). Routes to target

specific metabolites are complex as it is difficult to find an inhibitor that has high specificity for key enzymes.

Indeed the mechanistic actions for many of the neuropathic pain drugs currently available are incompletely understood. These drugs include tricyclic antidepressants, gabapentin, pregabalin, and serotonin-noradrenaline re-uptake inhibitors (SNRIs) (Moulin et al. 2014). Studies with gabapentin and duloxetine (a SNRI) failed to show efficacy for neuropathic pain. However, pregabalin which acts as a calcium channel α2-δ ligand, decreases the release of neurotransmitters including glutamate, noradrenaline, substance P and calcitonin gene related peptide (Ben-Menachem 2004; Finnerup and Baastrup 2012; Dalal et al. 2013), and has been proven to be an effective analgesic. The effect of pregabalin on the metabolome and DMS concentrations are not known and were a focus of our investigation here.

As aforementioned, we have hypothesized that acid ceramidase is involved in DMS production. Previously, we have shown that acid ceramidase expression is upregulated 21-days after peripheral-nerve injury. This indicated that this enzyme may be associated with neuronal reorganization and cellular ceramide degradation. Therefore, we chose to target ceramidase as a novel potential therapeutic. Acid ceramidase inhibitors B-13 and *N*-oleoylethanolamine (NOE) have IC_{50}'s of 10 μM and 500 μM respectively (Proksch et al. 2011), however NOE is an endocannabinoid-like, endogenous metabolite with a low risk of toxicity and was therefore chosen as a potential antimetabolite of interest to investigate here.

Materials and Methods

Animals and Surgery Adult male Sprague–Dawley rats were housed according to institutional animal care and use committee (IACUC) guidelines. TNT and chronic-constriction injury (CCI) surgery were performed as previously described (Hofmann et al. 2003; Patti et al. 2012). Rats were randomly assigned to vehicle, pregabalin, NOE or DMS groups (n=2–7). The sciatic or tibial nerve was exposed in anesthetized animals using a blunt-dissection technique. In the TNT model (used for vehicle, pregabalin and DMS treatment), the tibial nerve was transected distal to the trifurcation of the sciatic nerve. For CCI animals (used for vehicle and NOE treatment), (4–0) silk ligatures were loosely tied approximately 1 mm apart around the sciatic nerve at mid-thigh level. The wound was closed with staples and the animals were allowed to recover on a heating pad. Sham-operated animals were treated identically in both models, except their nerves were not transected or ligated. Rats were monitored for alterations in motor function and animals showing any movement deficits were excluded from the study. All pain measurements were measured by von Frey filaments to determine 50 % paw withdrawal thresholds (PWT) using the up-down method of Dixon (Dixon 1980).

Drug Treatment

Pregabalin, DMS and TNT Treatment A stock solution of pregabalin (Lyrica®, Pfizer) was made in 0.5 % methylcellulose/water, vehicle was methylcellulose/water (0.5%) alone. Stock solutions of pregabalin (Lyrica®, Pfizer) (5 μM), NOE (Sigma-Aldrich) (5 μM) and DMS (Cayman Chemical) (15 μM), were dissolved in sterile ethanol and diluted in sterile phosphate-buffer solution (PBS) for injection into animals. Vehicle was ethanol (0.1 %) in PBS alone. For pregabalin treatment, TNT rats were dosed P.O three weeks post-surgery at 0 h and 2 h (10 mg/Kg/dose), and animals were monitored for the development of mechanical allodynia using von Frey filaments as previously described (Patti et al. 2012), both before dosing and 3 hours post first dose. The rats were sacrificed 24 h later after anesthesia. Ipsilateral dorsal horn tissue was dissected and frozen in liquid nitrogen for metabolomics analysis. For DMS treatment rats were administered DMS in ethanol intrathecally on days 1,2,5 and 6, with 5 different doses; vehicle, 2.5 ng/Kg, 25 ng/Kg, 250 ng/Kg and 1600 ng/Kg. For NOE treatment, NOE was injected intrathecally starting on days 1–3 and 5–7 after surgery.

Metabolomic Analysis Untargeted metabolomic analysis was carried out on extracted ipsilateral horn tissues taken from vehicle and pregabalin treated rats, as previously described (Patti et al. 2012). For pregabalin dosing samples were analyzed by liquid chromatography mass spectrometry (LC/MS) using a reversed-phase C18 column (Zorbax C18, Agilent 5 μm, 150×0.5 mm) and Agilent 6210 TOF in positive mode. 4 μl of sample was injected onto the column at 0 min 90 % solvent A (0.1 % formic acid in H_2O) and 10 % solvent B (0.1 % formic acid in H_2O) at a flow rate of 20 μl/min. A linear gradient elution was used over 75 min as follows: 0 min 10 % B, 5 min 10 % B, 10 min 40 % B, 65 min 98 % B, 70 min 98 % B and 75 min 10 % B, with 10 mins equilibration. Mass spectrometry parameters were as follows: m/z range 100-1500, fragmentor 120 V, skimmer 60 V, gas temp 350 C, drying gas 5.0 l/min, nebulizer 20 psig, capillary 3500 V. The data were analyzed by XCMS Online with the "HPLC/Q-TOF" parameter set using Welch's t-test with unequal variances.

Statistics

Statistical analysis of the metabolomics data was performed by XCMS Online (employing a univariate t-test). The Student's t-test for unpaired data was used to compare DMS- and vehicle-treated controls, and CCI animals administered NOE or vehicle starting on day 2 after surgery where $p<0.05$ was considered statistically significant in all cases.

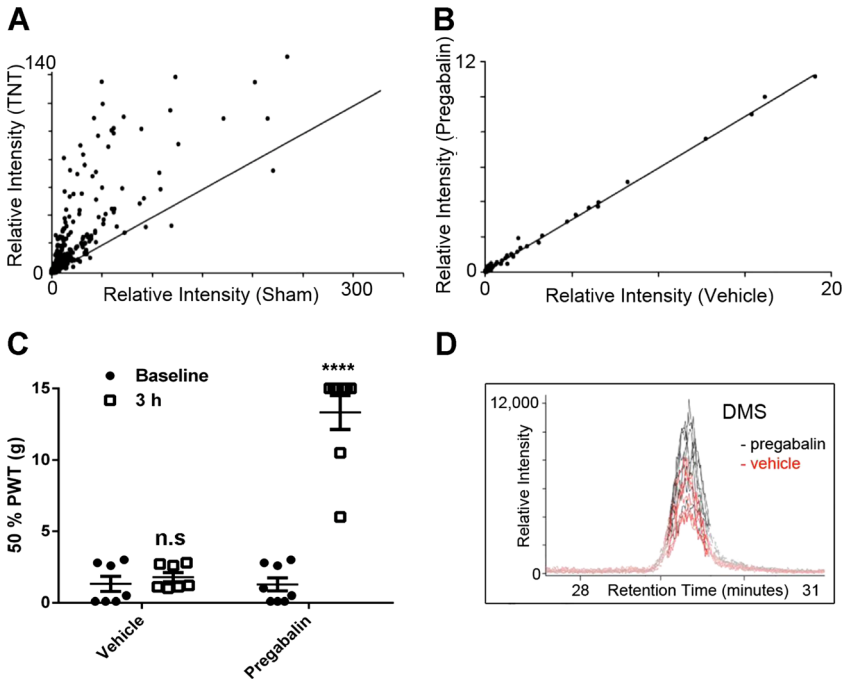

Fig. 2 Metabolic differences in the ipsilateral dorsal horn of **a** sham compared to tibial nerve transected (TNT) rats and **b** TNT rats treated with vehicle or pregabalin. **c** Effect of vehicle and pregabalin administration on the development of mechanical allodynia in healthy rats. Baseline measurements before dosing are compared to measurements 3 h postdose (after two doses given 0 h and 2 h), expressed as 50 % paw withdrawal threshold of mean ± SD by Student's t-test for unpaired data (n.s = not significant ****=p<0.0001). **d** Untargeted metabolomics of ipsilateral dorsal horn tissue from TNT rats treated with vehicle or pregabalin

Results and Discussion

Our previous studies have shown that the pain response observed in TNT rats is correlated with changes in lipid metabolism within the ipsilateral dorsal horn metabolome (Patti et al. 2012) (Fig. 1c and 2a). There were a number of metabolites from the same pathway similarly dysregulated, monohexosylceramide (d18:1/24:1), sphinganine, sphingosine and most interestingly DMS, upregulated 3.5-fold in TNT rats.

To study the metabolic effect of pregabalin treatment on the ipsilateral dorsal horn of TNT rats three weeks post-surgery, untargeted metabolomic analysis was carried out. It was revealed that there were no appreciable metabolic differences

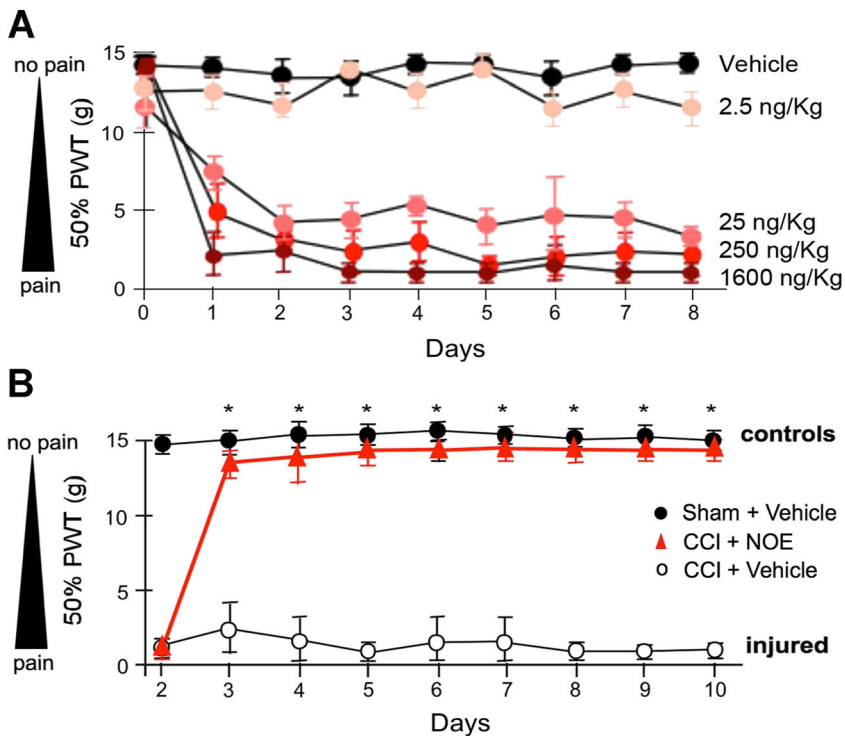

Fig. 3 **a** Effect of *N,N*-dimethylsphingosine (DMS) administration on the development of mechanical allodynia in healthy rats. Measurements expressed as 50 % by paw withdrawal threshold of mean±SD by Student's *t*-test for unpaired data ($p<0.05$). **b** *N*-oleylethanolamine (NOE) was administered on days 1–3 and 5–7 after chronic constriction injury (CCI). Measurements of mechanical allodynia in NOE-treated, vehicle-treated rats and sham controls treated with vehicle. Sham control rats treated with NOE were not significantly different from control rats treated with vehicle. Measurements expressed as 50 % paw withdrawal threshold of mean ± SD ($n=3$ per group). NOE rats have markedly reduced adverse responses to mechanical stimulation of the hind paw when compared to vehicle treated controls (*$p<0.05$)

between vehicle and pregabalin treatment in the TNT rat model (Fig. 2b). In addition we saw that pregabalin was capable of reducing allodynia in the TNT rats ($p<0.0001$) as shown in Fig. 2c. Given that pregabalin is capable of reducing neuropathic pain in TNT rats but does not change the metabolome on the ipsilateral horn, it is probable that pregabalin acts at an alternate region rather than the spinal cord, most likely in the brain. However, due to the size and respective dilution effects, the brain represents a significant challenge for measuring these metabolites (Ivanisevic et al. 2014). We also examined whether DMS itself was changed in the ipsilateral dorsal horn, a small but significant change in DMS was seen; a 1.4-fold ($p=0.013$) increase in pregabalin treated TNT rats versus vehicle treated (Fig. 2d).

To further investigate and corroborate our previous data we dosed DMS to healthy rats to determine its effect on mechanical allodynia (Fig. 3a). Similar to our previous studies (Patti et al. 2012) vehicle administration with ethanol (0.1 %) in PBS, resulted in no mechanical allodynia in the rats. However rats administered increased doses of DMS developed mechanical allodynia, similar to that seen with TNT injury (Fig. 3a). Since we speculated that DMS is a downstream product of ceramide resulting from the action of ceramidase, we investigated acid ceramidase as a possible novel therapeutic target to reduce mechanical allodynia associated with some forms of neuropathic pain. NOE is an inhibitor of ceramidase, and thus we hypothesized that it might be useful in this therapeutic context (Fig. 1a and b). We administered NOE to rats suffering from CCI. NOE was injected intrathecally on days 1–3 and 5–7 after sciatic-nerve ligation while monitoring for mechanical allodynia. Allodynia was reduced from day 3 in the NOE-injected rats relative to rats receiving vehicle treatment (Fig. 3b). Longer term monitoring of rats (n=2) up to 20 days post-surgery showed no further development of neuropathic symptoms. NOE administered to CCI rats (n=2) on day 10 post surgery (without prior administration) did not reduce allodynia, revealing the importance of treating neuropathic pain immediately after injury.

Concluding Remarks

One goal of metabolomic studies is to identify novel therapeutic avenues for disease intervention (Johnson et al. 2012). Following metabolite discovery and subsequent pathway analyses, antimetabolites can be used to inhibit the production of the dysregulated metabolite(s) and lead to the development of effective therapeutics through regaining metabostasis. However, there have been few metabolomic studies that have resulted in the discovery of endogenous metabolites that can be used as effective inhibitors. Here we have used NOE as a potential novel therapeutic for neuropathic pain. While it is exciting to consider the NOE as a possible antimetabolite to reduce DMS production, it is also possible that effects observed in NOE-treated animals are a result of properties of NOE unrelated to its inhibition of acid ceramidase. For example, NOE is also a ligand for peroxisome proliferator-activated receptor-alpha (PPAR-α) that may have antiinflammatory effects. Future work will be required with additional animals and additional models to determine the viability of NOE as a chronic pain therapeutic. Specifically, future studies will examine the effect of NOE on DMS production in the dorsal horn following injuries leading to neuropathic pain.

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Conflict of Interest The authors declare no conflicts of interest.

Ethical Approval Research animals were maintained according to institutional animal care and use committee (IACUC) guidelines. This article does not contain any studies with human participants performed by any of the authors.

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