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Decoy peptides that bind dynorphin noncovalently prevent NMDA receptor-mediated neurotoxicity

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Abstract

Prodynorphin - derived peptides elicit various pathological effects including neurological dysfunction and cell death. These actions are reduced by N-methyl-D-aspartate receptor (NMDAR) but not opioid receptor antagonists suggesting NMDAR - mediation. Here we show that a conserved epitope (KVNSEEEEEEDA) of the NR1 subunit of the NMDAR binds dynorphin peptides (DYNp) non-covalently. Synthetic peptides containing this epitope form stable complexes with DYNp and prevent the potentiation of NMDAR - gated currents produced by DYNp. They attenuate DYNp - evoked cell death in spinal cord and prevent, as well as reverse, DYNp - induced paralysis and allodynia. The data reveal a novel mechanism whereby prodynorphin-derived peptides facilitate NMDAR function and produce neurotoxicity. Furthermore, they suggest that synthetic peptides that bind DYNp, thus preventing their interaction with NMDAR, may be novel therapeutic agents for the treatment of spinal cord injury.

Keywords

NMDA receptor; Dynorphin; neurotoxicity; paralysis; noncovalent interaction

Increased production of the opioid peptide, dynorphin A 1–17 (DYN), following trauma to the spinal cord is implicated in the pathophysiology of spinal cord injury, and in neuropathic pain^{1–8}. Although intrathecal (i.t) infusion of low concentrations of DYN results in opioid-receptor mediated analgesia, higher concentrations produce allodynia (pain behaviour in response to innocuous stimuli), paralysis, and neuronal loss in the spinal cord of experimental animals^{4–7, 9–10}. These actions are not opioid receptor mediated as they are not blocked by

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opioid receptor antagonists. Moreover, deleterious effects are retained by truncated peptides that lack the N-terminus tyrosine residue that is essential for opioid receptor binding^{8–10}.

DYN and its major biotransformation product, DYN 2–17, bind to the NMDAR *in vitro* and can potentiate NMDA-evoked currents^{7,10,11}. NMDAR antagonists reduce DYN-induced tactile allodynia, paralysis and cell death suggesting that NMDAR mediates the pronociceptive and neurotoxic actions of DYN. The mechanisms by which DYNp modulate NMDAR function are unknown.

We show that adjacent arginine_{6,7} (Arg/R) in the DYN molecule enables DYNp to bind noncovalently to a conserved acidic epitope of the NR1 subunit (594–599, EEEEEED) *via* salt bridging^{12–15}. Synthetic peptides (590–600) containing the NR1 epitope form stable complexes with DYN and prevent the potentiation of NMDA-mediated currents elicited by DYN and DYN 2–17 *in-vitro*. The peptides reduce cell death in spinal cord cultures and their i.t. infusion attenuates the tactile allodynia, flaccid paralysis and loss of spinal cord motoneurons elicited by DYN 2–17. However the really striking effects are: when the peptides are administered after DYN 2–17, paralysis and motor dysfunction are significantly reduced.

Material and Methods

Peptides

The NR1 epitopes KVNSEEEEEEDA (590–600) [KVNE₅DA], and MLYLLDRFSP FGRFKVNSEE EEEDALTLSS AMWFSWGVLL NSGIGEGAPRS (576–626) were synthesized by the Sequencing and Synthesis Laboratory at the Johns Hopkins School of Medicine. DYN 1–17 (YGGFLRRIRPKLKWNDQ) and 2–17 were purchased from Sigma.

Mass spectrometry

Spectra were acquired on a MALDI DE-Pro,^{12,14} a TOF_TOF 4700¹⁵ and a Q-TOF¹³. The peptides mixtures were made of an equimolar amount of peptides, for a concentration of 1 pmole/μl. A saturated solution of 6-Aza-2-Thiothymine in 50% ethanol, was used as matrix. Digestion studies using an equimolar mixture of KVNSE₅DA, the 51 mer and DYN were conducted as previously described¹².

NMR

NMR experiments were performed on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe and z-gradient. Peptides were measured alone, to determine their monomeric state. Measured diffusion coefficients were corrected for viscosity due to D₂O contents. To examine the interactions between DYN and *l*- or *d*-peptide, increasing amounts of KVNSE₅DA were added to a 200 μM solution of DYN and the diffusion coefficient of DYN measured. Diffusion data were used as fitting parameters in calculating dissociation constants¹⁶.

Electrophysiology

Injection and recording from *Xenopus* oocytes were conducted as described^{11,17,18}. Oocytes were injected with mRNA encoding NR1 and NR2B or an equal volume of water and perfused with modified Barths medium (pH 7.5).

Spinal cord neuron cultures

Dorsal spinal cord neurons were isolated from embryonic day 14 ICR mouse fetuses and maintained *in vitro* as previously described⁸. Dorsal spinal cord neurons in these cultures have been characterized as neurofilament, PGP 9.5, and MAP2 immunoreactive,^{19,20} and the vast majority co-express -opioid receptors and NR1 subunits⁸. Neurons were photographed before

and for 72 h following addition of DYN 2–17, KVNSE₅DA or their combination. Neuronal losses were characterized by destruction of the cell body as evidenced by nuclear pyknosis, as well as neuritic beading, fragmentation, and subsequent dissolution⁸.

Tactile Allodynia

Male Sprague Dawley rats (300–350 g) were anesthetized with Equithesin (3 ml/kg, i.p.) and implanted with an i.t catheter (PE-10 tubing) directed to the lumbar spinal subarachnoid space. Five days later the influence of co-infusion of KVNSE₅DA (75 nmol; 10 μ l) infusion upon changes in mechanical threshold produced by i.t infusion of DYN 2–17 (0; 5 nmol; 10 μ l) was measured with Von Frey filaments. In parallel rats, motor performance on an accelerating rotor rod (Ugo Basile, Comerio, Italy) was tested 30 min and 1–21 days after drug injection²¹.

Hind-Limb Paralysis

We assessed paralysis induced by DYN 2–17 (75 nmol), KVNSE₅DA (250 nmol) or their combination in rats at various times after i.t infusion using a 5 point rating scale (4 normal motor function; 3 paraparesis with ability to support weight and walk with some impairment; 2 paraparesis with ability to make walking movements without supporting weight; 1 severe paraparesis in which animals could make hind limb movements but could not walk; 0 flaccid paralysis with absence of hind limb movement).

Immunocytochemistry

Rats were perfused intracardially with 4 % paraformaldehyde 72 hrs after i.t infusion of vehicle, DYN 2–17 (75 nmol), KVNSE₅DA (250 nmol) or their combination. Spinal cord tissue was removed, cryoprotected in 30 % sucrose, and 30 μ m serial frozen horizontal sections through the lumbosacral cord immunostained with a rabbit anti- CGRP antiserum (1: 10,000; Bachem AG, Belmont, CA) followed by a Cy-3 conjugated goat anti-rabbit secondary (1:1000; Jackson Labs). After capturing images, sections were counterstained with Cresyl Violet for Nissl staining.

Statistic

Data are expressed as mean \pm SE or as a percentage of control values. Electrophysiological data were analyzed using the Student's t-test and tissue culture data using one- or two-way ANOVA followed by post-hoc comparisons. Tactile allodynia and rotarod performance were analyzed using a two-factor repeated measures ANOVA, and Bonferroni corrections, followed by post-hoc comparisons. Paralysis scores were analyzed using the non-parametric Kruskal Wallis test. The Fisher's exact test was used to analyze motor neuron loss. For this analysis, spinal cords were scored as + or – for the presence of motor neuron loss. Sections were independently scored by two observers blind to treatment and whose results were completely concordant. $P \leq 0.05$ was considered significant.

Results

DYNp and NR1 form stable complexes via salt-bridging

Examination of the NMDAR revealed that the NR1 subunit contains five adjacent Glu and an Asp (594–599, EEEEEED). This sequence is conserved in all species. Since adjacent guanidinium groups on Arg_{6,7} interact with adjacent carboxyl groups of Asp or Glu^{12–15} resulting in formation of a powerful salt bridge, we hypothesized that the NR1 subunit epitope interacts similarly with DYN. Mass spectrometry confirmed this hypothesis. DYN or DYN 2–17 formed stable complexes with the NR1 epitope (590–600, KVNSE₅DA; Fig 1). Molecular ions [MH⁺] were seen at amu 1279.3 (KVNSE₅DA), 1985.3 (DYN 2–17), 2148.5 (DYN), as

well as at amu 3263.6 and 3426.8 corresponding to a noncovalent complex of DYN 2–17 or DYN and the NR1 epitope KVNSE₅DA.

Shorter neurotoxic fragments⁸ of DYN (containing Arg_{6,7}) also formed salt bridges with the epitope. Fragments that lack Arg_{6,7}, and are not neurotoxic did not. A larger epitope of NR1 (570–621, MH⁺ 5742.5) containing KVNSE₅DA also formed noncovalent complexes with DYN 2–17 and DYN at amu 7726.8 and 7890, again confirming complex formation. Complexes were observed after chymotryptic digestion of a solution containing an equimolar mixture of the 51 mer peptide (aa residues 570–621) of the NR1 epitope and DYN, indicating that once formed, complexes are stable and resistant to enzymatic attack (Table 1). The fragments forming complexes show that the DYN and NMDA residues likely to be involved in the interaction are RRIRPK and NSEEEEEED respectively. In addition, collision induced dissociation by both MALDI and ESI¹⁵ of the noncovalent complex of KVNSE₅DA and DYN did not result in fragmentation of the complex. Instead high fluency for MALDI and high collision voltage for ESI were needed to partially dissociate the complex.

To calculate the dissociation constant of the KVNSE₅DA-DYN complex we performed NMR diffusion measurements. Diffusion coefficients measured at 100 μM and 200 μM concentrations were $2.14 \pm 0.01 \times 10^{-10} \text{ m}^2/\text{s}$ for DYN and $2.62 \pm 0.02 \times 10^{-10} \text{ m}^2/\text{s}$ for *l*- and *d*-KVNSE₅DA. The values are in agreement with expected values for randomly coiled monomeric peptides of the same molecular weight¹⁶. Thus, at low concentrations the three peptides are found in aqueous solutions as randomly coiled monomers. NMR spectroscopy revealed a K_d of $0.25 \pm 0.05 \text{ mM}$ for DYN-*l*-KVNSE₅DA and $2.4 \pm 0.2 \text{ mM}$ for DYN-*d*-KVNSE₅DA indicating slow dissociation of the complexes. The diffusion coefficient for complexes of DYN and either *l*- or *d*-KVNSE₅DA was $1.46 \pm 0.03 \times 10^{-10} \text{ m}^2/\text{s}$.

KVNSE₅DA prevents DYNp facilitation of NMDAR function

Voltage clamp recording methods were used to measure glutamate-evoked currents in *Xenopus* oocytes expressing NR1/NR2B receptors. In the presence of glycine, application of glutamate (50 μM) rapidly activated inward currents and this was prevented by the NMDAR channel blocker MK-801. As reported¹⁷, glutamate (50 μM) elicited little current in the absence of added glycine. However, addition of either 10 μM DYN (Fig 2A), DYN or DYN 2–17 (Fig 2B) to the bathing solution significantly increased the amplitude of glutamate-evoked currents. Co-application of increasing concentrations of the synthetic peptides, *d*- or *l*-KVNSE₅DA, reduced the potentiation of glutamate currents evoked by DYNp in a concentration dependent manner (Fig 2C) whereas co-application of a scrambled sequence of KVNSE₅DA, (EKEVENESEAD) did not. KVNSE₅DA did not modify [³H] TCP binding to the NMDAR in cortical membranes indicating that KVNSE₅DA does alter channel number or affinity.

KVNSE₅DA exerts neuroprotective effects in-vitro and in-vivo

Increased DYN following spinal cord trauma is thought to contribute to neuronal death associated with secondary injury^{18–20, 22–25}. To determine whether KVNSE₅DA prevents the cytotoxic actions of DYN 2–17, we isolated neurons from spinal cords of embryonic mice and used time-lapse photography to examine neuronal viability following exposure to DYN 2–17 alone and in combination with KVNSE₅DA^{8,25}. In these and subsequent experiments, we used DYN 2–17 rather than DYN to avoid confounds resulting from concurrent activation of spinal cord opioid receptors. Continuous exposure of cultures to DYN 2–17 (1–100 μM) induces profound neuronal loss, which is significantly attenuated by NMDAR antagonists but not opioid receptor antagonists⁸. KVNSE₅DA (100 μM) prevented cell loss induced by 10 μM DYN 2–17 ($p < 0.05$) and significantly reduced cell loss produced by 100 μM DYN 2–17 ($p < 0.05$, Fig. 3A).

The i.t infusion of DYN or DYN 2–17 induces long-lasting allodynia^{9,26,27}. To determine whether KVNSE₅DA protects spinal cord neurons *in vivo*, we examined whether its co-infusion prevents tactile allodynia elicited by 5 nmol DYN 2–17. We observed a significant and long lasting decrease in nociceptive withdrawal threshold in all rats that received DYN 2–17. However, when DYN 2–17 was co-administered with KVNSE₅DA (250 nmol), nociceptive thresholds did not differ from controls (Fig. 3B).

Because higher doses of DYN or DYN 2–17 induce hind limb paralysis and loss of neuronal cell bodies in the lumbosacral spinal cord, we evaluated whether KVNSE₅DA could prevent motor dysfunction produced by DYN 2–17. Rats received an i.t. injection of either DYN 2–17 (75 nmol), KVNSE₅DA (250 nmol), or both. We assessed the presence or absence of hind-limb paralysis over 24 h using a 5-point scale²⁷. All DYN 2–17 treated rats exhibited hind limb paralysis within 5 min of infusion. However when KVNSE₅DA was administered with DYN 2–17 or 5 min after DYN 2–17, the magnitude and duration of paralysis was significantly reduced (Fig 3C; $p < 0.01$). By contrast, co-administration of a scrambled sequence of KVNSE₅DA did not affect paralysis produced by DYN 2–17. Similarly, when the Glu and Asp residues of KVNSE₅DA were replaced by Ala, no alteration in DYN 2–17 induced paralysis was seen. In other rats we infused DYN 2–17 alone or with *l*-KVNSE₅DA and assessed motor performance for 21 days using the rotating rod test²¹. Performance was significantly impaired in rats that had received DYN 2–17. However, no significant deficit occurred in animals that had received DYN 2–17 with KVNSE₅DA (Fig 3D; $p < 0.05$).

Motor dysfunction produced by DYN and DYN 2–17 is associated with a loss of motoneurons in the spinal cord¹⁸. To determine whether KVNSE₅DA antagonizes this effect, we examined serial horizontal sections of the lumbar spinal cords of rats using CGRP immunohistochemistry as a selective marker of motoneurons²⁸ and Nissl staining to monitor microglial infiltration. Fig. 4A shows that significant loss of motoneurons occurred in 9 of 12 rats, 72 h after DYN 2–17 infusion. Although i.t administered drugs are expected to be uniformly distributed, motoneuron loss was, in fact, patchy (Fig. 4A-C) precluding comparison of the magnitude of cell loss across animals. However, the protective effect of co-administered KVNSE₅DA was readily apparent. Only 2 of 13 animals showed motoneuron loss (Fig. 4D). Furthermore, the reduction of motoneuron loss exactly paralleled attenuation of paralysis (Fig. 4D).

Discussion

DYN and other opioid peptides are potent analgesics by virtue of their N-terminal Tyr. High concentrations of DYN or N-truncated cleavage products of DYN, which lack the opioid address sequence are excitotoxic producing neurological dysfunction and neuronal loss^{1–9}. Although increased DYN expression and a resulting enhancement of excitatory amino acid transmission have been implicated in the pathophysiology of spinal cord injury and nerve injury-induced neuropathic pain^{2–9}, the mechanisms underlying the neurotoxic effects of DYN have not been delineated.

Our studies demonstrate that DYN and DYN 2–17 bind noncovalently to a linear conserved acidic region of the NR1 subunit *via* salt bridging [a Coulombic interaction] ^{12–15, 29,30}. They further show that the Coulombic interaction of DYN with a short linear epitope (5 Glu and 1 Asp residues) of the NR1 that is contained within the much larger NMDAR complex is sufficient to facilitate NMDAR function and neurotoxicity. Although the critical role of such a short epitope in modulating protein function is at first glance surprising, recent studies have shown that short stretches of oppositely charged residues lead to receptor heteromerization^{15,29,30}.

Previous work has shown that DYN and its major biotransformation product DYN 2–17 potentiate NMDA-mediated cationic currents¹¹. Our in-vitro studies demonstrate that co-application of KVNSE₅DA with either of these prodynorphin derived peptide prevents this potentiation. KVNSE₅DA was also effective in reducing DYN 2–17 evoked cell death in spinal cord cultures. Importantly, the protective effects of this decoy peptide were also observed in-vivo. The i.t. infusion of KVNSE₅DA prevented the tactile allodynia and motor impairment produced by i.t. infusion of DYN 2–17. It also reduced the hindlimb paralysis and motoneuron loss in the spinal cord produced by infusion of a higher concentration of DYN 2–17, and reduced motoneuron loss in the spinal cord. Only peptides that contained the sequence of cognate amino acids corresponding to the NR1 epitope bound DYN and DYN 2–17 and prevented the pathological actions of DYN 2–17. Scrambled peptides or peptides where the Glu and Asp residues were replaced with Ala did not. We hypothesize that salt bridge formation^{12–15,29,30} between adjacent Arg_{6–7} in DYN and adjacent Glu of the NR1 epitope results in sequestration of toxic C-terminal peptides derived from exogenously or endogenously released DYN and that this mechanism underlies the neuroprotective effects of KVNSE₅DA.

Although our data are consistent with the hypothesis that the pathological actions of PDYNp result, in part, from their ability to bind to the NR1 subunit non-covalently, other targets must also be considered. DYNp stimulate glutamate release in the spinal cord and this action may contribute to their neurotoxicity. Furthermore, the NR1 subunit is widely expressed in spinal cord, yet neuronal loss induced by the i.t. infusion of DYN 2–17 is most pronounced in motoneurons and exhibits a patchy distribution. This pattern of loss cannot be attributed to inadequate tissue penetration by DYN because neurons in spinal cord segments rostral and caudal to the catheter tip were affected, while adjacent neurons appeared intact. Regional differences in the coupling of NR1 to the NR2A-D or NR3 subunits may confer enhanced susceptibility to the neurotoxic actions of DYN and DYN 2–17^{6,8,10}. Since, other membrane proteins possess stretches of adjacent acidic amino acids similar to the NR1 subunit, DYN sequestration by KVNSE₅DA may also prevent targeting of DYN to these proteins.

In summary, synthetic peptides that bind DYN and DYN 2–17 noncovalently, and prevent their interaction with the NR1 subunit, are neuroprotective. These peptides attenuate the neurotoxic effects of DYN 2–17 in the spinal cord and improve motor function even when administered after DYN 2–17. In view of the documented involvement of DYN systems in brain and spinal cord injury as well as in persistent pain, the present findings suggest that pharmaceuticals based on the structure of these peptides may be protective in various neuropathological states. Clearly, additional studies are needed to define the structure-activity-relationships of such “decoy” peptides, dosing regimens that produce maximal protection when administered post-insult and their potency following systemic (e.g. intranasal application). Their ability to reverse paralysis produced by exogenous DYN 2–17 and to improve motor function indicates that such studies are warranted. By reducing the deleterious consequences resulting from injury-induced increases in prodynorphin derived peptides³¹, KVNSE₅DA may afford neuroprotection and pain relief without the spectrum of side effects common to NMDAR antagonists.

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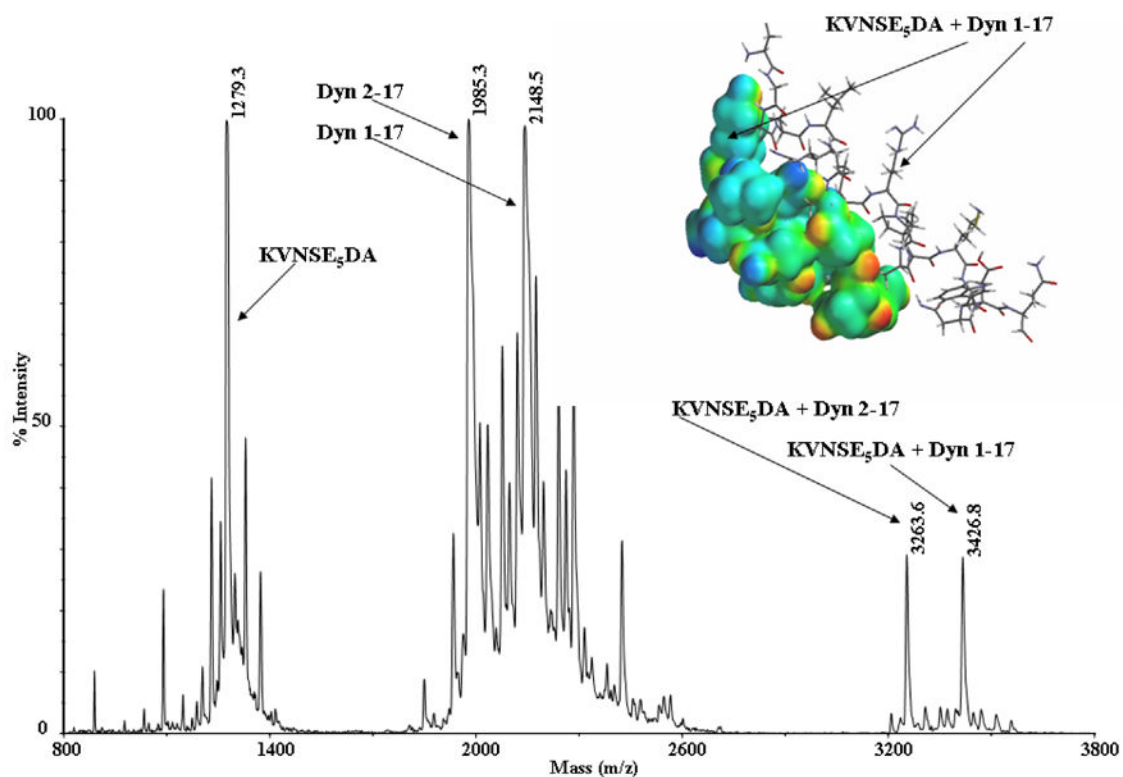


Fig. 1. Positive ion mode MALDI Mass spectrum of a solution containing an equimolar mixture of DYN (1 pmole), DYN 2-17 (1 pmole), and KVNSE₅DA (1 pmole). Complexes of DYN and DYN 2-17 with KVNSE₅DA are seen at amu 3426.8 and 3263.6. The insert shows a proposed model of the interaction of DYN and KVNSE₅DA.

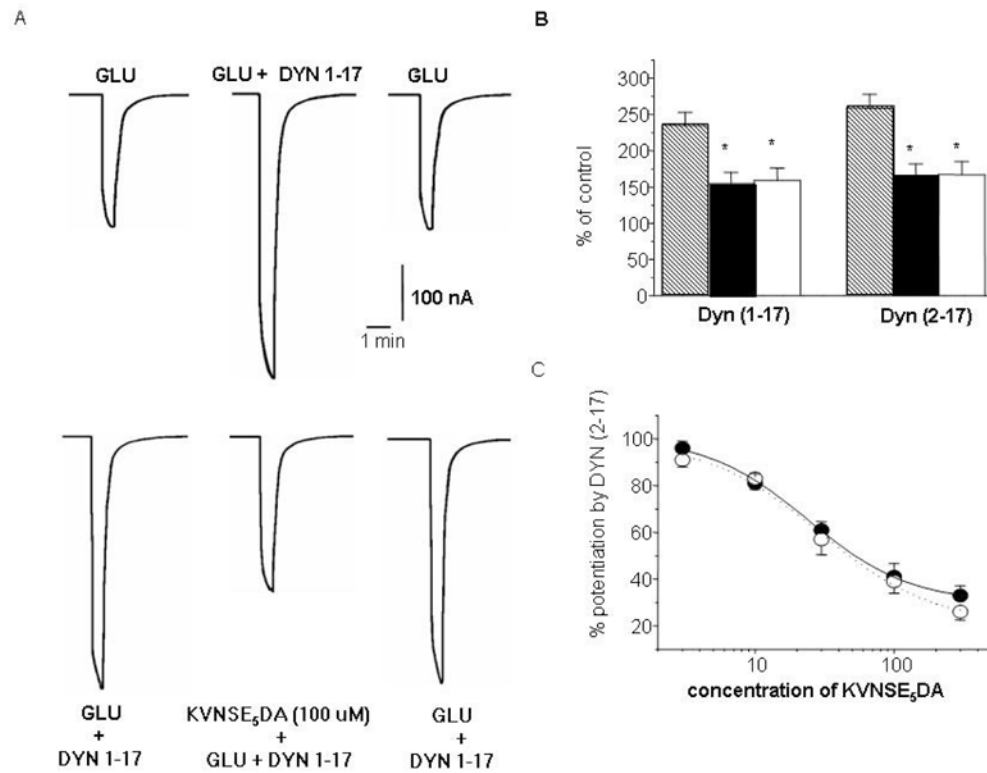


Fig. 2. KVNSE₅DA prevents the potentiation of glutamate activated currents produced by DYN and DYN 2–17 in *Xenopus* oocytes expressing NR1 and NR2B subunits. Potentiation of glutamate induced currents by DYN is reversibly reduced by co-application of *d*-KVNSE₅DA (100 μM) (A). Co-application of either *d*- (black symbols) or *l*-KVNSE₅DA (white symbols) significantly inhibits the potentiation of glutamate activated currents induced by DYN or DYN 2–17. [* p ≤ 0.05, n = 7–11; Student's t-test] (B). The inhibitory effect of *l*- and *d*-KVNSE₅DA upon DYN 2–17 is concentration-dependent (C).

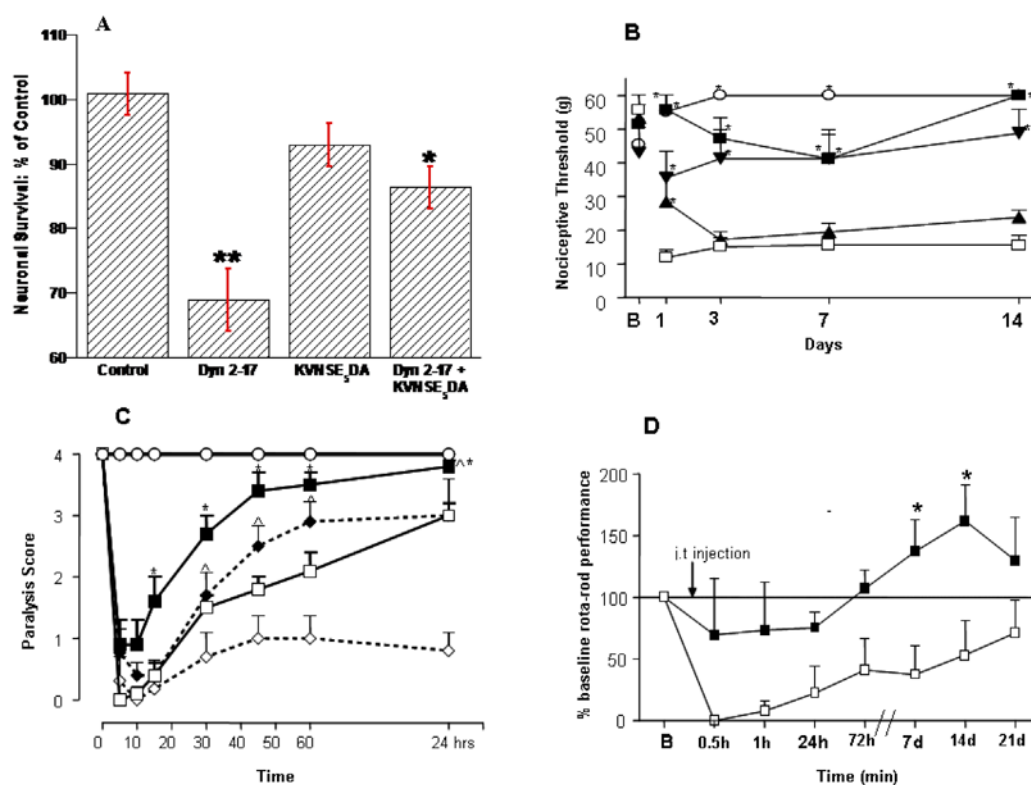


Fig. 3.

KVNSE₅DA attenuates the neurotoxic actions of DYN 2-17 in the spinal cord (A). DYN 2-17 (100 μ M) induced significant cell death in cultured spinal cord neurons at 72 h ($p < 0.01$, $F_{(1,8)} = 6.9$, ANOVA; ** $p < 0.01$ vs controls; Duncan test), which was significantly attenuated by co-administering an equimolar concentration of KVNSE₅DA (* $p < 0.01$ vs. DYN 2-17; Duncan test); KVNSE₅DA alone had no effect. i.t. infusion of DYN 2-17 (5 nmol) produces long-lasting tactile allodynia which is prevented by co-infusion of *l*-KVNSE₅DA ($p < 0.01$, $F_{(4,135)} = 15.8$, ANOVA; * $p < 0.05$ relative to Veh+DYN 2-17, Student-Newman-Keuls test: SNK). ○ Veh (n=7), □ Veh + DYN 2-17 (n=6), ▲ *l*-KVNSE₅DA 30 nmol + DYN 2-17 (n=5), ▼ *l*-KVNSE₅DA 100 nmol + DYN 2-17 (n=8). (C) DYN 2-17 induced motor paralysis is reduced by co- or post-infusion of *l*-KVNSE₅DA (250 nmol). *l*-KVNSE₅DA (250 nmol) was infused with DYN 2-17 (75 nmol) or 5 min after DYN 2-17 (100 nmol) infusion ($p \leq 0.01$, * $p < 0.05$ relative to Veh + DYN 2-17; Kruskal-Wallis test). ○ Control (n=9), □ Veh + 75 nmol DYN 2-17 (n=21), ■ *l*-KVNSE₅DA 250 nmol + 75 nmol DYN 2-17 (n=20). ◊ DYN 100 nmol, ◆ *l*-KVNSE₅DA 250 nmol + 100 nmol DYN (D) *l*-KVNSE₅DA (250 nmol) also prevents the impairment of motor function (rotor-rod performance) that is apparent 21 days after DYN 2-17 (75 nmol) infusion ($p < 0.01$, $F_{(1,89)} = 9.7$, ANOVA; * $p < 0.05$, SNK), □ Vehicle + DYN 2-17 (n=5), ■ *l*-KVNSE₅DA 250 nmol + *l*-KVNSE₅DA (n=5).

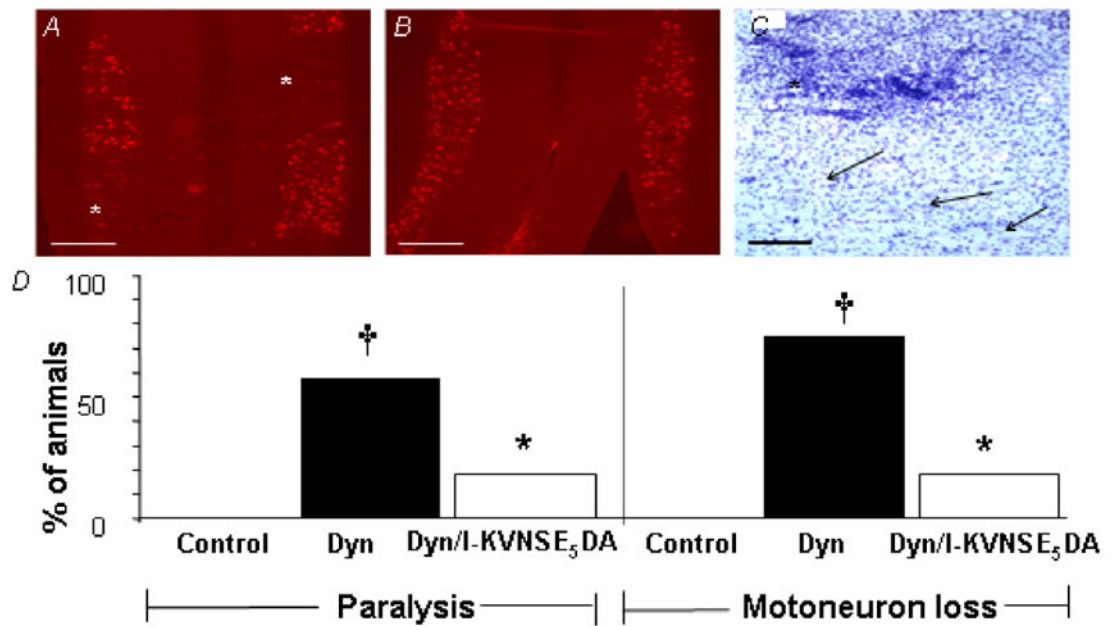


Fig. 4. KVNSE₅DA reduces DYN 2-17-induced paralysis and motor neuron loss. (A) CGRP immunostaining of motor neurons in horizontal section of DYN 2-17-treated rat illustrates patchy loss of motor neurons (*). (B) Co-administration of *l*-KVNSE₅DA (250 nmol) significantly protected against motor neuron loss. (C) Cresyl Violet Nissl stain illustrates shows microglial invasion (*) into an area of DYN 2-17-treated tissue where there is profound motor neuron loss. This area is adjacent to intact ventral horn tissue, with normal complement of motor neurons (arrows). Scale bars a,b: 500 μm; c: 100 μm. (D) Percentage of animals showing paralysis (score <3) or motor neuron loss, 72 h after infusion of vehicle (control; n=13), DYN 2-17 (DYN; n=12) or DYN + *l*-KVNSE₅DA (DYN/ *l*-KVNSE₅DA; n=11) (†).

Table 1

Chymotryptic fragments generated from the digest of the complex formed by the 51 mer epitope of the NMDA NR1 and DYN

Dyn aa residues + NR1 51mer aa residues	MW of Noncovalent Complex fragments	MH ⁺
5-16 + 15-25	1594.9 + 1278.3	2874.2
6-17 + 15-25	1609.9 + 1278.3	2889.2
3-17 + 17-25	1927.3 + 1050.9	2978.2
5-17 + 15-25	1723.1 + 1278.3	3002.4
2-17 + 17-25	1984.3 + 1050.9	3036.2
2-17 + 16-24	1984.3 + 1079.0	3064.3
1-17 + 18-25	2147.5 + 936.8	3085.3
1-16 + 16-25	2019.4 + 1150.1	3170.5
1-17 + 17-25	2147.5 + 1079.0	3199.4
1-16 + 15-24	2019.4 + 1207.2	3227.6
1-17 + 15-24	2147.5 + 1207.2	3355.7
1-17 + 15-25	2147.5 + 1278.3	3426.8
5-17 + 16-29	1723.1 + 1564.6	3288.7
2-11 + 14-32	1199.5 + 2129.3	3329.8
5-17 + 8-21	1723.1 + 1672.8	3396.9
5-17 + 16-31	1723.1 + 1722.7	3446.8
5-17 + 12-26	1723.1 + 1751.8	3475.9
1-17 + 12-26	2147.5 + 1751.8	3900.3
1-17 + 14-38	2147.5 + 2892.2	5040.7
1-17 + 1-25	2147.5 + 3022.3	5170.8