The Neuroprotective KDI Domain of γ1 Laminin is a Universal and Potent Inhibitor of Ionotropic Glutamate Receptors

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ABSTRACT

Previous work from this laboratory indicates that the KDI (Lys-Asp-Ile) domain of γ1 laminin promotes functional regeneration of adult rat spinal cord injuries and protects adult rat hippocampal neurons against massive neuronal death induced by intracerebral injection of the glutamate analogue kainic acid. In the present study, we used patch clamp recordings on cultured human embryonic neocortical neurons and HEK 293-cells expressing recombinant glutamate receptor subunits to study a putative interaction of the KDI with the glutamate system. We show that the KDI-domain of γ1 laminin is a universal and potent inhibitor of AMPA, kainate and NMDA subclasses of glutamate receptors with a non-competitive action on the AMPA receptor channel activity. As glutamate neurotoxicity plays a key role in both CNS trauma and neurodegenerative disorders, this unexpected novel function of the γ1 laminin derived tri-peptide may prove clinically valuable in treatment of CNS trauma and/or disease.

INTRODUCTION

Laminins were identified in late 1970's as extracellular matrix proteins and components of basement membranes (Martin and Timpl, 1987) and they presently form a growing family of glycoproteins with diverse functions (Tunggal et al., 2000; Li et al., 2003; Sasaki et al., 2004; Miner and Yurchenco, 2004). In recent years, the central nervous system (CNS) functions of various laminins have been extensively studied, and their multiple roles in the developing and mature CNS have started to emerge (Liesi, 1990; Luckenbill-Edds, 1997; Morgan and Inestrosa, 2001; Meiners and Mercado, 2003). Numerous studies have established that laminins are widely expressed in both CNS neurons and glial cells (Wiksten et al., 2004b; Liesi et al., 1983; Hagg et al., 1989; Liesi et al., 2001a). Specifically, the γ1-laminin has been linked to promotion of neurite outgrowth (Liesi et al., 1989; Liesi et al., 2001b), neuronal migration (Liesi, 1985; Liesi, 1990), and axon guidance (Cohen et al., 1987; Liesi and Silver, 1988; Wiksten et al., 2003). Interestingly, the neurite outgrowth function of γ1-laminin is mediated by a tri-peptide sequence KDI (Lys-Asp-Ile) located in the C-terminus of the protein (Liesi et al., 2001b). This tri-peptide enhances both viability and directional neurite outgrowth of human spinal cord neurons in vitro (Wiksten et al., 2003; Liebkind et al., 2003).

Recent data indicate that the KDI domain possesses dramatic neuroprotective functions in vivo: 1) it was shown to prevent kainic acid induced neuronal death in hippocampal and cortical areas of the rat (Wiksten et al., 2004b), and 2) to promote healing and functional regeneration of surgically induced spinal cord injury resulting in hind limb paralysis of adult rats (Wiksten et al., 2004a). Microscopic and molecular analyses of KDI-treated spinal cords and hippocampal tissues indicate that application of soluble KDI-peptide reduces tissue damage at the lesion site and enables both neurite outgrowth through the injured area and neuronal survival (Wiksten et al., 2004a,b).

In the present study we investigated the putative effect of γ1-laminin and its derivatives on the function of ionotropic glutamate receptors. We found that laminin-1, and peptide derivatives of the γ1-laminin, including its previously characterized biologically active KDI tri-peptide (Liesi et al., 2001), potently inhibit all known classes of

ionotropic glutamate receptors. Our present results elucidate a novel and unexpected function for γ1 laminin and provide one feasible mechanism for potent regenerative and neuroprotective actions (Wiksten et al., 2004 a,b) of the KDI domain.

MATERIALS AND METHODS

Human CNS-tissues

Human fetal CNS-tissues were obtained from 8-11 week old fetuses after legal abortion, and after informed consent from the patients. The tissues were collected within 2-4 hrs by the permission of the Ethics Committee of the Maternity Hospital of the Central University Hospital.

Neuronal Cultures

The CNS tissues were first placed in cold saline. The neocortical areas of 11-week embryos were identified under a stereomicroscope, and carefully freed of meningeal membranes. Neuronal cultures were prepared as described previously (Liesi et al., 2001) and cells were plated at a concentration of 20,000/dish on glass coverslips pre-coated with poly-D-lysine (10 μg/ml, Collaborative Research) in Neurobasal medium (Gibco, U.K.) with B27-supplement (Gibco, U.K.), antibiotics and 500 μM L-glutamine. After 14-30 days in culture, the cultures were fixed in 2% paraformaldehyde/PBS pH 7.4 for 15 min at room temperature (RT), and processed for immunocytochemistry.

Analysis of Glutamate Receptors in Cultured Human Neocortical Neurons

Immunocytochemistry for ionotropic glutamate receptors in cultured human neocortical neurons was done to demonstrate the presence/absence of receptor proteins and to complement for the electrophysiology by confirming that the cells were viable neurons with extensive neurite outgrowth. Highly specific rabbit polyclonal antibodies (Upstate, NY, USA) against AMPA, kainate and NMDA receptor subunits were used at 5-10 μg/ml. After fixation, the cultures were treated in 0.05% Tween-20 in PBS pH 7.4 for 30 min at RT, washed in PBS and incubated with polyclonal anti-glutamate receptor antibodies diluted in PBS for 1 hr at RT. After a brief wash in PBS, the cultures were incubated with anti-rabbit immunoglobulins coupled to FITC for 30 min at RT, washed once in PBS and mounted in PBS:Glycerol (1:1). The cultures were viewed using an

Olympus BX51 microscope with appropriate filters and photographed using an Olympus DP70 digital camera.

Electrophysiology of Human Neocortical Neurons and HEK 293 Cells

For whole cell patch clamp recordings, cultures were placed under an inverted microscope (Olympus IX71). During recordings neurons were continuously superfused with a external solution (pH 7.4) containing in mM: 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. Experiments were carried out at room temperature. Patch clamp pipettes had a resistance of 4 to 7 MΩ when filled with an internal solution containing in mM: 100 *N*-methyl-D-glucamine, 100 CH_3SO_3H, 40 CsF, 10 $MgCl_2$, 10 HEPES, and 5 EGTA, pH adjusted with CsOH to 7.4. Cells were clamped at a holding potential of -60 mV. Drugs were diluted in external solution and applied to cells with a multi-barrel fast solution application system (Warner Instrument, Hamden, CT). In most experiments, the test-drugs (e.g., laminin-1 and various laminin peptides) were first pre-applied to cells followed by co-application of the agonist and the test-drug. Currents were recorded using Axopatch 200B amplifier and pClamp 8.0 software (Axon Instruments, Inc., Foster City, CA) and stored on the hard drive of PC computer. Recordings were sampled at 20 Hz and filtered with 1Kz lowpass bessel filter. Data were analyzed with pClamp8.0 software. Statistical analysis of results was done with the Prism 3.02 software (GraphPad, San Diego, CA) using a repeated Measures ANOVA and Dunnett's post-test. L-glutamate and concanavalinA were from Sigma (St. Louis, MO) and SYM 2081 and NMDA were from Tocris (Avonmouth, UK). Mouse native laminin-1 was from Boehringer-Mannheim (Germany). All synthetic peptides were from Multiple Peptide Systems (San Diego, CA).

Human embryonic kidney cells (HEK 293 cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine and 1% penicillin-streptomycin solution. Cells were transfected with recombinant GluR4 (AMPA) and GluR6 (kainate) receptor subunit cDNA-clones using the calcium phosphate method as previously described (Pasternack et al., 2002).

RESULTS

To elucidate the possible interaction of the KDI-domain of γ1 laminin with the glutamate receptor function, we used immunocytochemistry and patch clamp recording

on human embryonic neocortical neurons cultured on a poly-D-lysine substratum. In addition, we applied patch clamp studies on HEK 293-cells expressing recombinant AMPA, and kainate receptor subunits. Immunocytochemically, neo-cortical neurons expressed subunits for all subclasses of glutamate receptors tested (Fig. 1a-b), but both expression patterns and subunit compositions showed considerable variation (Fig. 1a-b).

Application of glutamate to the cultured human neocortical neurons under conditions that inhibit NMDA receptor currents, e.g., in the presence of 1.0 mM Mg^{2+} in the external solution, produced a fast desensitizing current (Fig. 2a) that was effectively inhibited by 0.1-10 μg/ml of the KDI peptide (Fig. 2a). The glutamate-evoked current was solely mediated by AMPA receptors, since application of a selective kainate receptor agonist SYM 2081 together with 0.3 mg/ml of concanavalin A (Donevan et al., 1998) produced only a small (5 pA) insignificant current (data not shown). Thus, neocortical neurons on a poly-D-lysine substratum failed to express functional kainate receptors even though they showed expression of several kainate receptor subunit proteins (Fig. 1a-b).

Laminin-1 and various peptide derivatives of the neurite outgrowth domain of γ1-laminin (Liesi et al., 1989; Liesi et al., 2001b) inhibited AMPA receptor currents in these human neo-cortical neurons (Fig. 2b). Native laminin-1 (Martin and Timpl, 1987; 5 μg/ml=0.01 μM, α1β1γ1) inhibited currents evoked by 20 mM glutamate by 59 ± 5 % (Fig. 2b). Inhibition of a 10 amino acid long neurite outgrowth domain of γ1-laminin (Liesi et al., 1989; RDIAEIIKDI) was dependent on the form of the synthesized amino acid chain. An acid (-OH) form of the peptide inhibited the AMPA receptor currents at the concentration of 10 μg/ml (10 μM) by 72 ± 8 % (Fig. 2b) whereas the amide ($-NH_2$) form only by 22 ± 3 % (Fig. 2b). The previously characterized shortest active domain of γ1-laminin, KDI (Liesi et al., 2001b), at a concentration of 10 μg/ml (30 μM) was also found active in inhibiting AMPA receptor currents by 90±13%, and at a concentration of 3 μg/ml (10 μM) by 75% (Fig. 3a). The cell attachment peptide (CDPGYIGSR) from the β1 laminin (Graf et al., 1987) did not inhibit AMPA currents in a significant manner (Fig. 2b), and the previously identified active domain of the β2-laminin LRE (Hunter et al., 1991) was also inactive in modulating AMPA receptor currents of human neo-cortical neurons (Fig. 2b).

The acidic form of the KDI-peptide inhibited AMPA receptor currents of human neocortical neurons in a dose dependent manner with IC_{50} of 0.1 μg/ml (300 nM) of the tri-peptide (Fig. 3a). Glutamate dose response curves yielded EC_{50} values of 2.5 and 1.9 mM in the absence and presence of KDI were, respectively, indicating a non-competitive inhibition of the receptor by the KDI peptide (Fig. 3b). Inhibition of AMPA receptor currents by the KDI tri-peptide was a direct and fully reversible one. This was apparent

from the facts that only a short pre-application of the KDI-peptide (62 ± 22 ms) was required for maximal inhibition of the AMPA receptor currents (Fig. 3c), and that co-application of 3 μg/ml (10 μM) of the KDI with glutamate without KDI-pre-application produced only a small inhibition of 37 ± 20 % (Fig. 3c) as compared to the inhibition obtained with the KDI (3 μg/ml) pre-application (75 %; see Fig. 2b).

The effect of the KDI-peptide on NMDA receptor currents of human neocortical neurons was studied using 100 μM NMDA as an agonist in Mg^{2+}-free external solution (Fig. 4a). At 0.1-10 μg/ml (300 nM-30 μM), the KDI peptide inhibited NMDA receptor currents by 25-50% (Fig. 4a).

As we were unable to detect kainate receptor currents in human neocortical neurons even though they looked mature, healthy and expressed several kainate receptor subunits at the protein level (Fig. 1a), we used HEK 293 cells transfected with the recombinant GluR6 receptor subunit, also expressed by human cortical pyramidal neurons (Fig. 1a), to study the effects of the KDI-peptide on kainate receptor currents. Our results indicate that the GluR6 kainate receptors of the HEK 293 cells were equally sensitive to KDI-inhibition as the AMPA receptors of the human neocortical neurons showing an IC_{50} at 0.1 μg/ml of KDI peptide (Fig. 4b). Recombinant monomeric GluR4 AMPA receptors on transfected HEK 293 cells were highly sensitive to the inhibitory effect of KDI peptide, similar to human neocortical neurons (Figs. 2-3). The KDI peptide at 10 μg/ml (30 μM) inhibited GluR4 currents evoked by 10 mM glutamate by 83 ±14 %, IC_{50} being approximately 0.1 μg/ml (data not shown).

DISCUSSION

In the present study we show that laminin-1, and in particular the neurite outgrowth promoting KDI domain of the γ1-laminin, inhibit AMPA receptor currents in cultured human neo-cortical neurons in a dose-dependent, and non-competitive manner. Similarly, the KDI-domain also inhibits NMDA receptor currents in human neocortical neurons and recombinant kainate and AMPA receptor currents in transfected HEK 293-cells. As shown in Fig.1, cultured human neocortical neurons were mature-looking, extended long neurites, and expressed all subclasses of ionotropic glutamate receptor

proteins. Thus, the inability to detect kainite currents in human neocortical neurons may indicate a low level of kainite receptor activity in those cells. The KDI peptide produces almost complete inhibition of AMPA and kainate receptors at 10 μg/ml (30 μM) and a 50% inhibition of the NMDA receptor currents. Thus, the KDI domain of γ1 laminin is a novel, extremely potent and universal antagonist of the major subclasses of ionotropic glutamate receptors.

The direct inhibition of glutamate receptor function by the KDI tri-peptide shown here reveals an entirely novel and biologically highly relevant function for the γ1 laminin and its KDI domain. Until now, the effects of extracellular matrix proteins, including the γ1 laminin, in the nervous system have been considered indirect and mediated via signaling cascades initiated by cell-matrix contacts.

That laminin-1 (Martin and Timpl, 1987; composed of disulphide bonded α1β1γ1 laminins) and both the 10 amino acid neurite outgrowth domain (RDIAEIIKDI) of γ1 laminin (Liesi et al., 1989), e.g., the amphiphilic peak of the γ1 laminin (Liesi et al., 1989), and its shortest active KDI-domain (Liesi et al., 2001) all act as antagonists of AMPA-receptors indicate that the 10 amino acid neurite outgrowth domain, indeed, is facing the outside of the alphahelical domain I of the C-terminus of laminin-1. That the acid form of the 10 amino acid peptide is far more efficient than its amide form and the acid form of the KDI domain is the most efficient antagonist of glutamate receptors indicate that these domains are likely to be cleaved by proteolysis from the laminin-1 molecule rather than being synthesized and secreted as naturally occurring peptides. Indeed, expression and accumulation of such short peptides along neuronal surfaces have been reported (Murtomäki et al., 1995).

Never before has an extra-cellular matrix protein or its functionally active domain been shown to directly regulate the activity of a neurotransmitter-gated ion channel. Previous studies have shown aggregation of inwardly rectifying potassium channels (Guadagno and Moukhles, 2004) and most recently voltage gated calcium channels (Hiroshi et al., 2004) by binding of a particular laminin to the channel protein, but no electrical responses have been reported for this interaction (Guadagno and Moukhles, 2004; Hiroshi et al., 2004). Thus, the present results are revolutionary in showing that the role of γ1 laminin in the CNS is not limited to promotion of neurite outgrowth, neuronal migration and regeneration with the assumption that all these functions are mediated solely by its adhesive properties. Even though the adhesive properties have been shown to play an important role in these biological events, the present data indicate that the neurite outgrowth KDI domain of γ1 laminin has additional diverse and important functions that shed new relevance for expression of γ1 laminin in adult CNS neurons (Wiksten et al., 2004b; Hagg et al., 1989), and in CNS after trauma (Liesi and Kauppila,

2002) or in a neurological disorder, such as Alzheimer's disease (Murtomäki et al., 1992; Palu and Liesi, 2002). The inhibitory function of the KDI domain on glutamate receptor function indicates that γ1 laminin may be expressed in adult neocortical and hippocampal neurons for protective and regulatory reasons essential for normal functioning of the CNS. For example, degradation of laminin-1 and γ1 laminin by plasmin is known to impair maintenance of LTP (Nagami et al., 2000), and degradation of laminin from hippocampal areas is linked to induction of neuronal death (Wiksten et al., 2004b; Chen et al., 1997; Indyk et al., 2003; Chen et al., 2003).

Recent studies from this laboratory offer the first practical examples on the regulatory and neuroprotective potential of the KDI domain in vivo (Wiksten et al., 2004 a,b). These data, in particular the ability of the KDI peptide to protect adult hippocampus against kainic acid (Wiksten et al., 2004b), led us to study the possible direct interaction between the KDI domain and ionotropic glutamate receptor function. In the present paper we show that the KDI tri-peptide is the only known universal inhibitor of ionotropic glutamate receptor function with already demonstrated ability to protect adult rat CNS against excitotoxicity. As compelling evidence indicates that glutamate neurotoxicity is a major player in all CNS trauma, inflammation and neurodegenerative disorders (Choi, 1988; Choi, 1994; Sattler and Tymianski, 2001; Mattson, 2003), and some of the newest drugs to treat patients with Alzheimer's disease and ALS, such as memantine and riluzole, are inhibitors of the glutamate system, our present results strongly imply that the KDI peptide may become one of the most efficient targeted medications for CNS trauma and disease. Furthermore, the present finding of the KDI tri-peptide as a novel potent and possibly endogenous inhibitor of ionotropic glutamate receptors may help in designing novel compounds for more efficient treatment of degenerative and inflammatory brain diseases and CNS-trauma.

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FIGURE LEGENDS

Figure 1 In **a.** Immunocytochemistry for AMPA (GluR1, GluR2/3, GluR4), and kainate (GluR5, GluR6/7, KA2) glutamate receptor subunits ~~in~~ indicates that human embryonic neocortical neurons express several different ionotropic glutamate receptor proteins. For example, expression of the GluR1 receptor subunit is strongest in cell bodies, while the GluR2/3 subunit is strongly and specifically expressed along the long neurites as well as cell bodies. Expression of GluR4 receptor subunit is homogenous along both neurites and cell bodies, whereas GluR5 kainate subunit is being expressed as punctate deposits along the neurites. A weak but distinct expression of the GluR6/7 kainate receptors is visualized along the mature-looking pyramidal neurons, while KA2 receptors are

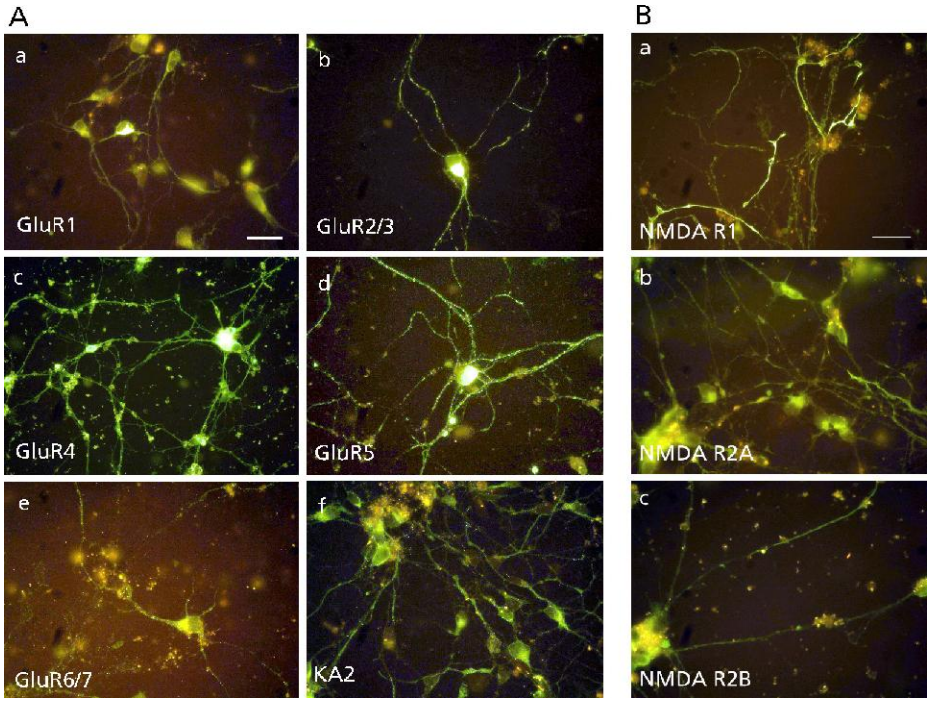
homogenously expressed along both neurites and cell bodies of the neocortical neurons. All cells are on poly-D-lysine at 14 days in vitro. Scale bar = 20 μm. In **b,** immunocytochemistry for NMDA receptor subunits in human embryonic neocortical neurons indicate that the NR1 subunit is expressed in a patchy fashion along neurites of the neocortical neurons. Both NR2A and NR2B subunits show weak expression patterns mainly in the cell bodies of neurons. Thus, the neocortical neurons express at least the NR1/NR2A and NR1/NR2B heterodimeric receptor proteins. All cells are on poly-D-lysine at 14 days in vitro. Scale bar = 20 μm.

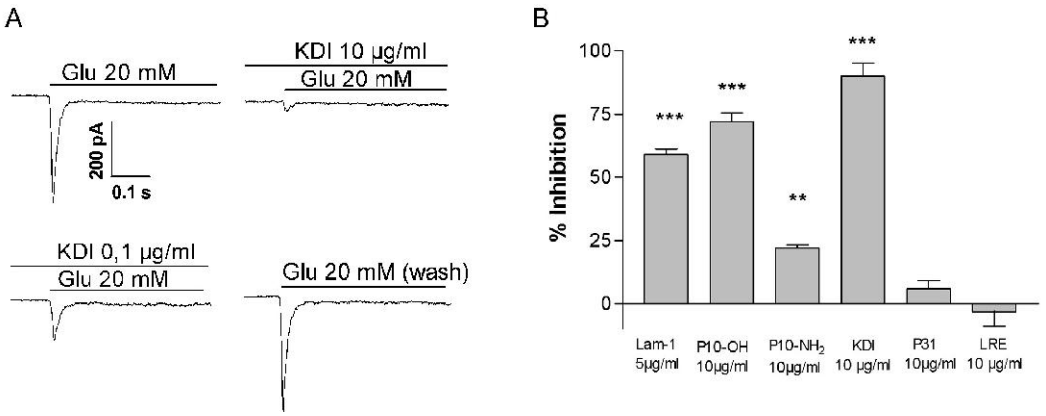
Figure 2 Example traces of 20 mM glutamate-evoked currents in human neocortical neurons (**a)** in the absence and presence of the KDI peptide (0.1-10 μg/ml) indicate that pre-application of KDI produces an inhibition, which is dose-dependent, washable and reproducible (n = 15). In **b**, a column presentation demonstrates a percentage of inhibition of laminin-1 (Lam-1) and its various derivatives on AMPA receptor currents of human neocortical neurons. Laminin-1 (Lam-1), purified from mouse EHS-tumor (Martin and Timpl, 1987), produced a significant (***) inhibition of AMPA currents. P10-OH (n=5), an acid form of the neurite outgrowth domain of the γ1 laminin (Liesi et al., 1989, RDIAEIIKDI) also produced effective (***) inhibition of AMPA receptors, whereas P10-NH_2 (n=5), an amide form, was much less efficient (**). The KDI peptide was most efficient (***) in inhibiting AMPA receptor currents whereas a control peptide from cell attachment domain of β1 laminin (Graf et al., 1987) (CDPGYIGSR, n=6), and a control tri-peptide LRE from β2 laminin (Hunter et al., 1991) (n=5) did not affect AMPA receptor currents. Asterisks indicate statistical significance of inhibition between the control and the peptide treatment tested by t- test, $p < 0.001$ (***), $p < 0.01$ (**).

Figure 3 Evaluation of KDI tri-peptide inhibition of AMPA currents of cultured human neocortical cells. In **a**, an inhibition curve of increasing concentrations of the KDI peptide on AMPA currents evoked by 20 mM glutamate indicate an IC_{50} of 0.1 μg/ml of the peptide (n=10). $P < 0.001$ (***), $p < 0.05$ (*). In **b**, the glutamate dose-response curves

indicate that 0.1 µg/ml of the KDI peptide does not essentially shift the EC_{50}, which indicates a non-competitive nature of inhibition (n=6). In **c**, the significance of the length of KDI pre-application time for inhibition of AMPA receptor currents is demonstrated (n=6). The results show that the pre-application time needed for maximal inhibition is short (62±22 ms). In some experiments, a direct co-application of KDI and glutamate was tested. Under those conditions, the KDI peptide produced only a 37±20% inhibition of AMPA currents, which indicates that the KDI peptide directly interacts with the AMPA receptor.

Figure 4 In **a**, both individual traces and column presentation indicate that the pre-applied KDI peptide is a potent inhibitor of NMDA currents at 0.1-10 µg/ml (n=7). $p<0.001$ (***), $p<0.05$ (*). In **b**, KDI inhibition of kainate receptor currents in HEK 293 cells transfected with GluR6 clones indicates almost 100% inhibition at 10 µg/ml ($p<0.001$ ***) of the KDI and an IC_{50} at 0.1 µg/ml of the KDI peptide (n=6).

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Glu 20 mM
KDI 10 µg/ml
Glu 20 mM
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100
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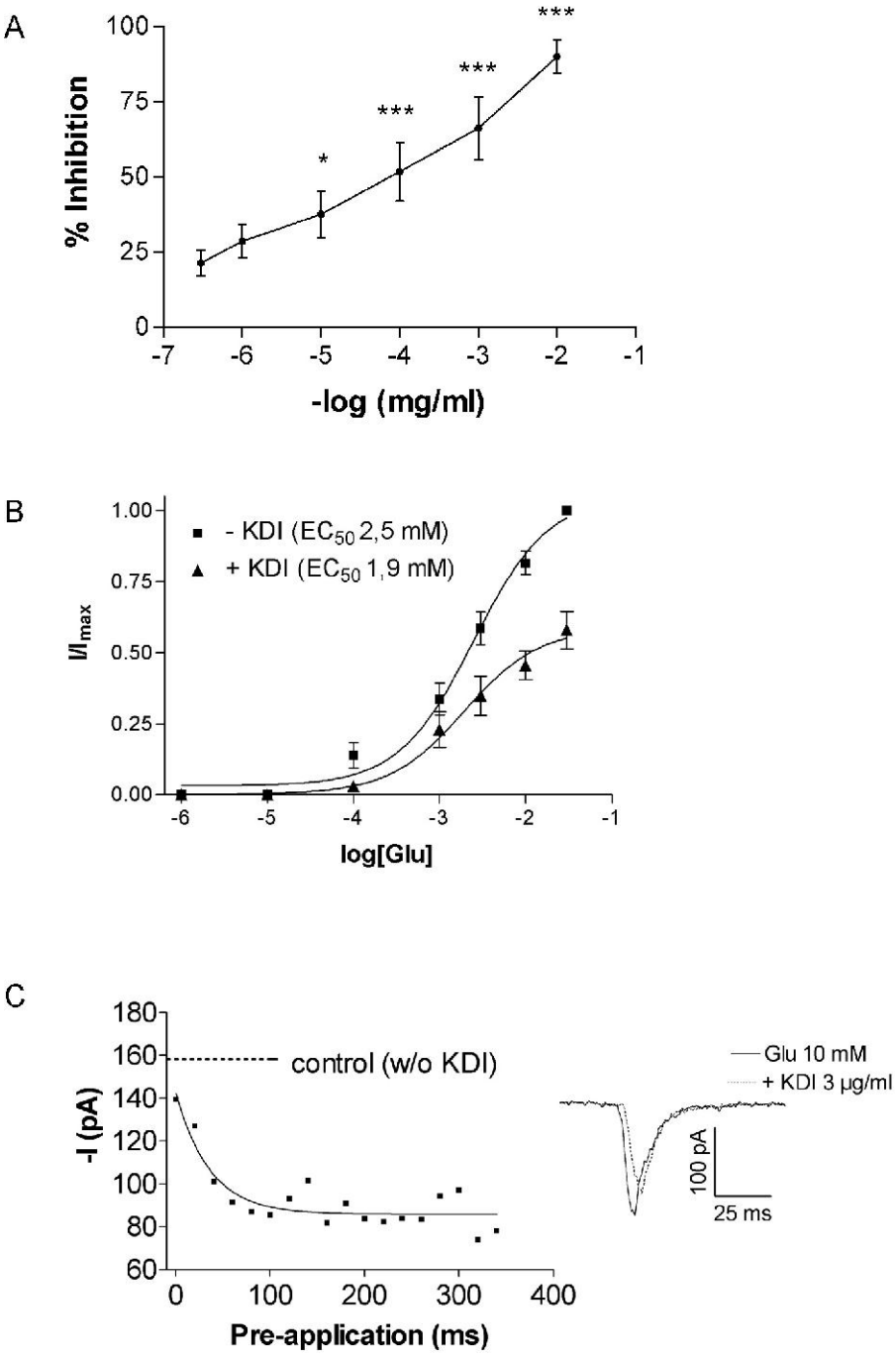
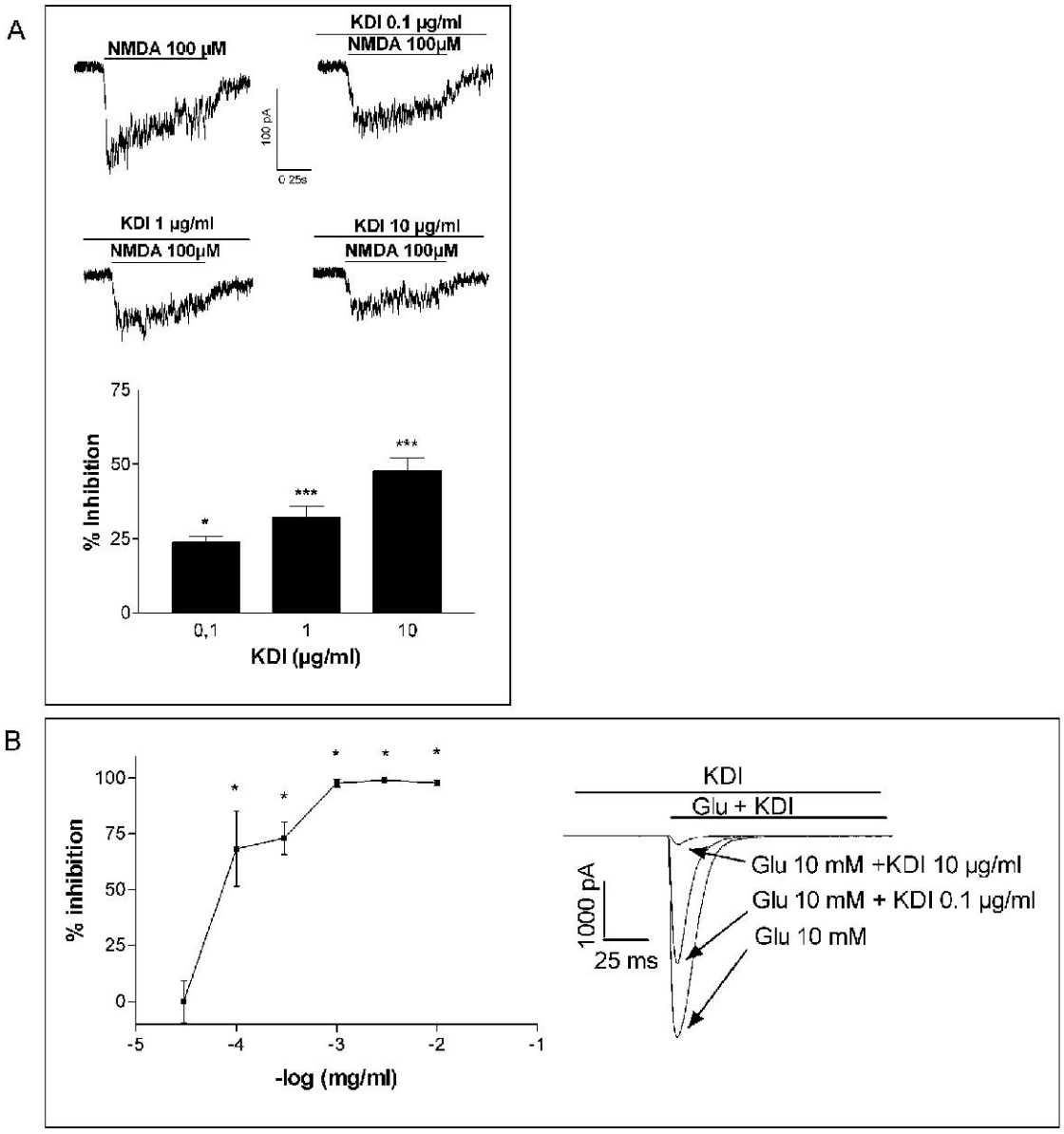
Lam-1 5µg/ml
P10-OH 10µg/ml
P10-NH2 10µg/ml
KDI 10 µg/ml
P31 10µg/ml
LRE 10 µg/ml


A
100
75
50
25
0
% Inhibition
*
***
***
***
-7
-6
-5
-4
-3
-2
-1
-log (mg/ml)
B
1.00
0.75
0.50
0.25
0.00
I/I max
- KDI (EC50 2,5 mM)
+ KDI (EC50 1,9 mM)
-6
-5
-4
-3
-2
-1
log[Glu]
C
180
160
140
120
100
80
60
-I (pA)
control (w/o KDI)
0
100
200
300
400
Pre-application (ms)
Glu 10 mM
+ KDI 3 µg/ml
100 pA
25 ms


A
NMDA 100 µM
KDI 0.1 µg/ml
NMDA 100µM
100 pA
0 25s
KDI 1 µg/ml
NMDA 100µM
KDI 10 µg/ml
NMDA 100µM
% Inhibition
75
50
25
0
*
***
***
0,1
1
10
KDI (µg/ml)
B
% inhibition
100
75
50
25
0
-5
-4
-3
-2
-1
-log (mg/ml)
KDI
Glu + KDI
Glu 10 mM +KDI 10 µg/ml
Glu 10 mM + KDI 0.1 µg/ml
Glu 10 mM
1000 pA
25 ms