Supplementary Information for

Nasally-delivered VEGFD mimetics mitigate stroke-induced dendrite loss and brain damage

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Methods relative to Supplementary Figures

Primary astrocyte cultures

Neurons and glial cells from new born C57BL/6N mice were isolated as described (1). Medium was changed 2.5 h after plating to DMEM supplemented with 10% FCS and Pen-Strep. Every 3 days, cultures were washed with ice-cold PBS to promote death and detachment of neuronal cells, and growth of astrocytes. When confluent, cultures were treated with 20 μM NMDA and then harvested for qRT-PCR analyses.

bEnd.3 cells culture

bEnd.3 cells were cultured in high-glucose-containing (4.5 g glucose/liter) Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, California, USA) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, Kawasaki, Kanagawa, Japan) on 0.1% gelatin coated flasks. When confluent, bEnd.3 cells were treated with 20 μM NMDA and then harvested for qRT-PCR analyses.

Oxygen-glucose-deprivation (OGD)

OGD was carried out as described (2). In brief, cells were washed with deoxygenated glucose-free salt solution (containing 140.1 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 10 mM glycine, and 0.5 mM sodium pyruvate) and then immediately transferred to an anaerobic chamber containing a 5% CO_2/95% N_2 atmosphere.

Quantification of vessel surface and length

Mice received intravenous injection of fluorescein-conjugated dextran (Thermo Fisher). After 15 min, mice were euthanized by intraperitoneal injection with 400 mg/kg BW pentobarbital (Narcoren; Merial GmbH, Hallbergmoos, Germany). Brains were quickly extracted and high-resolution pictures of the brains were taken. Pictures were used for quantification of the vessels

visible on the surface of brains. Quantification was perfomed using Fiji/ImageJ. Brains were cryosectioned and random images of FITC-dextran labeled vessels were acquired using a Leica SP2 confocal laser scanning SP2 microscope. Vessel surface in cortical coronal sections was measured with ImageJ/Fiji. Relative values were calculated by normalizing the mean values in the MCAO-aCSF mice.

Screening of peptide library

The efficiency of synthesized peptides in producing the same biological effects of recombinant VEGFD in neuronal cells was tested as follows: DIV10 hippocampal neurons were either left untreated or treated for 2 h with 7.7nM of rmVEGFD or with a VEGFD-mimetic peptides. The concentrations used were chosen based on previous results obtained with rmVEGFD (3-5). Neurons were harvested in boiling SDS sample buffer (160 mM Tris-HCl (pH 6.8), 4 % SDS, 30% glycerol, 10mM dithiothreitol, 0.02% bromophenolblue) and the lysates subjected to immunoblot analysis using antibodies specific for the phosphorylated (i.e., activated) form of p38 MAP kinase. In addition, samples were also tested for the activity of GSK-3 α/β; α-tubulin was used as loading control. Because GSK-3 α/β activity is not altered by treatment with rmVEGFD (3), all peptides that decrease or increase GSK-3 α/β activity by 25% or more compared to untreated controls were excluded. Positive selection of peptides required that their induction of p38 MAP kinase activity is at least as strong as that induced by rmVEGFD, which is know to activate p38 MAP kinase signaling (3). Antibodies used: Mouse anti-tubulin (Sigma Aldrich; T9026), HRP conjugated goat anti rabbit igG (Dianova, 111-035-144; Lot N 94406), HRP conjugated goat anti mouse igG (Dianova, 715-035-150; Lot N 85097), mouse monoclonal anti-phospho-p38 MAP kinase antibody (BD Biosciences 612288); rabbit polyclonal anti phospho-GSKα/β (Cell Signaling Technology 9331).

Morphometric analyses

The six most potent peptides (FMP6, FMP21, FMP59, FMP60, FMP65, FMP69) were tested for their efficacy to modulate dendritic length and complexity. It is known that blocking nuclear calcium signalling by over-expressing CaMBP4 (Calcium/ Calmodulin Binding Peptide 4) results in a decrease of dendritic length and complexity which can be rescued to normal levels by treating neurons with rmVEGFD (3). Primary hippocampal neurons were transfected on DIV8 with hrGFP (to visualize the entire dendritic arborization) and, when indicated, also with CaMBP4 to cause an impairment in the dendritic tree. At DIV10, neurons were treated with the indicated peptide or rmVEGFD (7.7nM). For morphometric analyses, neurons were analyzed 5 days after transfection. Total dendritic length was calculated using Fiji (6). Briefly, a z-stack acquisition was imported, calibrated and manually traced using the simple neurite tracer plugin (7). Total dendritic length was then computed. All analyses were performed blind. For each condition, 8 neurons from 3 independent preparations were analyzed.

In vitro toxicity assay

DIV10 primary hippocampal neurons were either left untreated or treated with increasing concentrations of FMP6 or FMP69 (7.7 nM, 100 nM, 1000 nM, 10000 nM). After 24h, cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS and counterstained with Hoechst 33258 (1 µg/ml) for 10 min. Cells were mounted in mowiol and examined by fluorescence microscopy. Dead neurons were identified by condensed chromatin and shrunken nuclei.

Legends to Supplementary Figures

Supplementary Figure 1

Characterization of eNMDAR-mediated VEGFD down-regulation

(A) QRT-PCR analysis of *VEGFD* expression in cultured hippocampal neurons with or without treatment of NMDA for 4 h at the indicated concentrations. N=3. **(B)** QRT-PCR analysis of *VEGFD* expression in cultured hippocampal neurons with or without treatment of NMDA for 10 min, 30' or 4 h. All cultures were harvested 4 h after the beginning of the NMDA exposure. N=3. **(C)** QRT-PCR analysis of *VEGFD* expression in cultured hippocampal neurons with or without treatment with actinomycin D (10µg/ml) followed by 20µM NMDA treatment for the indicated times. N=3. **(D)** QRT-PCR analysis of *cFos* expression in cultured hippocampal neurons with or without treatment of 50µM bicuculline/250 µM 4-amino pyridine (Bic/4AP) or 20 µM NMDA for the indicated times. N=3. **(E)** QRT-PCR analysis of *VEGFC*, *VEGF* or *flt4* expression in cultured hippocampal neurons with or without treatment of 20 µM NMDA for the indicated times. N=3. **(F)** QRT-PCR analysis of *VEGFD*, *VEGF* or *flt4* expression in cultured hippocampal neurons with or without OGD treatment. N=3. **(G)** QRT-PCR analysis of *VEGFD*, *VEGF* and *flt4* expression in contralateral cortex of mice, which underwent MCAO or sham surgery 24 h after surgery. **(H)** QRT-PCR analysis of *VEGFD*, *VEGF* and *flt4* expression in ipsilateral hippocampi of mice that underwent MCAO or sham surgery 24 h after surgery. N=6. **(I)** QRT-PCR analysis of *VEGFD, cFos, VEGFC, VEGF* and *flt4* expression in cultured bEnd.3 cells with or without treatment of 20 µM NMDA for 1, 2 or 4 h. Cultures were harvested 4 h after NMDA exposure (N=3). **(J)** QRT-PCR analysis of *VEGFD, cFos, VEGFC, VEGF* and *flt4* expression in primary astrocytes with or without treatment of 20 µM NMDA for 1, 2 or 4 h. All cultures were harvested 4 h after the beginning of the NMDA exposure (N=3). **(K)** Length of superficial vessels on the brains of mice which underwent MCAO surgery and i.c.v.-injected with aCSF or rmVEGFD (N=3). **(L)** Surface of vessels in coronal sections from brains of mice which underwent MCAO surgery and i.c.v.-injected with aCSF or rmVEGFD. Vessels were labeled with FITC-dextran. (N=3). A,B,C,D,E,I,J:

one-way ANOVA with Dunnett's correction for multiple comparisons; F,G,H,K,L: unpaired t-test. * p<0.05; ** p<0.01; *** p<0.001; **** p< 0.0001. Plotted data show mean ± SEM and individual values.

Supplementary Figure 2

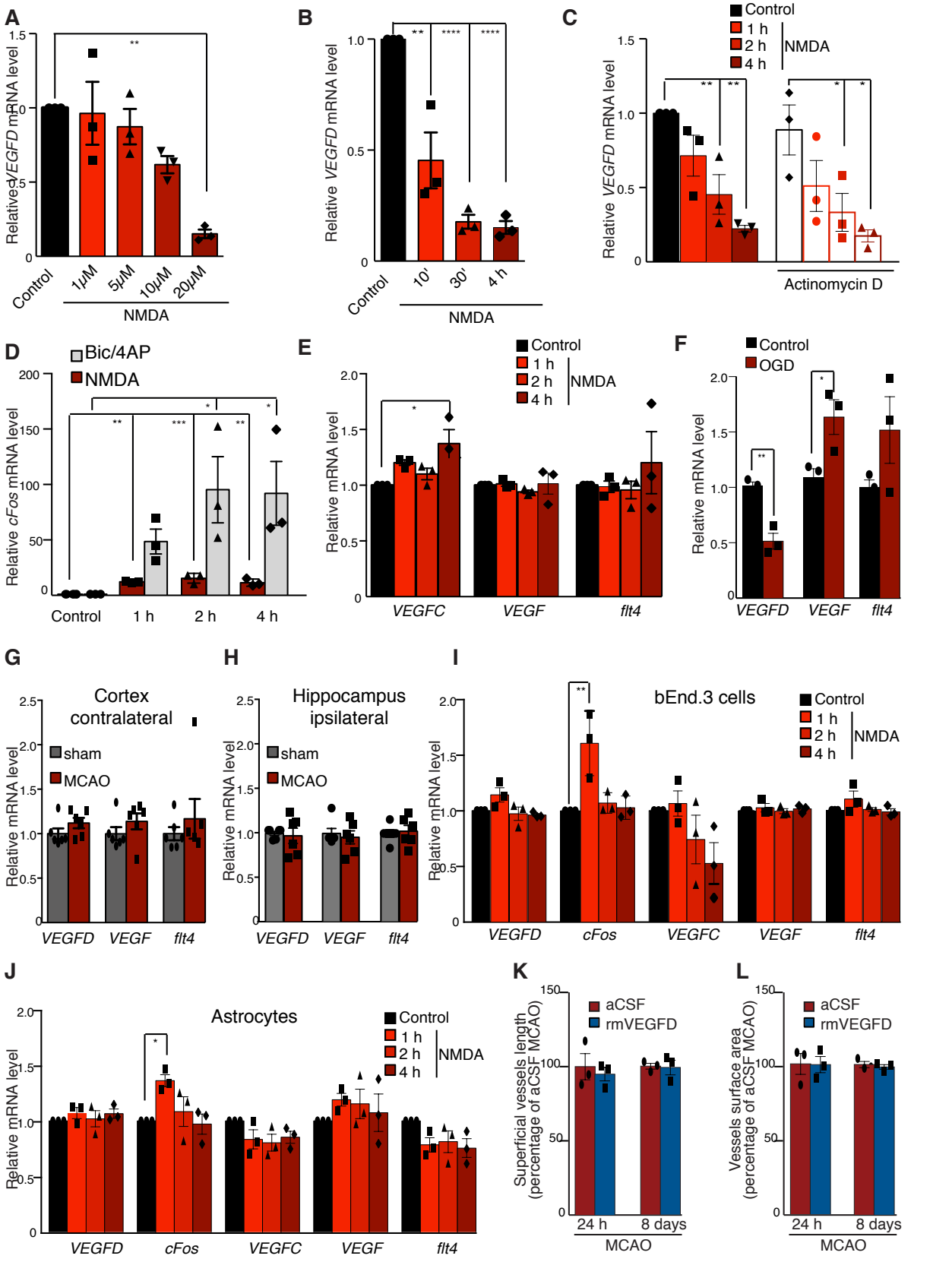
Design and screening of the VEGFD peptide mimetics.

Table representing the sequences and screening of the VEGFD peptide analogs designed based on the identified sequence motif of wild type VEGFD (WT) responsible for interaction with VEGFR3. Each peptide is named "FMP" followed by increasing numbers (column FMP id). Amino acids different from WT are shaded. Aib = alpha-amino-isobutyric acid (aib); gpn = gabapentine, two non-natural amino acids. Column "pGSK": levels of phosphorylated (i.e., activated) GSK-3 α/β in lysates from cultured hippocampal neurons treated with peptide (7.7 nM, 2 h) normalized to untreated controls. Since rmVEGFD treatment does not alter GSK-3 α/β activity in neurons (3), all peptides that decrease or increase GSK-3 α/β activity by 25% or more compared to control were excluded. Peptides that passed the negative selection are shaded in green. Column "pp38": phosphorylated (i.e., activated) form of p38 MAP kinase in lysates from cultured hippocampal neurons treated with peptide (7.7 nM, 2 h) normalized to untreated controls. RmVEGFD was used as positive control. P38 MAP kinase is a key player in the process that links VEGFD signaling to dendrite architecture (3). Peptides inducing p38 MAP kinase activity to at least the same extent as rmVEGFD are shaded in light blue. Column "morpho": The six most potent peptides (FMP6, FMP21, FMP59, FMP60, FMP65, FMP69) were further tested for their efficacy to modulate dendritic architecture and are shaded in dark blue.

Supplementary Figure 3

Characterization of six VEGFD peptide mimetics

(**A**,**B**) Structure of VEGFD homodimer in shown in dark blue, the portion used to design the VEGFD mimetic peptide library is highlighted and shown in light blue. (**C**) Blocking nuclear calcium signaling by over-expressing CaMBP4 (Calcium/ Calmodulin Binding Peptide 4) results in a decrease of dendritic length which can be rescued to normal levels by treating neurons with rmVEGFD (3). The ability of the six selected peptides to rescue the reduction of dendrite length and complexity caused by expression of CaMBP4 was tested. Analysis of total dendritic length of neurons transfected with expression vectors for hrGFP, CaMBP4 with or without treatment for 3 days with 7.7 nM rmVEGFD, FMP6, FMP21, FMP59, FMP60, FMP65 or FMP69 as indicated. N=8 (3). (**D**) Representative micrographs of neurons transfected and treated as in (C). Scale bar is 20 μm. (**E**) Mortality of cultured neurons treated for 24 h with increasing concentrations (7.7 nM, 100 nM, 1000 nM, 10000 nM) of FMP6 or FMP69. None of the treatments triggered neuronal death. N=3. C: one-way ANOVA with Bonferroni's correction for multiple comparisons; E: one-way ANOVA with Dunnett's correction for multiple comparisons. * $p<0.05$; *** $p<0.001$; **** $p< 0.0001$. Data show mean ± SEM and individual values.


A
Relative VEGFD mRNA level
Control
1μM
5μM
10μM
20μM
NMDA
B
Relative VEGFD mRNA level
Control
10'
30'
4 h
NMDA
C
Control
1 h
2 h
4 h
NMDA
Relative VEGFD mRNA level
Actinomycin D
D
Bic/4AP
NMDA
Relative cFos mRNA level
Control
1 h
2 h
4 h
E
Control
1 h
2 h
4 h
NMDA
Relative mRNA level
VEGFC
VEGF
flt4
F
Control
OGD
Relative mRNA level
VEGFD
VEGF
flt4
G
Cortex contralateral
sham
MCAO
Relative mRNA level
VEGFD
VEGF
flt4
H
Hippocampus ipsilateral
sham
MCAO
Relative mRNA level
VEGFD
VEGF
flt4
I
bEnd.3 cells
Control
1 h
2 h
4 h
NMDA
Relative mRNA level
VEGFD
cFos
VEGFC
VEGF
flt4
J
Astrocytes
Control
1 h
2 h
4 h
NMDA
Relative mRNA level
VEGFD
cFos
VEGFC
VEGF
flt4
K
aCSF
rmVEGFD
Superficial vessels length (percentage of aCSF MCAO)
24 h
8 days
MCAO
L
aCSF
rmVEGFD
Vessels surface area (percentage of aCSF MCAO)
24 h
8 days
MCAO

WT	I	E	T	L	K	V	I	D	E	E	W	Q	R	T	Q	C	WT			
FMP id																	FMP id	pGSK	pp38	morpho
1	K	L	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	1	1.41	2.10	
2	V	L	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	2	1.63	2.04	
3	R	L	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	3	1.26	1.83	
4	A	A	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	4	1.04	1.17	
5	A	E	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	5	1.43	1.96	
6	A	L	A	L	K	E	I	D	E	E	W	Q	R	K	G	I	6	1.15	2.89	
7	A	V	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	7	1.09	1.44	
8	A	L	S	L	K	E	I	D	E	E	W	Q	R	K	G	I	8	1.19	1.57	
9	A	L	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	9	0.73	1.87	
10	A	L	T	A	K	E	I	D	E	E	W	Q	R	K	G	I	10	0.60	1.73	
11	A	L	T	I	K	E	I	D	E	E	W	Q	R	K	G	I	11	1.11	1.91	
12	A	L	T	V	K	E	I	D	E	E	W	Q	R	K	G	I	12	0.90	1.52	
13	A	L	T	F	K	E	I	D	E	E	W	Q	R	K	G	I	13	0.90	1.53	
14	A	L	T	L	A	E	I	D	E	E	W	Q	R	K	G	I	14	0.83	1.44	
15	A	L	T	L	R	E	I	D	E	E	W	Q	R	K	G	I	15	1.27	1.61	
16	A	L	T	L	I	E	I	D	E	E	W	Q	R	K	G	I	16	0.66	3.13	
17	A	L	T	L	L	E	I	D	E	E	W	Q	R	K	G	I	17	1.13	1.46	
18	A	L	T	L	K	A	I	D	E	E	W	Q	R	K	G	I	18	0.76	0.88	
19	A	L	T	L	K	D	I	D	E	E	W	Q	R	K	G	I	19	0.78	0.95	
20	A	L	T	L	K	V	I	D	E	E	W	Q	R	K	G	I	20	0.94	1.12	
21	A	L	T	L	K	E	A	D	E	E	W	Q	R	K	G	I	21	0.80	2.27	
22	A	L	T	L	K	E	V	D	E	E	W	Q	R	K	G	I	22	0.76	1.91	
23	A	L	T	L	K	E	L	D	E	E	W	Q	R	K	G	I	23	0.62	1.22	
24	A	L	T	L	K	E	F	D	E	E	W	Q	R	K	G	I	24	0.66	0.86	
25	A	L	T	L	K	E	I	A	E	E	W	Q	R	K	G	I	25	0.67	2.58	
26	A	L	T	L	K	E	I	E	E	E	W	Q	R	K	G	I	26	0.59	1.73	
27	A	L	T	L	K	E	I	N	D	E	W	Q	R	K	G	I	27	0.98	1.20	
28	A	L	T	L	K	E	I	Q	D	E	W	Q	R	K	G	I	28	1.08	1.29	
29	A	L	T	L	K	E	I	D	A	E	W	Q	R	K	G	I	29	1.40	0.99	
31	A	L	T	L	K	E	I	D	N	E	W	Q	R	K	G	I	31	1.28	1.11	
32	A	L	T	L	K	E	I	D	Q	E	W	Q	R	K	G	I	32	0.87	1.42	
33	A	L	T	L	K	E	I	D	E	A	W	Q	R	K	G	I	33	0.95	0.90	
34	A	L	T	L	K	E	I	D	E	D	W	Q	R	K	G	I	34	0.90	1.26	
35	A	L	T	L	K	E	I	D	E	N	W	Q	R	K	G	I	35	0.83	1.91	
36	A	L	T	L	K	E	I	D	E	Q	W	Q	R	K	G	I	36	0.85	1.39	
37	A	L	T	L	K	E	I	D	E	E	A	Q	R	K	G	I	37	0.83	1.40	
38	A	L	T	L	K	E	I	D	E	E	F	Q	R	K	G	I	38	1.00	0.77	
39	A	L	T	L	K	E	I	D	E	E	I	Q	R	K	G	I	39	1.17	1.16	
40	A	L	T	L	K	E	I	D	E	E	L	Q	R	K	G	I	40	1.35	0.74	
41	A	L	T	L	K	E	I	D	E	E	W	A	R	K	G	I	41	1.32	1.01	
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43	A	L	T	L	K	E	I	D	E	E	W	D	R	K	G	I	43	1.29	1.43	
44	A	L	T	L	K	E	I	D	E	E	W	E	R	K	G	I	44	1.19	0.99	
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46	A	L	T	L	K	E	I	D	E	E	W	Q	A	K	G	I	46	1.12	0.79	
47	A	L	T	L	K	E	I	D	E	E	W	Q	K	K	G	I	47	0.88	1.19	
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62	A	L	T	L	K	E	I	D	E	E	W	Q	R	K	G	L	62	0.83	0.75	
64	A	aib	T	L	K	E	I	D	E	E	W	Q	R	K	G	I	64	0.84	1.12	
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70	A	L	T	L	K	E	I	aib	E	E	W	Q	R	K	G	I	70	1.11	1.83	
71	A	L	T	L	K	E	I	D	aib	E	W	Q	R	K	G	I	71	0.93	1.55	
72	A	L	T	L	K	E	I	D	E	aib	W	Q	R	K	G	I	72	0.75	0.50	
73	A	L	T	L	K	E	I	D	E	E	aib	Q	R	K	G	I	73	1.06	1.25	
74	A	L	T	L	K	E	I	D	E	E	W	aib	R	K	G	I	74	1.06	1.18	
75	A	L	T	L	K	E	I	D	E	E	W	Q	aib	K	G	I	75	0.95	1.37	
76	A	L	T	L	K	E	I	D	E	E	W	Q	R	aib	G	I	76	1.08	1.22	
77	A	L	T	L	K	E	I	D	E	E	W	Q	R	K	aib	I	77	1.20	1.24	
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79	A	gpn	L	K	E	I	D	E	E	W	Q	R	K	G	I	A	79	1.12	1.29	
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81	A	L	T	gpn	E	I	D	E	E	W	Q	R	K	G	I	A	81	1.10	1.00	
82	A	L	T	L	gpn	I	D	E	E	W	Q	R	K	G	I	A	82	0.74	0.42	
83	A	L	T	L	K	gpn	D	E	E	W	Q	R	K	G	I	A	83	0.92	0.65	
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89	A	L	T	L	K	E	I	D	E	E	W	gpn	K	G	I	A	89	0.96	1.43	
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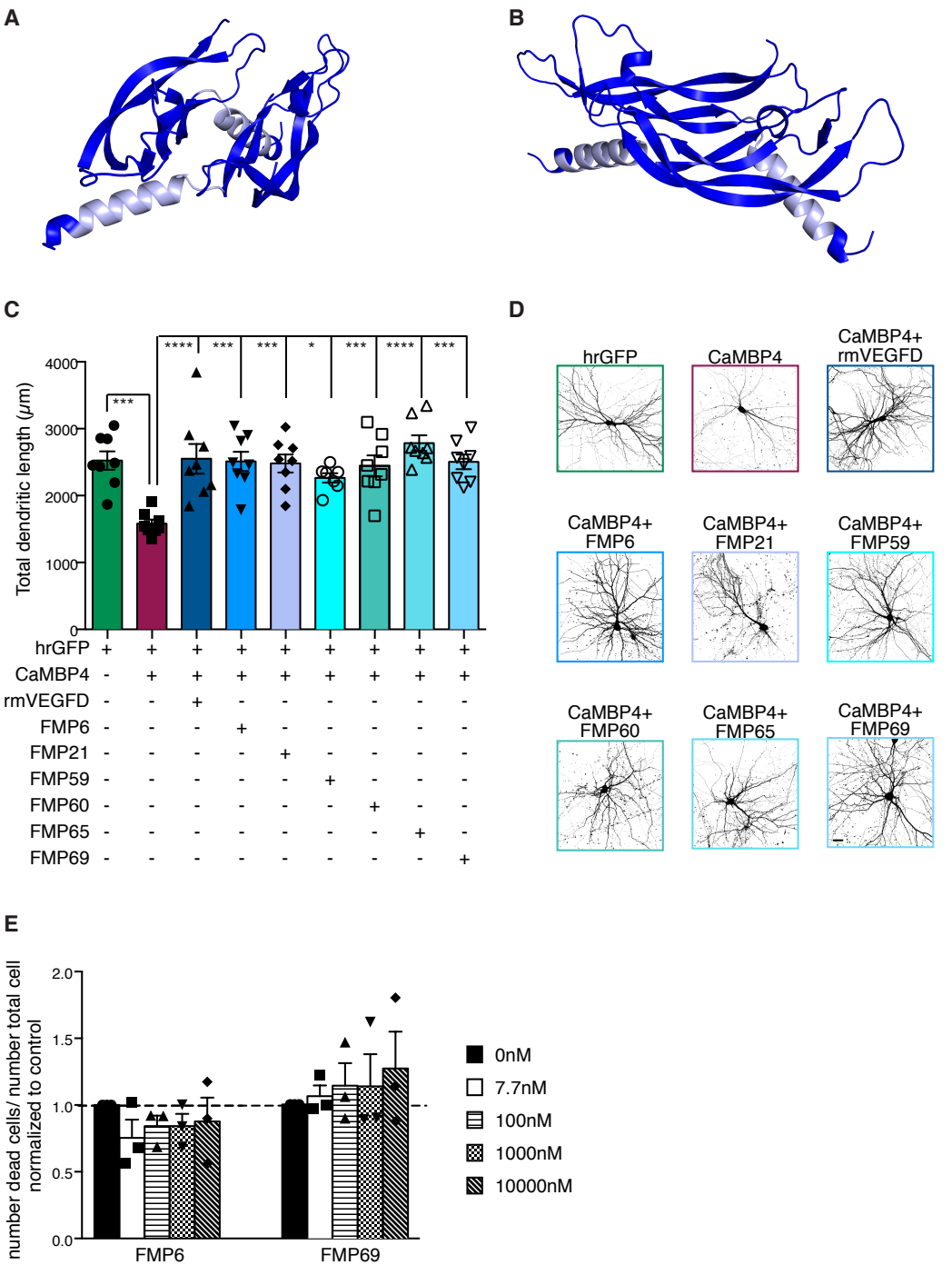
91	A	L	T	L	K	E	I	D	E	E	W	Q	R	gpn	I	A	91	0.94	1.10	
92	A	L	T	L	K	E	I	D	E	E	W	Q	R	K	gpn	A	92	0.80	1.52	
93	A	L	T	L	K	E	I	D	E	E	W	Q	R	K	I	gpn	93	0.89	1.94	
94	A	V	T	V	K	E	V	D	E	E	W	Q	R	K	G	V	94	0.75	0.93	
95	A	A	T	A	K	E	A	D	E	E	W	Q	R	K	G	A	95	0.84	1.59	
96	I	E	T	L	K	V	I	D	E	E	W	Q	R	T	Q	S	96	0.67	0.91	
FMP id																	FMP id	pGSK	pp38	morpho
WT	I	E	T	L	K	V	I	D	E	E	W	Q	R	T	Q	C	WT			


A
B
C
D
E
Total dendritic length (μm)
4000
3000
2000
1000
0
****
***
***
***
*
***
****
***
hrGFP + + + + + + + + +
CaMBP4 - + + + + + + + +
rmVEGFD - - + - - - - - -
FMP6 - - - + - - - - -
FMP21 - - - - + - - - -
FMP59 - - - - - + - - -
FMP60 - - - - - - + - -
FMP65 - - - - - - - + -
FMP69 - - - - - - - - +
hrGFP
CaMBP4
CaMBP4+ rmVEGFD
CaMBP4+ FMP6
CaMBP4+ FMP21
CaMBP4+ FMP59
CaMBP4+ FMP60
CaMBP4+ FMP65
CaMBP4+ FMP69
number dead cells/ number total cell normalized to control
2.0
1.5
1.0
0.5
0.0
FMP6
FMP69
0nM
7.7nM
100nM
1000nM
10000nM

References to additional methods

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