

Supplemental material for

Prolyl oligopeptidase is involved in the cellular signalling in human neuroblastoma SH-SY5Y cells

Abbreviated title: *POP in cell signalling*

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1. Endogenous PREP activity in SH-SY5Y after 24-h incubation with PREP inhibitors

One day old SH-SY5Y cells were incubated 24 hours with increasing concentrations of the PREP inhibitor (ZPP, KYP-2047, or JTP 4819). After incubation, cells were lysed as described in the main manuscript's materials and methods section, and PREP activity was assayed. The results obtained from titrations with ZPP and KYP-2047 are shown in Fig S1. ZPP seemed to be more potent as a result of better cell penetration and it was selected for the microarray experiments at a concentration of 10 μ M where PREP activity is undetectable.

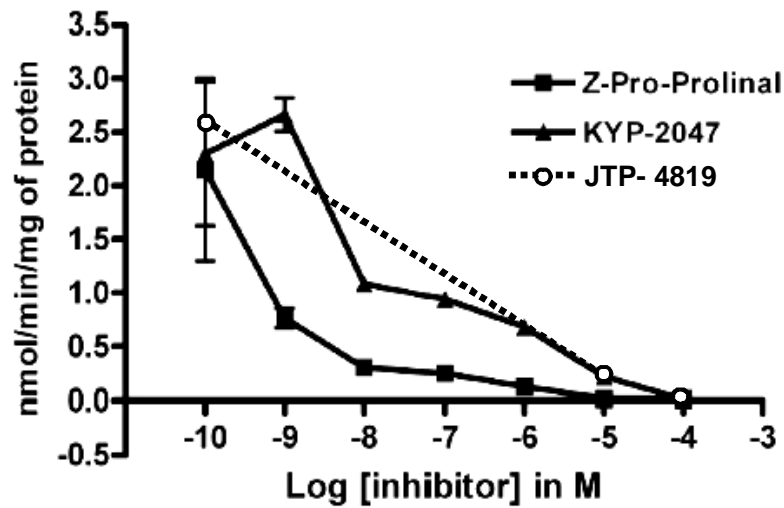


Figure S1. POP activities of SH-SY5Y cells treated with different concentrations of KYP-2047 and Z-Pro-Prolinal at 24 h timepoint. The experiment was repeated 3 times with duplicate samples. The results are expressed as mean \pm SD.

2. Microarray data.

Table SI. Up-regulated genes in SH-SY5Y cells in response to PREP inhibition.

Ratio	AFFY_ID	Gene Symbol	Gene Title
3,98	200908_s_at	RPLP2	Ribosomal protein, large, P2
3,91	213350_at	RPS11	Ribosomal protein S11
3,84	202648_at	RPS19	Ribosomal protein S19 pseudogene 3; ribosomal protein S19
2,62	213736_at	COX5B	Cytochrome c oxidase subunit Vb
2,59	M10098_M_at	GPR34	G protein-coupled receptor 34
2,56	213826_s_at	LOC100133109	Hypothetical similar to histone
2,55	1556316_s_at	LOC284889	Hypothetical protein LOC284889
2,27	228204_at	PSMB4	Proteasome (prosome, macropain) subunit, beta type, 4
2,26	213550_s_at	TMCO6	Transmembrane and coiled-coil domains 6
2,15	228251_at	UBXD1	UBX domain containing 1
2,07	214001_x_at	RPS10	Human ribosomal protein S10 mrna
2,05	213642_at	RPL27	Ribosomal protein L27
2,02	226014_at	EIF3F	Eukaryotic translation initiation factor 3, subunit F
2,02	212044_s_at	RPL27A	Ribosomal protein l27a
2,00	M27830_5_at	LOC100008589 /// MBOAT5 /// SOX18	Membrane bound O-acyltransferase domain containing 5 /// SRY (sex determining region Y)- box 18 /// 28S ribosomal RNA
1,88	218565_at	C9orf14	Chromosome 9 open reading frame 114
1,87	222968_at	C6orf48	Chromosome 6 open reading frame 48; small nucleolar RNA, C/D box 52
1,77	227744_s_at	HNRPD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kda)
1,76	224346_at	PRO1853	Homo sapiens PRO1853 mrna
1,76	233971_at	LOC401565	Similar to 4931415M17 protein
1,72	219138_at	RPL14	Ribosomal protein L14
1,71	213297_at	RMND5B	Required for meiotic nuclear division 5 homolog B (S. Cerevisiae)
1,71	213705_at	MAT2A	Methionine adenosyltransferase II, alpha
1,62	234464_s_at	EME1	Essential meiotic endonuclease 1 homolog 1 (S. Pombe)
1,62	221939_at	YIPF2	Yip1 domain family, member 2
1,60	222584_at	MSTO1	Similar to misato
1,59	213087_s_at	EEF1D	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)
1,56	213757_at	EIF5A	Eukaryotic translation initiation factor 5A
1,54	213296_at	RER1	RER1 retention in endoplasmic reticulum 1 homolog (S. Cerevisiae)
1,52	217734_s_at	WDR6	WD repeat domain 6
1,50	230894_s_at	MSI2	Musashi homolog 2
1,50	213813_x_at	FTL	Ferritin, light polypeptide
1,47	224680_at	TMED4	Transmembrane emp24 protein transport domain containing 4
1,46	224023_s_at	C3orf10	Chromosome 3 open reading frame 10
1,46	206352_s_at	PEX10	Peroxisome biogenesis factor 10
1,46	225990_at	BOC	Boc homolog (mouse)
1,46	216962_at	RPAIN	RPA interacting protein
1,45	219124_at	C8orf41	Chromosome 8 open reading frame 41
1,45	203679_at	TMED1	Transmembrane emp24 protein transport domain containing 1
1,45	1558702_at	TEX10	Testis expressed 10
1,44	213033_s_at	NFIB	Nuclear factor I/B
1,43	1552689_at	CASKIN1	CASK interacting protein 1
1,43	1565817_at	IKZF1	IKAROS family zinc finger 1 (Ikaros)
1,42	227299_at	CCNI	Cyclin I
1,41	216246_at	RPS20	Ribosomal protein S20
1,41	214041_x_at	RPL37A	Ribosomal protein l37a
1,40	49679_s_at	MT1P3	Metallothionein 1 pseudogene 3
1,39	229158_at	WNK4	WNK lysine deficient protein kinase 4

1,39	222487_s_at	RPS27L	Ribosomal protein S27-like
1,38	219788_at	PILRA	Paired immunoglobulin-like type 2 receptor alpha
1,38	213621_s_at	GUK1	Guanylate kinase 1
1,38	244150_at	SMC1A	Structural maintenance of chromosomes 1A
1,38	216609_at	TXN	Thioredoxin
1,37	1553538_s_at	COX1	Cytochrome c oxidase I
1,36	240821_at	PLCL1	Phospholipase C-like 1
1,35	209972_s_at	JTV1	JTV1 gene
1,34	234225_at	793658	Eukaryotic translation initiation factor 4H
1,34	221997_s_at	MRPL52	Mitochondrial ribosomal protein L52
1,34	229120_s_at	CDC42SE1	CDC42 small effector 1
1,34	AFFX-M27830_M_at	LOC100008589	28S ribosomal RNA
1,33	224062_x_at	KLK4	Kallikrein-related peptidase 4
1,33	238905_at	RHOJ	Ras homolog gene family, member J
1,33	1558109_x_at	LOC283788	Hypothetical protein LOC283788
1,33	203882_at	IRF9	Interferon regulatory factor 9
1,33	212356_at	KIAA0323	Kiaa0323
1,32	218808_at	DALRD3	DALR anticodon binding domain containing 3
1,32	202211_at	ARFGAP3	ADP-ribosylation factor gtpase activating protein 3
1,32	201836_s_at	SUPT7L	Suppressor of Ty 7 (S. Cerevisiae)-like
1,32	233135_at	---	CDNA clone IMAGE:4820713
1,32	232210_at	BCL2	Cell cycle/apoptotic gene
1,32	224823_at	MYLK	Myosin, light chain kinase
1,32	227760_at	IGFBPL1	Insulin-like growth factor binding protein-like 1
1,32	225444_at	FLJ25778	Hypothetical protein FLJ25778
1,31	213179_at	RQCD1	RCD1 required for cell differentiation1 homolog (S. Pombe)
1,31	206767_at	RBMS3	RNA binding motif, single stranded interacting protein
1,31	226216_at	INSR	Insulin receptor
1,31	203487_s_at	ARMC8	Armadillo repeat containing 8
1,31	235815_at	TSHZ2	Teashirt zinc finger homeobox 2
1,31	1555895_at	DNM2	Dynamin 2
1,31	216131_at	FRMD4B	FERM domain containing 4B
1,30	233332_at	---	Clone IMAGE:730443, mrna sequence
1,30	238644_at	MYSM1	Myb-like, SWIRM and MPN domains 1
1,30	221915_s_at	RANBP1	RAN binding protein 1

Table SII. Down regulated genes in SH-SY5Y in response to PREP inhibition.

Ratio	AFFY_ID	Gene Symbol	Gene Title
-1,54	202570_s_at	DLGAP4	Discs, large (Drosophila) homolog-associated protein 4
-1,49	227270_at	LOC285550	Hypothetical protein LOC285550
-1,49	211162_x_at	SCD	Stearoyl-coa desaturase (delta-9-desaturase)
-1,47	1552641_s_at	ATAD3A	Atpase family, AAA domain containing 3A
-1,47	207143_at	CDK6	Cyclin-dependent kinase 6
-1,45	211019_s_at	LSS	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)
-1,44	238347_at	MGC15523	Hypothetical protein MGC15523
-1,43	209512_at	HSDL2	Hydroxysteroid dehydrogenase like 2
-1,43	204120_s_at	ADK	Adenosine kinase
-1,43	214151_s_at	CCPG1	Cell cycle progression 1
-1,42	223126_s_at	C1orf21	Chromosome 1 open reading frame 21
-1,42	221078_s_at	CCDC88A	Coiled-coil domain containing 88A
-1,41	206943_at	TGFBR1	Transforming growth factor, beta receptor I
-1,41	227296_at	MFSD3	Major facilitator superfamily domain containing 3
-1,41	207702_s_at	MAGI2	Membrane associated guanylate kinase, WW and PDZ domain containing 2
-1,41	218197_s_at	OXR1	Oxidation resistance 1
-1,41	229399_at	C10orf118	Chromosome 10 open reading frame 118
-1,41	204728_s_at	WDHD1	WD repeat and HMG-box DNA binding protein 1
-1,41	235287_at	CDK6	Cyclin-dependent kinase 6
-1,40	227741_at	PTPLB	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
-1,40	212150_at	EFR3A	EFR3 homolog A (S. Cerevisiae)
-1,40	206527_at	ABAT	4-aminobutyrate aminotransferase
-1,40	235463_s_at	LASS6	LAG1 homolog, ceramide synthase 6
-1,40	1553106_at	C5orf24	Chromosome 5 open reading frame 24
-1,40	229068_at	CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)
-1,39	229010_at	CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence
-1,39	215649_s_at	MVK	Mevalonate kinase (mevalonic aciduria)
-1,39	235459_at	---	Transcribed locus
-1,38	227347_x_at	HES4	Hairy and enhancer of split 4 (Drosophila)
-1,38	242690_at	ATP11C	Atpase, class VI, type 11C
-1,38	215739_s_at	TUBGCP3	Tubulin, gamma complex associated protein 3
-1,38	210973_s_at	FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
-1,37	1554249_a_at	ZNF638	Zinc finger protein 638
-1,37	218433_at	PANK3	Pantothenate kinase 3
-1,37	227669_at	BRP44	Brain protein 44
-1,37	201626_at	INSIG1	Insulin induced gene 1
-1,37	243000_at	CDK6	Cyclin-dependent kinase 6
-1,36	213388_at	LOC727942	Similar to phosphodiesterase 4D interacting protein isoform 2
-1,36	1556283_s_at	FGFR1OP2	FGFR1 oncogene partner 2
-1,36	1568763_s_at	PDCD6	Programmed cell death 6
-1,36	227812_at	TNFRSF19	Tumor necrosis factor receptor superfamily, member 19
-1,36	66053_at	HNRPUL2	Heterogeneous nuclear ribonucleoprotein U-like 2
-1,36	1554780_a_at	PHTF2	Putative homeodomain transcription factor 2
-1,36	237215_s_at	TFRC	Transferrin receptor (p90, CD71)
-1,36	221851_at	LOC90379	Hypothetical protein BC002926
-1,35	1558279_a_at	FVT1	Follicular lymphoma variant translocation 1
-1,35	44040_at	FBXO41	F-box protein 41
-1,35	230213_at	C19orf43	Chromosome 19 open reading frame 43
-1,35	231300_at	LOC90835	Hypothetical protein LOC90835
-1,35	209227_at	TUSC3	Tumor suppressor candidate 3
-1,35	219268_at	ETNK2	Ethanolamine kinase 2

-1,35	218783_at	INTS7	Integrator complex subunit 7
-1,35	1555832_s_at	KLF6	Kruppel-like factor 6
-1,34	217663_at	---	Transcribed locus
-1,34	1570393_at	EML5	Echinoderm microtubule associated protein like 5
-1,34	1552812_a_at	SENP1	SUMO1/sentrin specific peptidase 1
-1,34	228333_at	---	Full length insert cdna clone YT94E02
-1,34	209083_at	CORO1A	Coronin, actin binding protein, 1A
-1,34	231090_s_at	ARID2	AT rich interactive domain 2 (ARID, RFX-like)
-1,34	235189_at	NARG2	NMDA receptor regulated 2
-1,34	202056_at	KPNA1	Karyopherin alpha 1 (importin alpha 5)
-1,34	208319_s_at	RBM3	RNA binding motif (RNP1, RRM) protein 3
-1,34	235931_at	---	Transcribed locus
-1,34	244688_at	---	Transcribed locus
-1,33	212902_at	SEC24A	SEC24 related gene family, member A (S. Cerevisiae)
-1,33	211139_s_at	NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)
-1,33	203242_s_at	PDLIM5	PDZ and LIM domain 5
-1,33	201791_s_at	DHCR7	7-dehydrocholesterol reductase
-1,33	221280_s_at	PARD3	Par-3 partitioning defective 3 homolog (C. Elegans)
-1,33	221845_s_at	CLPB	Clpb caseinolytic peptidase B homolog (E. Coli)
-1,33	204633_s_at	RPS6KA5	Ribosomal protein S6 kinase, 90kda, polypeptide 5
-1,33	235497_at	LOC643837	Hypothetical protein LOC643837
-1,33	214156_at	MYRIP	Myosin VIIA and Rab interacting protein
-1,33	229705_at	---	Transcribed locus
-1,33	210896_s_at	ASPH	Aspartate beta-hydroxylase
-1,33	210281_s_at	ZMYM2	Zinc finger, MYM-type 2
-1,33	219793_at	SNX16	Sorting nexin 16
-1,33	225412_at	---	Full-length cdna clone CS0DL008YP09 of B cells (Ramos cell line) Cot 25-normalized
-1,33	1554311_a_at	SUPT6H	Suppressor of Ty 6 homolog (S. Cerevisiae)
-1,33	221848_at	ZGPAT	Zinc finger, CCCH-type with G patch domain
-1,32	1552264_a_at	MAPK1	Mitogen-activated protein kinase 1
-1,32	214727_at	BRCA2	Breast cancer 2, early onset
-1,32	204088_at	P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4
-1,32	244486_at	---	FLJ00387 protein
-1,32	204325_s_at	NF1	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)
-1,32	235688_s_at	TRAF4	TNF receptor-associated factor 4
-1,32	225605_at	TP53I13	Tumor protein p53 inducible protein 13
-1,32	209753_s_at	TMPO	Thymopoietin
-1,32	211543_s_at	GRK6	G protein-coupled receptor kinase 6
-1,32	36711_at	MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
-1,31	230939_at	---	---
-1,31	203377_s_at	CDC40	Cell division cycle 40 homolog (S. Cerevisiae)
-1,31	1562699_at	NCAM1	Neural cell adhesion molecule 1
-1,31	211214_s_at	DAPK1	Death-associated protein kinase 1
-1,31	1554152_a_at	OGDH	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)
-1,31	209355_s_at	PPAP2B	Phosphatidic acid phosphatase type 2B
-1,31	1560562_a_at	ZNF677	Zinc finger protein 677
-1,31	214331_at	TFSM	Ts translation elongation factor, mitochondrial
-1,31	229114_at	---	CDNA clone IMAGE:4801326
-1,31	1555561_a_at	UGCGL2	UDP-glucose ceramide glucosyltransferase-like 2
-1,31	214546_s_at	P2RY11	Purinergic receptor P2Y, G-protein coupled, 11
-1,31	222220_s_at	TSNAXIP1	Translin-associated factor X interacting protein 1
-1,31	206233_at	B4GALT6	UDP-Gal:betaglcnac beta 1,4- galactosyltransferase, polypeptide 6
-1,31	1554633_a_at	MYT1L	Myelin transcription factor 1-like
-1,31	213555_at	RWDD2A	RWD domain containing 2A

-1,31	239792_at	LOC440288	Similar to FLJ16518 protein
-1,30	201627_s_at	INSIG1	Insulin induced gene 1
-1,30	204036_at	EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
-1,30	203845_at	PCAF	P300/CBP-associated factor
-1,30	228302_x_at	CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1
-1,30	222846_at	RAB8B	RAB8B, member RAS oncogene family
-1,30	1564601_at	LOC286123	Hypothetical protein LOC286123
-1,30	214231_s_at	KIAA0564	Kiaa0564
-1,30	208425_s_at	TANC2	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2

3. QRT-PCR validation of microarray results.

Cells were cultured under identical conditions as those used for the microarray experiment, but now we incubated the cells with two PREP inhibitors, JTP-4819 and ZPP, at 10 μ M. The results for JTP are show in figure S2. RNA from the cells used in the microarray study was used for verification of the microarray data. Reverse transcription (RT) was performed (iScriptTM cDNA Synthesis Kit, Biorad, California, USA) with random hexamer primers. PCR was performed in the MiniOpticon cyclor (BioRad, California, USA) with the iQ SYBR Green kit (BioRad, California, USA) following the manufacturers' protocol. Equivalent amounts of RNA from several treated samples were pooled to create standard curves (input RNA 1–16ng) that were assayed in parallel with replicate samples (5ng RNA) from control. A linear standard curve was generated and all samples fell within the range of the curve. For sample normalization, individual transcript values were divided by the β -actin values determined using the same RT reaction. β -Actin transcript levels did not differ between treated and control cells ($p=0.85$) or microarray ($p=0.10$). Differences in transcript abundance between treated and controls were assessed by paired t-tests. The analysis was performed on 2 of 3 pairs of samples. The PCR primers used for this study were: ribosomal S19 mRNA, forward (5'- AGG CCG CAC GAT GCC TGG AGT TAC TGT AAA AGA - 3') and reverse primer (5'- GCC AAC AAG AAG CAT TAG AAC AAA CCA TGC - 3'); elongation factor-1 delta, forward (5'-CCC GCG TCC GCC GAT TCC TC-3') and reverse primer (5'-CGC TGG CGC CGT TCT CCT G-3'); G protein-coupled receptor 34, forward (5'-GGT TCT GCT CCT TTT ACT TCA GGC G-3') and reverse primer (5'-AAA TAC ATA GAG GGC GAT TAT GTT CCC AAC CAG-3'); eukaryotic translation initiation factor 5A, forward (5'-TGC CAA GGT CCA TCT GGT T-3') and reverse (5'- CAG CTG CCT CCT CTG TCA TG-3'); fibroblast growth factor receptor 1, forward (5'- ACG GCC GAC TGC CTG TGA AG-3') and reverse (5'-AGC TCC GGG TGT CGG GAA AG-3'); MAPK1 (ERK2), forward (5'-GTT CCC AAA TGC TGA CTC CAA-3', and reverse (5'-CTC GGG TCG TAA TAC TGC TCC-3'); β -actin forward (5'-CTG GGA CGA CAT GGA GAA AA-3')and reverse (5'-AAG GAA GGC TGG AAG AGT GC-3').

Results are presented in Fig. S2.

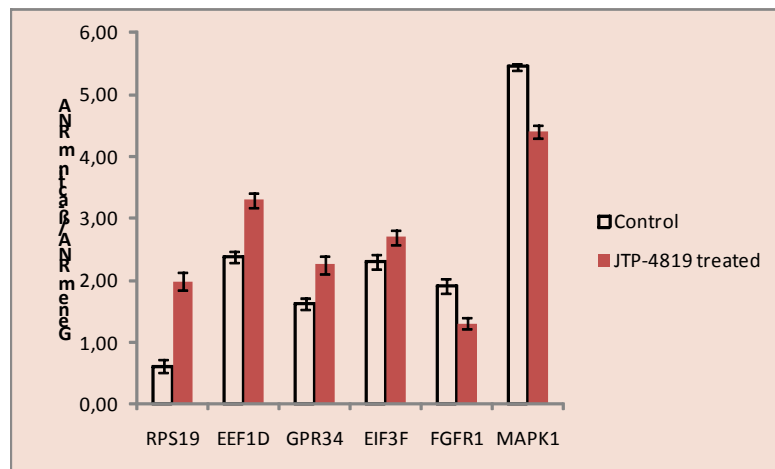


Figure S2. qRT-PCR validation of six RNAs differentially expressed between PREP inhibited (JTP-4819, red bars) and control (white bars). RNA abundances were corrected for β -actin transcript levels

in the same sample and RT reaction. White bars control, red bars treated. For all target genes, $p < 0.05$ by paired t-test. Gene/Actin arbitrary units. A similar trend was obtained using ZPP (not shown).

The results from the qrtPCR experiment are in accordance with the data obtained by microarray. Figure S3 shows the correlation of these results ($R^2 = 0.8794$) and confirms the conclusions from the array data.

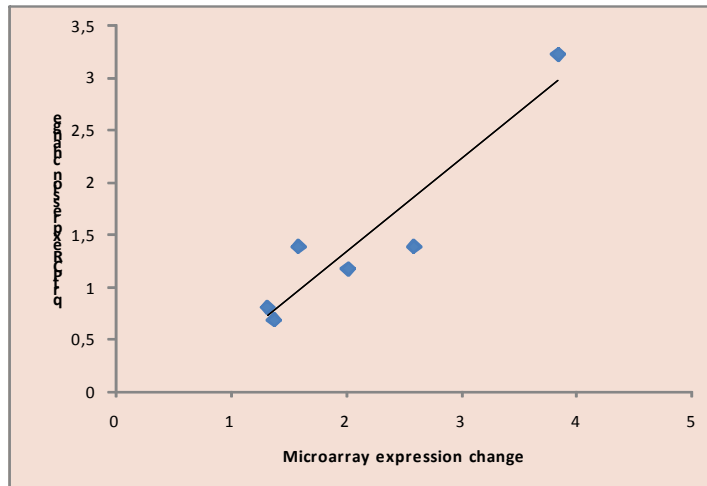


Figure S3. Correlation between the data obtained from Microarray analysis (Tables IS and 2S) and the data obtained by qrtPCR of the same transcripts plotted in Fig 1S.

4. Statistical analysis of morphological features during differentiation of SH-SY5Y cells upon retinoic acid treatment.

Cells were cultured in the conditions as stated in Materials and Methods section in the main text

Number of differentiated neurons (Fig. 5.A)

Poisson regression of number of differentiated neurons to the variable control versus treatment (tto) to evaluate POP inhibitor effect.

Parameters and results of the analysis on R project for statistical computing (<http://www.R-project.org>):

```
> neuronas.glm <- glm(Neuronas ~ tto, data=cnn, family=poisson())
> summary(neuronas.glm)
Call:
glm(formula = Neuronas ~ tto, family = poisson(), data = cnn)
```

Deviance Residuals:

Min	1Q	Median	3Q	Max
-2.1499	-1.8135	-0.5423	0.4330	5.2019

Coefficients:

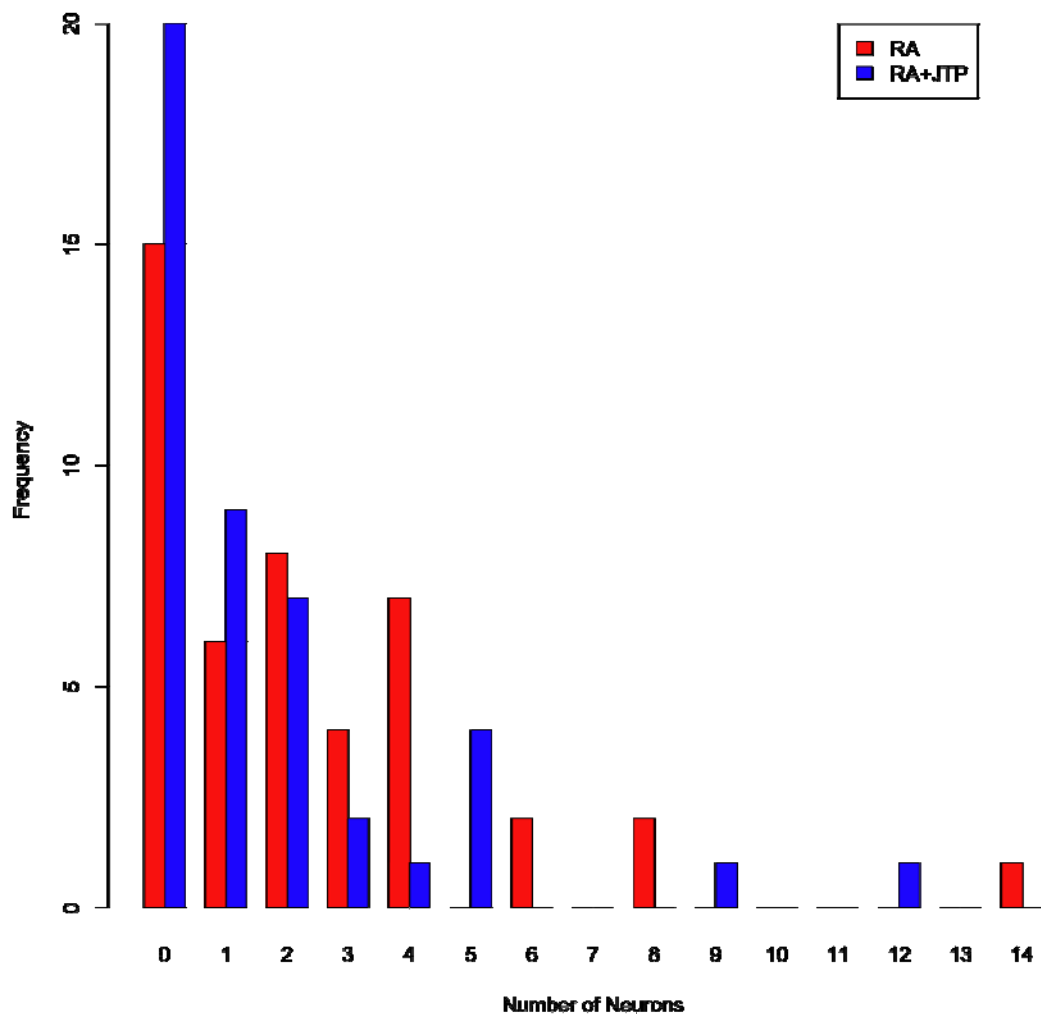
	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	0.83773	0.09806	8.543	<2e-16 ***
ttoTreatment	-0.34033	0.15208	-2.238	0.0252 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for poisson family taken to be 1)

Null deviance: 274.08 on 89 degrees of freedom
Residual deviance: 269.00 on 88 degrees of freedom
AIC: 428.28

Number of Fisher Scoring iterations: 6



Number of neurites (Fig. 5.B)

Poisson regression of number of neurites to the variable control versus treatment (tto) to evaluate POP inhibitor effect.

Parameters and results of the analysis on R project for statistical computing (<http://www.R-project.org>):

```
> neurites.glm <- glm(Neurites.total ~ tto, data=cnn, family=poisson())  
> summary(neurites.glm)
```

Call:

```
glm(formula = Neurites.total ~ tto, family = poisson(), data = cnn)
```

Deviance Residuals:

Min	1Q	Median	3Q	Max
-3.3133	-3.0984	-1.0232	0.9868	9.3129

Coefficients:

	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	1.70273	0.06363	26.76	<2e-16 ***
ttoTreatment	-0.13411	0.09316	-1.44	0.15

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

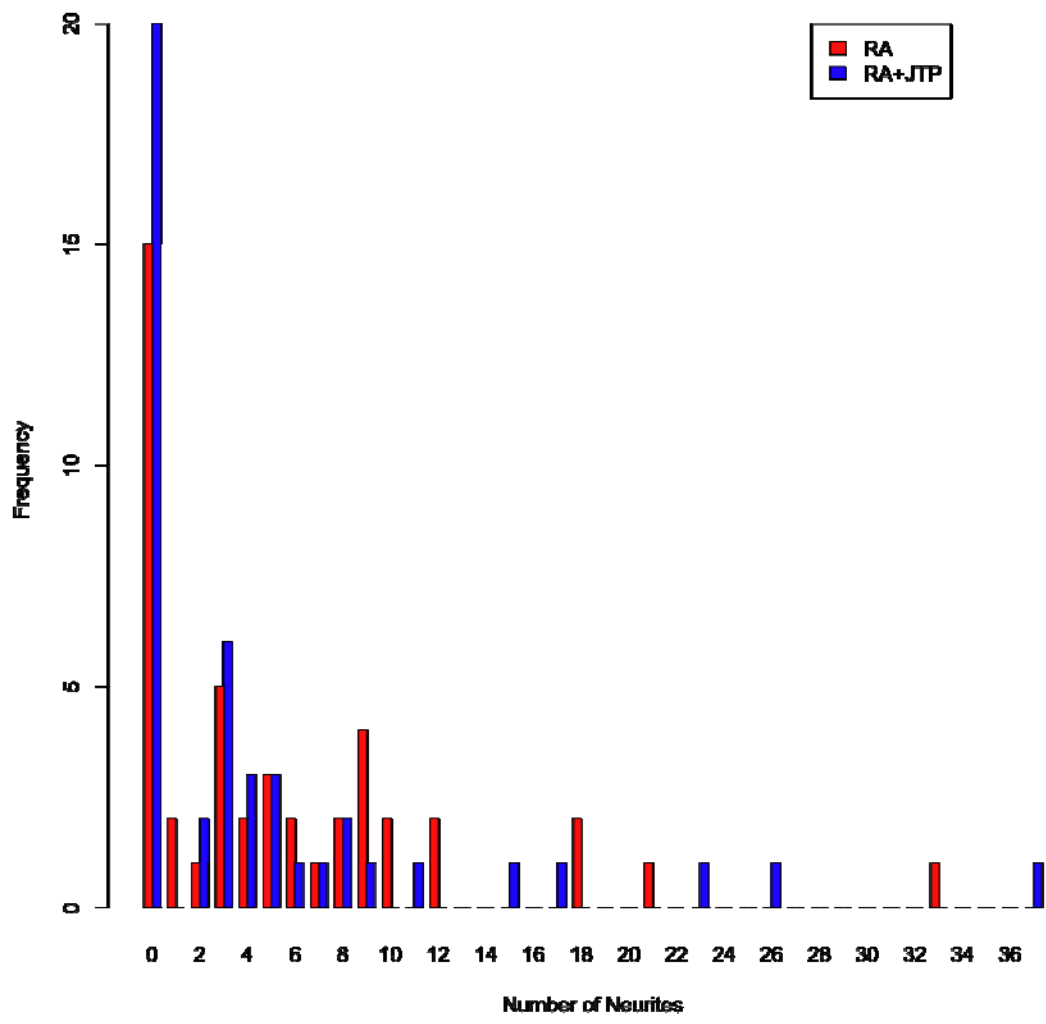
(Dispersion parameter for poisson family taken to be 1)

Null deviance: 755.27 on 89 degrees of freedom

Residual deviance: 753.19 on 88 degrees of freedom

AIC: 959.48

Number of Fisher Scoring iterations: 6



Neurite length (Fig. 5.A)

Gamma regression of neurite length (relative to cell soma size) to the variable control versus treatment (tto) to evaluate POP inhibitor effect.

Parameters and results of the analysis on R project for statistical computing (<http://www.R-project.org>):

```
> long.glm <- glm(Long ~ Sample, data=long.tr, weights, family=Gamma())  
> summary(long.glm)
```

Call:

```
glm(formula = Long ~ Sample, family = Gamma(), data = long.tr)
```

Deviance Residuals:

Min	1Q	Median	3Q	Max
-3.11484	-1.09053	0.04042	0.68069	2.65541

Coefficients:

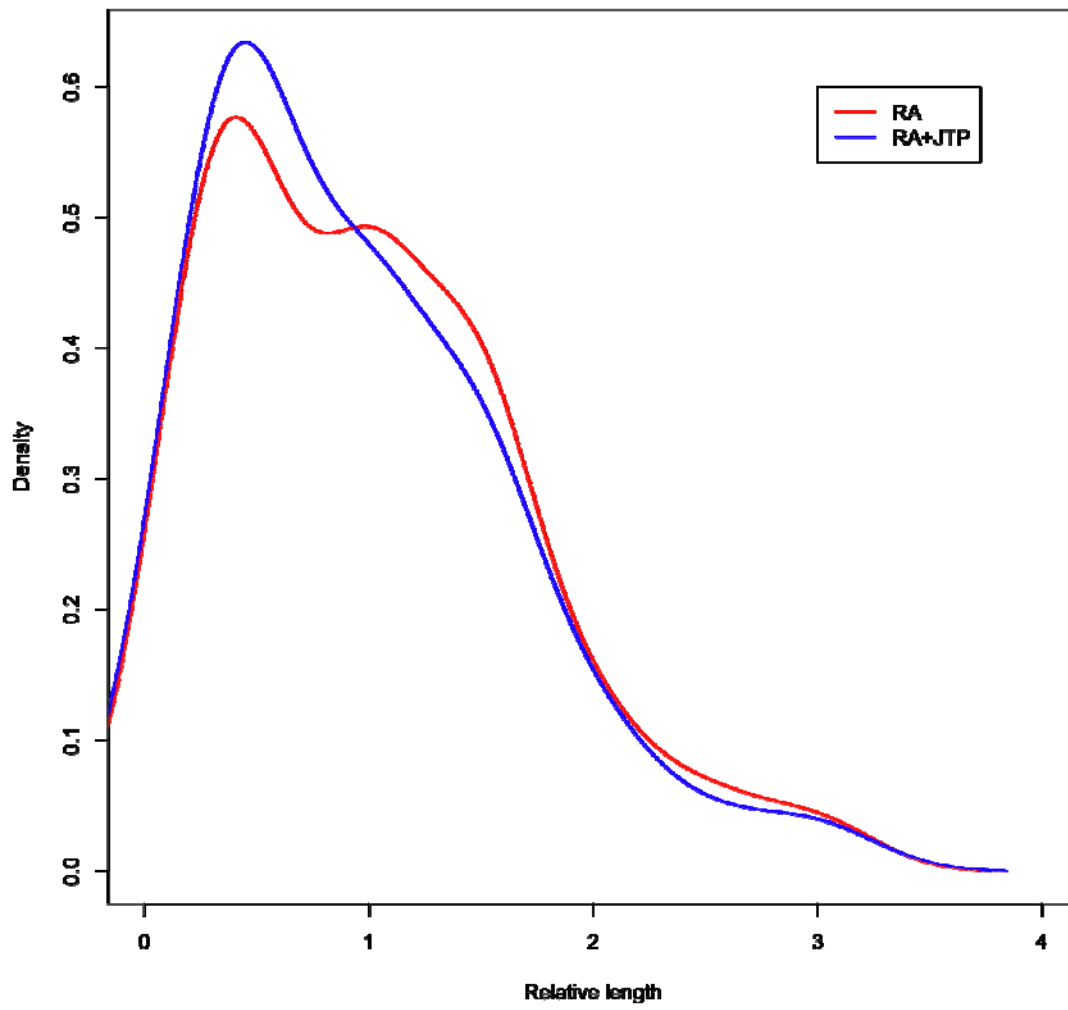
	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	0.98998	0.06983	14.178	<2e-16 ***
MuestraAR+JTP	0.05098	0.10507	0.485	0.628

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for Gamma family taken to be 1.228821)

Null deviance: 235.23 on 174 degrees of freedom
Residual deviance: 234.94 on 173 degrees of freedom
AIC: 797.37

Number of Fisher Scoring iterations: 6

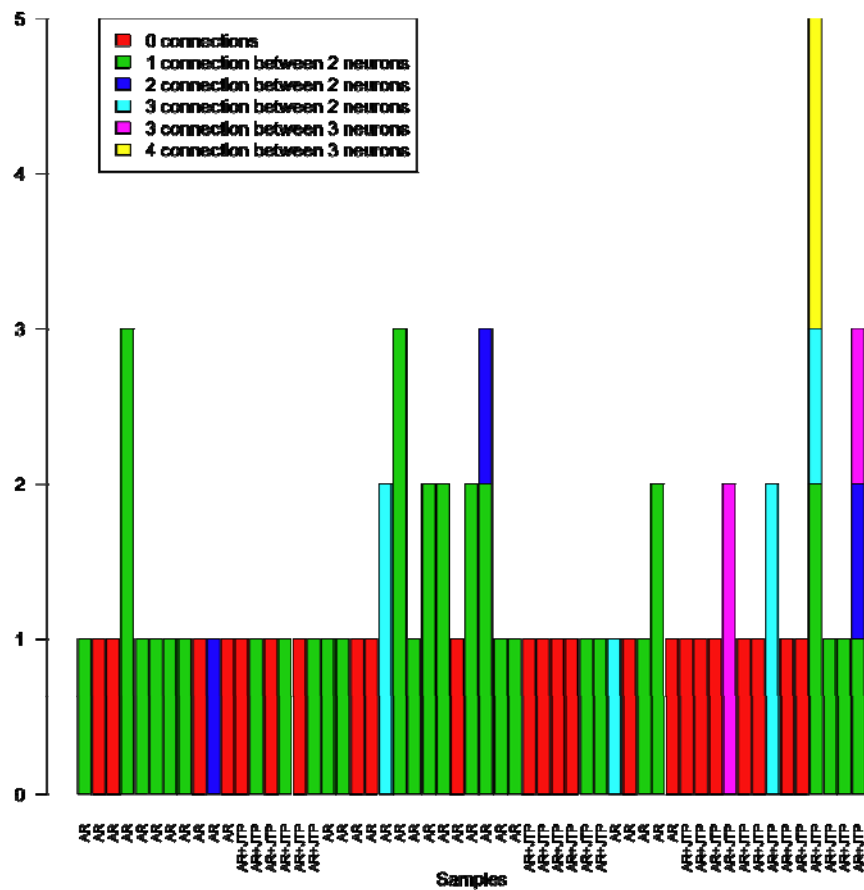


Number of connections per cell (Fig. 6)

Logistic multinomial regression of the distribution of cell connectivity considering the categories indicated in the table of results, relative to the treatment. Reference was the AR (retinoic acid) group.

Parameters and results of the analysis on R project for statistical computing (<http://www.R-project.org>):

	Coeff (AR+JTP)	SE	Pvalue
1 connection between 2 neurons	-1.44	0.57	0.01
2 connections between 2 neurons	-1.14	1.30	0.019
3 connections between 2 neurons	-0.44	0.92	0.032
3 connections between 3 neurons	10.50	137.36	0.047
4 connections between 3 neurons	9.95	127.49	0.047



5. Levels of PREP activity in control cells and after incubation with PREP inhibitors

The effect of the presence of PREP inhibitor in the culture media on the endogenous PREP of SH-SY5Y cells was tested using ZPP, JTP-4819 and KYP-2048. In all cases, the activity measured in the cells cultured in the presence of the inhibitor was less than 2% of that measured from cells cultured under control conditions. The data for JTP is presented in figure S4.

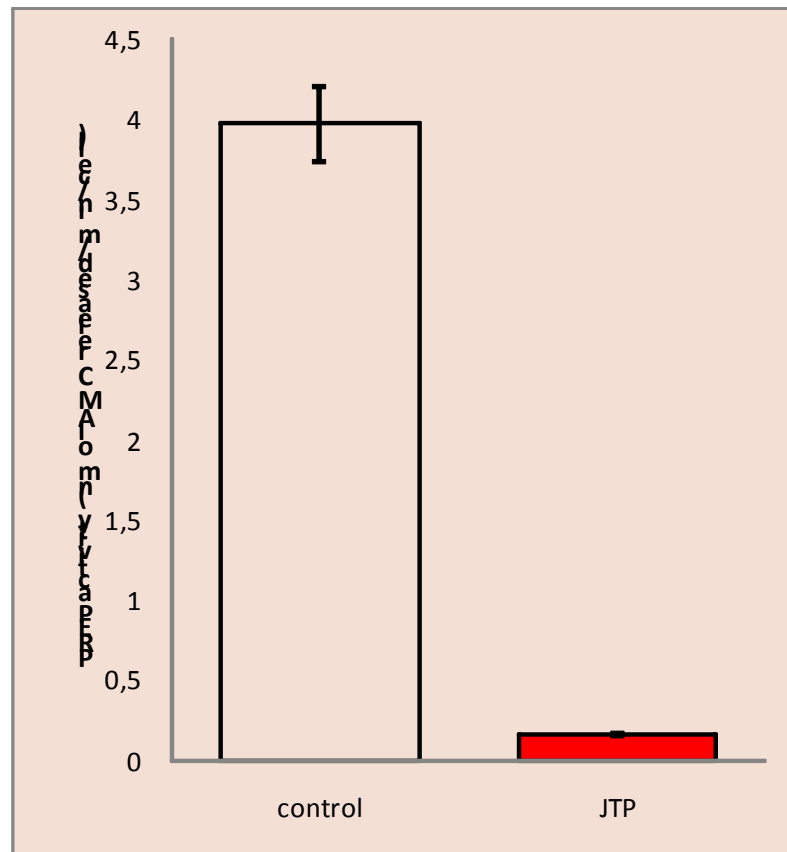


Figure S4. Representative figure of the effect of PREP inhibitors (10 μ M) in the culture medium over activity measured from cell lysates after 15 day incubation without (control) and with inhibitor. Data for JTP-4819 is shown. Data is from three different cell cultures (10^6 cells) conducted in duplicate. The activity assay was performed as stated in the main manuscript's materials and methods.

6. Rationale for selection of PREP inhibitor concentrations.

Besides the evidence presented in the control experiments in Figures S1 and S4, to ascertain the degree and specificity of PREP inhibition under the conditions described in this work, we would like to state that at the concentrations used in this study, KYP-2047, JTP-4819 and ZPP, specifically inhibit PREP without affecting the activity of other proline specific proteases (DPP2, DPP4, DPP8, DPP9, α -FAP, aminopeptidase P, prolidase, proline imino/aminopeptidase, and FKBP12 (data not shown) nor other serine proteases (Venäläinen, PhD Thesis, University of Kuopio, 2005). The PREP inhibitor concentrations needed to achieve 50 % inhibition of endogenous PREP activity in SH-SY5Y cells are slightly higher, especially those of KYP-2047, than previously determined IC₅₀ values in brain homogenates. This may be due to poor penetration of these inhibitors through the SH-SY5Y cell membrane or by an active pumping out from cells. The inhibitor concentrations used are comparable with those estimated in previous experiments in rodents ([1-3]). Up to 100 to 200 μ M of inhibitor did not affect the viability of SH-SY5Y cells in culture (AM Lambeir, personal communication).

References

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- [3] Jalkanen AJ; Puttonen KA; Venäläinen JI; Sinerva V; Mannila A; Ruotsalainen S; Jarho EM; Wallen EA; Mannistö PT. Beneficial effect of prolyl oligopeptidase inhibition on spatial memory in young but not in old scopolamine-treated rats. *Basic Clin Pharmacol Toxicol* **2007**, 100, (2), 132-8.