Conopeptide-Functionalized Nanoparticles Selectively Antagonize Extrasynaptic *N*-Methyl-D-aspartate Receptors and Protect Hippocampal Neurons from Excitotoxicity *In Vitro*

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Supporting Information

Table S1. Peptides used in this work.[a]

Abbreviation	Sequence[c]
Con-G'	G E γ γ L Q γ N Q γ L I R γ K S N C
Con-G'*	G E γ γ L Q γ N Q γ L I R γ K* S N C
Ctrl pept*	G E γE γE L Q γE N Q γE L I R γE K* S N C
Con-R'	G E γ γ V A K M A A γ L A R γ N I A K G C
Con-R'*	G E γ γ V A K M A A γ L A R γ N I A K* G C

[a)] All peptides used in this work are characterized by a free *N*-terminus and a *C*-terminal amide. γ= γ-carboxyglutamate; γE= γ-glutamate or isoglutamate; * indicate a fluorescent label: peptides carry 5(6)-carboxytetramethylrhodamine (TAMRA) on the side chain of Lys-15 (Con-G derivatives) or Lys-19 (Con-R derivative).

Preparation of conopeptide-functionalized gold nanoparticles (AuNPs).

Preparation of ~40 nm diameter AuNPs via seeded-growth. AuNPs were synthesized as previously reported[1-3] with small modifications. Briefly, AuNP seeds (10.0 mL, 13 nM, 13±1 nm diameter)[4] were added to 1980 g of ultrapure deionized water followed by 5.1 mL of 80 mM hydroxylamine sulfate (equivalent to 160 mM NH_2OH). An aqueous solution of $HAuCl_4$ (25.0 mL, 10.0 mM) was added at 12.5 mL/h constant flow to this mixture under continuous stirring. Once the addition of gold had terminated, a 100 mM solution of trisodium citrate was added dropwise to reach 1 mM final concentration (20.5 mL). The deep red solution was centrifuged in 200 mL polycarbonate tubes (45 min, 8000 × *g*). The supernatants were discarded and the AuNP pellets pooled together giving 11.5 mL of concentrated AuNPs solution (7.5 nM, ~75% yield). The nanoparticle size, determined *via* the processing of transmission electron microscopy (TEM) images[1], was 40 ± 3 nm and the average ellipticity of the nanoparticles, defined as the ratio between the major and the minor axis was ≤ 1.11, supporting the assumption of a spherical shape (Figure S1a – c)

Coating of the AuNP surface with alkyl-PEG600 thiols (passivation). The experimental procedure for coating the AuNP surface (passivation) has already been reported.[1] Briefly, (1-mercaptoundec-11-yl)PEG600)-acetic acid and (1-mercaptoundec-11-yl)-(ω-amino)PEG600

were present in 9:1 molar ratio in the passivation reaction with a total thiol concentration at 1.0 mM. The other components of the passivation mixture were: $NaHCO_3$ (25.0 mM), ethanol (20% v/v) and the AuNPs (3.9 nM) in a total volume of 22 mL. The passivation was carried out in a closed glass vial protected from light at r.t. for 72 h. Purification by ultrafiltration and gel filtration as earlier reported afforded 3.5 mL of PEGylated AuNPs (16.0 nM, 85% yield, Figure S1a).

Functionalization of AuNPs with the $SM(EG)_2$ linker. In a typical experiment, a freshly prepared 75.0 mM solution of the heterobifunctional linker succinimidyl-([*N*-maleimidopropionamido]-diethyleneglycol) ester ($SM(EG)_2$) in dry acetonitrile was added to a precooled 10.0 nM aqueous solution of PEGylated AuNPs in 30 mM phosphate buffer at pH 8.2 (3.4 mL). The linker concentration in the reaction mixture was 5.0 mM. The reaction mixture was shaken 4 h at 4 °C. The AuNPs were then purified from excess linker *via* ultrafiltration (washed with 2 × 4 mL 2:8 v/v EtOH/20 mM phosphate buffer pH 8.2 and 2 × 4 mL H_2O) performing all steps on ice, in a 4 °C-cooled centrifuge, and with ice cold wash solutions. Purified, linker-functionalized AuNPs (1.9 mL, 18 nM, 95% yield) were directly used in the following peptide conjugation reactions.

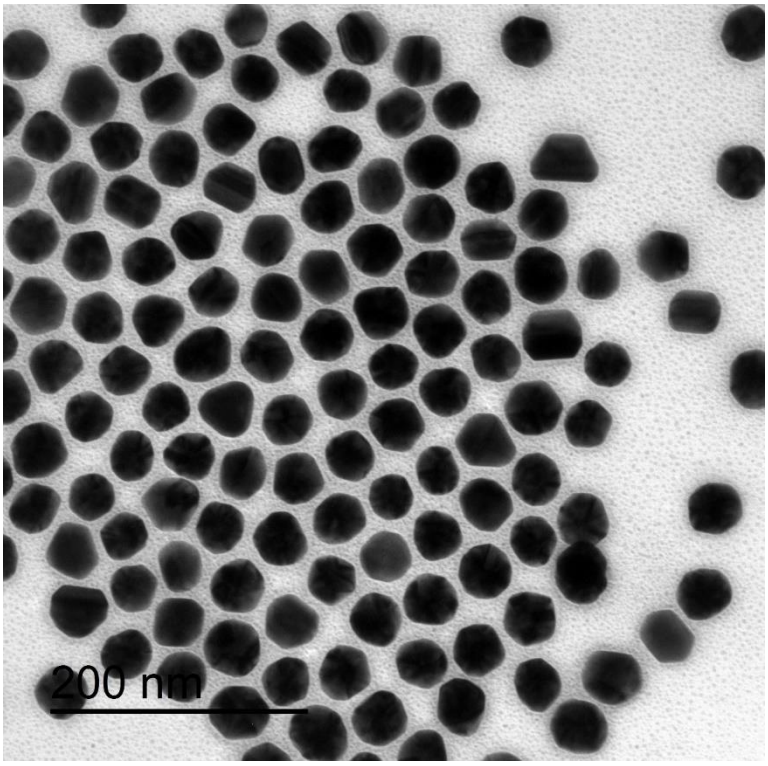
*Peptide conjugation reactions: preparation of AuNPs **1** – **3***. Conjugation of fluorescently labeled conopeptide derivatives **Con-G'***, **Con-R'***, and **Ctrl pept*** to linker-functionalized AuNPs was carried out overnight at 4 °C in 20 mM sodium phosphate buffer at pH 7.0.[5] Stock solutions of the peptides (220 μM in 50 mM $NaHCO_3$) were incubated with 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) on ice for 1 – 2 hours just before conjugation to reduce any present disulfide. The conjugation reaction mixtures (2.0 mL, 5.5 nM linker-functionalized AuNPs) contained 22 μM **Con-G'***, or 2 μM **Ctrl pept***, 8 μM **Con-R'***. Different peptide concentration were found necessary during optimization of the conjugation reaction to obtain AuNPs with similar peptide loading. Excess, unconjugated peptide was removed by centrifugation (10 min, 14000 ×

g, 4 °C) and resuspension in 2 mL 50 mM $NaHCO_3$ (2×), ultrafiltration (washed with 1×4 mL 10 mM $NaHCO_3$, 1×4 mL H_2O, 1×4 mL 10 mM $NaHCO_3$), and gel filtration eluting 10 mM $NaHCO_3$. The eluted AuNP solutions were centrifuged (5 min, 14000 × *g*, 4 °C). The supernatants were discarded and the pellets taken up in 400 μL of 10 mM $NaHCO_3$. The AuNP solutions were filtered sterile and the filter membranes rinsed with additional 300 μL of 10 mM $NaHCO_3$. This procedure afforded ~700 μL of 12 nM AuNPs **1** – **3**. The hydrodynamic diameter and the ζ-potential of these AuNPs are reported in Table 1, while the size distribution for all prepared nanoparticles, from the seed-growth step to the final peptide-conjugation, as determined by DLS are shown in Figure S1b.

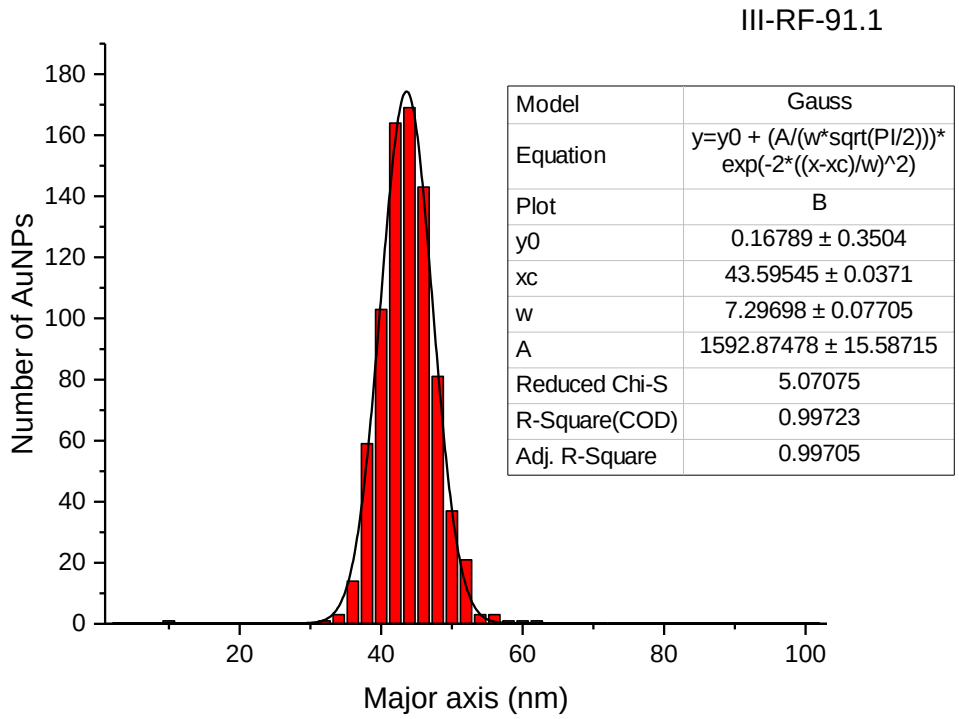
Peptide loading was determined by measuring the fluorescence signal (TAMRA-labeled peptides) after stripping off the passivation layer and the peptides from the AuNP surface with DTT-PB buffer (1M 1,4-dithiothreitol in 0.18 M sodium phosphate buffer pH 8.0) as previously reported.[5]

Peptide functionalized AuNPs **1** – **3** were freshly prepared before each series of experiments on cultured hippocampal neurons and used within 4 weeks. During this period of time, AuNPs were stored at 4 °C, protected from light and aliquoted in small portions to minimize the degradation of the self-assembled monolayer of alkyl-PEG600 thiols and the release of peptide in solution.[5] Under these conditions, the release of peptides in solution was less than 10% after 4 weeks as judged by the intensity of the fluorescence signal in the supernatants.

a)

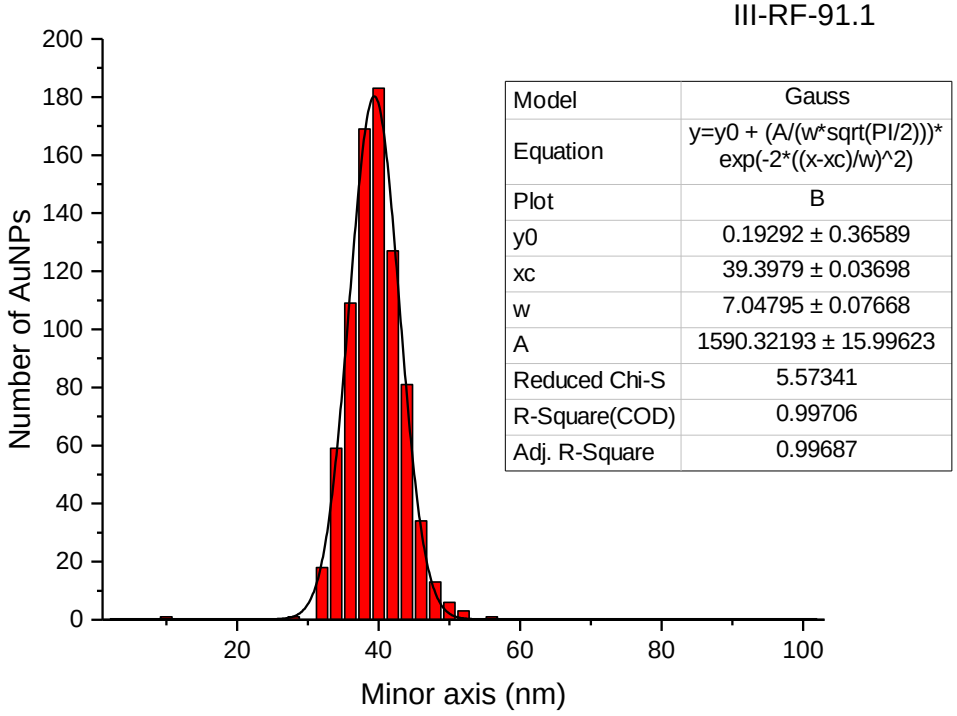

200 nm

b)


III-RF-91.1
Number of AuNPs
Major axis (nm)
0
20
40
60
80
100
120
140
160
180
20
40
60
80
100
Model Gauss
Equation y=y0 + (A/(w*sqrt(PI/2)))* exp(-2*((x-xc)/w)^2)
Plot B
y0 0.16789 ± 0.3504
xc 43.59545 ± 0.0371
w 7.29698 ± 0.07705
A 1592.87478 ± 15.58715
Reduced Chi-S 5.07075
R-Square(COD) 0.99723
Adj. R-Square 0.99705

b -*continues*)

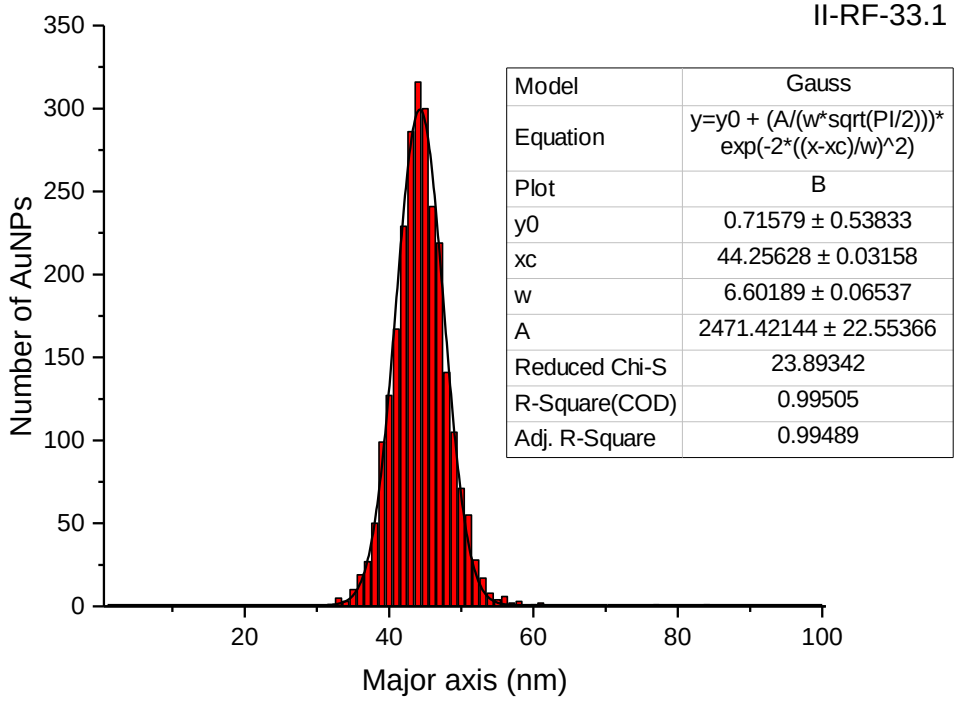
III-RF-91.1



Model	Gauss
Equation	y=y0 + (A/(w*sqrt(PI/2)))* exp(-2*((x-xc)/w)^2)
Plot	B
y0	0.19292 ± 0.36589
xc	39.3979 ± 0.03698
w	7.04795 ± 0.07668
A	1590.32193 ± 15.99623
Reduced Chi-S	5.57341
R-Square(COD)	0.99706
Adj. R-Square	0.99687

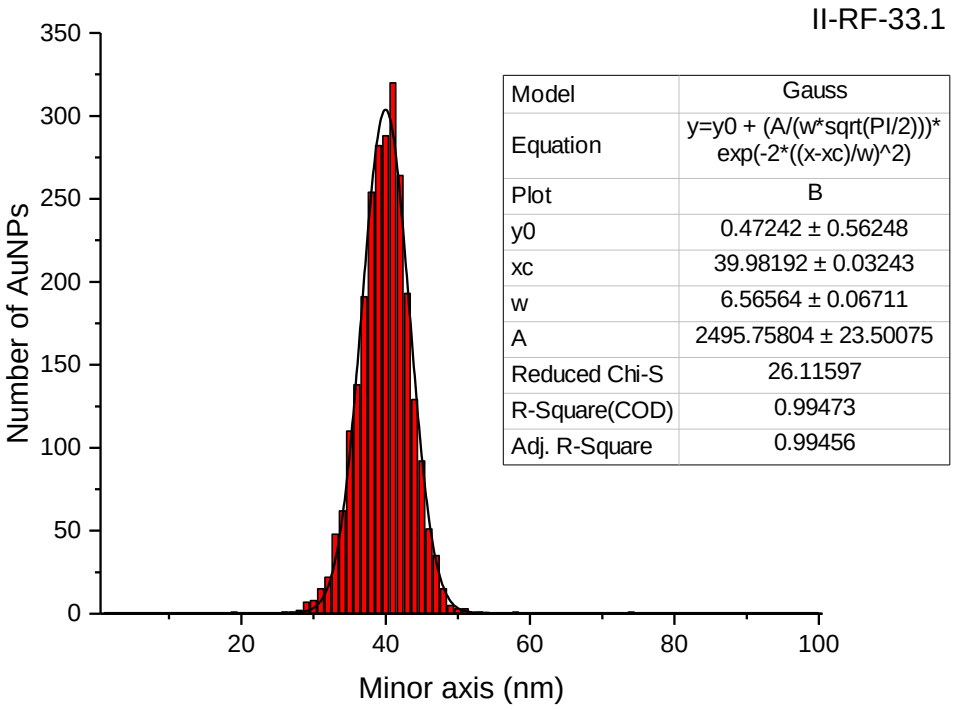
c)

II-RF-33.1



Model	Gauss
Equation	y=y0 + (A/(w*sqrt(PI/2)))* exp(-2*((x-xc)/w)^2)
Plot	B
y0	0.71579 ± 0.53833
xc	44.25628 ± 0.03158
w	6.60189 ± 0.06537
A	2471.42144 ± 22.55366
Reduced Chi-S	23.89342
R-Square(COD)	0.99505
Adj. R-Square	0.99489

c -*continues*)



d)

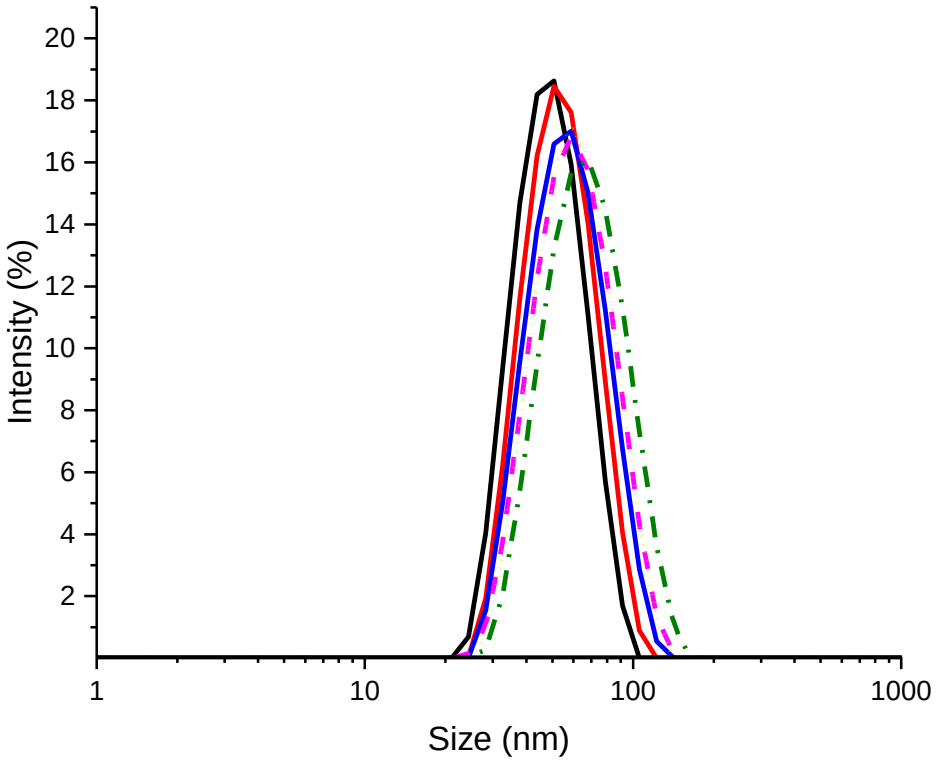


Figure S1. a) Representative TEM micrograph of the PEGylated AuNPs employed in this study. AuNP size distributions for two independent batches: b) analyzed 805 AuNPs from 11 TEM images, ellipticity 1.107 and

c) analyzed 2543 particles from 6 TEM images, ellipticity: 1.107. The TEM images are analysed by fitting ellipses to each particle contour and the analysis procedure returns the major and the minor axis for each particle. Both axis distributions are shown for each preparation. The ellipticity is defined the ratio between the average major and minor axes obtained by Gaussian fitting of the distributions. d) Representative DLS size distributions by intensity for AuNPs dispersed in 10 mM $NaHCO_3$. Black: citrate stabilized AuNPs after seeded-growth; red: PEGylated AuNPs; blue: linker functionalized PEGylated AuNPs; dashed magenta: **Con-G'***-AuNPs **1**; dotted yellow: control AuNPs **2**; dash-dotted olive green: **Con-R'***-AuNPs **3**.

Table S2. Physico-chemical properties of precursor AuNPs used in this work.[a]

AuNPs	DLS[b]		ζ-Potential[b]
	Ø (nm)[c]	PDI[d]	(mV)
citrate stabilized after seeded-growth	47 ± 1	0.13 ± 0.04	---
PEGylated	53 ± 2	0.11 ± 0.04	- 44 ± 4
linker functionalized, PEGylated	56 ± 3	0.13 ± 0.02	- 49 ± 2

[a)] Reported values are means ± standard deviation of at least 3 to 6 independent batches of AuNPs; [b)] Measurements in 10–50 mm $NaHCO_3$, pH 8.4 [c)] Diameter derived from intensity distributions. [d)] PDI = polydispersity index.

Preparation of primary hippocampal neurons.

All experiments were carried out in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU of 22 September 2010) and were approved by the Italian Ministry of Health. Hippocampal cultures (E18) were prepared from wild-type C57bl/6j mice (Charles River, Calco, Italy) or Wistar rats (Charles River, UK). Mice or rats were sacrificed by CO_2 inhalation, and 18-day embryos (E18) were removed immediately by cesarean section. Briefly, enzymatically dissociated hippocampal neurons were plated on poly-L-lysine-coated (0.1 mg/mL) glass coverslips (Thermo-Fischer Scientific, Waltham, MA, USA). Cultures were incubated at 37 °C, 5% CO_2, 90% humidity in medium consisting of Neurobasal (Gibco/Thermo-Fischer Scientific) supplemented to reach a final concentration of 5% glutamax, 5% penicillin/streptomycin, and 10% B27 supplement (Gibco/Thermo-Fischer Scientific). All chemicals for culture preparation were purchased from Life Technologies/Thermo-Fischer Scientific.

Primary culture of neurons plated as autaptic neurons or low-density networks were made as described previously[6] with slight modifications. For autapses, dissociated neurons were plated at

very low density (20 cells/mm^2) on microdots (40-300 μm in diameter) obtained by spraying a mixture of poly-D-lysine (0.1 mg/ml) and collagen (0.25 mg/ml) on Petri dishes, pretreated with 0.15% agarose. Under this culture condition, each Petri dish showed about 20 isolated single autaptic neurons grown on poly-D-lysine microdots. For low-density network, dissociated neurons were plated at 160 cells/mm^2 on Petri dishes, previously pretreated with poly-D-lysine (0.1 mg/ml). No antimitotic drugs were added to prevent glia proliferation. All electrophysiological measures were conducted at room temperature (22-24 °C).

Transmission electron microscopy on AuNP-treated neurons.

Hippocampal neurons (13 days *in vitro,* DIV) plated on glass coverslips (~3×10^5 cells/coverslip Ø=25 mm) were incubated for 1 h with 1 mL of 0.25 nM AuNP **1** or **3** in cell culture medium at 37° C. Cells were then washed with cell culture medium (2×1 mL) and phosphate-buffered saline (PBS 2×1 mL) and fixed for 45min in 2.5% Glutaraldehyde+2% formaldehyde (from paraformaldehyde, PFA) in 0.1M phosphate pH 7.4. Fixed samples were washed three times in distilled water and incubated in 1% aq. OsO_4 for 45min at room temperature, followed by three more washes in distilled water. En bloc staining was done with 1% aq. uranyl acetate for 30min at room temperature. The samples were further washed twice in distilled water and dehydrated in a graded series of ethanol (20%, 50%, 70%, three times 100%). The samples were infiltrated in epoxy resin and polymerized for 48 h at 60° C. Ultrathin sections were cut from the polymerized resin block and picked up on carbon and Formvar-coated slot grids. Samples were examined using a TEM operated at 120 kV (JEOL JEM 1400Plus, JEOL, Japan). Images were acquired with a 2k by 2k format CCD camera (JEOL Ruby CCD Camera, JEOL, Japan). The images were analyzed using the IMOD software package[7] to automatically find the positions of the AuNPs, while

synapses in the datasets where manually labelled. Each dataset was generated by sectioning at random positions in the coverslip and then imaging 450 fields of view (each 9.69 μm × 7.24 μm). Each field of view image had around 10% overlap with the neighboring fields of view, allowing the reconstruction of very large stitched images (see Figure S2).

Figure S2. TEM imaging datasets were acquired automatically and are composed of large arrays of images that can be displayed as montaged panoramas. In this figure, one example is shown corresponding to hippocampal neurons treated with 0.25 nM **Con-R'***-AuNPs **3** (scale bar 30 μm).

Table S3. Statistical analysis of the data shown in **Figure 5a-c** (two-way ANOVA followed by Dunnett's tests) choosing the *untreated group* as the control group.[a]

Dunnett's multiple comparisons test	Significant?[b]	Summary	Adjusted P Value
Con-G'*-AuNPs **1**			
untreated *vs.* 50 μM NMDA	Yes	****	<0.0001
untreated *vs.* 0.1 nM AuNPs **1**	Yes	***	0.0002
untreated *vs.* 0.5 nM AuNPs **1**	Yes	**	0.0099
untreated *vs.* 1.0 nM AuNPs **1**	No	ns	0.4007
untreated *vs.* 1.0 nM AuNPs **1** (no NMDA)	No	ns	0.3877
Control AuNPs **2**			
untreated *vs.* 50 μM NMDA	Yes	***	0.0002
untreated *vs.* 0.1 nM AuNPs **2**	Yes	**	0.0012
untreated *vs.* 0.5 nM AuNPs **2**	Yes	***	0.0008
untreated *vs.* 1.0 nM AuNPs **2**	Yes	***	0.0006
untreated *vs.* 1.0 nM AuNPs **2** (no NMDA)	Yes	ns	0.0533
Con-R'*-AuNPs **3**			
untreated *vs.* 50 μM NMDA	Yes	****	<0.0001
untreated *vs.* 0.1 nM AuNPs **3**	No	ns	0.1796
untreated *vs.* 0.5 nM AuNPs **3**	No	ns	0.3465
untreated *vs.* 1.0 nM AuNPs **3**	No	ns	0.0545
untreated *vs.* 1.0 nM AuNPs **3** (no NMDA)	No	ns	0.2867

[a)] For each antagonist (AuNP **1** – **3**), one experiment consisted of testing the complete series of conditions on sibling cultures (untreated, 50 μM NMDA, 50 μM NMDA + indicated antagonist concentration, antagonist-only (no NMDA) at the maximal concentration of 1.0 nM). The results of 5 independent experiments were averaged and statistically analyzed. [b)] Alpha=0.05.

The results show that **Con-G**'*-AuNPs **1** and **Con-R**'*-AuNPs **3** (but not control AuNPs **2**) protect neurons from the excitotoxic stimulus and increase the survival of NMDA-treated neurons to levels not significantly different from untreated cells.

Statistical analysis.

Data are expressed as means ± standard error of the mean (sem) or means ± standard deviation (sd) for number of cells (n) or mouse preparations as detailed in the figure legends. Normal distribution of data was tested using the D'Agostino–Pearson's normality test. To compare more than 2 normally distributed sample groups, we used one-way or two-way ANOVA (repeated

measures ANOVA) followed by either the Bonferroni's or the Dunnett's multiple comparison test. Alpha levels for all tests were 0.05% (95% confidence intervals). Statistical analysis was carried out using OriginPro-8 (OriginLab Corp., Northampton, MA, USA) and Prism (GraphPad Software, Inc.) softwares.

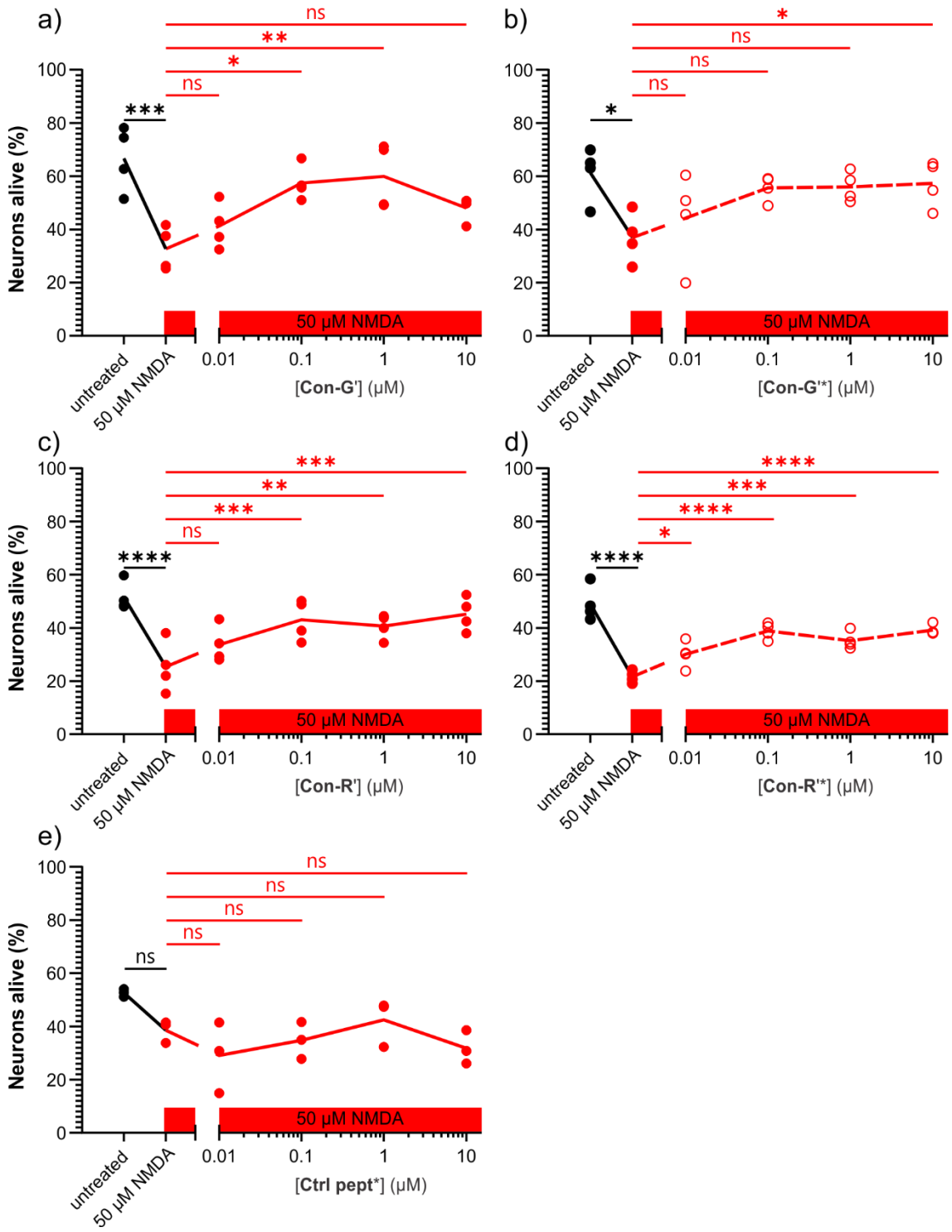


Figure S3. Conopeptides **Con-G'** (a), **Con-R'** (c), and their fluorescently labeled version **Con-G'*** (b), **Con-R'*** (d) protect cultured hippocampal neurons against NMDA-induced excitotoxicity, while control peptide **Ctrl pept*** does not (e). Solid lines connect the means of 4 independent experiments (a – d) or 3 independent experiments (e). Two-way ANOVA/Dunnett's tests: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

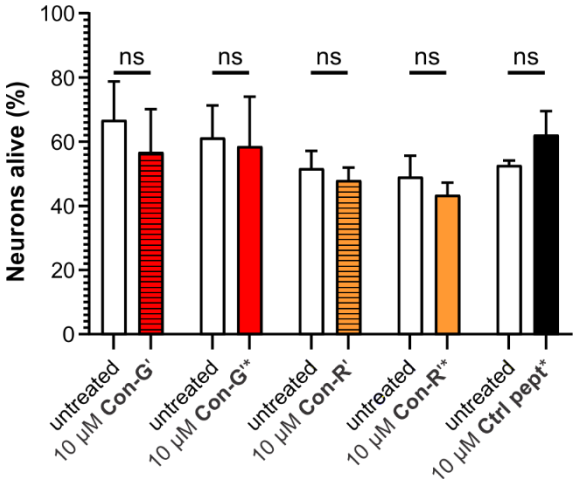


Figure S4. Incubation of neurons with 10 µM conopeptide derivatives for 1 h does not influence neuronal survival at 24 h. Shown data are means ± s.d.. Two-way ANOVA/Dunnett's tests (run together with data in Figure S3**)**.

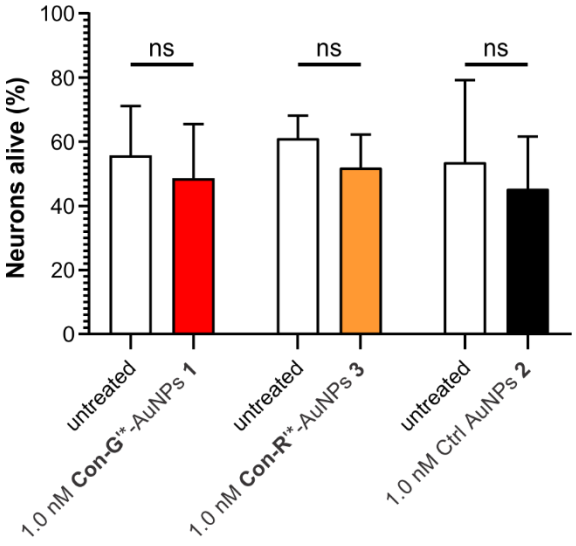


Figure S5. Incubation of neurons with 1.0 nM conopeptide-functionalized AuNPs **1** – **3** for 1 h does not influence neuronal survival at 24 h. Shown data are means ± s.d.. Two-way ANOVA/Dunnett's tests (run together with data in Figure 5).

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