Liquid Chromatography-Electrospray Linear Ion Trap Mass Spectrometry Analysis of Targeted Neuropeptides in Tac1$^{-/-}$ Mouse Spinal Cords Reveal Significant Lower Concentration of Opioid Peptides.

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Abstract

Tachykinin and opioid peptides play a central role in pain transmission, modulation and inhibition. The treatment of pain is very important in medicine and many studies using NK1 receptor antagonists failed to show significant analgesic effects in humans. Recent investigations suggest that both pronociceptive tachykinins and the analgesic opioid systems are important for normal pain sensation. The analysis of opioid peptides in $Tac1^{-/-}$ spinal cord tissues offers a great opportunity to verify the influence of the tachykinin system on specific opioid peptides. The objectives of this study were to develop a HPLC–MS/MRM assay to quantify targeted peptides in spinal cord tissues. Secondly, we wanted to verify if the $Tac1^{-/-}$ mouse endogenous opioid system is hampered and therefore affect significantly the pain modulatory pathways. Targeted neuropeptides were analyzed by high performance liquid chromatography linear ion trap mass spectrometry. Our results reveal that EM-2, Leu-Enk and Dyn A were down-regulated in $Tac1^{-/-}$ spinal cord tissues. Interestingly, Dyn A was almost 3 fold down-regulated ($p < 0.0001$). No significant concentration differences were observed in mouse $Tac1^{-/-}$ spinal cords for Met-Enk and CGRP. The analysis of $Tac1^{-/-}$ mouse spinal cords revealed noteworthy decreases of EM-2, Leu-Enk and Dyn A concentrations which strongly suggest a significant impact on the endogenous pain-relieving mechanisms. These observations may have insightful impact on future analgesic drug developments and therapeutic strategies.

1. Introduction

The sensation of pain produced by a noxious stimulus is not always consistent and depends on multiple factors influencing the neurophysiology of pain transmission. The nervous system has developed very complex mechanisms that control the way noxious sensory information is perceived by the organism [Basbaum *et al*., 2009]. It has been shown that considerable modulation of sensory information happens in the dorsal horn of the spinal cord [Honore *et al*., 2000, Levine *et al*., 1993]. There are various molecular events contributing to the transmission of the sensory information during the first synapse and several key neuropeptides were identified including tachykinin and opioid peptides [Kuner 2010, Mika *et al*. 2011, Felippotti *et al*. 2012].

Neuropeptides are either neurotransmitters or neuromodulators at various levels in the central nervous system and play a fundamental role in pain transmission [Levine *et al*., 1993; Seybold, 2009]. Recent studies described the central role of tachykinin and opioid related peptides [Pailleux *et al*., 2013; Felippotti *et al*., 2012; Ferland *et al*., 2011; Mika *et al*., 2011]. Many members of the tachykinin family (e.g. Substance P) are mostly pro-nociceptive neuropeptides and have been known to play an essential role in central sensitization leading to hyperalgesia and allodynia [Lecci *et al*., 2000]. Opioid peptides (i.e. endomorphins, enkephalins and dynorphins) have potent analgesic effects in the central nervous system (CNS) and play an important role in endogenous pain inhibition [Machelska, 2007, Wahlert *et al*. 2013, Bali *et al*., 2014]. They are interacting with μ, κ and δ opioid receptors expressed widely in the brain and in the spinal cord [Carr and Lovering, 2000; Stanojevic *et al*., 2008]. Tachykinin and opioid neuropeptides were extensively studied in the spinal cord, since they are major players in the synaptic processing of pain-related signals but research has been limited by several shortcomings inherent to *in vivo* neuropeptide studies.

Recently, it has been suggested that endogenous endomorphin-2 (EM-2) play an important role in the early stage of pain sensation, transmission, and modulation [Fichna *et al.*, 2007; Greco *et al.*, 2008, Wang *et al.*, 2013]. Immunohistochemistry and Immunocytochemistry analyses shown that endomorphins are largely distributed in the CNS. Endomorphins are abundant in areas such as the stria terminalis, the periaqueductal gray, the locus coeruleus, the parabrachial nucleus, and the nucleus of the solitary tract, but there are noteworthy distinctions in the neuroanatomical localization of these peptides [Pierce and Wessendorf, 2000]. Endomorphin-2 (EM-2) is primarily found in the spinal cord and lower brainstem [Martin-Schild et al., 1999; Pierce and Wessendorf, 2000]. It is largely observed in the hypothalamus, the nucleus of the solitary tract, the substantia gelatinosa of the medulla and the spinal cord dorsal horn. The participation of EM-2 in the modulation of pain transmission is important since EM-2 decreases excitability of postsynaptic receptors such as neurokinin 1 receptor (NK1) [Fichna *et al.*, 2007]. Moreover, the interaction of EM-2 with μ-opioid receptors is key to the modulation of the pain transmission [Wu *et al.*, 2015]. It regulates the release of dynorphin A (Dyn A) by stimulation of the descending dynorphinergic neurons resulting in the liberation of Dyn A. Thus, the interaction of Dyn A and κ-opioid receptors located on the presynaptic membrane inhibit the release of pronociceptive neuropeptides including Substance P (SP) [Fichna *et al.*, 2007; Zachariou and Goldstein, 1997].

Substance P is reported to play a critical role in nociceptive transmission in the CNS [Pailleux *et al.*, 2013 Gao and Peet, 1999]. Substance P is a pronociceptive peptide and agonist of NK1 located in the lamina I of the spinal cord [Teodoro *et al.*, 2013; Yu *et al.*, 1999]. Substance P is primarily synthesized in neurons and has a widespread distribution in both the central and peripheral nervous systems. More specifically, a significant proportion of primary afferent

neurons located in the dorsal root ganglia express high levels of SP and it is transported to both, the peripheral and central terminals. The expression of SP and NK1 correlates with intensity, frequency, and duration of pain [Sluka *et al*., 1997]. Agonists of NK1 receptors provoke a sustained slow depolarization that significantly contributes to the development of secondary hyperalgesia [Levine *et al*., 1993; Baumbauer *et al*., 2009; Dickenson, 1995]. Neuropeptides are derived from larger protein precursors recognized as proneuropeptides. The tachykinin precursor 1 gene (Tac1) encodes the protachykinin-1 protein containing the sequence of four tachykinin peptides, including SP [Basbaum et *al*., 1999]. The protachykinin-1 protein is cleaved by the action of specific proteases into active neuropeptides by post-translational proteolytic processing during axonal transport [Hook *et al*., 2008]. Interestingly, depolarization of a neuron containing proneuropeptides stimulates proprotein convertases processing, which occurs within synaptic vesicles in the presynaptic terminal [Yakovleva *et al*. 2006]. The stimulation of proprotein convertases processing will result in the liberation of higher concentration of neuropeptides into the intersynaptic space. Thus proprotein convertases are currently explored as a potential drug targets with the premise of partially inhibiting the release of pronociceptive peptides such as SP [Vivoli *et al*., 2012]. Tac1$^{-/-}$ mice showed significant decrease of nociceptive pain responses to moderate to intense stimuli [Cao et *al.,* 1998]. Conversely, Tac1$^{-/-}$ mice exhibited similar behavior following light or non-painful stimuli [Zimmer et *al*., 1998]. It is believe that pain perception is necessary to trigger the release of endomorphins. Endomorphin-2 is co-expressed with SP and both are found in dense core vesicles located in spinal cord primary afferent terminals suggesting concomitance release of excitatory and inhibitory neuropeptides [Sanderson *et al*., 2004; Wu *et al*., 2015]. Thus, we believe that Tac1$^{-/-}$ mice may also exhibit a significant deficit of inhibitory neuropeptides, including EM-2 and Dyn A. The objectives of this study were initially to develop and validate a HPLC–MS/MRM assay to quantify targeted peptides in spinal cord tissues.

Secondly, we wanted to verify if the Tac1$^{-/-}$ mouse endogenous opioid system is hampered and therefore affect significantly the pain modulatory pathways. This is an important consideration if new medicines are developed to specifically impede the release of SP.

2. Materials and Methods

2.1 Chemicals and reagents

Endomorphin 2 (EM-2), Leu-enkephalin (Leu-Enk), Met-enkephalin (Met-Enk), Dynorphin A (Dyn A), Substance P (SP) and Calcitonin gene related peptide (CGRP) were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Deuterium labeled analogue peptides were synthesized (CanPeptide, Inc., Pointe-Claire, QC, Canada) and used as internal standards. Acetonitrile was purchased from Fisher Scientific (NJ, USA) and trifluoroacetic acid (TFA) was obtained from BDH Laboratory supplies (Poole, England, UK). Hexane and formic acid (FA) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Standard solutions were prepared in 0.25% TFA solution as described previously [Beaudry et *al.*, 2009].

2.2 Sample Preparation

Spinal cord tissues (n=6 per genotypes) from male wild type (C57BL/6J) and male Tac1$^{-/-}$ mice (product # 004103) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and kept frozen at -80 °C until analysis. All mice were 8 weeks old at time of tissues collection. The animals from both groups were euthanized with an overdose of isoflurane followed by a transection of the cervical spine. A flush of saline was performed within the spinal canal to collect the spinal cord lumbar enlargement. Tissue sample was snap-frozen in cold hexane (-60 °C) and stored immediately at - 80 °C pending analyses. The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine of the

Université de Montréal and it was performed in accordance with the guidelines of the Canadian Council on Animal Care.

As we described previously [Beaudry, 2010], tissue processing is an important step in preserving neuropeptides from *in situ* degradation. Mouse tissues were weighed accurately and homogenized using a tissue tearor following the addition of 0.25 % TFA solution at a ratio of 1:5 (w/v) resulting in a pH < 3. At this pH, residual enzymatic activity is considerably reduced and peptides are stable under these conditions. The samples were sonicated for 20 min and 150 μL of the homogenate were mixed with 150 μL of acetonitrile to precipitate high molecular weight proteins. The samples were vortexed and centrifuged at 12,000 *g* for 10 min and 150 μL of the supernatant were transferred into an injection vial then spiked with 150 μL of the internal standard solution. Vials were capped and vortexed vigorously prior to analysis. All samples were analyzed in triplicates.

2.3 Instrumentation

The HPLC-MS/MS system included a Thermo Accela autosampler, a Thermo Accela pump and a Thermo LTQ-XL Linear Ion Trap Mass Spectrometer (San Jose, CA, USA). Linear ion trap instruments typically have unit mass resolution throughout the mass range. The instrument was calibrated and the resolution was set at 0.5-0.7 Da at full width at half maximum (FWHM). Data were acquired and analyzed with Xcalibur 2.2 (San Jose, CA, USA), and regression analyses were performed with PRISM (version 6.0d) GraphPad software (La Jolla, CA, USA) using nonlinear curve-fitting module with an estimation of the goodness of fit. The calibration lines were constructed from the peak-area ratios of targeted neuropeptides and corresponding deuterated labeled peptides used as internal standards.

2.4 Bioanalytical methods

The chromatography was achieved using a gradient mobile phase along with a microbore column Thermo Biobasic C8 100 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 1 min, the ratio was maintained at 5:95. From 1 to 12 min, a linear gradient was applied up to a ratio of 60:40 and maintained for 3 min. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 15 min for a total run time of 30 min. The flow rate was fixed at 75 µL/min and 2 µL of sample were injected using full loop mode. All targeted neuropeptides and deuterium labeled peptides eluted between 8.0 to 11.0 min. The mass spectrometer was coupled with the HPLC system using a pneumatically assisted electrospray ion source (ESI). The sheath gas was set to 25 units and the ESI electrode was set to 4000 V in positive mode. The capillary temperature was set at 300°C and the ion transfer tube voltage to 46 V. All scan events were acquired with a 100 ms maximum injection time. An activation q = 0.25 and activation time of 30 ms were used for all targeted peptides. The mass spectrometer operated for quantitative analyses in full scan MS/MS and the quantification was based on specific post-processing MRM extracted ion chromatograms. Specific analysis details are presented in Table 1. Two specific production ions were used to generate post acquisition MRM extracted ion chromatograms for quantification purposes. The method used an isotope dilution mass spectrometry (IDMS) strategy for the quantification of the targeted peptides. Due to synthesis yield issues related to label CGRP, a 37 amino acids peptide, deuterated SP was used as an internal standard for CGRP quantification along with the CGRP reference standard. The precision and accuracy was evaluated using dilution ratios of spinal cord homogenates with a solution of deuterium labeled peptide (i.e. 1:4, 1:2, 1:1, 2:1, 4:1). The labeled

peptides were used at a constant concentration of 50 pmol/mL. Absolute peptide quantification was performed using peak-area ratio of light (unlabeled) and heavy (labeled) isotope.

2.5 Statistical analysis

All data were analyzed using Student's t-test to compare mean WT and $Tac1^{-/-}$ mouse values. Significance was set a priori to $p < 0.05$. The statistical analyses were performed using PRISM (version 6.0d).

3. Results

3.1 Mass spectrometry

Full-scan and product ion mass spectra for all peptides and internal standards were obtained in positive ion mode. The full-scan electrospray mass spectrum of targeted peptides displayed the formation of characteristic pseudo molecular ions $[M+nH]^{n+}$ and the fragment ions observed in MS/MS spectra were annotated based on the Roepstorff and Fohlman nomenclature [Roepstorff and Fohlman, 1984]. Details on MS parameters and MRM transitions are reported in Table 1. Full-scan and product ion mass spectra are necessary to identify and characterize each neuropeptide. The full-scan electrospray mass spectra of targeted neuropeptides showed a base peak pseudo molecular ions at m/z 572.2 (1+) for EM-2 and 577.3 (1+) for d_5-EM-2; 556.2 (1+) for Leu-Enk and 561.3 (1+) for d_5-Leu-Enk; 574.2 (1+) for Met-Enk and 579.3 (2+) for d_5-Met-Enk; 716.4 (3+) for Dyn A and 718.1 (3+) for d_5-Dyn A; 674.4 (2+) for SP and 677.0 (2+) for d_5-SP; 952 (4+) for CGRP. Figure 1 presents product ion spectra (MS/MS) for targeted neuropeptides obtained and typical a, b, y and z positive ion fragments were observed. The observed collision-induced dissociation spectra were all compatible with the neuropeptide sequences. Additionally, we selected and optimized two of the most abundant and specific

product ions for each neuropeptide to generate post acquisition MRM extracted ion chromatograms to obtain the best sensitivity, selectivity and reproducibility. Furthermore, selected MRM transitions were monitored for extracted tissues and compared with reference materials. The overlay MRM extracted ion chromatograms display in Figure 2 demonstrate a good concordance between peptide reference standards and endogenous peptides observed in extracted tissues.

3.2 *Analytical performance*

The choice of analytical strategy is important in order to improve the precision and the accuracy of the data measurements, and consequently enhance the sensitivity of the assay. As demonstrated in a prior publication [Pailleux and Beaudry, 2012], normalization with stable isotope labeled internal standards provided the best approach for sample normalization and can be used for the absolute and relative quantification of peptides. Targeted neuropeptides were labeled on phenylalanine (d_5), glycine (d_2) and/or leucine (d_3) residues by incorporation of deuterium atoms. The instrument response linearity was tested using peak area ratio of targeted neuropeptides with corresponding deuterium labeled peptides. The linearity response was tested with dilution ratios (i.e Light/Heavy ratios) of spinal cord homogenates with a solution of deuterium labeled peptides at 1:4, 1:2, 1:1, 2:1, 4:1 to test the precision and accuracy of the isotopic dilution technique. Correlation assessments between measured peak area ratios and nominal dilution ratios were performed. As illustrated in Figure 3, the correlations were excellent (R^2 = 0.9909 to 0.9994). The precision (%CV) was comprised between 2.0% and 14.1% and the accuracy (%NOM) was contained between 86.6% and 111.4% for all targeted neuropeptides. Accordingly, the analytical method provided adequate figures of merit for targeted peptide analysis performed during this study.

3.3 *LC-MS/MS analysis of mouse spinal cords*

Following the analysis of EM-2, Dyn A, Leu-Enk, Met-Enk and CGRP in mouse spinal cords significantly altered concentrations were observed between Tac1$^{-/-}$ and WT groups for specific opioid peptides. Peptide concentrations were determined by IDMS and statistical comparisons between both groups were performed. As illustrated in Figure 4, EM-2, Leu-Enk and Dyn A were significantly down-regulated in Tac1$^{-/-}$ spinal cord tissues. EM-2 concentrations were 15.3% lower ($p < 0.05$), Leu-Enk were 20.7% lower ($p < 0.05$) and Dyn A concentrations were 62.3% lower ($p < 0.0001$) in Tac1$^{-/-}$ mice. However, concentrations for Met-Enk and CGRP were not statistically different. Also, as expected, SP was not detected in Tac1$^{-/-}$. These results clearly demonstrated that Tac1$^{-/-}$ mice exhibited significantly inferior EM-2, Leu-Enk and Dyn A concentrations in the spinal cord.

4. Discussion

Noxious sensory information is perceived by the nervous system following a cascade of complex physiological and biochemical processes [Basbaum, 1999; Basbaum *et al*., 2009]. Considerable deciphering and modulation of sensory information occurs in the spinal cord as it is relayed by peripheral sensory neurons [Moreira *et al*., 2009; Honore *et al*., 2000]. As discussed previously, neuropeptides are either neurotransmitters or neuromodulators at various levels in the CNS and play a fundamental role in pain transmission [Levine *et al*., 1993; Seybold, 2009]. Numerous neuropeptides were depicted, principally, tackykinin and opioid related peptides [Felippotti *et al*., 2012; Ferland *et al*., 2011; Mika *et al*., 2011; Bali *et al*., 2014]. Members of the tachykinin family are generally pronociceptive neuropeptides and have been known to play an important role in

central sensitization leading to hyperalgesia and allodynia [Lecci *et al*., 2000]. Opioid peptides (i.e. endomorphins and enkephalins) have potent analgesic effects in the CNS and play an essential role in endogenous pain inhibition [Machelska, 2007; Wang *et al*., 2013]. As illustrated in Figure 4, $Tac1^{-/-}$ mice showed a significant decrease of EM-2 and Leu-Enk concentrations in the spinal cord, but more importantly, an almost 3 fold decrease of Dyn A concentration. Interestingly, we have not observed any difference in CGRP concentration despite being coexpressed with SP. Likewise, we did not observed any significant differences for Met-Enk.

Endomorphin-2 is an endogenous opioid broadly distributed in the central nervous system playing an important role in the earliest stage of pain sensation, transmission and modulation. Additionally, the expression of EM-2 is closely linked with the expression of pronociceptive peptides (i.e. SP, CGRP) **[**Martin-Schild et *al*., 1999; Sanderson et *al*., 2004; Wang *et al*., 2013; Wu *et al*., 2015**]**. It has been demonstrated that EM-2 is co-localized with SP in large core dense vesicles (LCDV) present in primary afferent terminals suggesting concomitance release of excitatory and inhibitory neurotransmitters into the intersynaptic space. This may suggest that noxious stimulus evoke EM-2 release and regulate nociceptive processing by presynaptic and postsynaptic inhibitory actions [Sanderson et *al*., 2004]. Also, it is believe that pain perception is necessary to trigger the release of EM-2 into the intersynaptic space acting on opioid receptors [Williams, et *al*., 1999]. The nociceptive response of $Tac1^{-/-}$ mice is somehow hampered and could explain the lower EM-2 concentration found in the spinal cords.

Following nociceptive stimuli, SP is released from central terminal fibers into intersynaptic space and activates the NK1 receptor located at the postsynaptic membrane. SP induced Ca^{2+} mobilization is highly correlated with NK1 receptor activation, induction and internalization in the dorsal horn [Sahbaie et *al.,* 2012]. The Ca^{2+} influx and NK1 internalization induces an

increase of the expression of δ and μ-opioid receptors but not κ-opioid receptor [Aimone and Yaksh 1989, Yaksh 1988]. The activation of δ and μ-opioid receptors mediate analgesic effects. It has been demonstrated that EM-2 has an affinity and selectivity for μ-opioid receptors [Zadina et *al*., 1997; Wu *et al*., 2015]. Therefore, the significant decrease of EM-2 concentration in the spinal cord of Tac1$^{-/-}$ mice may have an impact on the endogenous pain-relieving mechanisms.

Also, protachykinin precursors of SP and δ-opioid receptors are co-localized in the LCDV and trafficking of δ-opioid receptors depends mainly on its interaction with SP domain of the protachykinin precursor [Guan et *al*., 2005]. The direct interaction between protachykinins and δ-opioid receptors is responsible for sorting δ-opioid receptors into LCDV, inducing stimulus surface insertion of δ-opioid receptors [Ueda et *al*., 1995; Zachariou and Goldstein, 1996]. However, SP is not considered to be a ligand for δ-opioid receptors, which is activated by endogenous opioid peptides such as Leu-Enk and Met-Enk. As previously established, Leu-Enk is principally localized in the spinal cord and Met-Enk in the brain [Anupama et *al*., 2009]. Proenkephalin encode both Leu-Enk and Met-Enk but prodynorphin, the precursor of Dyn A, has two copies of Leu-Enk encoded in its primary sequence. Moreover, the N-terminal sequence of Dyn A encodes specifically Leu-Enk. Proteolytic processing of prodynorphin and Dyn A can lead to the formation of Leu-Enk contributing to the observed endogenous levels. Proprotein convertase 1/3 and 2 can cleave Dyn A to form Dyn A_{1-7} and basic amino acid residues can be removed by the action of carboxypeptidase E (CPE) to form Leu-Enk [Hook *et al*., 2008]. As shown in Figure 4, the concentration of Dyn A is severely reduced in Tac1$^{-/-}$ mouse spinal cords. This result may explain the reason we observed a decrease concentration of Leu-Enk and not for Met-Enk. The interaction of EM-2 with μ-opioid receptors located on the interneurons in the dorsal horn play a central role in the release of Dyn A [Iadarola et *al*., 1988, Dubner and Ruda

1992, Bian et *al*., 1999, Malan et *al*., 2000, Bao et *al*., 2003; Bali *et al*. 2014; Wu *et al*., 2015]. The combined interaction of EM-2 with μ opioid receptors and Dyn A with κ-opioid receptors inhibits the release of SP, therefore contributing to alleviate pain [Li *et al*., 1998, Wang *et al*., 2000, Sanderson *et al*., 2004; Zadina *et al*., 1997; Przewlocka *et al*., 1999]. Morphine is a μ-opioid receptor agonist and it has been recently demonstrated that tolerance to morphine is not observed in $Tac1^{-/-}$ mice [Guan *et al.*, 2005]. Tolerance and addiction are intimately related to μ-opioid receptors expression in the brain and spinal cord [Contet *et al*., 2004] and these results suggest impaired μ-opioid receptor activities in $Tac1^{-/-}$ mice. Dynorphin A release might be significantly reduced since it is intimately related with the interaction of EM-2 and μ-opioid receptors located on the interneurons.

The $Tac1^{-/-}$ mice presented similar pain sensitivity compared with WT mice using mild thermal pain models, supporting the hypothesis that SP may not play an important role in the sensitivity to low and moderate pain. However, our results suggest that the absence of SP appears to have an impact on the endogenous pain-relieving mechanisms. This is important information since impairment of the endogenous opioid system, may have a significant impact on patients suffering of persistent low to moderate pain and on their well-being. The development of NK1 receptor antagonists did not produce clear analgesic effect for a variety of pain states during clinical trials [Hill, 2000]. The intimacy between the tachykinin and opioid systems may explain the lack of clinical efficacy of NK1 receptor antagonists. Additionally, new strategies targeting the processing of protachykinins are being developed [Vivoli *et al.*, 2012; Yongye *et al*., 2013], but may face similar limitations particularly for chronic treatments.

5. Conclusion

Tachykinin and opioid neuropeptides play a central role in pain transmission, modulation and inhibition. The treatment of pain is very important in medicine and studies using NK1 receptor antagonists failed to show significant analgesic effects in humans (e.g. post-operative and osteoarthritis pain, diabetic neuropathy and migraine). Thus, recent investigations suggest that both pronociceptive tachykinin system (SP-NK1) and the analgesic opioid system are important for normal pain sensation. The analysis of $Tac1^{-/-}$ mouse spinal cords revealed noteworthy decreases of EM-2, Leu-Enk and Dyn A concentrations which strongly suggest a significant impact on the endogenous pain-relieving mechanisms. These observations may have insightful impact on future analgesic drug developments and therapeutic strategies.

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Table 1. Summary of peptide quantification parameters used for HPLC-MS/MS analysis and post processing MRM

Peptides	Sequences	Precursor ions	Product ions	Collision energy (%)
EM-2	YPFF-NH_2	572.2 (z = 1)	408.3 (b_3)	30
			392.2 (z_3)	
(d_5)EM-2	YPF**F(**d_5**)**-NH_2	577.3 (z = 1)	408.3 (b_3)	30
			397.2 (z_3)	
Leu-Enk	YGGFL	556.2 (z = 1)	425.2 (b_4)	30
			397.3 (a_4)	
(d_5)Leu-Enk	YGG**F(**d_5)L	561.3 (z = 1)	430.2 (b_4)	30
			402.2 (a_4)	
Met-Enk	YGGFM	574.2 (z = 1)	425.2 (b_4)	30
			397.3 (a_4)	
(d_5)Met-Enk	YGG**F(**d_5)M	579.3 (z = 1)	430.2 (b_4)	30
			402.2 (a_4)	
Dyn A	YGGFLRRIRPKLKWDNQ	716.4 (z = 3)	944.3 (b_{15}^{2+})	30
			629.8 (b_{15}^{3+})	
(d_5)Dyn A	YGG**F**(d_5) LRRIRPKLKWDNQ	718.1 (z = 3)	946.6 (b_{15}^{2+})	30
			631.4 (b_{15}^{3+})	
SP	RPKPQQFFGLM-NH_2	674.4 (z = 2)	600.4 (b_{10}^{2+})	30
			254.0 (b_2)	
(d_5)SP	RPKPQQFF**G(**d_2**)L(**d_3**)** M-NH_2	677.0 (z = 2)	602.9 (b_{10}^{2+})	30
			254.0 (b_2)	
CGRP	S**C**NTAT**C**VTH RLAGLLSRSG GVVKDNFVPT NVGSEAF-NH_2	952.0 (z = 4)	1214.4 (b_{36}^{3+})	30
			962.6 (b_{28}^{3+})	

Figures legends

Figure 1. Product ion spectra (MS^2) of EM-2 (A), Leu-Enk (B), Met-Enk (C), Dyn A (D), SP (E) and CGRP (F).

Figure 2. **Analysis of targeted peptides in mouse spinal cord tissues.** EM-2 overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red) (A). Leu-Enk overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red) (B). Met-Enk overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red) (C). Dyn A overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red) (D). SP overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red) (E). CGRP overlay MRM extracted ion chromatograms of a reference (black) and a spinal cord tissue (red).

Figure 3. Calibration curve for targeted peptides. The peak area ratio between endogenous peptides and deuterium labeled peptides are display against five specific dilution ratios (e.g. 1:4, 1:2. 1:1, 2:1 and 4:1). Deuterium labeled peptides were used at a constant concentration of 50 pmol/mL.

Figure 4. Histograms of neuropeptide concentrations observed in WT (n = 6) and $Tac1^{-/-}$ (n = 6) mouse spinal cords (Mean ± SE). Peptide concentrations were determined using an isotope ratio mass spectrometry method. Concentrations observed in WT and $Tac1^{-/-}$ mouse spinal cords for EM-2 (A), Leu-Enk (B), Met-Enk (C), Dyn A (D) and CGRP (E) respectively.

Figure 1.

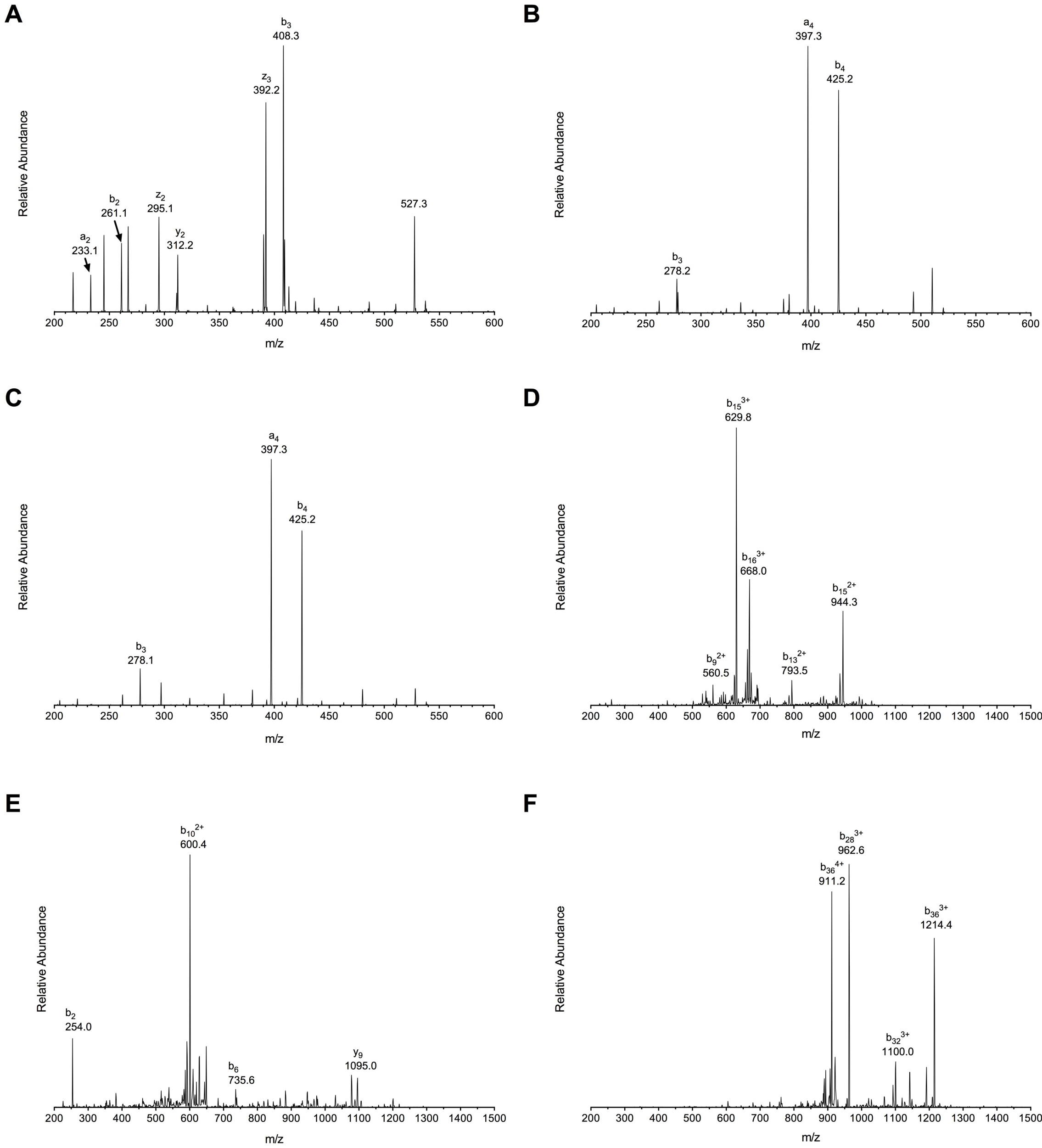


Figure 2.

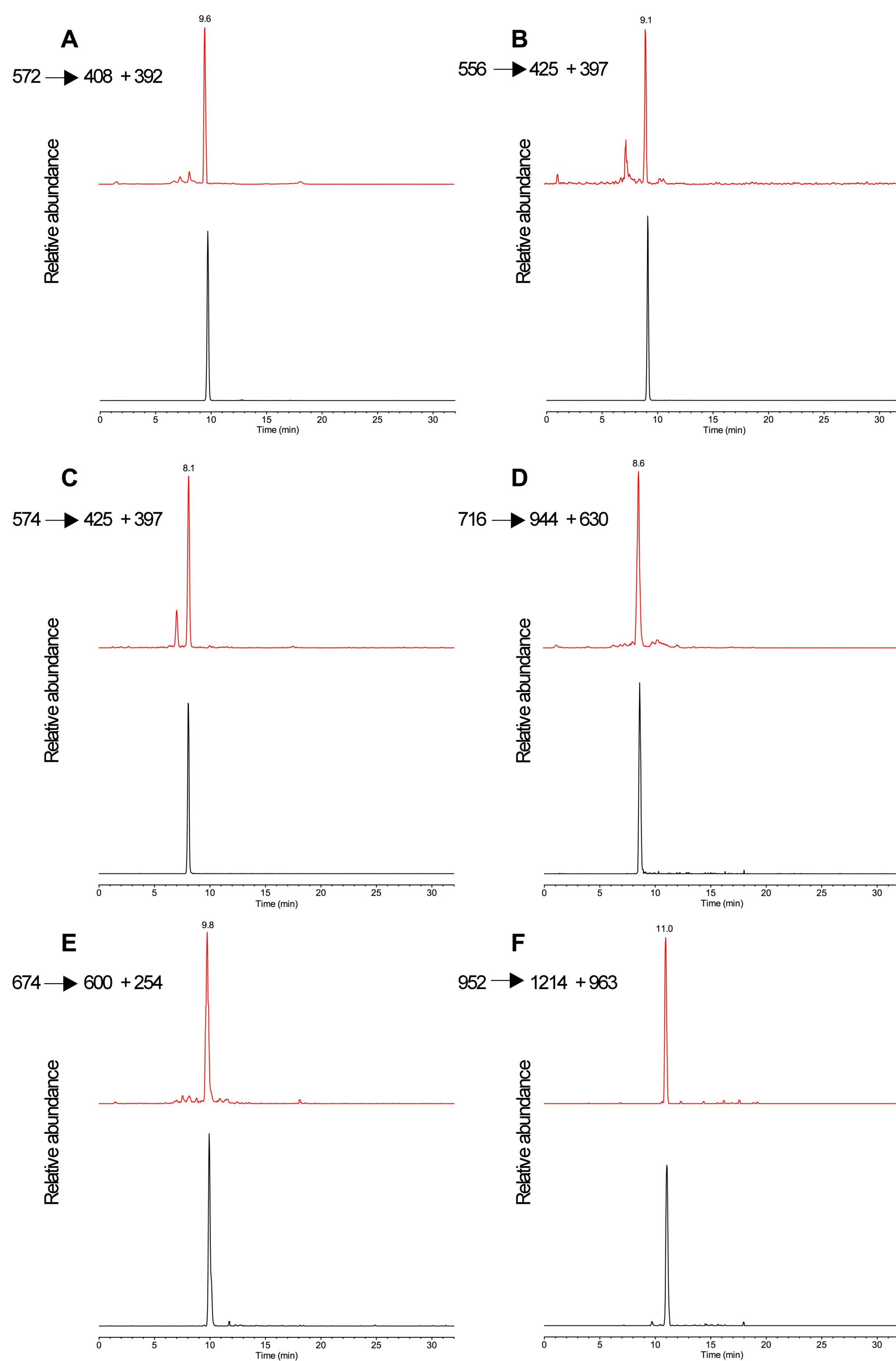
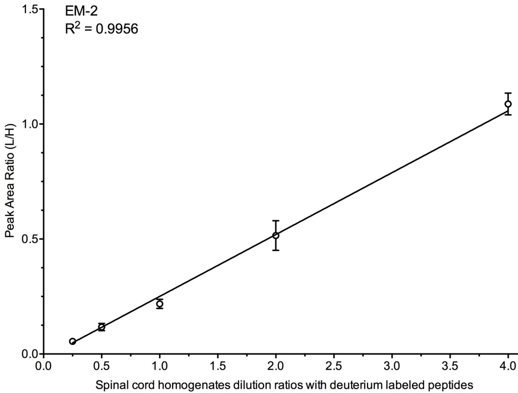
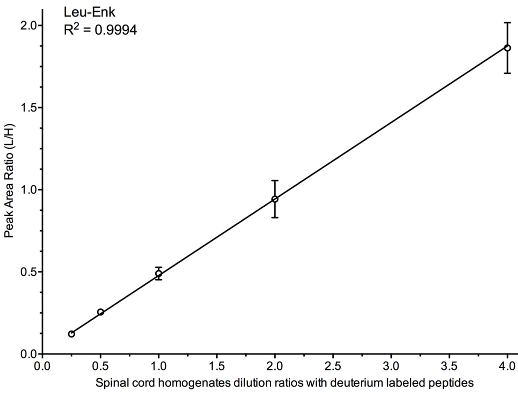
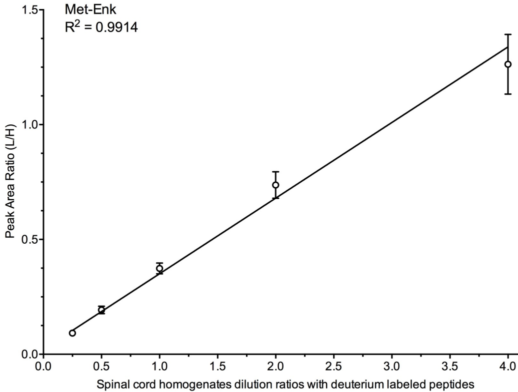
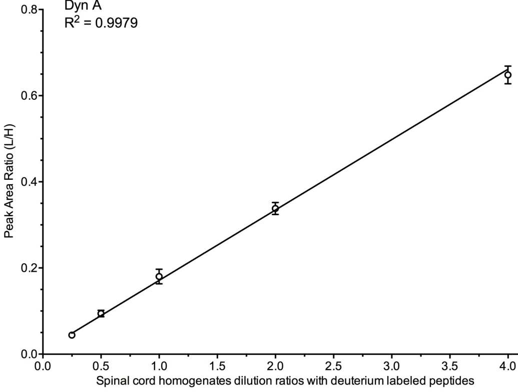


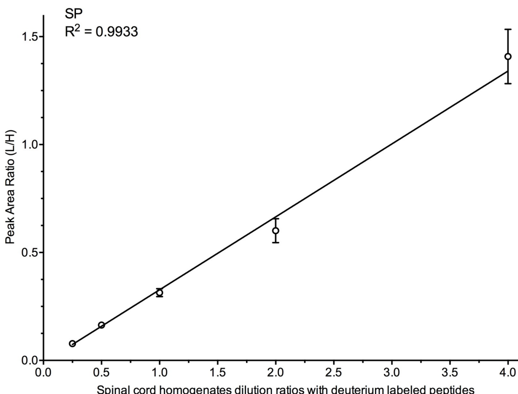
Figure 3.











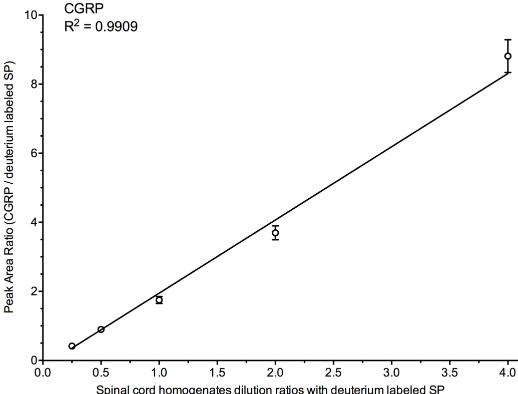


Figure 4.