Modified peptides as potent inhibitors of the psd-95/nmda receptor interaction

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ABSTRACT

The present invention is directed to the provision of small molecule inhibitors of the PSD-95/NMDA receptor interaction, employing an undecapeptide corresponding to the C-terminal of the NMDA as a template for finding lead candidates. A compound (NMDAR/PSD-95 inhibitor) of the invention includes a peptide or peptide analogue comprising at least four peptide bonded residues having the sequence YTXV or YSXV, wherein Y is selected from among E, Q, and A, or an analogue thereof, and X is selected from among A, Q, D, N, *N*-Me-A, N-Me-Q, *N*-Me-D, and *N*-Me-N or an analogue thereof, wherein an amino-terminal residue of the peptide is *N*-alkylated. Alternatively the compound of the invention comprises a first peptide or peptide analogue linked to a second peptide or peptide analogue by a linker, where the first and second peptide or peptide analogue each comprise at least four peptide bonded residues having the sequence YTXV or YSXV, wherein Y is selected from among E, Q, and A, or an analogue thereof, and X is selected from among A, Q, D, N, N-Me-A, *N*-Me-Q, N-Me-D, and *N*-Me-N or an analogue thereof.

DESCRIPTION (OCR text may contain errors)

Title: Modified peptides as potent inhibitors of the PSD-95/NMDA receptor interaction.

Field of the invention The protein-protein interaction between the NMDA receptor and its intracellular scaffolding protein, PSD-95, is a potential target for treatment of ischemic or traumatic injury of the central nervous system (CNS). The present invention is directed to the provision of small molecule inhibitors of the PSD-95/NMDA receptor interaction, employing an undecapeptide corresponding to the C-terminal of the NMDA as a template for finding lead candidates.

Background of the invention

Protein-protein interactions (PPIs) are essential to vital cellular processes, and are involved in numerous pathophysiological states where they serve as potential targets for therapeutic intervention. PPIs have generally been perceived as difficult to target with small organic molecules, since they are often characterized by large, flat, and hydrophobic interfaces.

A class of PPIs is one involving PDZ domains [PDZ is an abbreviation for postsynaptic density protein-95 (PSD-95), Drosophila homologue discs large tumor suppressor (DlgA) and zonula occludens-1 protein (ZO-l)]. PDZ domains often function as modules in scaffolding proteins that are involved in assembling large protein complexes in the cell, and are highly abundant in eukaryotic organisms. PDZ domains comprise about 90 amino acids and generally interact with only a few amino acids of the C-terminal part of the interacting protein. PDZ domains are typically divided into three classes according to the sequence of their ligands. PSD-95, contains three PDZ domains, PDZ 1-3, which bind peptide ligands with the consensus sequence Glu/Gln-Ser/Thr-X-Val-COOH, thus being designated class I PDZ domains. The structural basis for the interaction of PDZ domains with C-terminal peptides was first elucidated by an X-ray crystallographic structure of PDZ3 of PSD-95 complexed with a native peptide ligand, CRIPT. PDZ3 contains six antiparallel β- strands (βA-βF) and two α-helices (αA and αB), and the C-terminal peptide ligand binds as an additional

CLAIMS (OCR text may contain errors)

Claims:

1. A compound comprising a first peptide or peptide analogue linked to a second peptide or peptide analogue by a linker, wherein the first and the second peptide or peptide analogue comprise at least four amide-bonded residues having a sequence YTXV or YSXV, wherein a. Y is selected from among E, Q, and A, or an analogue thereof, and b. X is selected from among A, Q, D, N, N-Me-A, N-Me-Q, N-Me-D, and N-Me-N or an analogue thereof.

2. A compound comprising a peptide or peptide analogue, the peptide or peptide analogue comprising at least four amide-bonded residues having a sequence YTXV or YSXV, wherein a. Y is selected from among E, Q, and A, or an analogue thereof, and b. X is selected from among A, Q, D, N, N-Me-A, N-Me-Q, N-Me-D, and N-Me-N or an analogue thereof; wherein the peptide or peptide analogue is N-alkylated in position $P^{"3}$ in the sequence.

3. A compound according to claim 1 or 2, capable of inhibiting a protein-protein interaction between NMDAR and PSD-95.

4. A compound according to claim 1, wherein the linker is a PEG linker comprising

1 to 28 moieties (N=l -28) of ethylene glycol.

5. A compound according to claim 4, wherein the PEG linker comprises from 1 to

12 moieties (N=l-12) of ethylene glycol.

6. A compound according to any one of claims 1-5, wherein the peptide or peptide analogue is from 4 to 10 amide-bonded residues in length.

7. A compound according to any one of claims 1-6, wherein the peptide is comprised of at least 4 L-amino acid residues.

anti-parallel β-strand into a groove between the βB strand and αB helix. Two residues in the peptide ligand are considered particularly important for affinity and specificity, the first (P^0) and the third ($P^{"2}$) amino acids (counting from the C-terminal). The side chain of the amino acid in P^0 position projects into a hydrophobic pocket and an amino acid with an aliphatic side chains (Val, He and Leu) is required. In the PDZ3 -CRIPT structure, the hydroxyl oxygen of Thr ($P^{"2}$) forms a hydrogen bond with the nitrogen of an imidazole side chain of His372, which is a highly conserved residue in class I PDZ domains. A conserved Gly-Leu- Gly-Phe (position 322-325 in PDZ3) motif and a positively charged residue (Arg318 in PDZ3) of PDZ domains mediate binding to the C-terminal carboxylate group.

The PDZI and PDZ2 domains of PSD-95 interact with a number of proteins including a group of ionotropic glutamate receptors, the JV-methyl-D-aspartate (NMDA) receptor. This receptor is a hetero tetrameric ion channel generally formed by the two subunits, NRI and NR2, and gated by glutamate and glycine. The NMDA receptor (NMDAR) plays a key role in several diseases in the CNS brain, but development of drugs that directly interact with the NMDA receptor has been difficult. Therefore, there is a need for alternative approaches to modulate the NMDA receptor activity; one such approach is perturbation of the PSD-95/NMDA receptor interaction. PSD-95 simultaneously binds the NMDA receptor, primarily NR2A and NR2B subunits, and the enzyme neuronal nitric oxide synthase (nNOS) through PDZI or PDZ2 (Figure 1). Activation of the NMDA receptor causes an influx of Ca^{2+}, which activates nNOS thereby leading to nitric oxide (NO) generation. Thus, PSD-95 mediates a specific association between NMDA receptor activation and NO production, which can be detrimental for the cells if sustained for a longer period (Figure 1).

Inhibition of the PSD-95/NMDA receptor interaction is known to prevent ischemic brain damage in mice, presumably by impairing the functional link between Ca^{2+} entry and NO production, while the physiological function of the NMDA receptor remains intact.[1] Uncoupling of PSD-95 from the NR2B subunit was achieved by a nonapeptide, corresponding to the C-terminal of NR2B, fused to HIV-I Tat peptide, known for its ability to facilitate membrane permeability. This 20-mer peptide (Tat- NR2B, 3, Table 1) is currently in clinical trials as a potential drug for the treatment of cerebrovascular ischemia, as seen in stroke.[2"4] However, peptides are generally not attractive drug candidates due to their poor bioavailability, instability in vivo and low patient tolerance due to development of "immunogenicity" to the administered peptide.

The binding pocket of PDZ domains, which embeds a small, linear peptide motif, has a relatively small surface area and a non-favourable geometry, which makes PDZ domains difficult to target with small molecules. These difficulties are reflected by the very low number of small molecule inhibitors of PDZ domain interactions.

Summary of the invention

According to a first embodiment, the invention provides a compound comprising a (modified) peptide or (modified) peptide analogue, the peptide or peptide analogue comprising at least four amide-bonded residues having the sequence YTXV or

YSXV, wherein Y is selected from E, Q, and A, or an analogue of the selected residue; and X is selected from among A, Q, D, N, N-Me-A, N-Me-Q, N-Me-D, and

N-Me-N, or an analogue of the selected residue, and wherein a residue of the peptide or peptide analogue is N-alkylated and wherein the N-alkylated residue is at position

$P^{"3}$ corresponding to residue Y.

8. A compound according to any of claims 1-7, wherein X is selected from among A, Q, and D.

9. A compound according to any one of claims 2-8, wherein the peptide or peptide analogue is JV-alkylated with a cyclohexane substituent, and further comprises a spacer group between the substituent and the terminal amino group of the peptide or peptide analogue, wherein the spacer is an alkyl group, preferably selected from among methylene, ethylene, propylene and butylene.

10. A compound according to any one of claims 2-8, wherein the peptide or peptide analogue is JV-alkylated with an aromatic substituent, and further comprises a spacer group between the substituent and the terminal amino group of the peptide, wherein the spacer is an alkyl group, preferably selected from among methylene, ethylene, propylene and butylene.

11. A compound according to claim 10, wherein the aromatic substituent is a naphthalen-2-yl moiety.

12. A compound according to claim 10 or 11, wherein the aromatic substituent is an aromatic ring substituted with one or two halogen and/or alkyl group.

13. A compound according to any of claims 1-12, wherein the peptide or peptide analogue is covalently bonded to a polyamine or a diamine.

14. A complex comprising one or more PDZ domain and a compound according to any one of claims 1-13.

15. A complex according to claim 14, wherein the PDZ domain is a PDZI and/or a

PDZ2 domain comprised in a PSD-95 protein.

16. Use of JV-alkylation of a residue at position $P^{"3}$ of a peptide or peptide analogue to enhance binding affinity of the peptide or peptide analogue for a PDZ domain.

17. Use of a PEG linker to enhance binding affinity of a compound for a PDZ domain protein, wherein the compound comprises a first peptide or peptide analogue linked to a second peptide or peptide analogue.

18. Use of a compound according to any one of claims 1-13, to inhibit a protein- protein interaction between a protein and a PDZ domain.

19. A use according to claim 18, wherein said interaction is between an NMDAR and PSD-95 and wherein the NMDAR is comprised in a cell.

20. A pharmaceutical composition comprising a compound according to any one of claims 1-13 for use as a medicament.

21. A pharmaceutical composition comprising a compound according to any one of claims 1-13 for use in the prophylaxis and/or treatment of an excitotoxic-related disease in a subject.

22. A pharmaceutical composition according to claims 21, wherein the disease is ischemic or traumatic injury of the CNS.

23. A kit comprising the pharmaceutical composition according to any one of claims

20 to 22, further comprising means for delivering said composition to a subject.

24. A method of providing prophylaxis and/or treatment of an excitotoxic-related disease in a subject, comprising administering a pharmaceutical composition according to claim 20 to the subject.

25. A method according to claim 24, wherein said disease is ischemic or traumatic injury of the CNS.

According to a second embodiment, the invention provides a compound comprising a first peptide or peptide analogue linked to a second peptide or peptide analogue by a linker, wherein the first and the second peptide or peptide analogue comprise at least four amide-bonded residues having the sequence YTXV or YSXV, wherein Y is selected from among E, Q, and A, or an analogue of the selected residue, and X is selected from among A, Q, D, N, N-Me-A, N-Me-Q, N-Me-D, and N-Me-N or an analogue of the selected residue. The linker of the compound may comprise PEG, having a length of from 1 to 28 moieties (n=l-28) of ethylene glycol, preferably from 1 to 12 moieties (n=l-12), more preferably from 4 to 6 moieties (n=4-6). A compound according to the second embodiment is further provided, wherein an amino acid residue of the first and/or the second peptide or peptide analogue may, or may not additionally be, Λf-alkylated, at position $P^{"J}$.

A compound according to the first or second embodiment is preferably one that is capable of inhibiting protein-protein interaction between NMDAR and PSD-95 (i.e. NMDAR/PSD-95 inhibitor).

A compound according to the first or second embodiment is provided, wherein the peptide or peptide analogue is 10, 9, 8, 7 or 6 amide-bonded residues in length, more preferably 5 or 4 amide-bonded residues in length.

A compound according to the first or second embodiment is provided, wherein the peptide or peptide analogue comprises at least 4 L-amino acid residues. Preferably the residue X in the compound is selected from among A, Q, and D.

A compound according to the first embodiment is provided, wherein the peptide or peptide analogue is //-alkylated (at position $P^{"3}$) with a cycloalkyl substituent, and further comprises a spacer group between the substituent and the terminal amino group of the peptide or peptide analogue, wherein the spacer is an alkyl group. The alkyl group is preferably selected from among methylene, ethylene, propylene and butylene. The cycloalkyl substituent in the compound may be cyclohexane.

A compound according to the first embodiment is provided, wherein the peptide or peptide analogue is //-alkylated (at position $P^{"3}$) with an aromatic substituent, and further comprises a spacer group between the substituent and a terminal amino group of the peptide, wherein the spacer is an alkyl group. The alkyl group is preferably selected from among methylene, ethylene, propylene and butylene. The aromatic substituent in the compound may be a naphthalen-2-yl moiety. Where the aromatic substituent in the compound is an aromatic ring, the aromatic ring may be substituted with one or two halogen atoms (for example chlorine or fluorine) and/or alkyl group.

A compound according to the first or second embodiment is provided, wherein the amino acid residue V in the peptide is substituted by tert-leucine.

A compound according to the first or second embodiment is provided, wherein the peptide or peptide analogue is covalently bonded to a polyamine or a diamine.

The invention further provides a complex comprising a PDZ domain and a compound according to the first or second embodiment. Preferably, the PDZ domain in the complex is a PDZl and/or a PDZ2 domain comprised in a PSD-95 protein.

The invention is further directed to the use of iV-alkylation of an amino -terminal residue of a peptide or peptide analogue to enhance its affinity for a PDZ domain. Accordingly JV-alkylation may be used to enhance the affinity of a compound according to the first or second embodiment for a PDZ domain of PSD-95. Preferably, the interaction is between NMDAR and PSD-95 and the NMDAR is comprised in a cell.

The invention is further directed to the use of a linker to dimerize a PDZ binding ligand or peptide in order to enhance its affinity and selectivity towards proteins containing tandem PDZ domains. Preferably the linker is a PEG linker comprising 1 to 28 PEG moieties, more preferably 1 to 12 PEG moieties, and the interaction is between NMDAR and PSD-95 and the NMDAR is comprised in a cell.

The invention further provides a pharmaceutical composition comprising a compound according to the first or second embodiment, for use as a medicament. The pharmaceutical composition may be used in the prophylaxis and/or treatment of an excitotoxic-related disease, preferably ischemic or traumatic injury of the CNS. The invention further provides a kit comprising the pharmaceutical composition of the invention, further comprising means for delivering the composition to a subject.

Brief description of the drawings

Figure 1. Scheme showing NMDA receptor activation during ischemia. During ischemia an excessive amount of glutamate is released. This release activates the membrane bound NMDA receptors leading to Ca^{2+} ions entering the cells. Due to the co-localization of NMDA receptors and nNOS mediated by PSD-95, this Ca^{2+} influx is coupled to the potential harmful production of NO.

Figure 2. A) K_1 values of truncated peptides for the interaction with PDZl and PDZ2. B) K_1 values of Ala-scan performed

with pentapeptide (7) towards PDZI and PDZ2. Error bars indicate SEM based on at least four individual measurements. Figure 3. Silver-stained SDS PAGE gel showing PDZ2 domain peptide bound to an immobilised polyhistidine NR2B WT peptide bait following incubation in the presence or absence of the following peptide analogue inhibitors: Tat-NR2B (3), EMeTAV (40), TAV (49), SDV (9), ESDV (8), IESDV (7) and then recovered from the pull-down assay.

Figure 4. K_1 values of iV-methylated tetrapeptides and their corresponding non- methylated tetrapeptides towards PDZI and PDZ2, where individually favourable substitutions have been combined. Error bars indicate SEM based on at least four individual measurements.

Figure 5. Peptides docked into the PDZI homology model. A) IESDV (7), B) EMeTAV (40) and C) TAV (49). Figure 6. //-terminal alkylation of peptide analogues according to Scheme \[a]. Reagents and conditions: (a) o-Nitrobenzenesulfonyl chloride, DIPEA. (b) PI13P, ROH, DIAD (c) NaSPh, DMF (d) TFA, TIPS, H_2O (90:5:5).

Figure 7. Chemical structure of the dimeric compounds 74 and 75. For compound 75 the cyclohexylethyl group is positioned on the nitrogen of Glu (E) in P"[3]. Figure 8. Inhibition curves of dimeric peptide ligands 74 and 75 to PDZI, PDZ2, PDZ3 and PDZI -2 domains measured by fluorescence polarization (FP). Figure 9. Inhibition curves of dimeric peptide ligands 74, 76-81 to PDZI, PDZ2, and PDZ 1-2 domains measured by FP. Figure 10. Structure of compounds 56 and 83.

Figure 11. Stability of the compounds 74 and 76-78 in human blood plasma is shown and compared with the stability of the corresponding monomeric ligand (7), the clinical candidate Tat-N2B (3), and the pegylated, monomeric ligand 91. Figure 12. Effect on NMDA-mediated excitotoxicity in cultured cortical neurons of compound 3, 56, 63, 73 and 74 at a concentration of 500 nM.

Detailed description of the invention

I. Definition of abbreviations and terms:

"A" or "a" as used herein, can mean one or more, depending on the context in which it is used.

Abu, 2-aminobutanoic acid; Aib, α-aminoisobutyric acid;

Amide bond is formed by a reaction between a carboxylic acid and an amine. Where the reaction is between two amino acid residues, the bond formed as a result of the reaction is known as a peptide linkage (peptide bond);

Amino acid, that is naturally occurring, is named herein using either its 1 -letter or 3- letter code according to the recommendations from IUPAC, see for example http://www. chem.qmw.ac.uk/iupac. If nothing else is specified an amino acid may be of D or L- form. In the description (but not in the sequence listing) a 3-letter code starting with a capital letter indicates an amino acid of L-form, whereas a 3-letter code in small letters indicates an amino acid of D-form; cGMP, guanosine 3 ',5 '-monophosphate;

"comprising" should be understood in an inclusive manner. Hence, by way of example, a composition comprising compound X, may comprise compound X and optionally additional compounds;

CNS, central nervous sytem; CPP, cell penetrating peptide;

DAPI, 4', 6-diamidino-2-phenylindole; DIPEA, diisopropylethylamine;

DMF, Λ/,Λ/-Dimethylformamide;

FP, fluorescence polarization;

GFP, green fluorescent protein HATU, O-(7-azabenzotriazol-l-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate;

HBTU, O-(benzotriazol- 1 -yl)-Λ/,Λ/,Λf',Λf'-tetramethyluronium hexafluorophosphate

ITC, Isothermal Titration Calorimetry;

"mammalian cell" is intended to indicate any cell of mammalian origin. The cell may be an established cell line, many of which are available from The American

Type Culture Collection (ATCC, Virginia, USA) or a primary cell with a limited life span derived from a mammalian tissue, including tissues derived from a transgenic animal, or a newly established immortal cell line derived from a mammalian tissue including transgenic tissues, or a hybrid cell or cell line derived by fusing different cell types of mammalian origin e.g. hybridoma cell lines. The cells may optionally express one or more non-native gene products, e.g. receptors;

MCAO, middle cerebral artery occlusion; nNOS, neuronal nitric oxide synthase;

NO, nitric oxide; NMDA, Λ/-methyl-D-aspartate;

NMDAR, NMDA receptor;

NMDAR/PSD-95 inhibitor, is a compound comprising a peptide or peptide analogue capable of binding to, or interacting with, PDZ domains and thereby inhibiting the PSD-95/NMDAR interaction, where the peptide or peptide analogue may be modified by JV-alkylation (modified peptide inhibitor/ modified peptide analogue inhibitor) at position $P^{"}$. Alternatively, the inhibitor is a compound comprising two peptide or peptide analogues that are covalently linked by means of a linker;

P^0, Defined as the first amino acid residue or analogue corresponding to the C- terminal amino acid of the peptide/peptide analogue; $P^{"1}$, Defined as the second amino acid residue or analogue thereof counting from the

C-terminal amino acid of the peptide/peptide analogue;

$P^{"2}$, Defined as the third amino acid residue or analogue thereof counting from the C- terminal amino acid of the peptide/peptide analogue; $P^{"3}$, Defined as the fourth amino acid residue or analogue thereof counting from the

C-terminal amino acid of the peptide/peptide analogue.

PDZ, Postsynaptic density protein-95 (PSD-95), Drosophila homologue discs large tumor suppressor (DIgA), Zonula occludens-1 protein (zo-1);

PEG, polyethylene glycol; PEG is a polymer of ethylene glycol, where for example 12 PEG moieties, or PEG12, corresponds to a polymer of 12 moieties (n=12) of ethylene glycol.

PPIs, protein-protein interactions;

PSD-95, postsynaptic density protein-95; rLUC, cytosolic Renilla Luciferase; SEM, standard error of mean;

TIPS, 5% triisopropylsilane;

TMF, trifluoracetic acid;

WT, wild-type.

II. Chemical structure and properties of a modified peptide inhibitor (NMDAR/PSD-95 inhibitor) of the invention according to a first embodiment

The NR2B undecapeptide 1 (YEKLSSIESDV) has been used as a template for development of smaller non-peptide inhibitor molecules with the potential of uncoupling the PPI between PSD-95 and the NMDA receptor. By a number of modifications used for converting peptides into simplified non-peptide structures, a series of small peptide derivatives have been identified that demonstrate several fold improved affinities towards the PDZ domains of PSD-95, thereby providing drug- like inhibitors of the PSD-95/NMDA receptor interaction. Specifically, a peptidomimetic approach was followed, starting with truncation of 1 from its JV-terminus to a pentapeptide, IESDV (7), without loss of affinity towards PDZl and PDZ2 of PSD-95, whereas further deletion reduced affinity. The decrease in affinity could be compensated by substituting Glu and Ser of the tetrapeptide ESDV with //-Me-Glu and Thr, respectively, thereby resulting in //-methylated tetrapeptide, E_{Me}TDV (36), with improved affinity to PDZl and essentially WT affinity at PDZ2. The Asp residue could be replaced with the non-charged Gln or the hydrophobic Ala without affecting affinity significantly, providing E_{Me}TAV (40) as a promising lead. Interestingly, a tripeptide TAV (49) is still a reasonably potent inhibitor of PDZl and PDZ2 with K_1 values of 42 and 37 μM, respectively, thus only ca. 2- and 9-fold less potent than the NRB2B peptide (1), while still showing selectivity within the PDZ domains of PSD-95.

Guided by molecular modeling and docking of peptides and modified peptides into a homology model of PDZl, modification of the //-terminal methyl group of EMeTAV was pursued. Applying the Fukuyama protocol of the Mitsunobu reaction allowed the preparation of 11 //-alkylated tetrapeptides, which all had superior affinity at PDZl and PDZ2 compared to the lead peptide E_MeTAV. In particular, replacing the methyl group with either cyclohexylmethyl (55) or cyclohexylethyl (56) provided potent inhibitors of the PSD-95/NMDA receptor interaction, the most potent being (56) with K_1 values of 1 and 0.5 μM at PDZl and PDZ2, respectively (structures in figure 10). In addition, introduction of aromatic substituents provided almost equipotent compounds, as exemplified by compound 62 and 63. Although, PDZ3 binding was also increased by the //-terminal alkylations a considerable selectivity towards PDZl and 2 was still observed.

The present invention teaches that //-alkylation in position $P^{"3}$ can be used to increase the affinity of a peptide or peptide analogue for one or more target PDZ domain, thereby enhancing its ability to prevent PPI interactions occurring with said target.

Where the PPI interaction is between NMDAR/PSD-95 inhibitor, the peptide or peptide analogue preferably comprises at least 4 amide linked residues and the sequence YTXV or YSXV, wherein Y is selected from among E, Q, and A, or an

analogue thereof, and X is selected from among A, Q, D, N, N-MQ-A, N-MQ-Q, N-

Me-D, and //-Me-N or an analogue thereof. Suitable analogues of residue Y or X, or analogues of any of the 4 amide-linked residues (YTXV or YSXV), include: inverso- amides and/or thioamides (Chemistry & Biochemistry of amino acids, peptides, and proteins", vol 7, 1983, Boris Weinstein, Ch.5 by Arno F. Spatola); the aza-@-unit (5- dihydro-2(3H)-pyrazone moiety), particularly position $P"^1$ or $P"^3$ corresponding to residue X or Y (Hammond et al, Chemistry and Biology, 2006, p.1247); where the choice of analogue may be assisted by use of the tools and assays for a peptidomimetic approach as described herein.

III Optimizing modified peptide inhibitors (NMDAR/PSD-95 inhibitor) according to a first and second embodiment with regard to membrane permeability, selectivity and pharmacokinetic properties.

The probability that the modified peptide inhibitors penetrate cellular membranes would be increased by decreasing their polarity and charge. Surprisingly, modifications in the peptide sequence of //-alkylated YTXV (Y = Glu, Ala or Gln and X = Asp and Ala) to decrease polarity and/or charge do not significantly impair their affinity to PDZl and PDZ2 of PSD-95. The tetrapeptides were N-alkylated with cyclohexylethyl and compared with their non-alkylated versions with regards to affinity and selectivity for PSD-95 's PDZ domains. Non-charged and even small and hydrophobic amino acids could be positioned in these variable regions, individually or in combination, without significant loss in affinity (compounds 70-73). It was also noticed that the effect of N-alkylation was as significant as observed previously, thereby underlining the reproducibility and generality of N-alkylation as a modification that increases the affinity of the peptide inhibitors to PDZl and PDZ2 of PSD-95. The present invention thus provides N-alkylated peptide ligands whose size may be reduced to a tetrapeptide, and whose affinity for PDZl and PDZ2 is far greater than that of either the WT peptide sequence or Tat-NR2B (3). The N- alkylated peptide ligands of the invention, when compared to known peptide ligands, also have a reduced risk of immunogenicity and lower production cost, due to their smaller size. Furthermore, N-terminal alkylation of a peptide ligand of the invention (e.g. at $P"^3$), in contrast to other peptide derivatization techniques (such as the "natural occurring" acetylation, glycosylation or phosphorylation) serves to enhance its hydrophobicity and thereby increase its membrane permeability. Thereby the compounds are able to inhibit the PSD-95/NMDA receptor interaction intracellularly, thus reducing NMDA-mediated excitotoxicity in cultured cortical rat neurons.

According to the second embodiment, the invention further provides modified peptide inhibitors comprising two peptide (or peptide analogue) inhibitors linked together to form a dimeric ligand (as exemplified by 74 and 75). The two peptides or peptide analogues may have the same structure and composition or may have a different structure and composition. The peptide or peptide analogues in the dimeric ligand, in a further embodiment, may be additionally N-alkylated at $P"^3$ in the same chemical forms as described for a peptide of peptide analogue of the first embodiment. By binding to PDZl and PDZ2 of PDZ1-2 simultaneously, the affinity of the dimeric ligand to the tandem PSD-95 PDZ 1-2 construct is dramatically increased, while remaining constant with respect to the single PDZ domains PDZl, 2 and 3. The peptide inhibitors are linked together by means of a linker. Suitable linkers include a linker composed of polyethylene glycol (PEG) diacid; polyamine (Herve F et al, 2008, AAPS J, E-publ. August 26); peptide nucleic acid (PNA) (Egholm et al., 2005 Nature 365:566-568); locked nucleic acid (LNA) (Singh et al., 1998, Chem Comm, p.455). When the linker is a PEG linker it may also comprise an active functional group, such as an electrophilic or nucleophilic functional group (WO/2007/140282), which can be used to attach the PEG linker to each peptide (or peptide analogue) inhibitor. Suitable functional groups for attachment include amino- reactive electrophilic groups, selected from among N-hydroxysuccinimide (NHS) ester, p-nitrophenyl ester, succinimidyl carbonate, p-nitrophenyl carbonate, succinimidyl urethane, isocyanate, isothiocyanate, acyl azide, sulfonyl chloride, aldehyde, carbonate, imidioester or anhydride; and thio-reactive groups selected from among maleimide, haloacetyl, alkyl halide derivatives, aziridine, acryloyl derivatives arylating agents or thio-disulfide exchange reagents. Suitable nucleophilic functional groups include amine, hydrazide, carbazate, acyl hydrazide, semicarbamate or hydrazine, which can undergo reactions with aldehyde or carboxyl groups on the peptide or peptide analogue inhibitor.

The optimal length of linker in the dimeric ligand will depend on the selected linker. When the linker is PEG, the number of ethylene glycol moieties (n) of PEG may lie between n=l-28 or n=4-28, or the linker may have a length of n= 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12. According to the present invention, PEG-diacids are used to link ligands (peptides or peptide analogues), where the e.g. PEG12-linker is modified so that two carboxylic acid groups are present at each end of the linker. Thus, a PEG 12- linker prior to the dimerization process is named 4,7, 10, 13 , 16, 19,22,25 ,28,31 ,34,37,40-tridecaoxatritetracontane- 1 ,43-dioic acid.

During dimerization of the two peptide ligands with the linker, the two carboxylic acid groups react with the TV-terminal amino groups of the ligands to create amide bonds. The PEGO, 1,2,4,6, 8 linkers are in accordance with this description.

A PEG linker having 12 moieties (n=12) will, when stretched out, have a length of about 50 A, which will easily allow the ligands at each end of the linker to be in sufficiently close proximity with the tandem PDZ domain and thereby act together as a potent inhibitor. The distance between PDZl and PDZ2 in PSD-95 has been modeled and estimated to be around 20 A, (measured from the two conserved histidines on αB) (Long et al, JMB, 2003, p.203-214). Accordingly, the PEG linker in the dimeric ligand preferably comprises 1-12 moieties (n=l-12), more preferably 2-12 (n=2-4) and even more preferably 4-6 (n=4-

6) moieties. The dimeric ligands of the invention possess several advantages: Their affinity to the tandem PDZ 1-2 construct is increased significantly - up to 1000-fold compared to WT or Tat-NR2B (3). The affinity of these ligands to the tandem PDZ 1-2 protein, which shares the greatest structural similarity to the native PSD-95 protein target for these modified peptide inhibitors, is of key importance with respect to their efficacy during therapeutic use. Also, the PDZ 1-2 tandem corresponds to a much more distinctive protein target as compared to single PDZ domains, which are rather promiscuous in nature. Therefore, by providing dimerized ligand peptide inhibitors that specifically target the PDZ 1-2, an improvement in selectivity is obtained compared to monomeric ligands, which is potentially important for the therapeutic use of these inhibitors. In addition PEG modification of peptides offers several pharmacokinetic advantages such as decreasing immunogenicity, delaying clearance from the blood and reducing sensitivity towards proteases. Furthermore, PEG is non-toxic, highly soluble and known to facilitate cellular uptake, and the dimeric ligands are also shown to inhibit the PSD-95/NMDA receptor interaction intracellularly, hence reducing NMDA-mediated excitotoxicity in cultured cortical rat neurons. Thus besides functioning as a linker, PEG enhances the therapeutic properties of these modified peptide inhibitors.

The membrane permeability and blood-brain barrier permeability of the peptide or peptide analogues of the present invention may be further enhanced by cationization with a natural or synthetic polyamine or diamine (e.g. hexamethylenediamine; putresine; spermidine; spermine), which may be covalently linked to the peptide or peptide analogue (Herve F et al, 2008, AAPS J, p.455-472). Preferably the peptide or peptide analogues includes the cationization at its TV-terminus.

Linking PEG12-diacid (4,7,10,13,16,19,22,25,28,31,34,37,40- tridecaoxatritetracontane-l,43-dioic acid), PEG8-diacid (4,7,10,13,16,19,22,25,28- nonaoxahentriacontane-l,31-dioic acid), PEG6-diacid (4,7,10,13,16,19,22- heptaoxapentacosane-l,25-dioic acid), and PEG4-diacid (4,7,10,13,16- pentaoxanonadecane-l,19-dioic acid) to two pentapeptide molecules with the sequence IESDV, result in the dimeric ligands 74, 76, 77, and 78 respectively, which demonstrate one or more of the properties of high affinity binding to the PDZ12 protein target, complete resistance to proteolytic enzymes in blood plasma, membrane permeability and activity in a cell-based excitotoxicity ex vivo assay. Affinity is increased even further by dimerization of the pentapeptide having the sequence IETAV, to get compound 83, while plasma stability is still pronounced - especially compared to the monomeric peptide ligand. IV Tools for monitoring and evaluating the inhibitor properties of the NMDAR/PSD-95 inhibitor of the invention

A. Fluorescence Polarization (FP) assay: as described below in Example 1 and in the Methodology section, provides a convenient and reliable way to monitor and evaluate the inhibitor properties of an NMDAR/PSD-95 inhibitor of the invention. The FP assay allows a wide range of peptide analogues to be tested and compared with respect to their interaction with PDZ domains, and their specificity with respect to the three PDZ domains, PDZ 1-3, of PSD-95. PDZ domains may be expressed individually, or as tandem domain constructs, using standard recombinant DNA technology known to those skilled in the art. Purification of the expressed PDZ domains may be facilitated by the inclusion of an affinity tag (e.g. poly-histidine-tag, Glutathione-S-transferase-tag, or antibody-tag such as FLAG-tag) in the expressed protein comprising the PDZ domain (e.g. fusion protein), and the use of an affinity resin to selective purify tagged PDZ domain proteins. More specifically, the assay is based on a heterologous competition binding assay, where the affinity measured as IC50 of a given (non- fluorescent) peptide analogue for a PDZ domain is measured in the presence of a fixed concentration of a fluorescent labelled undecapeptide peptide corresponding to the wild-type (WT) C-terminal of the NR2B subunit (YEKLSSIESDV) and CRIPT (LDTKNYKQT SV). Determined IC50 values may be converted to K_Y values.[5] Suitable fluorophores include either 5- FAM or Cy5, which may be coupled to a tripeptide (KSG or CSG) linker attached to the JV-terminus of the undecapeptide, to give 5-FAM-NR2B, Cy5-NR2B, 5-FAM- CRIPT and Cy5-CRIPT. The 5-FAM fiuorophore may be attached to the undecapeptides by coupling with HATU. The Cy5 fiuorophore may be conjugated to the undecapeptides by coupling Cy5-maleimide to the cysteine side chain of the tripeptide sequence CSG attached to the N-terminus of the peptide, as detailed in the Methodology section.

B. Pull-down assay: The polyhistidine pull-down is an in vitro technique that consists of a polyhistidine-tagged bait NRB2 or CRIPT protein C-terminal domain

(e.g NR2B WT peptide with the sequence HHHHHHYEKLS SIESDV) that can be used to identify competitors of a PDZ domain-binding partner (PDZ domain of PSD- 95) (the prey). The bait protein is immobilized on cobalt chelated affinity gel. The bait serves as the secondary affinity support for the protein partner to the bait and for identifying competitors that can displace the prey from the bait. The competitors are peptide analogues, specifically an NMDAR/PSD-95 inhibitor of the present invention. PPIs, between the prey and bait are analyzed by chemically dissociating/eluting the bound prey (PDZ domain or peptide analogue) and visualizing the prey by SDS-PAGE and associated detection methods depending on the sensitivity requirements of the interacting proteins. These methods include Coomassie® Dye, silver, and zinc staining; western blotting; and [^{35}S] radioisotopic detection. Alternatively, the displaced prey detected on the gel, can be isolated from a polyacrylamide gel, and further analyzed. Suitable pull-down assay protocols are well known in the art (Sambrook, J. and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York. 3rd edition), while ProFoundiM Pull-Down PolyHis Protein:Protein Interaction Kit from Pierce Biotechnology, 3747 N. Meridian Road, P.O. Box 117 Rockford, IL 61105 contains a complete, validated set of reagents specifically developed for performing pull-down assays.

C. Isothermal Titration Calorimetry (ITC): This technique (Pierce, M. et al, 1999., Methods, p. 213-221) provides an exact determination of the affinity of a ligand for its target protein, as well as measuring the thermodynamic properties of the interaction, including enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) together with the stoichiometric relationship of ligand binding. In this method, un-labeled ligand is titrated into a solution of un-labeled PDZ protein located in the calorimeter. Heat generated upon protein recognition is measured (ΔH) during the titration of the ligand and the PDZ protein until saturation is achieved, and the heat-exchange upon further ligand addition is negligible. Based on the amount of ligand needed to achieve saturation an affinity constant (K_d) is calculated and in addition ΔH, ΔS, ΔG and stoichiometry are determined. D. Blood Plasma Stability Assay. In this assay, the half- life (Tm) of a compound in blood plasma is measured in vitro, whereby its susceptibility to degradation by proteases, peptidases and general plasma contents is quantified.

E. Neuronal NMDA Toxicity Assay. When cultured cortical neurons are exposed to high concentrations of NMDA, an excitoxic cascade is initiated, which leads to cellular damage and ultimately neuronal cell death. Consequently, the cell membranes are destroyed, and cytoplasmic enzymes are released into the cell surroundings. The extent of this neuronal cell death can be quantified by measuring the release of cytoplasmic lactate dehydrogenase (LDH) enzyme into the cell medium. The compounds of interest and control compound (3) are investigated with respect to their abilities to attenuate NMDA-mediated toxicity. Compounds that are active in this assay are both membrane permeable, and can prevent ex vivo excitotoxicity, which are important properties for therapeutics towards excitotoxicity mediated diseases.

F. Bioluminescence Resonance Energy Transfer2 (BRET): This assay can be used to assess the cell permeability of an NMDAR/PSD-95 inhibitor, which is important for its bioavailability required for the effective therapeutic treatment of a mammalian subject. A mammalian cell line can be transfected with pairs of DNA constructs encoding two fusion proteins, comprising a GFP-fusion protein (e.g. GFP- NR2B) and a rLUC-fusion protein (e.g. rLUC-PDZ2). Interaction between the two fusion proteins, when expressed in a transfected cell, can be monitored by virtue of the BRET between the two fusion proteins on binding to each other. The ability of an NMDAR/PSD-95 inhibitor, when added to extracellularly, to cross the cell membrane and inhibit the interaction between the two fusion proteins can be monitored with the BRET assay, providing a valuable measure of the therapeutic potential of the NMDAR/PSD-95 inhibitor of the invention. V Methods for synthesising the NMDAR/PSD-95 inhibitor of the invention and for verifying its composition and structure

A. Synthesis of peptide analogues: Fmoc-based solid-phase peptide synthesis (SPPS) provides a suitable procedure for the synthesis of the peptide analogues, whereby an NMDAR/PSD-95 inhibitor of the invention may be prepared. Peptides with a natural C-terminal amino acid residue, such as Val or Ala, may be synthesized starting with pre-loaded Wang resins. In the case of peptides having an unnatural C- terminal amino acid residue, then a 2-chloro-trityl resin may be used, where the residue may be loaded on the resin using diisopropylethylamine (DIPEA) (resin/amino acid/DIPEA in 1 :4:8) in DCM for 30 min., then capped with methanol, prior to Fmoc deprotection and coupling of the consecutive amino acid residue. TV-methylated amino acids and the amino acid following the TV-methylated amino acid may be coupled to the growing peptide using HATU. A detailed description of a suitable Fmoc-based SPPS protocol is given below in the Methodology section.

B. TV-terminal alkylation of peptide analogues: The Mitsunobu reaction using the Fukuyama protocol[6] provides a suitable procedure for the preparation of TV-alkylated peptides.[7] The terminal amino group of the peptide is activated as a nitrobenzyl sulfonamide, and subsequently reacted with a range of alcohols mediated by diisopropyl azadicarboxylate (DIAD) and PI13P to give the protected, resin-bound products (Figure 6/Scheme 1). The final TV-alkylated products, obtained by deprotection of the sulfonamide and cleavage from the resin, have the required purity and yield.

C. Synthesis of Dimeric ligands: PEG-diacids are activated as pentafluorophenyl (Pφ)-esters and reacted with the TV-terminal amino group of the resin-bound peptide ligand by using 1-hydroxybenzotriazole (HOBt) as a catalyst. Thereby, a dimeric ligand composed of two peptide ligands linked together by the PEG-linker through amide bonds is generated.[8] Alternatively, the dimeric ligands can be produced by activating the PEG-diacids in situ with coupling reagents such as HBTU and HATU, followed by incubation with the TV-terminal amino group of the resin-bound peptide ligand. Using this procedure, the dimerization procedure is limited to a one-step reaction and can be performed in one day instead of six.

D. Chemical analysis of peptide analogues: The peptide may be analyzed by ESI- LC/MS, and further characterised by proton (^{1}H) NMR spectra and high resolution mass spectrometry, employing techniques well-known to the skilled man, and exemplified in the Methodology section.

VI. NMDAR/PSD-95 inhibitors according to the first or second embodiment of the invention for therapeutic treatment of excitotoxic-related disorders ischemic or traumatic injury of the central nervous system

In neuronal synapses, the C-termini of NMDA receptor subunits interact with PDZ domains of PSD-95 linking them to downstream neurotoxic signaling molecules (e.g nNOS) leading to NO production and excitotoxicity. The present invention provides NMDAR/PSD-95 inhibitors that can inhibit the interaction between NMDA receptors and nNOS in a cell, without impairing the NMDAR ionic currents and calcium signalling functions of the NMDAR. Thus an NMDAR/PSD-95 inhibitor of the invention acts as a neuroprotectant of one or more cells or tissues providing a specific strategy for treating excitotoxic disorders, including spinal cord injury, stroke, traumatic brain injury, ischemic or traumatic injury of the central nervous

system (CNS), epilepsy, and neurodegenerative diseases of the CNS.

Therapeutic treatment of subjects at risk or presently suffering from the above disorders and diseases may be given either prophylactic treatment to reduce the risk of the disorder or disease onset or therapeutic treatment following the disorder or disease onset. The subject may be a mammalian or human patient.

VII. Manufacture of a pharmaceutical composition comprising NMDAR/PSD- 95 inhibitor according to the first or second embodiment of the invention Formulations of an NMDAR/PSD-95 inhibitor according to the first or second embodiment of the present invention into pharmaceutical compositions is well known in the art, and is further described in Gennaro (ed.), 2000, Remington: The Science and Practice of Pharmacy, 20th ed., Lippincott, Williams & Wilkins (2000); and Ansel et al., 1999, Pharmaceutical Dosage Forms and Drug Delivery Systems, 7th ed., Lippincott Williams & Wilkins Publishers.

Such a composition typically contains from about 0.1 to 90% by weight (such as about 1 to 20% or about 1 to 10%) of an NMDAR/PSD-95 inhibitor of the invention in a pharmaceutically accepted carrier.

Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Compositions suitable for oral administration can be formulated by combining an NMDAR/PSD-95 inhibitor of the invention with a suitable carrier as a tablet, pill, dragee, capsule, liquid, gel, syrup, slurry, suspension for oral ingestions by the subject to be treated. For solid oral/rectal formulations, suitable excipients include fillers such as sugars (e.g. lactose, sucrose, mannitol and sorbitol); cellulose preparations (e.g. maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidine; granulating agents; and binding agents. Optionally, disintegrating agents may be included, such as cross- linked polyvinylpyrrolidine, agar, or alginic acid or a salt of sodium alginate. The solid formulation may further include an enteric-coating.

For liquid oral formulations, suitable excipients or diluents include water, glycols, oils and alcohols.

Injectable formulations of the compositions can contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injections, water-soluble versions of the compounds can be administered by the drip method, whereby a pharmaceutical formulation containing the active agent (NMDAR/PSD-95 inhibitor) and a physiologically acceptable excipient is infused. Physiologically acceptable excipients can include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, e.g., a sterile formulation of a suitable soluble salt form of the compounds, can be dissolved and administered in a pharmaceutical excipient such as Water- for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound can be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long chain fatty acid (e.g., ethyl oleate).

An NMDAR/PSD-95 inhibitor of the invention may also be formulated as a long acting depot preparation. For example, the inhibitor may be formulated with suitable polymeric or hydrophobic materials (e.g. an emulsion of an acceptable oil) or ion exchange resin, or as a sparingly soluble derivative, such as a sparingly soluble salt.

Liposomes and emulsions may also be used to deliver the NMDAR/PSD-95 inhibitor. Additionally, the NMDAR/PSD-95 inhibitor may be delivered via a sustained release system, such as semi-permeable matrices of solid polymers comprising the inhibitor.

The optimal percentage of the therapeutic agent in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect desired in the specific pathologies and correlated therapeutic regimens.

VIII. Mode of administration of a pharmaceutical composition comprising an

NMDAR/PSD-95 inhibitor according to the first or second embodiment of the invention

Conventional methods, known to those of ordinary skill in the art of medicine, can be used to administer compositions to the subject or patient, and may be supplied for use in the form of a kit. These include but are not limited to subcutaneous, intrapulmonary, transmucosal, intraperitoneal, intrauterine, sublingual, intrathecal, or intramuscular routes, by using standard methods/means for delivery [including by injection, catheter , where the kit may include an injection devise, a devise for delivering an injectable depot, or a catheter]. In addition, the pharmaceutical formulations can be administered to the patient via injectable depot routes of administration such as by using 1-, 3-, or 6-month depot injectable or biodegradable materials and methods.

Regardless of the route of administration, an NMDAR/PSD-95 inhibitor of the present invention is typically administered at a daily dosage of about 0.01 mg to about 30 mg/kg of body weight of the patient (e.g., 1 mg/kg to 5 mg/kg). The pharmaceutical formulation can be administered in multiple doses per day, if desired, to achieve the total desired daily dose.

Conventional methods, known to those of ordinary skill in the art of medicine, can be used to administer the pharmaceutical formulation(s) of the present invention to the patient. The pharmaceutical compositions of the present invention can be administered alone, or in combination with other therapeutic agents or interventions. Specifically, the compositions of the present invention may further comprise a plurality of agents of the present invention.

Examples

Methodology

Chemical analysis: Proton (^{1}H) NMR spectra were recorded on Bruker spectrometers: Avance 300 NMR (300 MHz). Chemical shifts (δ) are reported in parts per million (ppm) with reference to tetramethylsilane (TMS) as internal standard. NMR experiments were carried out in CD_3OD. The following abbreviations are used for the proton spectra multiplicities: s, singlet; d, doublet; dd, double doublet, triplet; q, quartet; m, multiplet. Coupling constants (J) are reported in Hertz (Hz). Mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray (ESI-MS) coupled to an Agilent 1200 HPLC system with autosampler and diode-array detector using a linear gradient of the binary solvent system of water/acetonitrile/TFA (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 mL/min. During ESI-MS analysis evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector, which were used for estimation of the purity of the final products. High resolution mass spectra (HRMS) were obtained using a Q-Tof™ 2 instrument and were all within ± 5 ppm of theoretical values. Preparative HPLC was performed on a Agilent 1100 system using a Cl 8 reverse phase column (Zorbax 300 SB-C 18, 21.2 x 250 mm) with a linear gradient of the binary solvent system of water/acetonitrile/TFA (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 20 mL/min and UV detection at 230 nm.

Expression and Purification of PDZl, PDZ2, PDZ3 and PDZ1-2 of PSD-95:

The cDNA coding for PSD-95 PDZl (residues 61-151), PDZ2 (residues 155-249), PDZ3 (residues 309-401) and PDZ1-2 (residues 61-249) tandem were amplified by inverted PCR and cloned in modified His-tagged pRSET vector (Invitrogen, Carlsbad, CA, USA) (numbers in parenthesis refer to the residue numbers in the human full-length PSD95CC without exon 4b). All PDZ constructs contained an extra sequence, MHHHHHPRGS, which was used as a tag for purification (His-tag), and the DNA coding sequences and encoded proteins are designated as follows:

HIS-PDZl DNA [SEQ ID NO: 1] encoding HIS-PDZl protein [SEQ ID NO: 2] HIS-PDZ2 DNA [SEQ ID NO: 3] encoding HIS-PDZ2 protein [SEQ ID NO: 4] HIS-PDZ3 DNA [SEQ ID NO: 5] encoding HIS-PDZ3 protein [SEQ ID NO: 6] HIS-PDZ 1-2 DNA [SEQ ID NO: 7] encoding HIS-PDZ 1-2 protein [SEQ ID NO: 8] Competent E. coli bacteria (BL21 - DE3, pLysS) were transformed with PDZ expressing constructs and grown overnight on agar plates containing ampicillin (lOOμg/mL) and chloramphenicol (35 μg/mL) at 37°C. Colonies were picked and used to inoculate bacterial cultures (LB medium with 50 μg/mL ampicillin). These were stirred while being incubated at 37°C until Aβoo reached 0.95 for PDZl, PDZ2, and PDZ3 or 0.45 for PDZ 1-2, at which point 1 mM isopropyl β-D-1- thiogalactopyranoside was added. Induced cultures were incubated for 4 hours at 37°C (PDZl, 2, 3) or over night at 30^0C (PDZ 1-2). Cells were harvested by spinning at 10 00Og for 10 min at 4°C and re-suspension in lysis buffer (50 mM Tris/HCL pH 7.5, ImM PMSF, 25 μg/ml DNAse, 40 mM Mg_2SO_4). The cells were destroyed using a cell disruptor apparatus at 26 KPsi. The cell lysate was spun down at 35 00Og for 1 hour and the supernatant filtered with a 0.45 μm and a 0.22 μm filter. Purification was performed by first a nickel (II)-charged column (HisTrap™ HP, GE Healthcare, UK) equilibrated with Tris-buffer (Tris/HCl buffer 5OmM, pH 7.5) followed by anion-exchange chromatography for PDZl and 3 and gel- filtration for PDZ2 and 1-2. For anion-exchange chromatography, a MonoQ HR 5/5 column (GE Healthcare, UK) equilibrated with 50 mM Tris/HCL, pH 8.5 was used and elution was done with a gradient of 0-500 mM NaCl. For gel- filtration the PDZ sample was loaded on a Superdex™ 75 HR 10/30 column (GE Healthcare, UK) equilibrated with Tris buffer (20 mM Tris/HCL, pH 7.5) with a constant flow rate at 0.5 mL/min. The relevant fractions were analyzed on a SDS-PAGE gel stained by a standard silver staining protocol. The final purification was analyzed by electrospray ionization liquid chromatography-mass spectrometry (ESI-LC/MS) to get the exact molecular weight and thereby verify the identity of the PDZ domain. Molar extinction coefficients were found by amino acids analysis (Alphalyse, Odense, Denmark) and thereafter used for measuring protein concentrations. For Pull-down experiments, PDZ2 without His-tag was produced, to act a "prey". For this a slightly different construct was used that allowed the His-tag to be enzymatically cleaved off by bovine Thrombin (1 unit per 100 μg protein; Incubation over night at room temperature while rotating), followed by purification using "reverse purification" on the HisTrap™ HP column under the same condition as previously described.

Peptide Synthesis: Peptides were manually synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) using a MiniBlock™ (Mettler-Toledo, Columbus, OH, USA). Peptides with C-terminal Val or Ala were synthesized from pre-loaded Wang resins (Novabiochem, Darmstadt, Germany). For peptides with an unnatural amino acid in the C-terminal a 2-chloro-trityl resin was used and the first amino acid was loaded on the resin using diisopropylethylamine (DIPEA) (resin/amino acid/DIPEA in 1:4:8) in DCM for 30 min., followed by capping with methanol (DCM/MeOH/DIPEA 17:2:1). Fmoc deprotection was performed with 20% piperidine in DMF (2x10 min) and coupling of the consecutive amino acid was carried out with 0-(benzotriazol-l-yl)-Λ/,Λ/,Λf',Λf'-tetramethyluronium hexafiuorophosphate (HBTU) and DIPEA (resin/amino acid/HBTU/DIPEA 1 :4:4:4) and monitored by the ninhydrin test. The final peptide was cleaved from the resin by treatment

with 5% water and 5% triisopropylsilane (TIPS) in trifluoracetic acid (TFA) for 2 h. The crude peptide was purified by preparative HPLC to >98% purity. The peptide was analyzed by ESI-LC/MS and lyophilized. Quantification of the synthesized peptides was performed by weighing the peptide product. Key peptides (NR2B WT [1], 5-FAM-NR2B and Tat-NR2B [3]) were analyzed by amino acid analysis (Alphalyse, Odense, Denmark) and the molar extinction coefficients were thereby determined. The 5-FAM (Anaspec, San Jose, CA, USA) was attached to the peptides by coupling with 0-(7-azabenzotriazol-l-yl)-/V,/V,/V',/V'-tetramethyluronium hexafiuorophosphate (HATU). Similarly, /V-methylated amino acids and the amino acid following the /V-methylated amino acid were coupled to the growing peptide using HATU. Cy5 conjugated NR2B (Cy5-NR2B) was synthesized by coupling Cy5- maleimide (GE Healthcare, UK) to the cysteine side chain of the peptide sequence CSG-YEKLSSIESDV in solution followed by HPLC purification and ESI-LC/MS analysis. The reaction was performed in a 1 :1 ratio between peptide and Cy5- maleimide in IxTBS buffer for 2 hours at room temperature. Quantification of Cy 5- peptides was achieved by measuring absorbance using the molar extinction coefficient of Cy5. Two fluorescent PDZ3 binding peptides, based on the CRIPT sequence (KSG/CSG-LDTKNYKQTSV),[9] were synthesized with 5-FAM and Cy5, respectively, as described above for NR2B. //-terminal acetylated peptides were synthesized by treating the deprotected peptide with acetic anhydride in DIPEA and DMF (1 :2:3) for 1 hour followed by TFA cleavage, purification, and characterization as described above.

TV-terminal Alkylation of Peptides - General procedure: Peptide (0.25 mmol, 1 equiv) was synthesized on a 2-chloro-trityl resin as described above followed by Fmoc-deprotection, washing and drying of the resin. The resin was swelled in DIPEA (6 equiv) in THF (2.5 rnL) and 2-nitrobenzenesulfonyl chloride (4 equiv) in CH_2Cl_2 (1 mL) was added slowly while agitating the solution. After shaking at room temperature for 3 h the resin was drained and washed with THF, MeOH, DCM, and THF (flow washes for 2 min.). Subsequently, the resin was treated with triphenylphosphine (Ph_3P, 2 M in THF, 5 equiv) and the alcohol (ROH, 10 equiv) in dry THF (1.0 mL) under nitrogen. DIAD (1 M in THF, 5 equiv) was introduced slowly followed by agitation for 1 h at room temperature. The resin was then drained and washed with THF and DCM (flow washes). The resin-bound alkylated sulfonamide was swelled in 2 mL DMF and treated with NaSPh/DMF solution (I M, 2 mL) for 1 h. This was repeated four times to ascertain full deprotection, after which the resin was washed and the modified peptide cleaved from the resin, purified, and characterized as described previously.

7V-Methyl-ETAV (40). Yield: 80%. ESI-LC/MS: >99% (ELSD), 98% (UV). ^{1}H NMR (CD_3OD) δ (ppm): 4.47 (q, J= 7.2, IH), 4.44 (d, J= 4.8, IH), 4.30 (d, J= 5.6, IH), 4.18-4.14 (m, IH), 3.97 (dd, J= 7.2, 5.6 Hz, IH), 2.68 (s, 3H), 2.53 (t, J= 7.4, 2H), 2.19-2.14 (m, 3H), 1.39 (d, J = 7.2, 3H), 1.23 (d, J = 6.4, 3H), 0.97 (d, J= 6.6, 6H). HRMS (ES+) calcd for $Ci_8H_{33}N_4O_8$ [M + H]$^+$, 433.2298; found, m/z 433.2315.

7V-Ethyl-ETAV (52). Yield: 71%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}H NMR (CD_3OD) δ (ppm): 4.47 (q, J= 6.8, IH), 4.44 (d, J= 5.2, IH), 4.30 (d, J= 6.0, IH), 4.17-4.13 (m, IH), 4.03 (dd, J= 7.6, 5.2 Hz, IH), 3.06-3.03 (m, 2H), 2.53 (t, J = 7.4, 2H), 2.19-2.13 (m, 3H), 1.38 (d, J = 7.2, 3H), 1.32 (t, J = 7.4, 3H), 1.22 (d, J = 6.4, 3H), 0.97 (d, J = 6.8, 6H). HRMS (ESI+) calcd for $Ci_9H_{35}N_4O_8$ [M + Na]$^+$, 469.2274; found, m/z 469.2280.

7V-Propyl-ETAV (53). Yield: 95%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}U

NMR (CD_3OD) δ (ppm): 4.47 (q, J= 6.8, IH), 4.44 (d, J= 5.2, IH), 4.30 (d, J= 5.2, IH), 4.17-4.14 (m, IH), 4.02 (dd, J= 7.6, 5.2 Hz, IH), 2.98-2.88 (m, 2H), 2.54 (t, J =

7.4, 2H), 2.21-2.12 (m, 3H), 1.76-1.70 (m, 2H), 1.38 (d, J = 7.2, 3H), 1.22 (d, J = 6.4, 3H), 1.0 (t, J = 7.4, 3H), 0.97 (d, J = 6.8, 6H). HRMS (ESI+) calcd for $C_{20}H_{37}N_4O_8$ [M + H]$^+$, 461.2611; found, m/z 461.2624.

7V-Butyl-ETAV (54). Yield: 94%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 4.47 (q, J= 7.2, IH), 4.44 (d, J= 5.2, IH), 4.30 (d, J= 5.6, IH), 4.17-4.14 (m, IH), 4.02 (dd, J= 7.2, 5.2 Hz, IH), 3.00-2.93 (m, 2H), 2.54 (t, J = 7.4, 2H), 2.21-2.12 (m, 3H), 1.70-1.65 (m, 2H), 1.45-1.40 (m, 2H), 1.38 (d, J = 7.2, 3H), 1.22 (d, J = 6.4, 3H), 1.0 (t, J = 7.4, 3H), 0.97 (d, J = 6.8, 6H). HRMS (ESI+) calcd for $C_2IH_{39}N_4O_8$ [M + H]$^+$, 475.2768; found, m/z 475.2770.

TV-Cyclohexylmethyl-ETAV (55). Yield: 8%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 4.45 (q, J = 7.2, IH), 4.41 (d, J = 4.8, IH), 4.28 (d, J = 5.4, IH), 4.17-4.12 (m, IH), 3.94 (dd, J = 7.2, 5.6 Hz, IH), 2.82 (d, J = 6.6, IH), 2.76 (d, J = 7.2, IH), 2.56 (t, J = 7.4, 2H), 2.18-2.12 (m, 3H), 1.86-1.68 (m, 6H), 1.38 (d, J = 7.2, 3H), 1.32-1.25 and 1.08-1.01 (m, 5H), 1.22 (d, J = 6.0, 3H), 0.96 (d, J = 6.6, 6H). HRMS (ESI+) calcd for $C_{24}H_{43}N_4O_8$ [M + H]$^+$, 515.3081; found, m/z 515.3091.

TV-Cyclohexylethyl-ETAV (56). Yield: 56%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 4.45 (q, J = 7.2, IH), 4.42 (d, J = 4.8, IH), 4.29 (d, J= 5.4, IH), 4.16-4.12 (m, IH), 3.99 (dd, J= 7.2, 5.4 Hz, IH), 3.03-2.91 (m, 2H), 2.53 (t, J= 7.4, 2H), 2.22-2.06 (m, 3H), 1.76-1.66 and 1.32-1.25 (m, 10H), 1.58 (q, J = 7.2, IH), 1.38 (d, J = 7.2, 3H), 1.22 (d, J =

6.3, 3H), 0.97 (d, J= 6.9, 6H). HRMS (ESI+) calcd for $C_{25}H_{45}N_4O_8$ $[M + H]^+$, 529.3237; found, m/z 529.3214.

7V-Benzyl-ETAV (58). Yield: 66%. ESI-LC/MS: >99% (ELSD), 97% (UV). ^{1}H NMR (CD_3OD) δ (ppm): 7.49-7.41 (m, 5H), 4.48 (q, J = 7.2, IH), 4.44 (d, J = 4.8, IH), 4.28 (d, J = 5.4, IH), 4.16 (s, 2H), 4.12-4.01 (m, 2H), 2.54 (t, J = 6.9, 2H), 2.22-2.11 (m, 3H), 1.38 (d, J = 7.2, 3H), 1.24 (d, J = 6.3, 3H), 0.97 (d, J = 6.6, 6H). HRMS (ESI+) calcd for $C_{24}H_{37}N_4O_8$ $[M + H]^+$, 509.2611; found, m/z 509.2595 . 7V-Phenylethyl-ETAV (59). Yield: 11%. ESI-LC/MS: 98% (ELSD), 98% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 7.35-7.23 (m, 5H), 4.45 (q, J = 6.9, IH), 4.42 (d, J = 4.8, IH), 4.28 (d, J = 5.4, IH), 4.13-4.09 (m, IH), 4.05 (dd, J = 7.2, 5.1 Hz, IH), 3.23- 3.15 (m, 2H), 3.00 (t, J= 7.8, 2H), 2.54 (t, J= 7.2, 2H), 2.22-2.12 (m, 3H), 1.38 (d, J = 7.2, 3H), 1.20 (d, J = 6.3, 3H), 0.96 (d, J = 6.9, 6H). HRMS (ESI+) calcd for $C_{25}H_{39}N_4O_8$ $[M + H]^+$, 523.2768; found, m/z 523.2787.

7V-Phenylpropyl-ETAV (60). Yield: 30%. ESI-LC/MS: 98% (ELSD), 97% (UV). ^{1}H NMR (CD_3OD) δ (ppm): 7.48 (d, J = 8.1, IH), 7.46 (d, J= 1.2, IH), 7.20 (dd, J = 8.4, 2.1, IH), 4.45 (q, J= 6.9, IH), 4.41 (d, J= 5.1, IH), 4.28 (d, J= 5.4, IH), 4.15- 4.09 (m, IH), 4.05 (dd, J= 7.2, 5.4 Hz, IH), 3.27-3.13 (m, 2H), 2.99 (t, J= 7.8, 2H), 2.54 (t, J = 7.2, 2H), 2.22-2.09 (m, 3H), 1.38 (d, J = 7.5, 3H), 1.21 (d, J = 6.6, 3H), 0.96 (d, J = 6.6, 6H). HRMS (ESI+) calcd for $C_{26}H_4IN_4O_8$ $[M + H]^+$, 537.2924; found, m/z 537.2928 .

7V-(3,4-Dichlorophenyl)propyl-ETAV (61). Yield: 20%. ESI-LC/MS: 98% (ELSD), 97% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 126-1 Al (m, 2H), 7.09-7.04 (m, IH), 4.45 (q, J= 6.9, IH), 4.41 (d, J = 5.1, IH), 4.28 (d, J= 5.4, IH), 4.15-4.09 (m, IH), 4.05 (dd, J = 7.2, 5.4 Hz, IH), 3.27-3.13 (m, 2H), 2.99 (t, J = 7.8, 2H), 2.54 (t, J = 7.2, 2H), 2.22-2.09 (m, 3H), 1.38 (d, J = 7.5, 3H), 1.21 (d, J = 6.6, 3H), 0.96 (d, J= 6.6, 6H). HRMS (ESI+) calcd for $C_{25}H_{37}Cl_2N_4O_8$ $[M + H]^+$, 591.1988; found, m/z 591.1967.

7V-(3,4-Difluorophenyl)propyl-ETAV (62). Yield: 12%. ESI-LC/MS: 98% (ELSD), 98% (UV). ^{1}U NMR (CD_3OD) δ (ppm): 7.85-7.79 (m, 3H), 7.74-7.72 (m, IH), 7.47- 7.44 (m, 3H), 7.37 (dd, J= 8.7, 1.8, IH), 4.45 (q, J= 6.9, IH), 4.42 (d, J= 4.8, IH), 4.28 (d, J = 5.4, IH), 4.13-4.06 (m, 2H), 3.34-3.14 (m, 4H), 2.545 (t, J = 7.5, 2H), 2.21-2.15 (m, 3H), 1.38 (d, J = 7.2, 3H), 1.19 (d, J = 6.3, 3H), 0.96 (d, J = 6.9, 6H). HRMS (ESI+) calcd for $C_{25}H_{37}F_2N_4O_8$ $[M + H]^+$, 559.2579; found, m/z 559.2591. 7V-(Naphtalene-2-yl)ethyl-ETAV (63). Yield: 49%. ESI-LC/MS: >99% (ELSD), >99% (UV). ^{1}H NMR (CD_3OD) δ (ppm): 7.29-7.15 (m, 5H), 4.45 (q, J = 6.9, IH), 4.40 (d, J= 4.8, IH), 4.28 (d, J= 5.4, IH), 4.14-4.10 (m, IH), 3.99 (dd, J= 7.2, 5.1 Hz, IH), 2.96-2.91 (m, 2H), 2.70 (t, J= 7.2, 2H), 2.52 (t, J= 7.2, 2H), 2.19-2.08 (m, 3H), 2.06-1.98 (m, 2H), 1.38 (d, J = 7.2, 3H), 1.17 (d, J = 6.6, 3H), 0.96 (d, J= 6.9, 6H). HRMS (ESI+) calcd !Or$C_{29}H_4IN_4O_8$ $[M + H]^+$, 573.2898; found, m/z 573.2897.

Synthesis of Dimeric ligands. General procedure 1 ("Pfp-Method"):

HOOC-PEG(12)-COOH (PEG12 diacid, Iris Biotechnology, Germany) (0.691 g, 1 mmol) was dissolved in ethyl acetate (100 mL) at 0 ^{0}C by vigorously stirring for 25 min. Pentafluorophenol (pfp) (0.368 g, 2 mmol) and Λ^iV-dicyclohexylcarbodiimide (DCC) (0.413 g, 2 mmol) was added and the reaction mixture was stirred for 2 h at 0 ^{0}C. The mixture was filtered and the filtrate evaporated in vacuo. The resulting Pfp_2-PEG-diester was used without further purification. Peptides were synthesized as previously described The Pfp_2-PEG-diester (0.125 mmol) and HOBt (0.625 mmol) was added to the resin-bound peptide (0.25 mmol) in 5 portions each dissolved in dry DMF (4 mL) over five days The resin was drained and washed with DMF prior to coupling the next portion. The dimeric peptide was cleaved off the resin with TFA/H_2O/TIPS (90/5/5) at rt for 2 h. After removal of solvents in vacuo the dimeric peptide was purified by preparative HPLC and characterized by LC/MS.

Synthesis of Dimeric ligands. General procedure 2 ("HBTU-Method"):

PEG-diacid (0.1 eq.) is pre-activated with HBTU (0.2 eq) and DIPEA (0.4 eq) and added to the peptide-resin (1 eq, 0.25 mmol) in DMF (2 mL), and incubated for 45 min. This step is repeated five times, and the resin is washed in between with DMF. The dimeric peptide is cleaved off the resin by treatment with TFA/H_2O/TIPS (90/5/5) at room temperature for 2 h. After removal of solvents in vacuo, the dimeric peptide is purified by preparative HPLC and characterized by LC/MS.

$(IESDV)_2$PEG12 (74). Pentapeptide, IESDV, was synthesized as described above and 74 was generated by using the general protocol for dimerizations (using the "Pφ-method"). Yield: 40%. ESI-LC/MS: >99% (ELSD), 98% (UV). MS (ESI+)

calcd for $C_{76}HI_{33}NI_0O_{37}$ $[M + H]^+$, 1778.9; found, m/z 1779.

(GE [JV-Cyclohexylethyl] TDV)$_2$PEGH (75). Tetrapeptide ETDV was synthesized and //-alkylated with cyclohexylethyl as described in the general procedure of TV- terminal alkylation of peptides. Gly was then added to the sequence and dimerization was conducted by using the general protocol (using the "Pφ-method"). Yield: 18%. ESI-LC/MS: 99% (ELSD), 94% (UV). MS (ESI+) calcd for $C_{86}Hi_{49}Ni_0O_{37}$ $[M + H]^+$, 1915.2; found, m/z 1915.

(IESDV)$_2$PEG8 (76). Pentapeptide, IESDV, was synthesized as described above and

76 was generated by using the general protocol for dimerizations (using the "HBTU- method"). Yield: 23%. ESI-LC/MS: >99% (ELSD), 98% (UV). MS (ESI+) calcd for $C_{68}HiI_7Ni_0O_{33}$ $[M + H]^+$, 1601.7; found, m/z 1602.

(IESDV)$_2$PEG6 (77). Pentapeptide, IESDV, was synthesized as described above and

77 was generated by using the general protocol for dimerizations (using the "HBTU- method"). Yield: 23%. ESI-LC/MS: >99% (ELSD), 99% (UV). MS (ESI+) calcd for $C_{64}Hi_{09}Ni_0O_3I$ $[M + H]^+$, 1513.7; found, m/z 1514.

(IESDV)$_2$PEG4 (78). Pentapeptide, IESDV, was synthesized as described above and

78 was generated by using the general protocol for dimerizations (using the "HBTU- method"). Yield: 24%. ESI-LC/MS: 98% (ELSD), 98% (UV). MS (ESI+) calcd for $C_{60}Hi_0INi_0O_{29}$ $[M + H]^+$, 1425.7; found, m/z 1426.

(IETAV)$_2$PEG4 (83). Pentapeptide, IESDV, was synthesized as described above and 83 was generated by using the general protocol for dimerizations (using the "HBTU- method"). Yield: 35%. ESI-LC/MS: >99% (ELSD), 99% (UV). MS (ESI+) calcd for $C_{60}Hi_{04}Ni_0O_{25}$ $[M + H]^+$, 1365.6; found, m/z 1366. Fluorescence Polarization (FP) Assay: PDZ saturation binding assays were used to measure the binding affinity between the fluorescent peptides (Cy5-NR2B, 5 -FAM- NR2B, Cy5-CRIPT or 5-FAM-CRIPT). To a fixed concentration of Cy5-NR2B, 5- FAM-NR2B, Cy5 -CRIPT or 5-FAM-CRIPT (50 nM) increasing concentrations of PDZ was added to get a saturation binding curve. The assay was performed in a IxTBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4) including 1% BSA in black flat bottom 384-well plates (Corning Life Sciences, NY, USA). After incubation for 20- 30 mins at rt, the FP of the samples was measured on a Safire^2 plate-reader (Tecan, Mannedorf, Switzerland), but before reading the samples the g-factor was adjusted such that 50 nM probe without any PDZ present would give a FP value of 20 mP. Cy5-NR2B and Cy5-CRIP, as well as 5-FAM-NR2B and 5-FAM-CRIPT were measured at excitation/emission values of 635/670 nm and 470/525 nm, respectively. The FP values were fitted to the equation $Y = B_{max}*X / (Kd + X)$, where B_{max} is the maximal FP value, X is the PDZ concentration, and Y is variable FP values. As long as the concentration of labeled peptide is well below the true Kd during the assay, the K_d can be directly derived from this saturation curve as being equal to the PDZ concentration where the curve is half saturated (at these conditions EC50 = [total

PDZJhalf saturation = [free PDZJhalf saturation = ^d)-

To measure the affinities between non-fluorescent peptides and PDZ domains, heterologous competition bindings assay were performed. This was done by adding increasing concentrations of peptide to a fixed concentration of Cy5-

NR2B, 5-FAM-NR2B, Cy5-CRIPT or 5-FAM-CRIPT (50 nM) and PDZ (20 μM for

PDZl, 3 μM for PDZ2 and 1-2 and 5 μM for PDZ3) in the same TBS buffer and conditions as described above. FP values were then fitted to the general equation: $Y = Bottom + (Top-Bottom)/[l+(10^{\Lambda X\ logIC50})]$, where X is the logarithmic value of peptide concentration. Hereby the IC50 value was obtained, which is used to calculate the theoretical competitive inhibition constant, K_1.[5]

Stopped- flow fluorimetry: The HIS-PDZ2 and HIS-PDZ 1-2 expression constructs of PSD-95 were genetically manipulated to encode a tryptophan, (in position 51 of SEQ ID NO: 4 and position 145 in SEQ ID NO: 8, respectively) instead of isoleucine. Binding between PDZ2 and PDZ1-2 and peptide ligand 74 was then monitored and quantified, by measuring the fluorescence increase from this tryptophan upon ligand recognition, (excitation λ at 280 nm, emission λ > 320 nm). Stopped-flow measurements were performed at $1O^0$C or 25^0C on an SX-20MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK). The peptide, in varying amounts, was rapidly mixed with a constant amount of PDZ and the change in fluorescence was measured over time. The fluorescence versus time plots was fitted to a single or double exponential equation, from which one or two observed rate constants were obtained. Observed rate constants were then plotted against the concentration of the varied species and microscopic rate constants determined by fitting the data to the equation for a bimolecular association (Eq. 1) $kobs = ((kon^2\ (n-[A]of + Kff +2\ k_{on}k_{off}(n+[A]o)f^5$ (Eq. 1) kon is the association rate constant, k_os is the dissociation rate constant, and [A]o and n are the initial concentrations of the varied and constants species,

respectively.

To determine k_os rates, a chase experiment was conducted. In this experiment, PDZ 1-21195 W in complex with 74 was mixed with non-mutated PDZ 1-2, whereby 74 was competed out. At high concentrations of non-mutated PDZ 1-2, the observed rate constant approaches the net off-rate constant for the binding reaction (£$_o$ff^{app}) between 74 and PDZ1-2. Observed rate constants were fitted to Eq. 2 to estimate the apparent, or net, off-rate constant for the peptide, $k_o$fi^{vv}. Us = £off^{app} +

$$k_{on}' \times K_D/(K_D + [\text{unlabeled peptide}]) \quad \text{(Eq. 2)}$$

The equilibrium dissociation constant K_d of the PDZ-peptide complex was calculated by the experimental determined k_{on} and k_os.

Pull-down assay: The assay was performed with a ProFound™ Pull-down PolyHis Protein-protein Interaction Kit purchased from Pierce and performed according to the manufacturer's instructions.

Molecular Modeling: PDZl and PDZ2 were aligned with PDZ3 using Prime (Schródinger, Portland, OR, USA). From these sequences homology models were created using the PDZ3 X-ray crystal structure (PDB structure 1BE9)[10] as template in Prime with standard parameters. The peptide ligand from 1BE9, KQTSV, was rebuilt to IESDV in the homology models. The side chains of the PDZ domain and the peptide were then minimized in Macromodel (Schródinger, Portland, OR, USA) using the force field OPLS2005 and by constraining the backbone. A grid around the peptide was generated in Glide (Schródinger, Portland, OR, USA) and used for docking. Relevant peptides were docked flexibly in Glide using default parameters, and the best scoring poses were energy minimized. The conserved water molecule seen in the binding pocket was kept constant during docking and minimization. Pymol, version 0.97, was used for creating figures.[11]

Isothermal Titration Calorimetry (ITC): Calorimetry experiments were performed on a microcalorimeter (Microcal, MA, USA). The ligands were titrated directly into the PDZ solution. All ligands and PDZ proteins were solubilized in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and when necessary pH was adjusted to 7.4 within 0.02 pH units after solubilization. The concentration of peptide and PDZ in solution was quantified by amino acid analysis. The calorimetry experiments were conducted at 25 ^{0}C, where the PDZ 1-2 concentration was generally adjusted to about 20 µM (200 µL in calorimeter) and the ligand concentration to between 200-2500 µM (injected to the protein as 20 x 2 µL). Experiments were designed so that c-value (c-value = K_A* [Protein] *n; where $K_A$is the affinity association constant, [Protein] means protein concentration, and n is the stoichiometry of the reaction) was within 1-1000. The analysed solution was stirred at 1000 rpm, with a reference power of 6 µcal/s. Heats of dilution were initially determined by titrating the ligand into buffer and subtracting these baseline values from the observed "heat values". The data from each titration experiment were collected by ORIGIN 7.0 (Microcal, MA, USA), which was also used to determine the thermodynamic properties of ligand binding using non-linear least-squares fitting assuming a single-site model. Human Blood Plasma Stability Assay. Compounds of interest were dissolved in human blood plasma (270 µl; 3H Biomedical, Sweden, cat no 1300-1-P50) to a concentration of 0.25 mM (30 µL of 2.5 niM) and incubated at 37°C. Aliquots (30 µl) were removed at various time intervals (0, 5, 10, 20, 40, 80, 160, 320, 960, 1280, 2550, 4560 and 7240 min) and quenched with 60 µL trichloroacetic acid (aq., 5 %). The aliquots were vortexed, and incubated for at least 15 min, at 4 ^{0}C prior to centrifugation at 18,000 g for 2 min and the supernatant was analyzed by RP-HPLC to quantify the remaining compound (absorbance at 218 nm). The RP-HPLC analysis was conducted on an Agilent 1100 system using a Zorbax 300 SB-C 18 column (5 µm, 4.6x150 mm, Agilent Technologies, USA), flow rate of 1 niL/min, and a gradient starting from 100 % buffer A (95 % water, 5 % acetonitrile, 1 % TFA) to 40 % buffer B (95 % acetonitrile, 5 % water, 1 % TFA) and 60 % buffer A over 40 min.

Neuronal Excitotoxicity Assay. Cortical neurons were surgically removed from rats (e.g. E21) and then cultured for 7-9 days in vitro in Neurobasal A medium with B-27 supplement (each supplied by Invitrogen), further supplemented with ImM glutamine, 50 units/ml penicillin, and 50µg/ml streptomycin. The following steps were then performed: 1. Samples of the cultured cortical neurons were removed from the Neurobasal-A based culture medium, at various time points prior to exposure to compound, and placed into "transfection medium" (TH; as defined by Bading et al (1993) Science 260, 181-186) comprising: 10% modified Minimum Essential Medium (supplied by Invitrogen; product no: 21090022; containing Earles salt, but no L-glutamine), 90% Salt-Glucose-Glycine (SGG) medium: (114 mM NaCl, 0.219% $NaHCO_3$, 5.292 mM KCL, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM Glycine, 30 mM glucose, 0.5 mM sodium pyruvate, 0.1% Phenol Red), insulin-transferrin-selenite supplement (supplied by Sigma: 7.5 µg insulin/ml, 7.5 µg transferring/ml and 7.5 ng sodium selenite/ml); with a final osmolarity of 325 mosm/1. All subsequent steps were performed in this TM medium. 2. Samples of neurons are exposed to an NMDAR/PSD-95 inhibitor of the invention (compounds 40, 52-90) at a final concentration of between 0.05 - 50 µM for a period of between 0.5 to 1.5 hours. In control assays, the samples were exposed to NR2B (1), or Tat-NR2B (3) or TM medium alone.

3. Each sample of neurons was washed at least one time in TM medium to remove the NMDAR/PSD-95 inhibitor, and placed in TM medium. 4. After various periods of time, the samples of neurons were exposed to NMDA at a concentration of between 10-100 μM for a period of 0.5 to 1.5 hours, preferably 1 hour, after which the samples were again placed in TM medium for 24 hours. 5. LDH in the cell medium was quantified using commercial available kits (e.g. Promega Biotech AB, cat no G 1780).

Example 1. A Fluorescence Polarization Assay for detecting interactions between peptide analogues and PDZ domains. A convenient and reliable way to examine interactions between peptide analogues and PDZ domains is by using a fluorescence polarization (FP) assay. The three PDZ domains, PDZ1-3, of PSD-95 were expressed individually as set out above, while PDZ 1-2 was also expressed as a tandem construct. As set out above, fluorescent peptides were synthesized by labelling the undecapeptide peptides corresponding to the wild-type (WT) C-terminal of the NR2B subunit (YEKLSSIESDV) and CPJPT (LDTKNYKQTSV) with either 5-FAM or Cy5, through a tripeptide (KSG or CSG) linker, at the //-terminus (designated 5-FAM-NR2B, Cy5-NR2B, 5-FAM-CPJPT and Cy5-CRIPT).[12] K_d values were then determined. A competition binding assay was also implemented, to measure the affinity as IC_{50} values between PDZ domains and non-fluorescent NR2B (1, KSG-YEKLSSIESDV) and CRIPT (2, KS G-LDTKNYKQT SV) peptides. IC_{50} values were then converted to K_1 values.[5]

Since K^ and K_1 values should be similar when measuring the same PDZ-peptide interaction, these values were compared for the NR2B derived peptides (PDZl, PDZ2, and PDZ 1-2) and CRIPT derived peptides (PDZ3). The K_d and K_1 values were found to be very similar when measured against PDZl, PDZ2 and PDZ3, although the K_1 for the tandem construct PDZ 1-2 is somewhat higher than K_d (Table 1). Table 1. Validation of FP assay: $K_{\&}$ and K_1 values of WT probes and peptides." Compound PDZl PDZ2 PDZ3 PDZ1-2

5-FAM/Cy5- 20 ± 1.6 3.0 ± 0. 16 NA[rf] 1.7 ± 0.11

NR2B*

5-FAM/Cy5- ND[rf] ND[rf] 33..55 = ± 0.18 ND"

CRIPT[0]

NR2B m[c] 18 ± 0.92 4.1 ± 0. 17 NA[rf] 7.0 ± 0.19

CRIPT (2)[c] 97 ± 18 25 ± 1.6 2.1 ± 0.15 45 ± 4.1

Tat-NR2B (3)[C] 14 ± 1.9 4.4 ± 0.32 NA[d] 9.8 ± 0.35

" Ki and K_1 values are shown as mean ± SEM (standard error of mean) in μM based on at least four individual measurements. * K^ values. [c] K_1 values. [d] ND: Not determined, NA: No activity.

Furthermore, the Kd values are independent of whether 5-FAM or Cy5 is used as a fluorophore (data not shown). The K_A for 5-Fam-NR2B peptide was also determined for PDZ2 with and without His-tag and, as expected, no difference was observed (data not shown).[13, 14] Thus, the results obtained in the assay represent accurate measurements, consistent with the literature values for similar measurements.[15"17] The NR2B peptide (1) shows no binding to PDZ3 (Table 1), which is expected,[9, n] hence activity at PDZ3 was used as a measure of selectivity in the examples. All compounds were tested for PDZ3 binding and unless otherwise stated, compounds had no affinity towards this domain.

The 20-mer Tat-NR2B peptide (3, YGRKKRRQRRR-KLSSIESDV) is reported to have >100-fold increased affinity to PDZ2 compared to the NR2B peptide alone, as shown by a solid-phase ELISA based assay.[3] However, the FP assay reveals, K_1 values for PSD-95 PDZ domains are similar for NR2B (1) and Tat-NR2B (3) (Table 1). Thus, the notable in vivo effects demonstrated for this Tat-NR2B peptide[1] are more likely due to the ability of the Tat-moiety to improve membrane permeability of peptides, rather than increasing the affinity between peptide ligand and the PDZ domains of PSD-95.

Example 2. Identification of the essential features of a NR2B peptide required for binding to PDZ domains to obtain an NMDAR/PSD-95 inhibitor of the invention. The minimal sequence of the NR2B peptide (1) required for binding to PDZl and PDZ2 was identified using the FP assay. The NR2B peptide was truncated sequentially from the JV-terminus and notably it was possible to reduce it to a pentapeptide, IESDV (7), without losing affinity (Figure 2A). Truncation to the tetrapeptide ESDV (8) showed 3.1 and 6.8 fold increase in K_1 values for PDZl and PDZ2, respectively, and the tripeptide, SDV, demonstrated >20 and >30 fold increase in K_1 compared to peptide 1. In order to examine the generality of this approach to PDZ domain proteins, the activity of the CRIPT peptide (2) at PDZ3 was compared with a truncated CRIPT pentapeptide

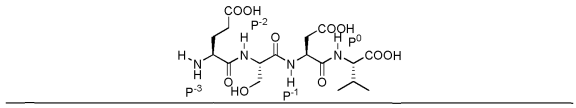
(KQTSV); in contrast to the NR2B peptide (1) and PDZI and PDZ2, a 14-fold reduction in K_1 was observed for this pentapeptide (data not shown).

To investigate the individual importance of the five amino acids in the pentapeptide (7) an alanine scan was performed on this peptide (Figure 2B). For both PDZI and PDZ2, almost complete loss of affinity was observed when substituting Val in the P^0 position (14), thereby underlining the crucial importance of the isopropyl side chain. A similar effect was observed for PDZI, when exchanging Ser ($P"^2$) (12), while a 7- fold increase in the K_1 value was seen for PDZ2. Thus positions P^0 and $P"^2$ are particularly critical for affinity in the pentapeptide, corresponding to a truncated NR2B peptide (1). Two other positions, Glu ($P"^3$) and lie ($P"^4$) displayed less sensitivity to Ala substitutions (10 and 11, respectively), but K_1 values were still somewhat higher than 1 towards PDZI and PDZ2. On the other hand, replacing Asp ($P"^1$) (13) did not affect affinity to PDZI or PDZ2, however some activity at PDZ3 appeared. Although the affinity of 13 towards PDZ3 is low (K_1 >30 fold higher than CRIPT), this suggest that Asp ($P"^1$) plays a role in determining selectivity between the PDZ domains of PSD-95.
Example 3. Enhancing the affinity of peptide ligands for PDZI and 2 domains by single modifications to obtain an NMDAR/PSD-95 inhibitor of the invention.

Substitutions in the truncated NR2B peptide ligand were tested to improve the affinity of the ligand, and at the same time reduce its polarity. The tetrapeptide, ESDV (8) was used as a template, rather than the pentapeptide, because it is a better starting point for development of small molecules, and it is still relatively potent at both PDZI and PDZ2. Introduction of D- and //-methyl amino acids can often induce resistance to enzymatic cleavage. Moreover, D-amino acids provide information on the importance of stereochemical arrangement of the side chains of the amino acids, while //-methyl amino acids are known to stabilize certain amide bond conformations.[18]

Introduction of D-Ser, D-Asp and D-Val instead of their respective L-amino acids one at a time into ESDV (8) abolished affinity to both PDZI and PDZ2, whereas D-Glu impaired the affinity >25 times for PDZI and PDZ2 relative to peptide 1 (data not shown). Thus, L-amino acids are essential for affinity. Substitution with TV-methyl amino acids was better tolerated (Table 2). /V-methylation of Asp in $P"^1$ (16) showed some loss in affinity compared to the NR2B peptide (1) for both PDZI and PDZ2 (Table 2), whereas, /V-methylation of Val (P^0) (15) and Ser ($P"^2$) (17) resulted in inactive peptides. However, substituting the terminal amino acid Glu ($P"^3$) with TV- methylated Glu (18) improved the affinity relative to the reference tetrapeptide (8) and compared to peptide 1, peptide 18 showed only 1.3 and 3.7-fold higher K_1 values for PDZI and PDZ2, respectively (Table 2).

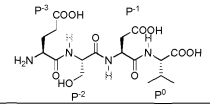
Table 2. K_1 values of N-methylated ESDV analogues [a]



Compound	Position of modification	Structure of modification	PDZI	PDZ2
NR2B (1)			18 ± 0.92	4.1 ± 0.17
ESDV (8)	-		56 ±2.4	28 ± 0.92
15	P^0	CH_3	NA^b	NA^b
16	CH_3		87 ±3.1	78 ±4.5
17	CH_3		NA*	NA*
18	CH_3		24 ±1.8	15 ±1.2

a K_1 values are shown as mean ± SEM in μM based on at least four individual measurements. [6] NA: No activity.

The effects of modifying side-chains in ESDV (8) were investigated by substituting with proteinogenic and non-proteinogenic amino acids (Table 3) with focus on changes in size and/or polarity in an attempt to optimize PDZ binding and drug-likeness. Initially, Val (P^0) was substituted with relatively conservative unnatural amino acid analogues. Replacing with either 2-aminobutanoic acid (Abu) or α- aminoisobutyric acid (Aib) to give 19 and 20, respectively reduced affinity dramatically (Table 3). When substituting with te/t-leucine (tLeu) (21), affinity to PDZI was reduced only 4.2 fold whereas affinity to PDZ2 was reduced ~40-fold relative to the NR2B peptide (1) (Table 3), providing means for differentially inhibiting the activity at PDZI and PDZ2. Table 3. K_1 values of single modifications of tetrapeptide, ESDV.[α]



Compound Position of Structure of PDZl PDZ2 modification modification

NR2B (1) - 18 ± 0.92 4.1 ± 0.17

ESDV (8) - 56 ± 2.4 28 ± 0.92

19 CH_2CH_3 230 ± 7.8 NA*

20 $(CH_3)_2$ NA* NA*

21 $C(CH_3)_3$ 76 ± 3.2 160 ± 5.0

22 $(CH_2)_2CONH_2$ 57 ± 2.3 29 ± 4.1

23 $(CH_2)_2COOH$ 67 ± 3.6 29 ± 2.1

24 CH_2CONH_2 88 ± 5.4 67 ± 7.1

25 $(CH_2)_2OH$ NA* NA*

26 $CH(CH_3)CH_2OH$ NA* NA*

27 $C(CH_3)_2CH_2OH$ NA* NA*

28 $CH(CH_3)CH_2OH$ 22 ± 0.95 16 ± 0.85

29 p-J $(CH_2)_2CONH_2$ 130 ± l l 100 ± 7.1

a K_1 values are shown as mean ± SEM in μM based on at least four individual measurements. * NA: No activity.

In the $P^{"1}$ position, Asp was substituted with Gln, Glu and Asn (22-24) and extending the acid side chain with one methylene group, as in Glu (23), did not reduce affinity significantly, but amidation of the acid, as in Asn (24), reduced affinity for PDZl and PDZ2. However, a combination of the two modifications, substituting Asp with the uncharged Gln (22) did not affect the affinity to either PDZl or PDZ2, thus peptide 22 has an affinity similar to ESDV (8) (Table 3).

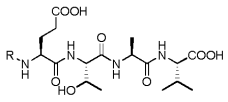
Substitution of Ser ($P^{"2}$) with the non-proteinogenic amino acids homo-serine, allo- threonine and hydroxy- valine, compounds 25-27, led to inactive peptides (Table 3), but substitution with Thr (28) increased the affinity compared to lead peptide 8, and only 1.2 and 4-fold increased K_1 values compared to the NR2B peptide (1) for PDZl and PDZ2, respectively. Substituting Glu ($P^{"3}$) with Gln (29) resulted in impairment of binding (Table 3). It is well-established that one of the primary issues in the search for new ligands for PPIs is the occurrence of false positives. Therefore, the relative affinities for NR2B (1), Tat-NR2B (3), IESDV (7), ESDV (8), and E_{Me}TAV (40) analysed with the FP assay in the present examples were validated and confirmed by a pull-down assay (Figure 3) using the following protocol (see also methodology section). As bait, a polyhistidine NR2B WT peptide with the sequence HHHHHHYEKLS SIESDV was synthesized. This peptide binds to the resin (immobilized cobalt chelate optimized for polyhistidine binding) whereby it itself is immobilized. PSD-95's PDZ2 domain without the His-tag was produced as described above in the methodology section (prey protein). Concentrations of bait and prey were optimized to be 25 μg and 100 μg pr. sample vial, respectively. Each peptide analogue was then tested (in triplicate) at a final concentration of 200 μM (except for Tat-NR2B that was tested in 150 μM) for the ability to displace bait and prey protein from each other, followed by a wash procedure. Finally, resin-attached PDZ2 (bound through the bait) was eluted with 250 mM imidazole. Eluted PDZ2 was analyzed by SDS-PAGE and silver staining. Figure 3, shows the silver stained SDS gel of the eluted PDZ2 domain, where the negative control indicates the amount of PDZ2 bound to the bait, in the absence of a peptide analogue inhibitor. The NMDAR/PSD-95 inhibitor E_{Me}TAV (40) is shown to be a very effective inhibitor of NMDAR/PSD-95 interactions. Example 4. Enhancing the affinity of peptide ligands for PDZl and 2 domains by a combination of substitutions to obtain an NMDAR/PSD-95 inhibitor of the invention. The above tests to improve peptide ligand affinity showed that Thr (28) and JV-methyl Glu (E_{Me}) (18) in positions $P^{"2}$ and $P^{"3}$, respectively of the tetrapeptide ESDV (8) are favorable. Modifications in position P^0 are generally not allowed, whereas a greater degree of freedom for modification is permitted in position $P^{"1}$. Based on these observations three series of compounds were designed and synthesized: Two series of compounds, ETXV and EMeSXV, with X being Gln, Asn, or N-MQ- Asp, and a series of compounds, E_{Me}TXV, where X is Gln, Asn, N-MQ- Asp, Ala, or Asp.

A combination of the two favorable modifications, EMe at $P^{"3}$ and Thr at $P^{"2}$, led to compounds with K_1 values significantly lower than the NR2B peptide (1) at PDZI and only slightly higher at PDZ2 (Figure 4). In particular, the modified tetrapeptides, E_{Me}TDV (36) and E_{Me}TQV (39) are ca. 3-fold more potent than the WT peptide 1 at PDZI and only slightly less potent at PDZ2. Introducing the small, non-polar amino acid Ala in $P^{"1}$ giving E_{MC}TAV (40) provided an inhibitor that was more potent than 1 at PDZI and only 2-fold less potent for PDZ2. Although, 40 demonstrated some activity at PDZ3, the K_1 value at PDZ3 was still considerably higher (-79 μM) relative to that of PDZI and PDZ2, thus 40 is considered selective towards PDZI and PDZ2 relative to PDZ3. Substituting in the $P^{"1}$ position with N-MQ- Asp (37) or Asn (38) reduced affinity compared to E_{Me}TDV (36), but these peptides were still equally potent towards PDZI compared to peptide 1 (Figure 4). In conclusion, TV-terminal methylation of tetrapeptides increases their binding affinity for PDZI and PDZ2 domains, as seen for all JV-methylated ligands in figure 4, as compared to their corresponding non-JV-methylated ligands. Furthermore, the increase in affinity due to methylating the //-terminal amine of the tetrapeptides is sufficiently great that it can compensate for the introduction of amino acid analogues e.g. position $P^{"1}$ that increase the in vivo stability of the ligand, such as iV-Me-Asp, even when this reduces ligand affinity. Example 5: Molecular Modeling and Docking of a peptide ligand in the binding pocket of the PDZ domain. Homology models of PDZI and PDZ2 were generated using Prime based on the X-ray crystallographic structure of PDZ3 in complex with CRIPT peptide,[10] and flexible docking of key peptides was performed using Glide. Initially, the pentapeptide IESDV (7) was docked into PDZI, resulting in a binding mode where the backbone of 7 superimposes with the backbone of the CRIPT peptide in PDZ3. However, an electrostatic interaction not found in the PD3 -CRIPT structure, emerges between the TV-terminal amino group of 7 and Asp90 in the large βB-βC loop of PDZI (Figure 5A). A hydrophobic pocket is formed by Thr 83, Gly89 and HisI30 in PDZI (PDZ2: His225, ValI78 and ProI84), which is partially filled by the side chain of He in position $P^{"4}$. The Glu residue in $P^{"3}$ interacts with Thr97 and Lys98 from the βC strand of PDZI (PDZ2: ThrI92 and LysI93) through hydrogen bonding and electrostatic forces, explaining the importance of this residue for affinity (Figure 5A). The hydroxyl group of Ser in $P^{"2}$ forms a hydrogen bond with His 130, similar to that observed in the PDZ3-CRIPT structure. Replacing Ser ($P^{"2}$) with Thr, allows the additional methyl group to interact with a hydrophobic area created by Val 134 in PDZI (PDZ2: Val229); which may explain the increased affinity of the Ser-to-Thr substitution. Substitutions with larger unnatural amino acids in $P^{"2}$ are probably not allowed due to steric clashes with the PDZ residues.

In the PDZ3 -CRIPT structure the side chain of $P^{"1}$ does not interact with a residue in PDZ3.[10] In our model, we see an electrostatic interaction between Asp ($P^{"1}$) and Lys98 (Figure 5A). We observed that Asp could be replaced with GIn or Ala indicating that neither charge nor hydrogen bonding is crucial for affinity. On the other hand, introduction of Asn in the $P^{"1}$ position reduced the affinity more extensively, demonstrating that not all side chain substitutions are equally allowed. Methylation of the TV-terminal amino group enhanced the affinities for the tetrapeptides. Docking of E_{Me}TAV (40) into PDZI reveals that the JV-terminal amino group is in close proximity to HisI30, thereby creating a favorable cation-π interaction (Figure 5B). The interaction is further stabilized by the JV-methyl group, which is situated in the π-system of the imidazole ring of HisI30 (Figure 5B), which explains the increased affinity upon JV-methylation. Side chain groups of position P^0, $P^{"2}$, and $P^{"3}$ and ligand backbone are bound to PDZ in the same configuration as for the pentapeptide 7. Docking of TAV (49) into the PDZI homology model show that the TV-terminal amino group mediates charge-assisted hydrogen bonding to the hydroxyl oxygen of Ser78 (PDZ2: SerI73) and a backbone carbonyl group (Figure 5C).

Example 6. Enhancing the affinity of the peptide ligand ETAV for PDZI and 2 domains by TV-terminal modification to obtain an NMDAR/PSD-95 inhibitor of the invention. The molecular modeling studies suggest that the methyl group in EMeTAV (40) only partially overlaps with the His 130 aromatic side-chain in PDZI (Figure 5B), indicating that replacing the methyl group with larger groups could improve affinity through increased hydrophobic interactions and/or aromatic stacking interactions. A series ETAV (51) analogues were therefore designed and synthesized where the JV-methyl group was replaced with larger aliphatic and aromatic substituents (Table 4). JV-alkylation of ETAV was facilitated by a Mitsunobu reaction using the Fukuyama protocol,[6] as previously applied for preparation of //-alkylated peptides.[7] The terminal amino group of ETAV was activated as a nitrobenzyl sulfonamide, and subsequently reacted with a range of alcohols mediated by diisopropyl azadicarboxylate (DIAD) and Ph_3P to give the protected, resin-bound products (Scheme 1 in Figure 6). The final products were obtained by deprotection of the sulfonamide and cleavage from the resin providing the desired products, compounds 52-56 and 58-63, in excellent purity and generally high yield (Table 4).

Initially, the methyl group of EMeTAV (40) was replaced with ethyl (52), propyl (53) and butyl (54); molecular docking studies specifically suggested that ethylated ETAV (52) would have similar affinity to E_{Me}TAV (40), whereas propylated and butylated ETAV (53 and 54) should have increased affinity. Biological evaluation of the compounds confirmed this prediction, particularly for affinity at PDZ2 (Table 4). Table 4. Rvalues of N-terminally modified ETAV peptides."



Compound R PDZI PDZ2 PDZ3

NR2B (1) 18 ±0.92 4.1 ±0.17 NA"

CRIPT (2) 97 ± 18 25 ± 1.6 2.1i : 0.15

ETAV (51) 29 ± 1.6 22 ± 0.92 260 ± 20

40 CH_3 11 ±0.47 8.9 ±0.50 78 ± 1.5

52 CH_2CH_3 9.3 ±0.55 9.6 ±0.80 69 ± 2.8

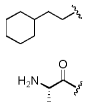
53 $(CH_2)_2CH_3$ 7.0 ±0.28 4.2 ± 0.20 35 ± 1.0

54 $(CH_2)_3CH_3$ 8.4 ±0.37 4.0 ±0.36 35 ± 0.82

55 1.0 ±0.24 1.3 ±0.094 21 ± 0.64

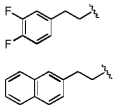
56 0.94 ±0.13 0.45 ±0.13 ll± 0.43

57 6.3 ±0.38 2.3 ± 0.27 35 ± 1.3



58 7.2 ±0.39 5.2 ± 0.22 63 ± 2.4 59 4.4 ± 0.49 1.6 ±0.19 14± 0.54

60 2.1 ±0.35 3.1 ±0.31 44 ± 2.5 61 ^{01}W 2.4 ±0.25 1.1 ±0.11 6.1 ± :0.20 62 0.98 ±0.06 1.0 ±0.043 7.8 ± :0.34 63 1.0 ±0.33 0.95 ±0.05 IO± 0.22



a K_1 values are shown as mean ± SEM in µM based on at least four individual measurements. * NA: No activity.

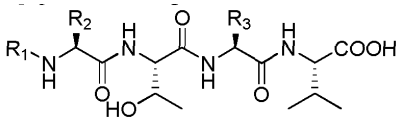
Generally, the increased affinity was more pronounced for PDZ2 than PDZI, and compounds 53 and 54 were equipotent to the NR2B peptide (1) at PDZ2, and ca.2- fold more potent at PDZI. In addition affinity towards PDZ3 was also increased, although a significant selectivity for PDZI and PDZ2 remain (Table 4). Increasing the bulk and hydrophobicity of the JV-alkyl substituents even further, modifying ETAV with methylene- and ethylene-cyclohexane (55 and 56) led to dramatic increases in affinity. Compared to the starting point, the NR2B peptide (1), compounds 55 and 56 shows a ca. 19-fold increase in affinity towards PDZI and 56 shows a 9-fold increase in affinity towards PDZ2. Thus, peptide derivative 56 is a very potent inhibitor of the PSD-95/NMDA receptor interaction and significantly more potent compound that those reported to date.

In view of this improvement of affinity, attention was focussed on substitutions with an aromatic group, which the modeling studies suggested would also improve affinities compared to 40 and 51-54. Initially, the pentapeptide, FETAV (57) was prepared, with an aromatic side-chain in the $P^{"4}$ position. The pentapeptide 57 had a 3- and 2-fold increase in affinity at PDZI and PDZ2, respectively compared to the NR2B peptide (1), thus confirming that an aromatic substituent in $P^{"4}$ is favorable. This prompted the synthesis of peptide derivatives, where the JV-terminus of ETAV was alkylated with aromatic substituents as in compounds 58-63 (Table 4). All of the six peptide derivatives (58-63) had improved affinity compared to both the NR2B peptide (1) and E_{Me}TAV (40). The length of the spacer between the aromatic group and the terminal amino group is important, and a gradual increase in affinity for PDZI is seen when extending from methylene (58), ethylene (59) to propylene spacer (60), whereas the ethylene spacer (59) is the most potent at PDZ2 (Table 4). Next, the effect of substituting the aromatic ring was investigated; substitution with two chloride atoms (61) increased affinity compared to 59 at both PDZI and PDZ2. Replacing chloride with fluorine (62) increased affinity even further, and 62 showed K_1 values around 1 µM at both PDZI and PDZ2, thus being almost equipotent to the ethylenecyclohexane derivative 56. Thus changing the electronic properties of the aromatic ring affect affinity, which is suggested by modeling studies, to be due to the interaction of the aromatic group with His 130 of PDZI. Docking compounds 59 and 61-62 into the homology model of PDZI

suggest a perpendicular stacking between the aromatic group from the peptides and His 130. Increasing the bulk of the aromatic group further to a naphthalene-2-yl moiety (63) resulted in a compound equipotent to 62. For all compounds a considerable increase in affinity towards PDZ3 was observed. However, a significant selectivity is preserved and for the most potent inhibitor, compound (56), a 10- and 20-fold selectivity towards PDZl and PDZ2 relatively to PDZ3 is seen (Table 3). In summary, by rational design guided by molecular docking techniques, it was possible to optimize the alkyl group used to N- alkylate the tetrapeptides, whereby surprisingly potent ligands towards the PDZl and PDZ2 domains of PSD-95 were generated.

Example 7. Substitution of amino acids in the $P"^{1}$ and $P"^{3}$ position in alkylated tetrapeptide ligands to obtain an NMDAR/PSD-95 inhibitor of the invention. Tetrapeptides and alkylated tetrapeptides in which the amino acid in P" was either E, Q, A and the amino acid at $P"^{1}$ was either D or A, were investigated to determine whether amino acids with a lesser charge and/or hydrophilicity could be combined in the alkylated tetrapeptide YTXV, and whether alkylation would compensate for any reduction in affinity due to these amino acid substitutions (Table 5). As seen in Table 5, it is possible to substitute the negatively charged Glu in $P"^{3}$ and Asp in $P"^{1}$ in the N- alkylated tetrapeptide with neutral or even hydrophobic amino acids, such as GIn and Ala, even in combination, and maintain an affinity to the PDZl and 2 domain close to that of the control peptide (1). This is an important finding since the cell membrane is more permeable to tetrapeptides with a reduced charge and hydrophilicity, which will enhance their uptake by a cell.

Table 5. K_1 values of modified peptides with the general structure:"



Compound R i Ri R, PDZl PDZ2 PDZ3

NR2B (1) 18 ± 0.92 4.1 ± 0.17 NA[b]

CRIPT (2) 97 ± 18 25 ± 1.6 2.1 ± 0.2

ETDV (64) H $(CH2)_2COOH$ CH_2COOH 22 ± 0.95 16 ± 0.88 NA[b]

QTDV (65) H $(CH_2)_2CONH_2$ CH_2COOH 58 ± 3.5 60 ± 1.9 NA[b]

ATDV (66) H CH_3 CH_2COOH 30 ± 0.72 38 ± 4.2 NA[b]

ETAV (51) H $(CH2)_2COOH$ CH_3 29 ± 1.6 22 ± 0.92 260 ± 20

QTAV (67) H $(CH_2)_2CONH_2$ CH_3 112 ± 4.4 108 ± 6.3 >400

ATAV (68) H CH_3 CH_3 64 ± 1.7 73 ± 3.8 400

ETDV (69) Cyclohexylethyl $(CH2)_2COOH$ CH_2COOH 1.8 ± 0.23 0.75 ± 0.1 NA[b]

QTDV (70) Cyclohexylethyl $(CH_2)_2CONH_2$ CH_2COOH 6.2 ± 0.93 4.4 ± 0.59 NA[b]

ATDV (71) Cyclohexylethyl CH_3 CH_2COOH 5.8 ± 0.33 3.6 ± 0.30 >400

ETAV (56) Cyclohexylethyl $(CH2)_2COOH$ CH_3 0.94 ± 0.1 0.45 ± 0.1 11 ± 0.43

QTAV (72) Cyclohexylethyl $(CH_2)_2CONH_2$ CH_3 21 ± 1.1 12 ± 0.36 141 ± 14

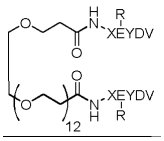
ATAV (73) Cyclohexylethyl CH_3 CH_3 8.9 ± 1.0 8.1 ± 0.12 89 ± 3.3 [a] K_1 values are shown as mean ± SEM in µM based on at least three individual measurements. * NA: No affinity.

Example 8. Enhancing the affinity against PDZ1-2 by linking modified peptide ligands with a PEG-linker, thereby increasing the affinity towards this PDZ motif but not the single PDZ domains.

Dimerization of the modified peptide ligands of the invention with a PEG-linker provides a NMDAR/PSD-95 inhibitor with several pharmacokinetic advantages. PEG (polyethylene glycol) is selected as the linker because it is a non-toxic chemical and when linked to the modified peptide ligands will serve to lower their renal clearance, provide protection against enzyme cleavage, reduce immunogenicity, and enhance their solubility and cell membrane permeability. Furthermore, these dimeric ligands, linked by PEG, are shown to have greatly increased affinity towards PDZ 1-2 of PSD-95. Initially, this is

demonstrated by the use of PEG molecules to chemically link two identical peptide ligands (IESDV) as in compound 74 or the alkylated GE(R)TDV (R=cyclohexylethyl; JV-alkylated in P"[3]) pentapeptide as in compound 75 (see structures in figure 7). In both these examples a dramatic increase in affinity for the tandem construct PDZ 1-2 is seen (See Figure 8 and K_1 values in table 6), reflecting the synergistic effect that is obtained by linking two ligands together.

Table 7. Rvalues for dimeric peptide ligands measured by FP. [a]



Compound R, X, Y PDZl PDZ2 PDZ1-2 PDZ3

NR2B (1) 18 ± 0.92 4.1 ± 0.17 6.8 ± 0.20 NA[b]

$(IESDV)_2$- R = H PEG12 (74) X = IIe 22 ± 1.4 2.8 ± 0.32 ■ O.I' NA[b]

Y = Ser

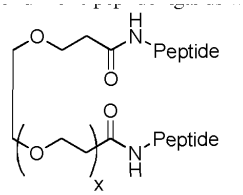
R =

$(GE[R]TDV)_2$- Cyclohexylethyl PEG12 (75) X = Gly 83 ± 6.2 55 ± 2.1 5.5 ± 0.46 NA"

Y = Thr a K_1 values are shown as mean ± SEM in uM based on at least three individual measurements. * NA: No affinity. [c] Because of the very low affinity of this ligand, an exact K_1 value can not be calculated in the FP assay (See text).

An exact K_1 value for 74 could not be calculated using the FP assay, due to its very low K_1. However, the K_1 was found to be about 100 nM in a fluorescence titration assay (stopped- flow binding assay[25]).

In order to optimize the length of the PEG-linker, IESDV was further dimerized with a linker comprising PEG8-, 6-, 4-, 2-, 1-, or 0-diacids (76-81) and tested in FP and ITC. It was found that from PEG4 to PEG6 is the optimal linker size range (where PEG4 (78) has a K_d = 32 nM towards PDZ 1-2) and that further shortening of the linker resulted in lower affinity (Figure 9, Table 8). It was also noticed that dimeric ligand and PDZ 1-2 binds in a 1:1 ratio as expected. Table 8. FP and ITC measurements for dimeric peptide li ands with the general structure:



		FP (K_i/μM)[a]		**ITC (K_d/μM)[a]**	
Compound	Peptide	X	PDZ2	PDZ12	PDZ2	PDZ12
7	IESDV	monomeric	3.1 ±0.15	3.9 ±0.12	2.6 ±0.19	3.2 ±0.16
8	ESDV	monomeric	28 ± 0.92	28 ±2.9	ND	ND
82	IETAV	monomeric	1.7 ±0.2	2.5 ±0.13	ND	1.9 ±0.11
86	IATAV	monomeric	16 ±0.33	18 ±0.47	ND	ND
89	$IATA_{Mc}V$	monomeric	104 ±9	142 ±5	ND	ND
74	IESDV	12	3.1 ±0.13	-0.1*	ND[C]	0.09 ±0.014
76	IESDV	8	2.6 ±0.13	-0.1*	ND	0.12 ±0.027
77	IESDV	6	2.0 ± 0.02	-0.1*	ND	0.039 ±0.01
78	IESDV	4	2.7 ±0.12	-0.1*	3.3 ± 0.02	0.032 ±0.01
79	IESDV	2	4.7 ±0.21	0.27 ± 0.03	ND	0.22 ±0.01

80 IESDV 1 4.7 ±0.16 0.56 ±0.06 ND 0.46 ± 0.02

81 IESDV O 3.1 ±0.17 1.5 ±0.09 ND 1.1 ±0.09

83 IETAV 4 1.1 ±0.07 -0.1* ND 0.011 ±0.01

84 ESDV 12 22 ±0.18 0.92 ± 0.03 ND ND

85 ESDV 8 19 ± 1.2 1.0 ±0.05 ND ND

87 IATAV 12 13 ± 1.1 1.0 ±0.06 ND ND

88 IATAV 4 25 ±0.88 1.1 ±0.07 ND ND

90 $IATA_{Mc}V$ 4 103 ± 1.1 13 ±0.6 ND ND [a] KJK_1 values are shown as mean ± SEM based on at least three individual measurements. Because of the very low affinity of this ligand, an exact K_1 value can not be calculated in the FP assay (See text). [c] ND: Not determined.

The potency of the ligand was further enhanced by combining the PEG4 linker with an optimized peptide sequence, for example the ligand ($(IETAV)_2$PEG4, 83) which has a K_d = 11 nM towards PDZ 1-2 of PSD-95. The exceptionally strong binding affinity of this ligand (Structure in figure 10) towards the tandem PDZ 1-2 construct was achieved without compromising its affinity to the single PDZl and PDZ2 domains. As compared to the monomeric ligand IETAV (82), dimerization increased ligand affinity by 176 fold (Figure 9, Table 8). Compared to the clinical candidate Tat-NR2B (3, Table 1), 83 has about 1000-fold greater affinity towards PDZ1-2 of PSD-95, thereby demonstrating that 83 has the affinity required for a strong drug candidate (Table 8).

Since dimerization enhances the binding affinity of a ligand towards PDZ 1-2, it can also be used to transform low affinity PDZ1/PDZ2 ligands into high affinity PDZ 1-2 ligands. Thereby, shorter peptides (e.g. ESDV), hydrophobic and more cell permeable peptides (e.g. IATAV and $IATA_{Me}V$), and proteolytically stable peptides (e.g. $IATA_{Me}V$) can be dimerized to provide potent ligands in which their respective beneficial properties are preserved (Table 8).

In conclusion, dimeric linkage of peptide-ligands (//-alkylated or not) using PEG molecules facilitates simultaneous binding of the ligand to PDZl and 2 of PSD-95, whereby binding affinity is increased. Thereby, a potent ligand, specific for the PDZ 1-2 protein motif is obtained, having increased selectivity towards PSD-95, together with improved pharmacokinetic properties.

Example 9. PEG-based dimerization of peptide ligands leads to human blood plasma stability.

Stability is a general concern in relation to peptide-based drugs, as proteases in human blood facilitate degradation of peptides. Accordingly, the time-dependent stability of key compounds was tested in a human blood plasma stability assay.

Monomeric peptides were degraded relatively fast (compounds 7, 29, 82 and 86 had a half- life of about 17-55 minutes; Table 9), but PEG-based dimerization of these peptide ligands leads to highly plasma- stabile compounds (83, 87, 88) and in some cases (74, 76-78, 90) complete resistance towards degradation was achieved, as no significant degradation could be detected over a time period of 6 days (Table 9 and Figure 11). For both the monomeric and dimeric ligands it is seen that the peptide sequence, IESDV, has a greater degree of stability compared to IETAV and IATAV, and that JV-methylation of $P^{"1}$ increases stability, since $IATA_{Me}V$ demonstrates a longer half-life compared to IATAV (Table 9). Finally, it is noticed that the dimeric compounds, 74-76-78, and 90 display a longer half-life compared to the clinical candidate Tat-N2B (3) (Table 9 and figure 11).

Table 9. Blood plasma half life (T_{1Z2}) of key compounds

ComP°und (minutes)

Tat-N2B (3) 1468

IESDV (7) 53

IETAV (82) 50

IATAV (86) 17

$IATA_{Mc}V$ (89) 32

$(IESDV)_2$PEG12 (74) > 8000

$(IESDV)_2$PEG8 (76) > 8000

$(IESDV)_2PEGo$ (77) > 8000

$(IESDV)_2PEG4$ (78) > 8000

$(IETAV)_2PEG4$ (83) 409

$(IATAV)_2PEG12$ (87) 183

$(IATAV)_2PEG4$ (88) 123

$(IATA_{Mc}V)_2PEG4$ (90) > 8000

IESDV-PEG4 (91) 602

The pegylated, monomeric ligand (91) also demonstrates an increased half life (about 10 fold) compared to non-pegylated peptide 7 (Table 9), thus the PEG-linker is instrumental for this effect. However, 91 is not completely resistant to plasma treatment, as seen for the IESDV-based dimeric ligands (74, 76-78; Table 9), hence having two peptide ligands in one molecule contributes to improving stability in blood plasma. Example 10. Analysis of the neuroprotective effect of an NMDAR/PSD-95 inhibitor of the invention in mammalian neurons ex vivo.

In order to evaluate the therapeutical potential of dimeric ligands and //-alkylated tetrapeptides key compounds from each series were tested in a neuronal cell based ex vz'vo assay. This assay evaluates membrane permeability together with compound specificity and ability to attenuate NMDA- induced excitotoxicity. This assay thereby constitutes a direct model of the pathogenesis of e.g. stroke or traumatic brain injuries and measures important compound properties. These experiments show that both JV-alkylated tetrapeptides (56, 63 and 73) and dimeric ligands (74), at a concentration of 500 nM, were able to permeate the neuronal plasma membrane and inhibit neuronal excitotoxicity, as efficiently as the clinical candidate Tat-N2B (3) (Figure 12).

Example 11. Prevention of NMDA evoked nNOS activation. NMDA evoke changes in cGMP levels in neurons which provide a surrogate measure of NO production by nNOS. Samples of cortical neurons are prepared, cultured and incubated as previously described Then, the level of cGMP in the neuron samples is measured, thereby providing a surrogate measure of the prevention of NOS activation by the NMDAR/PSD-95 inhibitor of the invention.

Example 12. Analysis of the membrane permeability of an NMDAR/PSD-95 inhibitor of the invention in mammalian cells.

A $BRET^2$ assay is used to analyse the ability of an NMDAR/PSD-95 inhibitor to permeate mammalian cells. Accordingly, 0.5 million COS-7 cells per well are seeded into a 6-well plate (in replicates) and grown in 10% fetal calf serum/Dulbecco's modified Eagle's medium (DMEM) and transfected with 3.7 µg of each DNA construct per well using COS7 transfection reagent (Altogen) according to the manufacturer's protocol. DNA constructs for transfection comprise a DNA sequence encoding a fusion protein designated as follows: GFP-NR2B DNA [SEQ ID NO: 9] encoding GFP-NR2B protein [SEQ ID NO: 10];

GFP-NR2B AA DNA [SEQ ID NO: 11] encoding GFP-NR2B AA protein [SEQ ID NO: 12] GFP-PDZ2 DNA [SEQ ID NO: 13] encoding GFP-PDZ2 protein [SEQ ID NO: 14] PDZ2-GFP [SEQ ID NO: 15] encoding PDZ2-GFP protein [SEQ ID NO: 16] Rluc-NR2B DNA [SEQ ID NO: 17] encoding Rluc-NR2B protein [SEQ ID NO: 18] Rluc-NR2B AA DNA [SEQ ID NO: 19] encoding Rluc-NR2B AA protein [SEQ ID NO: 20] Rluc-PDZ2 DNA [SEQ ID NO: 21] encoding Rluc-PDZ2 protein [SEQ ID NO: 22] PDZ2-Rluc DNA [SEQ ID NO: 23] encoding PDZ2-Rluc DNA [SEQ ID NO: 24], each of which are individually cloned in the vector pcDNA3.1 vector (Invitrogen). Cells in each well are transfected with a pair of vectors, encoding one of the following alternative pairs of fusion proteins: • wild-type GFP-NR2B or mutant GFP-NR2B AA with Rluc-PDZ2 or PDZ2-

Rluc; • wild-type Rluc-NR2B or mutant Rluc-NR2B AA with GFP-PDZ2 or PDZ2-

GFP; The total amount of transfection DNA is kept constant by adding empty pcDNA3.1 vector (Invitrogen). The cells are then incubated for 2 days, during which period samples of an NMDAR/PSD-95 inhibitor of the invention are added to the wells in replicate plates, at a final concentration of at least 1 µM, more than 1 µM, or between 5µM and 1.0 mM. The constructs encoding fusion with the mutant NR2B AA are included as controls, since the mutant is unable to bind to a PDZ domain of PSD-95.

After 2 days, the cells are washed, detached and re-suspended in 666 µL Ix PBS (8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH2PO_4 in 800 ml of distilled H2O, adjusted to pH 7.4 with HCl). The cells are then spilt into two portions in 96 well plates (1.5- 10^6 cells/well): The first portion (in black plates) is used to determine the GFP levels and RLUC expression levels using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany). Fluorescence excitation is performed at 425/20 nm and emission is measured at 530/10 nm. Luminescence is assayed by addition of Coelenterazine h (Biotium Inc., Hayward CA, USA) to a final concentration of 5 µM. Background values obtained with cells transfected with

empty pcDNA3.1 vector only are subtracted, and the mean luminesence of triplicate wells/sample are calculated.

The second portion of the cells (in white plates) is submitted to DeepBlueC (Coelenterazine 400a; Biotium Inc., Hayward CA, USA) excitation at a final concentration of 5μM, and the luminescence at the dual bands (515/30 nm and 410/80) is measured on the Mithras LB 940 plate reader. Background values are obtained with cells transfected with empty pcDNA3.1 vector only, and the means of triplicate wells/sample are calculated. BRET ratios are calculated as (emission515 nm - background515 nm)/(emission410 nm - background410 nm).

The background signal from RLUC is determined by co-expressing the Rluc construct with empty vector, and the $BRET^2$ ratio generated from this transfection is subtracted from all other $BRET^2$ ratios.

For BRET2 saturation experiments, a range of transfections are made with a stable amount of PDZ2-RLUC cDNA and increasing amounts of cDNA encoding the GFP2-NR2B protein. All measurements are made at room temperature.

Example 13. NMDAR/PSD-95 inhibitors of the invention have a neuroprotective effect against stroke damage in mammalian in vivo

A. Neuroprotection against stroke damage by pretreatment with NMDAR/PSD- 95 inhibitors.

Adult male Spraque-Dawley rats are subjected to transient MCAO for approximately 90 minutes by the intraluminal suture method, as set out in (29). The animal subjects are pretreated with a single intravenous bolus injection with either saline alone (control), saline supplemented with either NR2B peptide or an NMDAR/PSD-95 inhibitor of the invention at a final concentration of 3 nM/g body weight, 45 minutes prior to MCAO. Body temperature, blood pressure and blood gases are monitored and maintained in the animal subjects. The extent of cerebral infarction is measured after about 24 hours after MCAO onset. Tests include the postural reflex test and the forelimb-placing test to establish the degree of neurological function in the animal subjects during and following MCAO.

B. Attenuation of stroke damage by post-treatment with NMDAR/PSD-95 inhibitors.

Adult male Spraque-Dawley rats are subjected to transient MCAO as set out above (A), but the single intravenous bolus injection with saline alone or supplemented with either NR2B peptide or an NMDAR/PSD-95 inhibitor is first administered one hour after the onset of MCAO. The animal subjects are then monitored over the 24- hour period following onset of MCAO, as set out above.

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NON-PATENT CITATIONS

Reference		
1	*	See references of WO2010004003A2

* Cited by examiner

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