Non-opioid effects of dynorphins: possible role of the NMDA receptor

Vijay K. Shukla and Simon Lemaire

Dynorphin A (dynA) and related opioid peptides produce moderate analgesic effects with restricted types of pain stimuli that are often accompanied by a large variety of naloxone-insensitive biochemical and behavioural effects. In binding assays *in vitro*, dynA possesses a high affinity for μ-, δ- and κ- opioid receptors with some selectivity for κ sites, but it also binds to specific non-opioid sites. The involvement of the NMDA receptor has been suggested to explain some of the non-opioid effects of dynA and related peptides. In this article, Vijay Shukla and Simon Lemaire review the experimental evidence that suggests a role for the NMDA receptor in some of the pharmacological effects of dynA and related peptides.

Dynorphin A (dynA), an endogenous heptadecapeptide, was first isolated from porcine pituitary by Goldstein and co-workers[1]. Dynorphin-like peptides have since been identified by chemical and immunohistochemical studies in various tissues, including the brain, the hypothalamus, the spinal cord, the autonomic nervous system and the adrenal medulla[2,3].

Some correlation was noticed between the distribution of central dynA and that of the κ-opioid receptor[4]. However, the functional significance of such correlation is lacking, since dynA administered i.c.v. displays poor analgesia with only very restricted types of pain stimuli[5,6], whereas i.c.v. administration of nonpeptide κ-receptor agonists produces strong analgesia in a large variety of pain assays[7]. Binding studies *in vitro* indicate that dynA possesses a high affinity for all opioid receptors, with some selectivity for the κ type[8]. In addition, dynA and related peptides are thought to bind to non-opioid sites, since the specific binding of [^{3}H]dynA is not totally displaced by the opioid receptor antagonist naloxone or other opioid alkaloids[9]. Therefore, the physiological and/or pathophysiological function of dynA and related peptides may not only depend upon its interaction with opioid receptors but also with non-opioid binding sites.

The naloxone-resistant effects of dynA and related peptides such as hindlimb paralysis, motor disturbance and hyperalgesia are not mimicked by U50488H, a nonpeptide κ-opioid agonist[10–12]. On the other hand, some of these effects are partially or totally blocked by the presence of NMDA antagonists[13,14]. Walker and co-workers[6] suggested that two biological active sequences exist within the dynA molecule, one opioid and the other non-opioid, and both are capable of producing significant behavioural activity.

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Analgesia

Dynorphin A is the most potent opioid peptide in the guinea-pig ileum assay[1], but reports concerning its analgesic efficacy have been contradictory and controversial. Unlike other opioid peptides, dynA and related peptides when administered i.c.v. have been found to be ineffective in thermal analgesic assays using heat, such as rat tail flick[6] or hot-plate tests[15]. However, in nonthermal and mechanical analgesic assays such as flinch-jump response to foot shock[16], the paw-pressure test[15] or the tail-pinch test[10], these peptides (i.c.v.) have been reported to have a moderate analgesic effect. Since dynA (i.c.v.) can also induce various motor effects such as 'barrel-rolling', circling, jumping, ataxia and catatonia in animals[6,10], its analgesic response in mechanical analgesic assays is questionable. In some studies, dynA and dynA(1–13) (i.c.v.) did not show any analgesic activity, even at very high doses[6,17].

This lack of clear analgesic effect was initially ascribed to a possible rapid degradation by peptidases present *in vivo*[17]. However, dynA(1–13) (i.c.v.) antagonizes morphine-induced analgesia in the tail flick test[5]. Similar effects are also observed with i.c.v. administration of the non-opioid peptides [Des-Tyr^1]dynA(1–13) and Boc-[Arg^{11,13}]dynA(1–13)-Gly-NH$(CH_2)_5$-NH_2 (Boc-DAKLI) in rats[12,18], suggesting that the modulatory effect of dynA on morphine-induced analgesia is non-opioid in nature.

Several investigators have reported that dynA and related peptides are more effective in producing analgesia at a spinal level than at a supraspinal level[19]. Intrathecal (i.t.) administration of dynA(1–13) produces analgesia in tail flick and hot-plate analgesic assays, although concomitant neurological impairments obscure the interpretation of these findings[11,20]. Low doses of dynA(1–13) (i.t.) (at which no neurological impairment is observed) do not produce analgesia[11] but higher doses of dynA(1–13) (i.t.) in rats caused irreversible loss of the thermally evoked tail flick reflex as a result of hindlimb paralysis[14]. Interestingly, this loss of tail flick reflex was resistant to naloxone and blocked by preadministration of the NMDA receptor antagonist dizocilpine[14] (see below).

Hyperalgesia and inflammation

Non-opioid fragments of dynA-related peptides such as dynA(2–17) and Boc-DAKLI (i.c.v) produced hyperalgesia in rats[12], which was a longer lasting response than the analgesia observed when dynA-related peptides were administered i.c.v. in the writhing test in mice (<10 min) (Ref. 21). Since dynA undergoes rapid degradation by aminopeptidase and carboxypeptidase after i.c.v. administration[17], the short analgesic response[21] may be via opioid receptors, while the longer lasting hyperalgesia[12] may depend on the interaction of dynA with non-opioid

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receptors. The hyperalgesic response to dynA suggests that the peptide may play a role in the pathologies of inflammation. Further evidence to support this suggestion was obtained in rats subjected to chronic pain by administration of inflammatory agents: they displayed hyperalgesia and a pronounced increase in spinal levels of dynA and preprodynorphin mRNA without any change in the number and affinity of κ-opioid receptors[22].

Involvement of NMDA receptors

Recently, it has been demonstrated that dynA augments nociceptive behaviour induced by administration of NMDA i.t. (Ref. 23). Dubner and Ruda[24] proposed that dynA may contribute to spinal hyperexcitability and excitotoxicity by producing specific facilitation of NMDA receptor activity in rats subjected to peripheral inflammation (Fig. 1). The involvement of the NMDA receptor in inflammatory processes is also supported by the study of Ren and co-workers[25] who demonstrated that the hyperalgesic response to inflammation is attenuated by dizocilpine (i.p. or i.t.).

Furthermore, it is possible that the hyperalgesic response associated with chronic pain is due to the elevated concentration of dynA (Ref. 26). In this regard, dynA(1–13) has potent stimulatory effects on the C-fibre reflex by non-opioid mechanisms[26,27]. The C-fibre reflex is an NMDA receptor-mediated polysynaptic reflex and i.t. administration of the NMDA antagonist AP5 blocks this reflex[27]. Thus, the dynA-evoked nociceptive reflexes manifested by a forceful removal of a limb from a painful stimulus may be mediated by an NMDA receptor-related mechanism (Fig. 1).

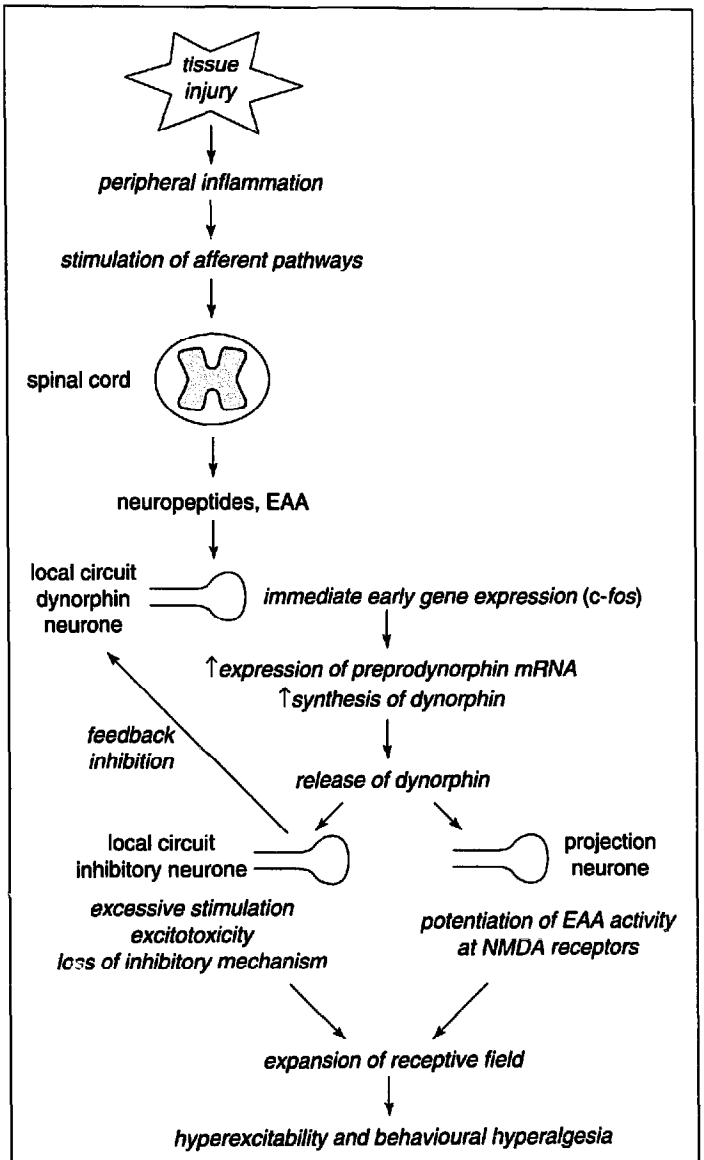

Fig. 1. Hypothetical sequence of events involving dynorphin in the production of hyperalgesia in response to peripheral inflammation. EAA, excitatory amino acids.

Spinal cord injury

Immunoreactive dynA in the spinal cord of rats is elevated after spinal trauma, the increase in its concentration being proportional to the severity of trauma[28]. Faden[29] observed that dynA treatment of rats subjected to spinal trauma exacerbated the post-traumatic behavioural outcome while treatment with dynA antiserum improved this condition. Moreover, i.t. injection of dynA or its non-opioid fragments dynA(2–17) ([Des-Tyr^1]dynA) or dynA(3–13) into the spinal subarachnoid space of rats produced a flaccid hindlimb paralysis in a dose-related manner, an effect that can be compared to post-traumatic paraparesis in humans[20,23,29]. This response was not blocked by naloxone.

Recent studies indicate that the neurological dysfunction induced by dynA and related peptides is blocked by NMDA receptor antagonists. Treatment with competitive and noncompetitive NMDA receptor antagonists blocked the loss of tail flick reflex resulting from i.t. administration of dynA(1–13) (Refs 14,27) and attenuated dynA- and dynA(2–17)-induced hindlimb paralysis[13]. In addition, dizocilpine improved neurological and neuropathological behavioural outcome in dynA treated animals[14].

The mechanism by which the NMDA receptor mediates the neurotoxic effects of dynA and related peptides remains to be determined. However, it is possible that the local reduction in spinal cord blood flow subsequent to i.t. administration of dynA (Refs 30,31) may induce spinal ischaemia leading to secretion of excitatory amino acids (EAA) such as glutamate and aspartate, overstimulation of the NMDA receptor, excessive entry of Ca^{2+} in postsynaptic neurones, increases in neuronal cell-free fatty acid contents, destabilization of the cell membrane network and neuronal cell death[32]. It is also possible that dynA causes the release of EAA through a presynaptic mechanism that is independent of ischaemia[33]. However, it has recently been indicated that paralysis and neurotoxicity resulting from i.t. administration of dynA(1–13) involve two separate mechanisms[23]: (1) a direct potentiation of

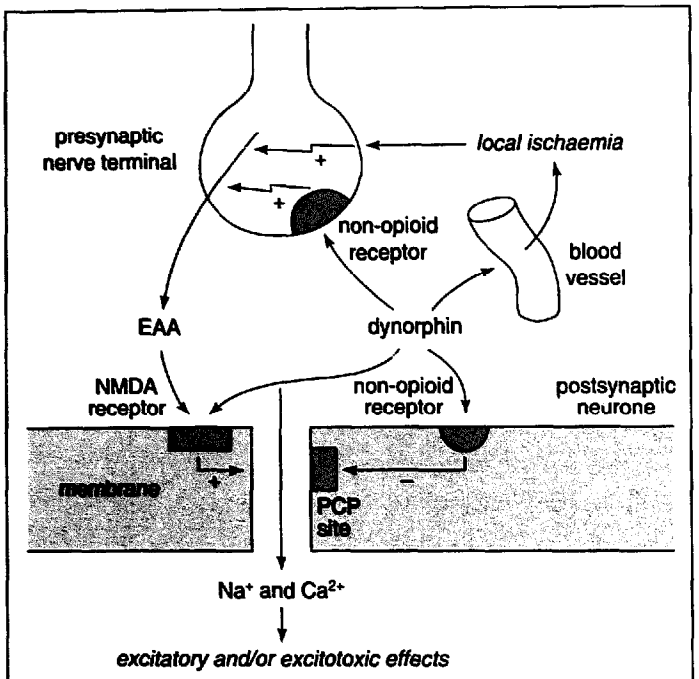

Fig. 2. Possible mechanisms of action of dynorphins on the NMDA receptor complex. The facilitatory effects of dynorphin A and related peptides on NMDA receptor-mediated activity may be due to a direct interaction of the peptide with the receptor complex. Dynorphin was found to interact with both phencyclidine (PCP) and NMDA receptors on the postsynaptic neurone. Dynorphin may also enhance the release of glutamate either by a direct interaction with presynaptic neurones or as a result of local ischaemia caused by the constriction of small blood vessels. EAA, excitatory amino acids.

EAA-mediated activity, and (2) a blockade of NMDA receptor desensitization. The confirmation of the involvement of one and/or the other mechanism will necessitate further experimentation.

Motor effects

Dynorphin A has been demonstrated to function as a hippocampal neurotransmitter[34]. The mossy fibre pathway in the hippocampus releases dynA and related peptides together with glutamate[35], and administration of dynA, dynA(2–17), dynA(1–13) and dynA(2–13) through a microdialysis probe stereotaxically placed into rat hippocampus caused marked increases in extracellular levels of glutamate and aspartate[33].

Both excitatory and inhibitory effects of dynA and related peptides have been described in rat hippocampus[6,36]. Iontophoretic application of dynA, dynA(1–13), dynA(1–8), and α-neo-endorphin on CA1 and CA3 hippocampal neurones of the rat generates excitatory responses when single-cell activity and hippocampal field potentials are recorded[36]. These excitatory responses are blocked by relatively high doses of naloxone as well as by Mg^{2+}, suggesting the possible involvement of both opioid and non-opioid receptors (since Mg^{2+} is a strong blocker of NMDA receptor-mediated activity). In similar studies, dynA and dynA(2–17) also displayed a dose-dependent depression of both spontaneous and glutamate-evoked discharges in a majority of CA1 and CA3 cells tested by single-unit extracellular recording technique[6]. The inhibitory effects of dynA were not blocked by naloxone.

Relevance of changes in dynorphin levels in hippocampus

The hippocampus has been implicated in the generation of epileptic phenomena in humans and in a variety of animal models of epileptic seizures. Seizures induced by intrahippocampal administration of NMDA, quisqualate or kainate were associated with marked increases in proenkephalin and prodynorphin gene expression[37]. By contrast, decreases in dynA-, dynA(1–8)- and dynB-like immunoreactivity have been observed in the hippocampal mossy fibre system after amygdaloid kindling and electric shock seizures in rats and mice[38,39], and the functional significance of such changes in dynorphin levels is yet to be determined. Dynorphin A and related peptides when directly injected i.c.v. or into other brain regions produce motor dysfunction such as wild running, jumping, circling, barrel-rolling, ataxia and unusual contorted posture[6,10]. Furthermore, dynA or [Des-Tyr^1]dynA (i.c.v.) induced large-amplitude slow-wave cortical EEG activity in rats[6]. These responses were not antagonized by the preadministration of naloxone, suggesting the involvement of non-opioid mechanisms[6]. However, wild running, pop-corn jumping, hind-limb jerking and barrel rolling resulting from the administration of a potent analogue of dynA (i.c.v.) in mice were blocked by the noncompetitive NMDA receptor antagonists metaphit, dextromethorphan and ketamine[21].

Suppression of opiate withdrawal and tolerance

Dynorphin A(1–13) has been observed to suppress the expression of opiate withdrawal and tolerance in animals[40,41], and opiate-withdrawal symptoms in heroin addicts[42]. Both opioid and non-opioid mechanisms appear to be involved in the suppressive effects of dynA on opiate withdrawal and tolerance in morphine-dependent mice[41]. Structure–activity studies reveal that the minimal amino acid sequence that is required for this activity is dynA(2–8) (Ref. 41). The exact mechanism of action is not known, but indirect evidence suggests that NMDA receptor-related mechanisms may be involved, since the NMDA receptor antagonists dizocilpine and LY274614 also suppressed opiate tolerance and dependence in morphine-dependent rats[43].

Possible role of the NMDA receptor complex

The possible modes of action of dynA on the NMDA receptor complex are illustrated in Fig. 2. The NMDA receptor is an EAA binding site linked to an ion channel that allows the entry of Na^+ and Ca^{2+} into postsynaptic neurones[44]. The NMDA receptor-linked ion channel is subject to voltage-dependent Mg^{2+} blockade. Stimulation of the NMDA receptor also involves a strychnine-insensitive glycine-binding domain, which facilitates the

opening of the ion channel. The entry of Ca^{2+} and Na^{+} through this channel is modulated by phencyclidine (PCP), Zn^{2+}, polyamines and other regulatory agents. Hence, the function of the NMDA receptor must depend on the dynamic equilibrium between multiple facilitatory and inhibitory factors.

Relationship between dynA and the NMDA receptor

The relationship between dynA and the NMDA receptor complex is not clear and may differ across various experimental models. On the basis of the model of neuronal injury induced by dynA and related peptides, it has been proposed that dynorphin-related peptides (i.t.) stimulate the NMDA receptor indirectly by a non-opioid mechanism (see above)[30–32]. Intrathecal administration of dynA produced local ischaemia at the site of injection and this effect was not blocked by naloxone[31], but was accompanied by the release of EAA (Ref. 32). Contrary to this, a recent study demonstrated that the motor dysfunction induced by dynA(1–13) appears before any change in cerebrospinal fluid levels of EAA, suggesting a possible direct interaction between the peptide and the NMDA receptor complex[23]. Furthermore, dynA(1–13) (i.t.) has been shown to first produce direct potentiation of NMDA mediated C-fibre reflex followed by complete loss of C-fibre reflex owing to NMDA receptor-mediated excitotoxicity[27]. Similarly, in behavioural studies, coadministration of dynA(1–13) (i.t.) with NMDA facilitates NMDA-induced nociceptive responses in mice[23].

Site of action of dynA on the NMDA receptor

These biochemical, electrophysiological and behavioural studies indicate a direct facilitatory action of dynA and related peptides on NMDA receptor-mediated responses, but they do not demonstrate how these peptides interact with the NMDA receptor. In receptor binding studies using rat brain membranes, Massardier and Hunt[45] suggested that dynA(1–13) acts as an antagonist at the glutamate site on the NMDA-receptor complex. However, these results are not consistent with the predominantly excitatory effects of dynA(1–13) that are observed in most studies. A competitive interaction between dynA(1–13) and the PCP receptor has been observed by measuring the displacement of [^{3}H]dizocilpine binding[21]. The involvement of the PCP site in the action of dynorphin is also supported by the blockade of motor effects by metaphit, dextro-methorphan and ketamine[21]. The action of dynA on the non-opioid PCP site may promote an open conformation of the NMDA receptor-linked ion channel following the interaction of EAA, and result in facilitatory activity[44].

Concluding remarks

In conclusion, different studies (Table 1) support the concept that the non-opioid effects of dynA and related peptides are physiologically and pathophysiologically relevant and most likely involve a modulation of NMDA receptor activity. No clear picture has yet emerged to explain the mechanism of action of dynorphin on the NMDA receptor complex (Fig. 2). The elucidation of the type of interaction (direct or indirect) between dynA and the NMDA receptor and the full characterization of the non-opioid dynorphin binding sites will necessitate further experimentation.

Table 1. Non-opioid effects of dynorphin A and related peptides

Effect	Receptor involved	Refs
Hyperalgesia and inflammation	Non-opioid NMDA	12 23,24
Antagonism of morphine analgesia	Non-opioid	6,12,25
Spinal cord injury and neurological dysfunction	Non-opioid NMDA	11,29–31 13,14,24,27
Central motor effects	Non-opioid NMDA	6,10 21
Suppression of opiate withdrawal and tolerance	Non-opioid	41

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Chemical names

LY274614: (±)-6-phosphonomethyl-decahydroisoquinolin-3-carboxylic acid

U50488H: *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulphonate

Toxins from mamba venoms: small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors

Diana Jerusalinsky and Alan L. Harvey

Muscarinic acetylcholine receptors exist as five subtypes that are widely distributed throughout the body. Conventional pharmacological agents are not highly selective for particular subtypes, making investigations on the functional significance of the subtypes difficult. Recent findings indicate that mamba snake venoms contain several small proteins ('muscarinic toxins') that are highly specific for muscarinic receptors, and are discussed in this review by Diana Jerusalinsky and Alan Harvey. Some of these toxins act selectively and irreversibly on individual subtypes of receptor, and some are antagonists, while others activate muscarinic receptors. The toxins should be useful tools in studies of the functions of individual receptor subtypes, and comparisons of their three-dimensional structures should give clues about how selective binding to muscarinic receptor subtypes can be obtained.

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Snake venoms have provided a variety of toxins that have been used as pharmacological tools, and mamba (*Dendroaspis*) venoms[1] are a particularly rich source of highly specific toxins that act on different target molecules. For example, dendrotoxins block some voltage-dependent K^+ channels in neurones[2], fasciculins are specific, noncompetitive inhibitors of acetylcholinesterase[3], calciseptine[4] and calcicludine[5] block some Ca^{2+} channels, and the mambins inhibit platelet aggregation and integrin binding[6]. A number of mamba venoms have curaremimetic α-neurotoxins (homologous to the well-known α-bungarotoxin) that bind tightly to nicotinic acetylcholine receptors, but mamba venoms also contain several proteins whose structure has been determined but whose function was unknown[1]. However, some of these mystery proteins are now known to bind to muscarinic acetylcholine receptors[7]. Since these 'muscarinic toxins' show selectivity for different subtypes of muscarinic receptors[8], they may be useful in studies of the functional roles of particular muscarinic receptor subtypes since the available pharmacological agents do not show high enough selectivity to be completely satisfactory in such studies[9,10].

Five different muscarinic receptors (M_1–M_5) are expressed in mammalian brain, including human brain. These intrinsic membrane proteins are examples of G protein-coupled receptors with seven transmembrane domains, and are found in neurones, cardiac and smooth muscle cells, and in other peripheral tissues. It is well established that the M_1, M_3 and M_5 subtypes are associated with an agonist-induced increase in the production of inositol (1,4,5)-trisphosphate resulting from activation of phospholipase C (via the G_q family), whereas the M_2 and M_4 subtypes mediate a decrease in cAMP production resulting from inhibition of activated adenylate cyclase (via G_i and G_o)[11,12]. In addition, myocardial M_2 receptors couple to the stimulation of K^+ channels via the βδ-subunit of G proteins[9].