Review

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# Dynorphin A analogs for the treatment of chronic neuropathic pain

Chronic pain is one of the most ubiquitous diseases in the world, but treatment is difficult with conventional methods, due to undesirable side effects of treatments and unknown mechanisms of pathological pain states. The endogenous peptide, dynorphin A has long been established as a target for the treatment of pain. Interestingly, this unique peptide has both inhibitory (opioid in nature) and excitatory activities (nonopioid) in the CNS. Both of these effects have been found to play a role in pain and much work has been done to develop therapeutics to enhance the inhibitory effects. Here we will review the dynorphin A compounds that have been designed for the modulation of pain and will discuss where the field stands today.

First draft submitted: 10 July 2015; Accepted for publication: 6 October 2015; Published online: 29 January 2016

Sara M Hall[1], Yeon Sun Lee[1] & Victor J Hruby*,[1]

[1]Department of Chemistry & Biochemistry, University of Arizona, Tucson, AZ 85721, USA

*Author for correspondence:
Tel.: +1 520 621 6332
Fax: +1 520 621 8407
hruby@email.arizona.edu

## Chronic neuropathic pain

Pain is an unpleasant feeling that has been categorized as acute (lasting a few days or weeks) or chronic (lasting months/years) [2]. Acute pain arises from tissue injury and usually dissipates once the injury is healed, and this pain is useful in alerting the body of damage [2]. On the other hand, chronic pain has no purpose and exists in the absence of tissue injury. Although chronic pain is one of the most prevalent diseases, affecting 100 million Americans [2] (more than heart disease, cancer and diabetes combined), funding from the NIH is miniscule when compared with the other diseases due to rare fatality (Figure 1). Chronic pain greatly limits the patient's quality of life and costs an estimated US$61 billion/year in lost productive time alone [3]. Neuropathic pain, a type of chronic pain, results from the dysfunction of the CNS or the peripheral nervous system (PNS) that can occur in the presence or absence of an initial injury. The hallmarks of this type of pain are hyperalgesia, increased sensitivity to painful stimuli, and allodynia, increased sensitivity to nonpainful stimuli [4].

## Therapeutics for chronic neuropathic pain

There are currently many therapeutics for inflammatory acute pain, but unfortunately, there are not many effective treatments for chronic neuropathic pain. Even with quite different mechanisms, acute and chronic pain symptoms are treated with the same therapeutics. Acetaminophen and NSAIDs are generally prescribed for mild-to-moderate pain states and have relatively little side effects and work well for postoperative pain but not for neuropathic pain. For severe pain, opioids are often prescribed and are useful in acute pain states but are not effective in chronic pain states because of serious side effects caused by long-term administration [2]. Anticonvulsants such as pregablin and gabapentin are often prescribed and are effective for patients with postherpetic neuralgia, fibromyalgia and diabetic peripheral neuropathy [5]. For over 40 years, antidepressants such as tricycles, selective serotonin reuptake inhibitors and serotonin norepinephrine reuptake inhibitors have been used for the treatment of neuropathic pain and show efficacy for some patients [6]. Steroids are generally used

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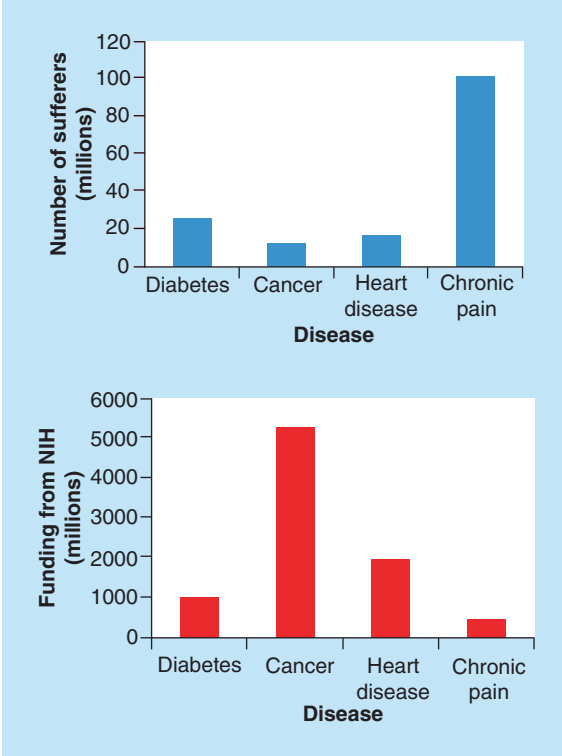



**Figure 1. Estimates of the number of people suffering from the most common diseases in the USA and estimates of funding from the NIH.** Data taken from [2], American Diabetes Association, American Cancer Society and the American Heart Association.

as an adjunct therapy with opioids and are beneficial to patients with metastatic bone pain, visceral pain and neuropathic pain [7]. Ziconotide, a natural peptide from snail venom, is approved for the treatment of intractable chronic pain [8,9]. Ziconotide administration is limited to intrathecal because of cardiovascular liabilities caused by the intravenous route [10]. There appears to be many treatments for neuropathic pain, but most of them are not effective in some patients showing high numbers needed to treat (NNT) and/or develop serious side effects (Table 1). Therefore, there is still a pressing need for novel therapeutics for the management of chronic neuropathic pain.

## Dynorphin A, an extraordinary opioid peptide

In 1979, Goldstein *et al.* discovered a peptide from the porcine pituitary that was termed Dynorphin A (Dyn A), from the Greek word dynamis (power) [15]. The peptide was found to contain the Leu-enkephalin structure and was 730-fold and threefold more potent than Leu-enkephalin in the guinea pig ileum (GPI) and mouse vas deferens (MVD) contractility assays, respectively. The effects of the peptide in tissues were blocked by an opioid antagonist, naloxone, suggesting the opioid receptors as a target [15]. A few years later, the full structure of this peptide was determined to be a heptadecapeptide, H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH, with the Dyn A-(1–13) fragment crucial for its potency [16]. Later studies found that Dyn A is derived from prodynorphin [17]. After processing, the inactive precursor protein, prodynorphin, consists of α-neoendorphin and Big Dyn. Cleavage via prohormone convertase 2 affords the bioactive peptides Leu-enkephalin (5 residues) from α-neoendorphin and Dyn A (17 residues) and Dyn B (13 residues) from Big Dyn (Figure 2) [17].

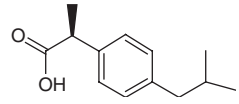
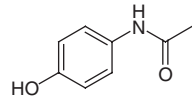
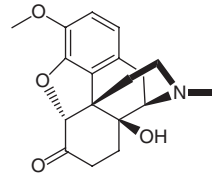
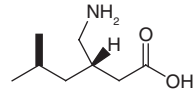
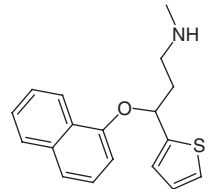
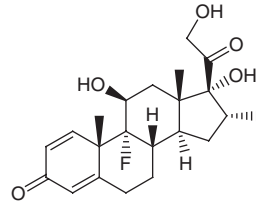
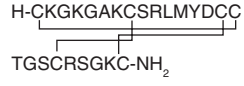
## Dyn A, an endogenous ligand for the κ-opioid receptor (KOR)

Although Dyn A has affinity for all three opioid receptors (Table 2), μ-opioid receptor (MOR), KOR and δ-opioid receptor (DOR), it may act primarily at the KOR considering the similar activity with the KOR-selective agonist, ethylketocyclazocine [18]. The KOR is a $G_{i/o}$-coupled G-protein coupled receptor (GPCR), in which agonists inhibit adenylate cyclase. There have been some publications suggesting different subtypes of the KOR (subtype 1–3), but to date only the KOR1 has been cloned [19–21]. Work by Devi and colleagues found that KOR, MOR and/or DOR can form heteromeric complexes that produce the pharmacological profiles of the previously reported KOR subtypes [22–24]. For the purpose of this review, we will only discuss ligands designed for the homomeric KOR1. For information involving bivalent ligands designed for the heteromers see the current work done by Portoghese [25,26]. The receptor is widely expressed in the brain, spinal cord and peripheral tissues [27], and the expression of the receptor is found in areas related to pain circuitry; dorsal root ganglia (DRG), dorsal spinal cord, rostral ventromedial medulla and periaqueductal gray [28–30]. Studies have shown that Dyn A interacting with the KOR has inhibitory effects on the brain reward circuit by inhibiting dopamine in brain regions that are associated with drug dependence [31,32]. In terms of pain, there has been some evidence that activation of KOR antagonizes the analgesic effects of the MOR [33]. Other studies have shown that selective KOR agonists have antinociceptive effects as well as being free of the adverse side effects (constipation, respiratory depression, tolerance, addiction) that are mediated by the MOR [34]. These selective KOR agonists have been found to be effective analgesics in visceral and inflammatory pain models [35,36]. Although KOR agonists are free of the side effects from MOR agonists, they have been found to have dysphoria and psychotomimetic effects that limit their use in the clinic [37].

## Structure–activity relationship of Dyn A at KOR

An early structure–activity relationship (SAR) study by Chavkin and Goldstein examined truncations of Dyn (1–13), since this fragment was found to have the same inhibitory effects as Dyn A [39]. Truncations of the *N*-terminal tyrosine residue abolished opioid activity in the GPI assay, and therefore, the studies focused on truncations at the *C*-terminus. From these studies, it was found that a *C*-terminal modification to an amide retained high affinity [39]. It was also found that $Lys^{13}$, $Lys^{11}$ and $Arg^{7}$ residues were important for the biological activity at the KOR, with $Arg^{7}$ being the most important residue [39]. From this work, it was suggested that the first four residues of Dyn A contain the message region, whereas residues 5–13 are the address region which is responsible for the potency and specificity at the KOR. Further studies have been performed in many laboratories to discover key residues that imparted selectivity for the KOR over MOR and DOR. Substitution of $Gly^{2}$ with DAla in Dyn A-(1–11)$NH_2$ to prevent aminopeptidase activity was found to not be a viable option, as this substitution decreased biological activity. Instead, *N*-terminal methylation, another strategy to prevent aminopeptidase activity, was well tolerated resulting in similar potency ($IC_{50}$ = 0.42 nM) as parent ligand ($IC_{50}$ = 0.23 nM) [39]. Our previous studies found that when $Gly^{3}$ of Dyn-(1–11)$NH_2$ was replaced with DAla, this led to greater selectivity at

**Table 1. Commonly prescribed drugs for chronic neuropathic pain.**

| Class of drug/example | Mechanism of action | Structure | Side effects† | NNT | Ref. |
|---|---|---|---|---|---|
| NSAIDs/ibuprofen | Nonselective inhibitors for COX |  | Ulcers, stomach bleeding, high blood pressure | 2 | [11] |
| Acetaminophen | Inhibition of prostaglandin synthesis. Unknown |  | Nausea, loss of appetite, jaundice, stomach pain | 4.6 | [11] |
| Opioids/oxycodone | Opioid receptors (mainly MOR) |  | Addiction, tolerance, constipation, respiratory depression | 2.6 | [12] |
| Anticonvulsant/ pregablin | $Ca^{2+}$ channel $\alpha_2$-$\delta$ subunit |  | Dizziness, somnolence, ataxia, tremor | 4.5 | [12] |
| Antidepressants/ duloxetine | Selective SNRI |  | Drowsiness, dry mouth, nausea, constipation | 5.0 | [12] |
| Steroids/ dexamethasone | Inhibits prostaglandin synthesis |  | Upset stomach, vomiting, headache, increased hair growth | N.D. | |
| Toxin/ziconotide | *N*-type $Ca^{2+}$ blocker |  | Dizziness, drowsiness, headache, nausea | 2‡ | [13] |

†Side effects found at [14].
‡Calculated for postoperation pain.
MOR: μ-opioid receptor; N.D: Not determined; NNT: Numbers needed to treat; SNRI: Serotonin norepinephrine reuptake inhibitor.

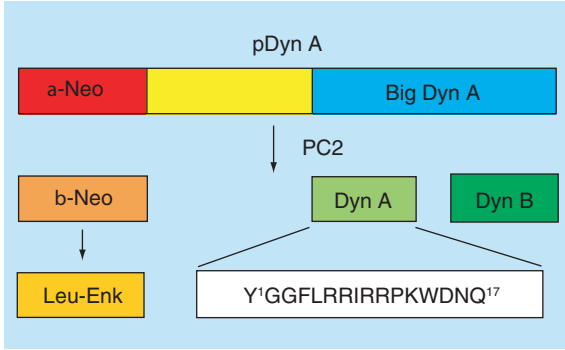



**Figure 2. Cleavage of prodynorphin into the active peptides Dyn A, Dyn B and Leu-enkephalin.**

the KOR over the MOR (350-fold) and DOR (1300-fold) [40]. Our group extensively studied the effects of cyclization between Leu$^5$ and Lys$^{11}$ in Dyn-(1–11)$NH_2$ and identified a selective KOR ligand [Pen$^5$, Cys$^{11}$] Dyn-(1–11)$NH_2$ (KOR/MOR/DOR = 1/2.4/165) in binding assays but was inactive in the GPI and MVD assays [41–43]. Similar results were seen with substitutions of Tyr$^1$ with $N^{\alpha}$-AcTyr$^1$, DTyr$^1$, Phe$^1$, or Phe(p-Br)$^1$, and Ile$^8$ with DAla$^8$. All the previous compounds were found to be up to 576-fold more selective at KOR than DOR but all had no activity in GPI or MVD assays so were not pursued any further [44]. An enhancement of KOR selectivity was also seen with substitution of DPro in position 10 of Dyn (1–11)$NH_2$ (KOR/MOR/DOR = 1/8/70, Table 3) [45]. Aldrich and colleagues used [DPro$^{10}$] Dyn A-(1–11) as a template to investigate *N*-monoalkylated and *N,N*-dialkylated tyrosine derivatives. They found that all of the *N*-monoalkylated analogs had greater KOR selectivity when compared with the *N,N*-dialkylated analogs, with the greatest KOR selectivity shown by *N*-allyl substitution (KOR/MOR/DOR = 1/220/9200, Table 3) [46]. Many of these analogs did not show efficacy in *in vivo* models of pain, and one reason may be because of low stability of the analogs. It has been found that degradation of Dyn A occurs at Tyr$^1$-Gly$^2$, Arg$^6$-Arg$^7$ and Pro$^{10}$-Lys$^{11}$ [47]. In an effort to increase the stability of the analogs as well as their blood–brain barrier (BBB) penetration, E-2078, was synthesized (H-MeTyr-Gly-Gly-Phe-Leu-Arg-NMeArg-DLeuNHEt). This analog was found to have a half-life ($t_{1/2}$) of 4 h (cf, Dyn A $t_{1/2}$ = 0.5 h), and had binding affinities at the opioid receptors comparable to Dyn A (KOR/MOR/DOR = 1/2.4/14, Table 3) [48,49]. E-2078 was found to be analgesic following subcutaneous administration in the formalin and tail flick tests in mice and crossed the BBB [47,50]. Another analog that was designed for an increase in stability was, SK-9709 (H-Tyr-DAla-Phe-Leu-Arg$\Psi$($CH_2$NH)-Arg-$NH_2$), in which the peptide bond was replaced by a $\Psi$ ($CH_2$NH) bond. SK-9709 showed antinociceptive effects in the acetic acid induced writhing test after subcutaneous, intracerebroventricular and intrathecal injections and was able to cross the BBB [51]. Although E-2078 and SK-9709 both appear to be promising, selective and stable KOR agonists, they were found to be effective in inflammatory pain models, the formalin test and acetic acid-induced writhing test. It is well known that opioids are effective in inflammatory pain states but are less effective in neuropathic pain states [52]. Therefore, tests with these compounds in neuropathic pain states, such as, spinal nerve ligation, diabetes-induced neuropathy or neuropathic bone cancer pain will need to be performed.

Although the gold standard for relief of severe pain is MOR agonists, there are serious side effects caused by long-term administration [53]. KOR agonists were pursued as a pain therapeutic that would not have the adverse side effects as MOR agonists. Unfortunately, it was found that while centrally acting KOR agonists did not have addictive properties, they had unpleasant side effects such as dysphoria, sedation and diuresis [37]. These side effects are mediated through the CNS and limit the use of KOR agonists for treatment of pain at the CNS but may be beneficial in pain at the PNS. Since KOR agonists have effects on mood, they are being examined as a treatment of the manic phase in bipolar disorder [54]. KOR agonists have been found to decrease dopamine levels and are also being examined as a treatment for drug abuse, in particular cocaine [55,56].

## KOR antagonists for the treatment of pain

Since Dyn A and the KOR are known to be involved in the body's response to stress, KOR antagonists could have some benefit in anxiety and depression. KOR antagonists have been found to be anxiolytic [57], prevent stress-induced reinstatement of cocaine-seeking behavior [58] and be beneficial in the treatment of opiate addiction [59]. KOR antagonists are also being investigated as a combination therapy with MOR/DOR agonists for the treatment of pain. In an effort to develop KOR antagonists, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

**Table 2. Binding and function of Dynorphin A at the opioid receptors.**

| | hMOR | hDOR | hKOR |
|---|---|---|---|
| $K_i$ (nM) | 1.60 ± 0.18 | 1.25 ± 0.12 | 0.05 ± 0.01 |
| $EC_{50}$ (nM) | 30 ± 5 | 84 ± 11 | 0.43 ± 0.08 |

hDOR; human δ-opioid receptor; hKOR: human κ-opioid receptor hMOR: human μ-opioid receptor.
Data taken from Zhang *et al.* 1998 [38].

Table 3. Common Dynorphin A agonists at the KOR.

| Compound | Structure | Selectivity (KOR/MOR/DOR) | GPI (nM) | Ref. |
|---|---|---|---|---|
| Dyn A- (1–13) | H-Y-G-G-F-L-R-R-I-R-P-K-L-K-OH | 1/17/N.D. | 0.63 | [15,51] |
| Dyn A-(1–11)$NH_2$ | H-Y-G-G-F-L-R-R-I-R-P-K-$NH_2$ | 1/17/44 | 1.1 ± 0.3 | [40] |
| [DAla$^3$] DynA-(1–11) $NH_2$ | H-Y-G-a-F-L-R-R-I-R-P-K-$NH_2$ | 1/350/1300 | 8.1 ± 2.3 | [40] |
| [DPro] DynA-(1–11) | H-Y-G-G-F-L-R-R-I-R-p-K-OH | 1/8/70 | 0.22 | [46] |
| N-allyl [DPro$^{10}$] DynA-(1–11) | N-allyl,Y-G-G-F-L-R-R-I-R-p-K-OH | 1/220/920 | 18 | [46] |
| E-2078 | H-MeY-G-G-F-L-R-NMeR-I-NHEt | 1/2.4/14 | 0.3 ± 0.03 | [49] |
| SK-9709 | H-Y-a-F-L-RΨ($CH_2$NH)-Arg-$NH_2$ | 1/15/350 | N.D. | [51] |

N.D.: Not determined.

(Tic) was replaced for Gly$^2$ in Dyn A-(1–11)$NH_2$ since it is known from dermorphin and Leu-enkephalin that this substitution can convert agonists to antagonists [60]. This substitution did cause antagonism at all three opioid receptors, but the selectivity for KOR was reduced (KOR/MOR/DOR = 1/0.5/0.14, Table 4) [60]. The Schiller group designed (2′,6′-dimethyltyrosine [Dmt]$^1$) Dyn A-(1–11)$NH_2$ analogs with the *N*-terminal amino group deleted or replaced with a methyl group using 3,4-Dihydro-2H-pyran-2-methanol (Dhp) or (2*S*)-2-methyl-3-(2′,6′-dimethyl-4′-hydroxyphenyl)-propionic acid (Mdp) to test for KOR antagonism. All of the analogs were potent KOR antagonists with weak MOR and DOR activity, with dynantin ([(2*S*)-Mdp$^1$] Dyn A-[1–11]$NH_2$) being the most selective for KOR, with subnanomolar binding affinity and antagonist activity [61]. [Pro$^3$] DynA-(1–11)$NH_2$ is one of the most selective KOR analogs (KOR/MOR/DOR =1/2100/3330, Table 4) but is a weak antagonist in both the GPI assay as well as the ($^{35}$S)GTPγS assay [62]. Aldrich and colleagues identified aromatic dynorphin (arodyn) starting from a novel chimeric peptide, extacet (a MOR-selective analog), substituted at the *N*-terminus in the message region. Arodyn was found to be selective for KOR (KOR/MOR/DOR = 1/170/580, Table 4) and was able to completely reverse the agonist activity of Dyn A-(1–11)$NH_2$ in the cAMP assay [63]. Aldrich and colleagues also synthesized, cyclodyn, (cyclo$^{N,5}$[Trp$^3$, Trp$^4$, Glu$^5$] DynA-[1–11] $NH_2$) by connecting the *N*-terminus to the side chain of the Glu$^5$ residue. Cyclodyn was found to be selective for KOR (KOR/MOR/DOR = 1/12/330, Table 4) and was able to block the agonist activity of Dyn A-(1–11) $NH_2$ in the cAMP assay [64]. Another cyclic Dyn A analog is zyklophin, ($N^{\alpha}$-benzyl)Tyr$^1$ cyclo(DAsp$^5$, Dap$^8$) DynA-(1–11)$NH_2$, which was selective for KOR (KOR/MOR/DOR = 1/190/330, Table 4) [65]. In the cAMP assay, zyklophin antagonized Dyn A-(1–11)$NH_2$ with $K_b$ = 84 nM, and also antagonized the antinocicpetive actions induced by U50,488 in the warm water tail withdrawal assay following subcutaneous injection [65,66]. Zyklophin was found to be more metabolically stable than other analogs, was able to cross the BBB and had a shorter duration of action (12 h) when compared with other KOR antagonists (e.g., JDTic several weeks) (for a complete review on KOR ligands see [67]).

## Future directions for Dynorphin A-based KOR ligands

KOR agonists have serious side effects (dysphoria and psychotomimetic effects) that limit their therapeutic use for pain, but have potential to treat bipolar disorder [54]. Since the psychotomimetic side effects are

Table 4. Common Dynorphin A antagonists at the KOR.

| Compound | Structure | Selectivity (KOR/MOR/DOR) | $K_e$ (nM) | Ref. |
|---|---|---|---|---|
| [Tic$^2$] DynA-(1–11)$NH_2$ | H-Y-Tic-G-F-L-R-R-I-R-P-K-$NH_2$ | 1/0.5/0.14 | 460 | [60] |
| Dynantin | (2*S*)-Mdp-G-G-F-L-R-R-I-R-P-K-$NH_2$ | 1/260/200 | 3.9 ± 0.7 | [61] |
| [Pro$^3$] DynA-(1–11)$NH_2$ | H-Y-G-P-F-L-R-R-I-R-P-K-$NH_2$ | 1/2100/3300 | 240 ± 51 | [62] |
| Arodyn | Ac-F-F-F-R-L-R-R-a-R-P-K-$NH_2$ | 1/170/580 | N.D. | [63] |
| Cyclodyn | c[Y-G-W-W-E]-R-R-I-R-P-K-$NH_2$ | 1/12/330 | N.D. | [64] |
| Zyklophin | Bz-Y-G-G-F-c[d-R-R-Dap]-R-P-K-$NH_2$ | 1/190/330 | 84$^{\dagger}$ | [65] |

$^{\dagger}K_b$ calculated from cAMP assay.
$K_e$ calculated from GPI assay, see specific citation for further details.
N.D.: not determined

mediated by the CNS, peripherally acting KOR agonists may not have these side effects and thus are currently being investigated as well as mixed MOR/KOR agonists. It has been suggested that the dysphoric effects of KOR agonists occurs through the recruitment of β-arrestin, whereas the analgesic properties do not [68]. Therefore, analogs that are biased and do not recruit β-arrestin may not have the dysphoric effects and this may be another avenue for pain management.

Another potential therapeutic for pain states that has recently been explored in our laboratory is the development of mixed MOR/DOR agonists with KOR antagonism that can be analgesics with limited side effects. One disadvantage of KOR antagonism is the long duration of action (lasting more than 21 days *in vivo*) [69]. Work is currently being carried out to develop ligands that do not have a long duration of action at the KOR.

## Nonopioid effects of Dynorphin A

Although Dyn A has well documented opioid effects that cause analgesia, there are also some effects of Dyn A that cannot be explained by the opioid receptors (see [70] for review on nonopioid effects). Walker and colleagues were one of the first to document the nonopioid effects of the [des-Tyr$^1$]-Dyn A fragments [71]. They observed that when Dyn A-(1–13) was injected into the brain, it produced dramatic motor and behavioral effects that were different than those produced by enkephalin-containing endorphins and these effects could not be blocked by naloxone [72]. These effects of Dyn-(1–13) were similar to the effects of the (des-Tyr$^1$)Dyn A fragment, Dyn A-(2–13), which was found not to interact with the opioid receptors. It has been found that upon release in the synapse, aminopeptidases rapidly degrade Dyn A to the des-tyrosyl fragments [73]. It is well established that Dyn A has serious motor effects when injected into rats, intracerebroventricular injection into the brain (either Dyn A-[1–17] or Dyn A-[1–13]) induced a barrel rotation in rats [74–76] and intrathecal injection at high doses caused paralysis [77–79]. All of these motor effects were not blocked by the opioid antagonist naloxone and are therefore nonopioid in nature. Levels of Dyn A have been shown to increase in the response to stressors, including neuropathic pain [80] and inflammatory pain [81]. An increase in Dyn A was seen in the anterior pituitary, thalamus and spinal cord in a chronic arthritic pain model [82]. Dyn A may also play a role in the inflammatory response, as evident in that Dyn A induced the release of histamine from rat mast cells [83] and induced plasma extravasation [84]. Both of these inflammatory responses were not blocked by naloxone, providing further evidence for the nonopioid effect. An increase in Dyn A has also been found in other pathological pain states such as chronic pancreatitis [85], opioid overuse induced hyperalgesia [86], bone cancer pain [87] and spinal cord trauma [88].

It has been demonstrated that Dyn A is not required for the initiation of pain but instead for the maintenance of pain, which was shown in prodynorphin knockout (KO) mice that had spinal nerve ligation. These KO mice had similar paw withdrawal latency and thresholds when compared with wild-type littermates days 2–6 after injury [89]. After day 10 post-surgery, the KO mice latency and thresholds were reduced to baseline while their wild-type littermates stayed below baseline [89]. Neuropathic pain is characterized by hyperalgesia and allodynia, and it has been demonstrated that intrathecal injection of Dyn A produces long-lasting allodynia (>60 days) that resembles a neuropathic pain state [90]. The administration of a Dyn A antiserum to nerve-injured rats reversed the neuropathic pain state [80]. Similar results were seen in an inflammatory pain model, thus giving more evidence that the higher levels of Dyn A contribute to different pain states [91]. All of these data have led to the hypothesis that during chronic pain states there is an upregulation of Dyn A which results in pronociceptive effects through a nonopioid mechanism to maintain the pain state.

## *N*-Methyl-D-aspartate receptor as the target for nonopioid effects

The *N*-Methyl-D-aspartate receptor (NMDAR) is a channel that consists of a tetrameric structure of seven subunits [92]. Upon binding of its endogenous agonists, glutamate and glycine, the channel opens and allows the influx of positive ions $Na^+$ and $Ca^{2+}$. Many studies have found that NMDAR antagonists can prevent Dyn A-induced neurological dysfunctions such as loss of tail-flick reflex [93,94], hindlimb paralysis and mortality [94–96]. Pretreatment of the NMDAR antagonist, **MK-801**, prevented Dyn A-induced allodynia whereas naloxone did not [90]. Further evidence for the NMDAR as the nonopioid target is that after spinal infusion of Dyn A-(2–13), an increase in prostaglandin E ($PGE_2$) and excitatory amino acids were measured and were blocked by the NMDAR antagonist, amino-5-phosphonovalerate, **AP5** [97]. The release of excitatory amino acids was also seen after the addition of Dyn A to the rat hippocampus [98].

Direct evidence for Dyn A interacting at the NMDAR was obtained from competitive binding assays, showing that Dyn A-(1–13) could displace part but not all of [$^3$H] glutamate in rat brain membranes [99]. Further competitive binding of Dyn A with NMDAR antagonists, [$^3$H] **MK-801** and [$^3$H]

CGP39, 653, showed moderate-to-low affinity [100,101]. Dyn A's affinity at the NMDAR was directly measured using [$^{125}$I] Dyn A-(2–17) with $K_d$ = 10 nM [102]. In the same study, it was also found that NMDAR antagonists that occupy the closed state of the channel (**AP-5**, **ifenprodil** and 7-chlorokynurenic acid) potentiated the affinity of [$^{125}$I] Dyn A-(2–17) [102]. Therefore, this suggests that Dyn A prefers the closed inactive state of the receptor and may behave as an antagonist. An inhibitory action of Dyn A at the NMDAR does not explain the excitatory effects of Dyn A shown *in vivo*. Also, it was found that in cortical neurons, Dyn A-(2–17) induced an increase in $Ca^{2+}$ which cannot be blocked by **MK-801**, and therefore, suggests a different mechanism [103]. One possible mechanism may be that a different splice variant or combination of splice variants of the NMDAR are responsible for Dyn A's excitatory effects. There are many different splice variants that make up the NMDAR which leads to thousands of possible combinations of receptors that may have Dyn A binding sites. In particular the NR1 subtype has been studied and it was demonstrated that both Dyn A and Dyn A-(2–17) were able to directly bind to the NR1 subunit on the NMDAR [104,105]. Using a decoy peptide that could bind Dyn A-(2–17), Shippenberg *et al.* were able to prevent the potentiation of NMDA-mediated currents by Dyn A-(2–17) [106]. This decoy was also able to reduce Dyn A-(2–17) evoked cell death in spinal cord neurons *in vitro* [106]. More interestingly, intrathecal administration of this decoy peptide was able to prevent the motor impairment and tactile allodynia from intrathecal Dyn A-(2–17) [106]. Therefore, peptides that can bind to Dyn A, such as the decoy peptide, would prevent binding to the NMDAR and may be used as a therapeutic.

## Non-neuronal cells as the target for the nonopioid effects

There is also some evidence that the neurotoxic effects of Dyn A arise from interaction with microglia. Studies by Mika *et al.* found that intrathecal minocycline, a microglia inhibitor, prevented Dyn A-induced paralysis in rats. After sciatic nerve injury, minocycline reduced the elevated levels of prodynorphin mRNA [107]. The authors suggest that to control neuropathic pain, drugs that alter the prodynorphin system could be used. More work will need to be carried out to determine if microglia are indeed a target for Dyn A.

## Bradykinin receptors as the target for nonopioid effects

Our collaborator, Lai, found that in cultured neurons of neonatal rat cortex Dyn A-(2–17) induced an increase in $Ca^{2+}$ that was not blocked by naloxone or **MK-801** [103]. The Lai group later discovered that the nonopioid excitatory effects of Dyn A were mediated by the bradykinin receptors (BRs) [108]. In their study, Dyn A-(2–13) was shown to induce a transient increase in $Ca^{2+}$ in rat DRG as well as the F11 cell line (hybridoma of rat DRG and mouse neuroblastoma cells). By using $Ca^{2+}$ channel blockers, they found that this effect was mediated by P/Q and L type $Ca^{2+}$ channels [108]. In an effort to determine what receptor Dyn A-(2–13) was interacting with to cause the $Ca^{2+}$ influx, antagonists for each receptor known to be present in the F11 cell line were tested. The bradykinin 2 receptor (B2R) antagonist, **HOE140**, was the only antagonist that had an ability to abolish Dyn A-(2–13)-induced $Ca^{2+}$ influx, whereas, the other antagonists including **MK-801**, an NMDAR antagonist, had no effect [108]. Competitive radioligand binding assays showed that Dyn A-(2–13) displaced [$^3$H] bradykinin (BK) with moderate affinity ($IC_{50}$ = 4 μM) [108]. Recently, we have found that Dyn A-(2–13) binds with high affinity to BRs in rat brain membranes ($IC_{50}$ = 170 nM), and an underestimation of the affinity was caused by changes in pH [109]. H89, a PKA inhibitor, was able to abolish Dyn A-(2–13)'s neuroexcitatory effects. On the basis of this, it was proposed that the pronociceptive effects of Dyn A arise from activation of the BRs that couples a PKA pathway to activate L and P/Q type $Ca^{2+}$ channels [108]. Further study showed that **HOE140** was able to block Dyn A-(2–13)'s neuroexcitatory effects in rats [108], supporting the notion that Dyn A's pronociceptive effects are mediated through the BRs.

The mRNA for the B2R was measured in the DRG, and a higher expression of transcript was measured after nerve injury. In the spinal cord of nerve-injured rats, low levels of the precursor to BK, kininogen, was measured. In contrast, high levels of transcript for the precursor of Dyn A, prodynorphin, was measured [108]. Since the BRs are present in the spinal cord but the endogeneous ligand, BK, or its precursor are not, it is hypothesized that Dyn A may be the endogenous ligand for the BRs in the spinal cord under pathological pain conditions. Taken together, it is now believed that the development of BRs antagonists to block Dyn A's pronociceptive actions can be an avenue for the treatment of chronic neuropathic pain (Figure 3).

The BRs are $G_q$-coupled GPCRs, and signal through phospholipase C [110]. The endogenous ligands for the BRs, BK and kallidin, have been found to be mediators of inflammation, and therefore, antagonists have been designed to block the hyperalgesic effects caused by activation of the BRs (in particular the B2R) [111]. In addi-

tion to the inflammatory response of BK, the ligand has also been found to be cardioprotective and a regulator of blood pressure [112]. Early studies aimed to develop B2R antagonists based on BK for the treatment of pain had undesirable cardiovascular side effects (see [113] for review on BR antagonists). However, considering the cardiovascular effects are limited to the peripheral B2Rs, centrally acting B2R antagonists can avoid these serious side effects. Since Dyn A's nonopioid effects are mediated through CNS BRs, the inhibition by BR antagonists in the CNS will result in antihyperalgesic effects without cardiovascular liabilities.

## Structure–activity relationship of Dynorphin A analogs at the bradykinin receptors

To determine the minimum pharmacophore at the BRs, truncations were made at the *N*- and *C*- termini of Dyn A-(2–13) and a distinct SAR was identified. It was found that analogs that had a basic amino acid at the *C*-terminus such as Dyn A-(3–11) and Dyn A-(4–11) had higher affinity ($IC_{50}$ = 130 and 140 nM, respectively, Table 5) at the BRs than analogs with hydrophobic residues, Dyn A-(3–8) and Dyn A-(3–10), ($IC_{50}$ = 2300 and 810 nM, respectively, Table 5) [109]. Truncations at the *N*-terminus until position 4 did not affect binding, and therefore, the *N*-terminus is not considered to be important in the binding at the BRs. *N*-terminal acetylation was well-tolerated ($IC_{50}$ = 120 nM) which further demonstrates that the *N*-terminus is not important for BRs recognition. Interestingly, when $Arg^7$, which is known to be an important residue for binding at the KOR was deleted, binding at the BRs was retained [114]. In an effort to improve stability, diverse modifications were performed, in which substitution of the non-natural amino acid, norleucine, for Leu and/or Ile was tolerated. However, *C*-terminal amidation caused a reduction in affinity at the BRs ($IC_{50}$ = 6500 nM, Table 5), which suggests that along with a basic amino acid at the *C*-terminus, the *C*-terminal carboxylate is also important [109]. The results suggest that amphipathicity, alternating hydrophobic residue, basic residue, is a key component for recognition at the BRs [115]. Nuclear Magnetic Resonance (NMR) studies of selected high-affinity ligands demonstrated a distorted type I β-turn structure at the *C*-terminus [109]. From the SAR studies, we have identified key structural features for the BRs; basic amino acid at the *C*-terminus, presence of *C*-terminal acid, combination of basic and hydrophobic amino acids and presence of Pro residue to make a turn structure. One of the lead ligands, [des-$Arg^7$] Dyn A-(4–11) which retained high affinity ($IC_{50}$ = 190 nM, Table 5) was tested for analgesic effects *in vivo*. This ligand blocked Dyn A-(2–13)-induced hyperalgesia providing evidence that this ligand is an antagonist [109]. In nerve-injured rats, intrathecal administration of the ligand reversed thermal hyperalgesia and mechanical hypersensitivity. Importantly, this ligand did not block BK-induced peripheral effects, so it may avoid the cardiovascular liabilities. A main advantage of peptides as therapeutics is that they have high specificity for their target. Further evidence of this was shown in an off-target screening assay of the lead

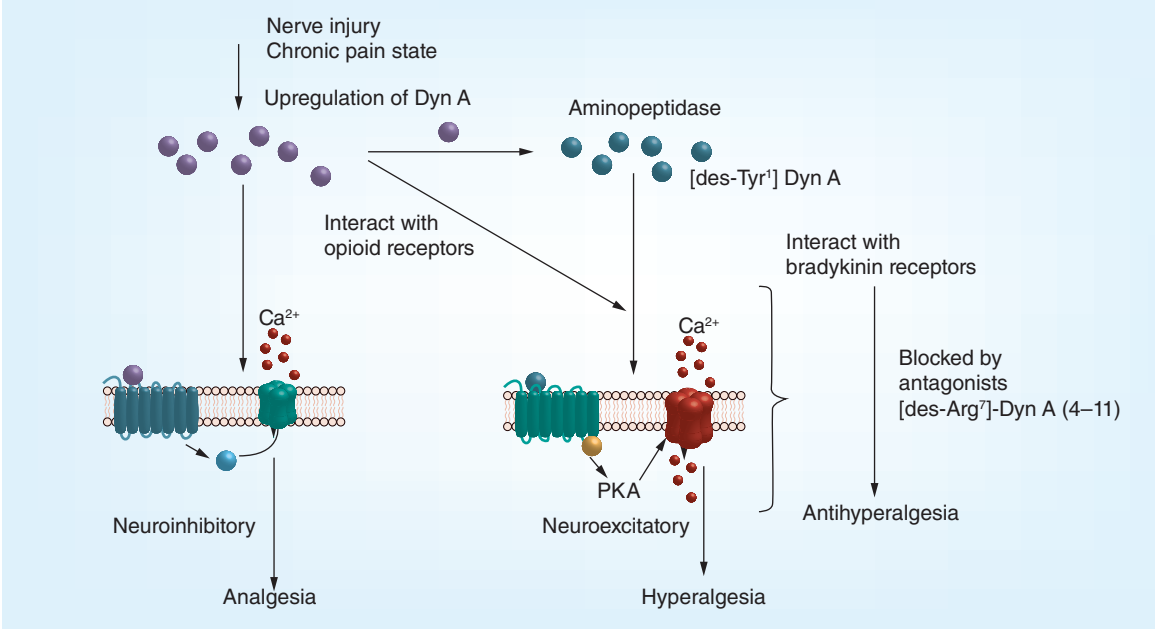



**Figure 3. Dynorphin A in the spinal cord during nerve injury or chronic pain state.**

Table 5. Structure–activity relationship of Dynorphin A analogs at the bradykinin receptors.

| Fragment | Structure | $IC_{50}$ (nM)† |
|---|---|---|
| Dyn A-(2–13) | H-G-G-F-L-R-R-I-R-P-K-L-K-OH | 170 |
| Dyn A-(3–11) | H-G-F-L-R-R-I-R-P-K-OH | 130 |
| Dyn A-(4–11) | H-F-L-R-R-I-R-P-K-OH | 140 |
| Dyn A-(3–10) | H-G-F-L-R-R-I-R-P-OH | 810 |
| Dyn A-(3–8) | H-G-F-L-R-R-I-OH | 2300 |
| [des-Arg$^7$] Dyn A-(4–11) | H-F-L-R-I-R-P-K-OH | 190 |
| Dyn A-(4–11)$NH_2$ | H-F-L-R-R-I-R-P-K-$NH_2$ | 6500 |
| [des-Arg$^7$] Ac-Dyn A-(4–11) | Ac-F-L-R-I-R-P-K-OH | 120 |

†Assay carried out according to the conditions in [109].

ligand, in which the ligand did not interact with any of the 43 receptors tested [109].

## Future directions for Dynorphin A analogs at bradykinin receptors

Currently, a lead ligand that binds with high affinity to the BRs in the CNS, blocks Dyn A-(2–13)-induced neuroexcitatory effects in naive animals, and reversed hyperalgesia and hypersensitivity in nerve-injured animals has been discovered. Future work will involve modifications of the lead ligand to increase metabolic stability due to the short half-life. Although the lead ligand is effective, it is composed of all natural amino acids and therefore, susceptible to enzymatic degradation. Substitution of unnatural amino acids will be tested in binding as well as stability assays.

## Conclusion

Chronic pain, and in particular neuropathic pain, continues to be a disease in which there are no effective treatments. Many of the approved therapeutics for this disease have serious side effects that limit their use. The inhibitory effects of Dyn A were first discovered and were found to be mediated through the opioid receptors. In an effort to obtain a therapeutic with analgesic properties and limited side effects, extensive SAR studies on Dyn A were done for the KOR. Although KOR agonists were found to be analgesics, they had dysphoric side effects that limited their use. KOR antagonists with shorter durations of action are currently being developed and may be used as analgesics in combination with MOR/DOR agonists. Dyn A has been found to be upregulated in many pain states; chronic pancreatitis, bone cancer pain, arthritis, spinal cord trauma, inflammatory pain and neuropathic pain. There has also been evidence about the excitatory effects of Dyn A that cannot be blocked by naloxone and are therefore, nonopioid. The NMDAR has been suggested as the target for the nonopioid effects of Dyn A but the evidence suggests an inhibitory action at the NMDAR which does not explain the neuroexcitatory effects of Dyn A seen *in vivo*. Instead there is substantial evidence for Dyn A's nonopioid excitatory effects to be mediated through the BRs which through a PKA-mediated pathway activate L and P/Q type calcium channels to cause an influx of $Ca^{2+}$. Dyn A may be the endogenous ligand for BRs in the spinal cord, as transcripts for the BRs and Dyn A have been measured spinally but the endogenous ligand, BK has not. Extensive SAR studies of Dyn A analogs at the BRs have discovered a lead ligand, (des-Arg$^7$) Dyn A-(4–11) that is able to antagonize Dyn A-(2–13)-induced neuroexcitatory effects and attenuate hyperalgesic effects in a neuropathic pain model. This ligand also does not show antagonist activity at the peripheral BRs that BK has shown and thus, can be a safe drug without the cardiovascular liabilities.

## Future perspective

Future advances in this field would greatly benefit from determining the mechanisms of neuropathic pain. These mechanisms are still unknown but it is known that it is different than acute pain and therefore, the treatment for the two pain states should not be the same. One potential mechanism of neuropathic pain is the upregulation of Dyn A which has been found to contribute to the maintenance of a neuropathic pain state. Developing antagonists that block this interaction may provide novel therapeutics that do not have the liabilities of opioids.

### Financial & competing interests disclosure

This work has been supported by U.S. Public Health Services, NIH, and NIDA P01DA006284 and R01DA013449. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

## Executive summary

- Neuropathic pain is a debilitating disease that results from the dysfunction of the CNS or peripheral nervous system. There are no effective treatments and a better understanding of the underlying mechanisms is needed.
- Dynorphin A (Dyn A) shows neuroinhibitory effects through the opioid receptors, specifically the κ-opioid receptor (KOR). The peptide also has neuroexcitatory effects that are thought to be mediated through the *N*-Methyl-D-aspartate receptor (NMDAR) and/or bradykinin receptors (BRs).
- Extensive structure–activity relationship studies have been completed on Dyn A and key pharmacophores have been found to be important for agonist and antagonist function at the KOR.
- KOR agonists based on Dyn A are effective analgesics that do not have μ-opioid receptor induced side effects but have dysphoric side effects that limit their use.
- KOR antagonists based on Dyn A structure that have a short duration of action are currently being pursued in combination with μ-opioid receptor/δ-opioid receptor agonists for analgesics with limited side effects.
- Blockade of the neuroexcitatory effects of nonopioid Dyn A by the NMDAR antagonist, MK-801, suggest that these effects are mediated through the NMDAR. Evidence of a direct interaction of nonopioid Dyn A with the NMDAR suggests an inhibitory effect at the receptor, which does not account for the excitatory effects seen *in vivo*.
- Recent studies discovered that the BRs are the nonopioid target for Dyn A. Dyn A-(2–13) has been found to bind with high affinity at the BRs and cause an increase in $Ca^{2+}$.
- An antagonist that blocked Dyn A's excitatory actions at the BRs has been identified and was effective in a neuropathic pain model.

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