Published in final edited form as:

Neuropharmacology. 2014 October ; 85: 375–383. doi:10.1016/j.neuropharm.2014.06.005.

Neuropathic plasticity in the opioid and non-opioid actions of dynorphin A fragments and their interactions with bradykinin B2 receptors on neuronal activity in the rat spinal cord

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Abstract

Dynorphin A is an endogenous opioid peptide derived from the precursor prodynorphin. The proteolytic fragment dynorphin A (1–17) exhibits inhibitory effects via opioid receptors. Paradoxically, the activity of the dynorphin system increases with chronic pain and neuropathy is associated with the up-regulation of dynorphin biosynthesis. Dynorphin A (1–17) is cleaved *in vivo* to produce a non-opioid fragment, dynorphin A (2–17). Previously, a mechanism by which the non-opioid fragment promotes pain through agonist action at bradykinin receptors was revealed. Bradykinin receptor expression is up-regulated after nerve injury and both a truncated version of non-opioid fragment dynorphin A (2–17), referred to as ‘Ligand 10’, and novel bradykinin receptor antagonist ‘Ligand 14’, are known to bind to the bradykinin receptor. Here we show that Ligand 10 facilitates the response of wide dynamic range (WDR) neurons to innocuous and noxious mechanical stimuli in neuropathic, but not naïve, animals, while Ligand 14 exhibits inhibitory effects in neuropathic animals only. Furthermore, we reveal an inhibitory effect of Ligand 14 in naïve animals by pre-dosing with either Ligand 10 or a 5-HT₃ receptor agonist to reflect activation of descending excitatory drives. Thus remarkably, by mimicking pro-excitatory pharmacological changes that occur after nerve injury in a naïve animal, we induce a state whereby the inhibitory actions of Ligand 14 are now effective. Ultimately our data support an increasing number of studies that suggest that blocking spinal bradykinin receptors may have a therapeutic potential in chronic pain states, here, in particular, in neuropathic pain.

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1. Introduction

The opioid polypeptide hormone prodynorphin is cleaved by pro-protein convertase 2 to release multiple active peptides including dynorphin A (Day, Lazure et al. 1998). The cysteine protease cathepsin L also participates in dynorphin production in the brain cortex (Minokadeh, Funkelstein et al. 2010). The endogenous dynorphin A opioid fragment is 17 amino acids long and, at physiological levels in the spinal cord, has a slight preferential binding affinity for the kappa opioid receptor (KOR) (Corbett, Paterson et al. 1982). Intrathecal or systemic injections of dynorphin A have previously demonstrated little or no anti-nociceptive effect (Walker, Moises et al. 1982; Faden 1990) despite the fact that, in many experimental animal models of chronic pain, it has been documented that dynorphin A is up-regulated in the spinal cord (Kajander, Sahara et al. 1990; Faden, Molineaux et al. 1985; Malan, Ossipov et al. 2000). Upon release, dynorphin A (1–17) is rapidly broken down by *in vivo* aminopeptidases to a non-opioid *des*-tyrosyl fragment, dynorphin A (2–17), which has low affinity for the kappa opioid receptor (Young, Walker et al. 1987; Lai, Ossipov et al. 2001). Previously, spinal dynorphin was shown to produce a significant elevation of the nociceptive threshold, measured by the tail flick test, but was associated with hind limb paralysis (Spampinato, Candeletti et al. 1984), and dynorphin has been shown to be neurotoxic in a dose dependent manner (Faden and Jacobs 1983; Skilling, Sun et al. 1992). In more recent years it was shown that administration of the opioid or non-opioid fragment of dynorphin A to the lumbar spinal cord induced transient tactile and thermal hypersensitivity in the hind limbs of naïve rats. Intriguingly, this pronociceptive effect was not blocked by the opioid antagonist naloxone and so an underlying non-opioid pronociceptive mechanism of dynorphin A was suggested along with a pathologically relevant role in chronic pain states such as neuropathy (Vanderah, Laughlin et al. 1996). Since then, data in support of spinal dynorphin promoting abnormal pain has been accumulating (Vanderah, Gardell et al. 2000) and in 2006 this pronociceptive effect was linked to activation of bradykinin receptors: A seminal study showed both that non-opioid and opioid dynorphin A fragments bind to B1 and B2 bradykinin receptors and that blockade of the receptors using the bradykinin receptor antagonist HOE-140 reversed nerve injury induced nociception, but only at times when spinal dynorphin was elevated (Lai, Luo et al. 2006). Activation of spinal bradykinin receptors by elevated spinal dynorphin has been shown to be essential in the maintenance of inflammatory hyperalgesia (Luo, Chen et al. 2008), and evidence that dynorphin A is a ligand of the B2 bradykinin receptor has been substantiated (Lee, Muthu et al. 2014). Pain processing may consequently be reduced or enhanced depending on the activity of particular fragments of dynorphin through both opioid and non-opioid mechanisms. The complex role of endogenous dynorphin in producing pronociception and antinociception after nerve injury has been investigated in knock out mice lacking prodynorphin (Xu, Petraschka et al. 2004).

As a basis for therapeutics using fragments of dynorphin A as new modulators of pain becomes increasingly attractive, a recent study describes the production of a series of non-opioid dynorphin A analogues as putative antagonists for the bradykinin receptors. Amongst these are fragments of dynorphin A, Ligand 10 and Ligand 14. Ligand 10 is a non-opioid peptide with pro-nociceptive effects whereas Ligand 14 retains high affinity for bradykinin

receptors with no agonist activity and neither fragment interacts with mu or delta opioid receptors owing to their *des*-tyrosyl status (Lee, Muthu et al. 2014).

Recording spinal neuronal activity allows for testing the therapeutic potential of dynorphin A at spinal bradykinin receptors in persistent pain states using such novel bradykinin receptor antagonists on high intensity suprathreshold activity, produced by different modalities, thus adding to behavioural approaches which generally focus on threshold changes. Here, we describe and compare the facilitatory and inhibitory effect of non-opioid and opioid dynorphin A fragments respectively on wide dynamic range (WDR) neuronal responses in naïve and nerve-injured animals. To validate the function of bradykinin receptors, for which dynorphin fragments appear to be ligands, in the spinal cord in a persistent pain state, notably neuropathy, we examine the therapeutic potential of the lead ligand, Ligand 14, *in vivo* and compare to ligand 10.

2. Materials and Methods

All animal experiments were approved by the United Kingdom Home Office and were carried out in accordance with guidelines set by personal and project licenses and thus complied with the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimize animal suffering and to reduce the number of animals used in this study.

2.1 Spinal nerve ligation

Neuropathic surgery was carried out as previously described (Suzuki, Rahman et al. 2004). Briefly, the left L5 and L6 spinal nerves of 120–130g male Sprague Dawley rats were isolated and tightly ligated with 6-0 silk thread under halothane anaesthesia (50% O₂:50% N₂O). Haemostasis was confirmed and the wound was sutured. After surgery, the foot posture and general behaviour of the operated rats were monitored throughout the post-operative period.

2.2 Electrophysiology

In vivo electrophysiology experiments were conducted on post-operative days 14–18 in anaesthetised rats as previously described (Urch and Dickenson 2003). Briefly, animals were anaesthetised and maintained for the duration of the experiment with isoflurane (1.5%) delivered in a gaseous mix of N₂O (66%) and O₂ (33%). A laminectomy was performed to expose the L4-5 segments of the spinal cord. Extracellular recordings were made from deep dorsal horn neurons (lamina V–VI) using parylene coated tungsten electrodes (A-M systems, USA). All the neurons recorded were wide dynamic range (WDR) and responded to natural stimuli including brush, low and high intensity mechanical and thermal stimuli in a graded manner with coding of increasing intensity.

A train of 16 transcutaneous electrical stimuli, delivered via stimulating needles inserted into the peripheral receptive field, was applied at 3 times the threshold current for C-fibre activation of the dorsal horn WDR cell. A post-stimulus histogram was constructed and responses evoked by A β - (0–20 ms), A δ - (20–90 ms) and C-fibres (90–350 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fibre latency band were quantified as post-discharge (350–800 ms). Activity dependent

hyperexcitability was measured as 'wind-up', calculated as the difference between the total number of action potentials at C-fibre latency produced by the train of 16 electrical stimuli, and 'input', which represented the post-synaptic C-fibre-evoked dorsal horn neuronal response following the first of the 16 electrical stimuli in the electrical train.

The peripheral receptive field was stimulated using mechanical punctate (von Frey filaments 2, 8, 26 and 60 g) and thermal (42, 45 and 48 °C applied with a constant water jet) stimuli. All natural stimuli were applied for 10 s each. Data was captured and analysed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions).

Three baseline responses to peripheral stimuli as detailed above were characterised for each neuron before pharmacological assessment (a drug study was carried out on one neuron per animal only) following 3 consecutive stable control trials (10% variation for C-fibre evoked responses, < 20% variation for all other parameters). Neuron values were averaged to give the pre-drug control values.

2.3 Drug administration

Dynorphin A (1–13), Ligand 10 (peptide sequence H-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH) and Ligand 14 (peptide sequence H-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH) (kind gifts from Dr. YeonSun Lee, University of Arizona) and 2-Methyl-5-hydroxytryptamine hydrochloride (2-Me 5-HT) (Tocris) were dissolved in H₂O. Drug was administered topically (directly onto the spinal cord) in volumes of 50 µl over the exposed surface corresponding to L3-5. Each individual drug dose effect was followed for 50 minutes, with tests carried out at 10, 30 and 50 minutes. For those experiments investigating the effect of high dose Ligand 14 on WDR neuronal responses following pre-treatment with high dose Ligand 10 or 2-Me 5-HT, Ligand 14 was administered at 40 minutes, with tests subsequently carried out at 10, 30 and 50 minutes. The maximal evoked reduction in neuronal response seen during the period 10 to 50 minutes was plotted and used for graph illustration and subsequent analysis.

2.4 Statistics

Statistical differences in fibre threshold, electrical parameters and neuronal responses to dynamic brush stimulation were determined using a paired Student's T test. Differences in mechanical and thermal coding were determined using a 2-way repeated-measures ANOVA, followed by a Bonferroni correction for multiple paired comparisons. Sphericity was tested with Mauchly's test, Greenhouse-Geiser corrections were used where required.

Data represents mean ± SEM. Asterisks denote statistically significant differences (*^{/^}P<0.05, **^{/^^}P<0.01, ***^{/^^^}P<0.001). Statistical analyses were performed using SPSS v21 (IBM).

3. Results

3.1 Dynorphin A (1–13) is anti-nociceptive in naïve and SNL animals

We evaluated the effect of intrathecal dynorphin A (1–13) (100nM and 250nM), which has the same inhibitory opioid properties as dynorphin A (1–17), on spinal neuron responses in naïve ($n = 9$) and spinal nerve ligated (SNL) ($n = 6$) animals (14–18 days). Having obtained the pre-drug control values for one neuron per animal, low dose dynorphin A (1–13) was administered and the effects followed for 50 minutes with tests carried out at 10, 30 and 50 minutes. At this time, the higher dose of dynorphin A (1–13) was administered and the process repeated. In both animals groups, both doses of dynorphin A (1–13) significantly reduced the neuronal response to natural (mechanical and electrical) stimuli, and we also observed significantly reduced C-fibre and brush evoked responses, as well as reduced wind-up (Fig. 1).

3.2 The pro-nociceptive effect of Ligand 10 is state dependent

We evaluated the effect of intrathecal non-opioid truncated dynorphin A (2–13) fragment Ligand 10 (100nM and 250nM) on spinal neuron responses in naïve ($n = 8$) and SNL ($n = 6$) animals. Having obtained the pre-drug control values for one neuron per animal, low dose Ligand 10 was administered and the effects followed for 50 minutes with tests carried out at 10, 30 and 50 minutes. At this time, the next dose of Ligand 10 was administered and the process repeated. There was no drug effect on the mean baseline evoked response to non-noxious or noxious mechanical stimuli in naïve animals, which also serves as a control for non-specific effects of application spinally. However, by complete contrast, in SNL animals, Ligand 10 significantly and markedly increased the mean maximal evoked response recorded to non-noxious (250nM Ligand 10 only) and noxious (both doses) mechanical stimuli (Fig. 2A and B). In the case of the thermal stimuli, in the absence or presence of a peripheral neuropathy, the higher dose of Ligand 10 significantly increased the mean maximal evoked response to threshold noxious and noxious stimuli (Fig. 2C and D). Ligand 10 did not significantly increase the electrically evoked responses in either animal group (Fig. 2E and F).

3.3 The anti-nociceptive effects of Ligand 14 are state dependent

We evaluated the effect of intrathecal bradykinin receptor antagonist Ligand 14 (50nM and 250nM) on spinal neuron responses in naïve ($n = 5$) and SNL ($n = 6$) animals. Having obtained the pre-drug control values for one neuron per animal, low dose Ligand 14 was administered and the effects followed for 50 minutes with tests carried out at 10, 30 and 50 minutes. At this time, the next dose of Ligand 14 was administered and the process repeated. There was no drug effect on the mean baseline evoked responses to any of the electrical, mechanical and thermal stimuli in naïve animals. But remarkably, in the presence of a peripheral neuropathy, 250nM Ligand 14 significantly and markedly attenuated the neuronal responses to low von Frey and noxious forces, as well as thermal responses from threshold to higher intensities (Fig. 3B and D). Now, we also observed significantly reduced wind-up (Fig. 3F).

3.4 The anti-nociceptive effects of Ligand 14 are revealed in naïve animals following pre-treatment with Ligand 10

We evaluated the effect of pre-treatment of intrathecal high dose Ligand 10 (250nM), and subsequent intrathecal administration of high dose Ligand 14 (250nM), on spinal neuron responses in naïve ($n = 6$) animals. Having obtained the pre-drug control values for one neuron per animal, Ligand 10 was administered and the effects followed for 40 minutes with tests carried out at 10, 30 and 40 minutes. At this time, Ligand 14 was administered and tests were carried out at 10, 30 and 50 minutes. In this naïve animal group, Ligand 10 significantly increased the mean baseline maximal evoked response to 26g von Frey stimulation and, as observed previously, significantly increased the mean maximal evoked response to threshold and noxious thermal stimuli, but did not significantly increase the electrically evoked response. Interestingly, in contrast to the effect of Ligand 14 alone in naïve animals, Ligand 14 now significantly decreased the WDR neuronal response to non-noxious and noxious mechanical, as well as to threshold and noxious thermal, stimuli (Fig. 4A and C). Further, we now observed significantly reduced input and brush evoked responses (Fig. 4E). The experiment was also performed in SNL animals ($n = 6$). Similar to the effect of 250nM Ligand 14 alone in these animals, we observed a marked facilitatory effect of ligand 10 on responses to mechanical stimulation that was significantly reduced by ligand 14 (Fig. 4B). However now, Ligand 14 did not significantly influence the facilitatory effect of ligand 10 on the evoked response to thermal, nor electrical, stimuli although there was a trend (Fig. 4D and F).

3.5 The anti-nociceptive effects of Ligand 14 are revealed in naïve animals following pre-treatment with 2-Me 5-HT

Neuropathy induced plasticity in the neuronal effects of these dynorphin fragments, and an enhancement of descending 5-HT₃ receptor mediated activity, is consistently seen in animal models of nerve injury (Bannister, Bee et al. 2009). We therefore mimicked this alteration in spinal cord processing by administering the 5HT₃ receptor agonist 2-Me 5-HT.

We evaluated the effect of pre-treatment of intrathecal 2-Me 5-HT (0.1µg in 50µl), and subsequent intrathecal administration of Ligand 14 (250nM) on spinal neuron responses in naïve ($n = 6$) animals. Having obtained the pre-drug control values for one neuron per animal, 2-Me 5-HT was administered and the effects followed for 40 minutes with tests carried out at 10, 30 and 40 minutes. At this time, Ligand 14 was administered and tests were carried out at 10, 30 and 50 minutes. In naïve animals, 2-Me 5-HT significantly increased the mean baseline evoked response to noxious natural stimuli. Remarkably, in contrast to the total lack of any effect of high dose Ligand 14 alone observed in naïve animals, Ligand 14 was now shown to significantly decrease the WDR neuronal response to noxious natural stimuli, both mechanical and thermal (Fig. 5A and B). Further, we also observed significantly reduced C-fibre, post-discharge, input and brush evoked responses (Fig. 5C).

4. Discussion

Elucidating the myriad of cellular and molecular processes that contribute to neuropathic pain, as well as discovering new optimal ways to inhibit neuronal responses in the spinal cord on neuropathy, remains a key objective in chronic pain research. A significant elevation of dynorphin A in the spinal cord in experimental models of nerve injury is well recognized (Kajander, Sahara et al.; Malan, Ossipov et al. 2000) whilst mechanistically, an excitatory pronociceptive effect of dynorphin A via direct agonist actions at spinal bradykinin receptors has been proposed (Lai, Luo et al. 2006). The involvement of NMDA receptors in mediating neuroexcitatory effects of dynorphin A has been widely investigated. Antagonising the NMDA receptor is shown to 1) prevent dynorphin A induced neurological damage, 2) block dynorphin A-evoked long lasting tactile allodynia in rats (specifically with antagonist pre-treatment) and 3) block the stimulated release of excitatory amino acids and PGE₂ by spinally infused dynorphin A (2–13) (Bakshi and Faden 1990; Vanderah, Laughlin et al. 1996; Koetzner, Hua et al. 2004). However, dynorphin A is shown to stimulate neuronal cells via a non-opioid non-NMDA mechanism (Tang, Lynch et al. 2000) and the evidence accumulated is not sufficient to differentiate between direct versus indirect actions of dynorphin A at the NMDA receptor (Lai, Luo et al. 2008).

Behavioural data in support of a pronociceptive role for spinal dynorphin A in chronic pain states is compelling. In rats, intrathecal administration of non-opioid dynorphin A into the lumbar region induced tactile hypersensitivity and thermal hyperalgesia in the hind paw. Subsequent intrathecal injection of bradykinin receptor antagonist HOE-140 produced a significant dose-dependent reversal of this effect, as well as a maximal reversal of tactile hypersensitivity in nerve injured rats in the absence of *de novo* bradykinin, providing evidence that the pronociceptive effect is mediated via bradykinin receptors (Lai, Luo et al. 2006). In the complete Freund's adjuvant (CFA) model of inflammatory pain, tactile hypersensitivity and thermal hyperalgesia in the rat hind paw is blocked by intrathecal anti-dynorphin serum and bradykinin receptor antagonist application, and elevated spinal dynorphin is suggested to maintain the persistent hyperalgesic state (Luo, Chen et al. 2008).

The spinal nerve ligation (SNL) rodent model is a well-known paradigm of neuropathic pain. In the present study, we used electrophysiology to investigate and compare the effect of non-opioid and opioid dynorphin A fragments on wide dynamic range (WDR) neuron responses to stimulus intensities above the behavioural withdrawal threshold in SNL and naïve animals. We showed that the opioid dynorphin A fragment, which predominantly exerts its effects through the kappa opioid receptor (KOR), was equally effective at inhibiting WDR neuron responses in both animal groups (section 3.1) suggesting that there is no alteration in the function of the KOR following nerve injury. Previously, an up-regulation of bradykinin B2 receptor expression was observed in L5 dorsal root ganglia (DRG) two days after nerve injury (Lai, Luo et al. 2006) which supports our finding that the non-opioid dynorphin A fragment Ligand 10 (which binds to the bradykinin receptor) facilitates the response of dorsal horn neurons to mechanical stimuli in SNL, but not naïve, animals (section 3.2).

Since stimulus intensities around the withdrawal threshold do not represent the high levels of pain reported by patients with neuropathy, electrophysiology represents a useful tool for determining the potential benefit of novel compounds in analgesia. One such compound, Ligand 14, is an amphipathic non-opioid dynorphin A analogue acting as a putative antagonist at the bradykinin receptor (Lee, Muthu et al. 2014). We investigated the therapeutic potential of Ligand 14 in SNL and naïve animals and showed it to be state-dependent, in that the drug lacked inhibitory effects in naïve animals (section 3.3). There is strong evidence to suggest that a spinal-bulbo-spinal loop involving 5-HT₃ receptor-mediated descending facilitations, which produces central excitability, exists in pain states like neuropathy (Suzuki, Morcuende et al. 2002). Remarkably, by pre-dosing naïve animals with 5-HT₃ receptor agonist 2-Me 5-HT, the inhibitory effects of Ligand 14 now became clear and prolonged for the natural stimuli, and we also observed significantly reduced electrical activity in these animals. This was also true when pre-dosing naïve animals with the non-opioid dynorphin A fragment Ligand 10 (sections 3.4 and 3.5). Thus we created a sensitized state in the spinal cord that was permissive for the inhibitory actions of Ligand 14 by simulating a serotonergic input to the spinal cord or by elevating the level of non-opioid dynorphin A. Thus, for comparable levels of neuronal firing (when comparing area under the curve data) Ligand 14 alone in naïve animals reduced the overall mechanical response by only 8% (% change from naïve baseline) compared to the marked effects seen in the presence of Ligand 10 or by activation of the 5HT-3 receptor - 42% and 48% (% change from dynorphin A (2–13) or 2-Me 5-HT baseline respectively) (Fig. 6).

The effect of novel bradykinin receptor antagonist Ligand 14, taken together with the effect of non-opioid dynorphin A fragment Ligand 10, in SNL animals suggests that bradykinin receptor up-regulation in the neuropathic animals may lead to a greater inhibitory and facilitatory role of Ligand 14 and Ligand 10 respectively, compared to their role in control animals. By contrast, after pre-treating SNL animals with non-opioid dynorphin A, the inhibitory effect of Ligand 14 persisted for the mechanical responses but was otherwise attenuated since now, no thermal or electrical responses were significantly reduced (section 3.4). The Ligand 14 fragment has no endogenous agonist activity at bradykinin receptors but shares a single binding site with Ligand 10 with very similar binding affinity (Lee, Muthu et al. 2014). Dynorphin is consistently upregulated in the spinal cord upon nerve injury, and the non-opioid actions of dynorphin are pronociceptive (effects that are consistent with excitatory effects on neurons). In theory, since we now introduce non-opioid dynorphin A to a system already more saturated with endogenous non-opioid dynorphin A, the competition for binding between this and Ligand 14 would be tipped in favour of dynorphin A, thus reducing Ligand 14's inhibitory capabilities. There is plenty of evidence that says that the effect of dynorphin A (2–13) (ligand 10) is via the B₂ receptors (Lai, Luo et al. 2006). In vitro binding studies show that ligand 10 has high affinity for the human B₂ receptor and that when rigorously controlled to pH 6.8, ligand 10's affinity is consistently in nM at the B₂ receptor (Lee, Muthu et al. 2014).

In all, coupled with 1) in vitro data showing that dynorphin A directly activates bradykinin receptors, 2) the lack of pronociceptive activity of dynorphin A in transgenic mice with a null mutation of the B₂ bradykinin receptor (*Bdkrb2*^{-/-}), and 3) the results of previous behavioural studies including those that use prodynorphin knock out mice to demonstrate

that upregulated spinal dynorphin is pronociceptive and required for the maintenance of persistent neuropathic pain (Wang, Gardell et al. 2001), our data support and extend the findings by demonstrating a physiological relevance of the action of dynorphin A and its putative analogue, bradykinin receptor antagonist Ligand 14, at WDR neurons in neuropathic pain states. The pronociceptive effects of ligand 10 and the ability of ligand 14 to both block the effects of ligand 10 and possess inhibitory effects alone in the presence of neuropathy could be observed on the whole range of these evoked neuronal responses. The data strongly support and extend the previous concept that dynorphin and its fragments play key bi-directional roles at the spinal cord level. A plausible substrate for these effects would be agonist (eg ligand 10) and antagonist (eg ligand 14) actions at bradykinin receptors alongside weak opioid mediated inhibitions of dynorphin A (1–13) itself.

Acknowledgments

Supported by the University College London, Grant no. FENDV, and National Institutes of Health (Bethesda, Maryland) Grant no. FEPB (to Dr. Porreca).

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Abbreviations

WDR	Wide dynamic range
DRG	Dorsal root ganglia
B2 receptor	Bradykinin 2 receptor
SNL	Spinal nerve ligation
CFA	Complete Freund's adjuvant
KOR	Kappa opioid receptor
5-HT	5-hydroxytryptamine
2-Me 5-HT	2-Methyl-5-hydroxytryptamine hydrochloride
DyA	Dynorphin A

5-HT₃ R 5-hydroxytryptamine 3 receptor

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Highlights

- Non-opioid Dy-A fragment L-10 facilitates WDR neuronal responses in neuropathy
- While novel bradykinin receptor antagonist L-14 inhibits the response in neuropathy
- An inhibitory role for L-14 is revealed in naïve animals after pre-dosing with L-10
- L-14 is also inhibitory in naïve animals after pre-dosing with 5-HT₃ R agonist
- Blocking spinal bradykinin receptors offers a therapeutic potential in neuropathy

Figure 1.

The effect of intrathecal dynorphin A (1–13) (low dose 100nM and high dose 250nM) on the mean baseline response profiles of WDR spinal neurons in naive ($n = 9$) and spinal-nerve ligated (SNL) ($n = 6$) animals. All data are presented as mean $\pm$ standard error of the mean (SEM). Here, drug effect is expressed as the mean maximal evoked reduction in neuronal response. In both animal groups the evoked responses to mechanical (A and B), thermal (C and D) and electrical (E and F) stimuli were recorded before and after drug treatment. Both doses of dynorphin A (1–13) significantly reduced the excitability of spinal neurons to

peripherally applied noxious natural, and a subset of electrical, stimuli (C-fibre, wind-up and brush evoked responses were reduced). In addition, in SNL animals, both doses of dynorphin A (1–13) significantly reduced the excitability of spinal neurons to a peripherally applied non-noxious mechanical stimulus. Significant differences from baseline responses to low dose dynorphin A (1–13): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Significant differences from baseline responses to high dose dynorphin A (1–13): $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$. PD = post-discharge, IP = input, WU = wind up, DyA = dynorphin A. Note, vertical axis is set to 800.

Figure 2.

The effect of intrathecal Ligand 10 (low dose 100nM and high dose 250nM) on the mean baseline response profiles of WDR spinal neurons in naive ($n = 8$) and SNL ($n = 6$) animals. All data are presented as mean $\pm$ standard error of the mean (SEM). Here drug effect is expressed as the mean maximal evoked increase in neuronal response. In both animal groups the evoked responses to mechanical (A and B), thermal (C and D) and electrical (E and F) stimuli were recorded before and after drug treatment. In SNL animals, but not naïve animals, high dose Ligand 10 significantly increased the excitability of spinal neurons to

peripherally applied non-noxious and noxious mechanical stimuli. In both animals groups high dose Ligand 10 significantly increased the excitability of spinal neurons to peripherally applied noxious thermal stimuli while the neuronal responses to peripherally applied electrical stimuli were not significantly affected. Significant differences from baseline responses to low dose Ligand 10: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Significant differences from baseline responses to high dose Ligand 10: $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$. PD = post-discharge, IP = input, WU = wind up.

Figure 3.

The effect of intrathecal Ligand 14 (low dose 50nM and high dose 250nM) on the mean baseline response profiles of WDR spinal neurons in naive ($n = 5$) and SNL ($n = 6$) animals. All data are presented as mean $\pm$ standard error of the mean (SEM). Here drug effect is expressed as the mean maximal evoked reduction in neuronal response. In both animal groups the evoked responses to mechanical (A and B), thermal (C and D) and electrical (E and F) stimuli were recorded before and after drug treatment. In SNL animals, but not naïve animals, high dose Ligand 14 significantly reduced the excitability of spinal neurons to

peripherally applied non-noxious and noxious mechanical, as well as to noxious thermal, stimuli. Wind-up was now also significantly reduced. Significant differences from baseline responses to low dose Ligand 14: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences from baseline responses to high dose Ligand 14: ^ $P < 0.05$, ^^ $P < 0.01$, ^^ $P < 0.001$. PD = post-discharge, IP = input, WU = wind up. Note vertical axis is set to 1000.

Figure 4.

The effect of high dose intrathecal Ligand 10 (250nM), and subsequent application of high dose intrathecal Ligand 14 (250nM), on the mean baseline response profiles of WDR spinal neurons in naive ($n = 6$) and SNL ($n = 6$) animals. All data are presented as mean $\pm$ standard error of the mean (SEM). Here drug effect is expressed as the mean maximal evoked increase in neuronal response following treatment with Ligand 10, and as the mean maximal evoked decrease in response following treatment with Ligand 14. In naive animals the evoked response to mechanical (A), thermal (C) and electrical (E) stimuli were recorded

before and after drug treatment. Following pre-treatment with Ligand 10, Ligand 14 significantly reduced the excitability of spinal neurons to peripherally applied non-noxious and noxious mechanical stimuli as well as to noxious thermal stimuli. Input and brush-evoked responses were also significantly reduced. In SNL animals, following pre-treatment with Ligand 10, Ligand 14 significantly reduced the excitability of spinal neurons to peripherally applied noxious mechanical stimuli only (B, D and F). Significant differences from baseline responses to Ligand 10: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences from Ligand 10 response to Ligand 14: ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.001$. PD = post-discharge, IP = input, WU = wind up.

Figure 5.

The effect of intrathecal 2-Methyl 5-HT (0.1µg in 50ul saline), and subsequent application of high dose intrathecal Ligand 14 (250nM), on the mean baseline response profiles of WDR spinal neurons in naive (n = 6) animals. All data are presented as mean ± standard error of the mean (SEM). Here drug effect is expressed as the mean maximal evoked increase in neuronal response following treatment with 2-Me 5-HT and as the mean maximal evoked decrease in response following treatment with Ligand 14. The evoked responses to mechanical (A), thermal (B) and electrical (C) stimuli were recorded before and after drug

treatment. Following pre-treatment with 2-Me 5-HT, Ligand 14 significantly reduced the excitability of spinal neurons to peripherally applied noxious natural, and a subset of electrical, stimuli (C-fibre, post-discharge, input and brush-evoked responses were significantly reduced). Significant differences from baseline responses to 2-Me 5-HT: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Significant differences from 2-Me 5-HT response to high dose Ligand 14: $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$. PD = post-discharge, IP = input, WU = wind up.

Figure 6.

Area-under-curve comparisons of the evoked neuronal response to non-noxious and noxious mechanical stimulation in SNL and naive animal groups. There is a greater overall inhibitory effect of high dose Ligand 14 (250nM) in naïve animals following pre-treatment with Ligand 10 or 2-Me 5-HT, compared to the effect of Ligand 14 alone. Thus, the inhibitory effects are revealed in control animals by activation of the neurones by Ligand 10 and also by activation of the 5HT-3 receptor by 2-Me 5-HT.