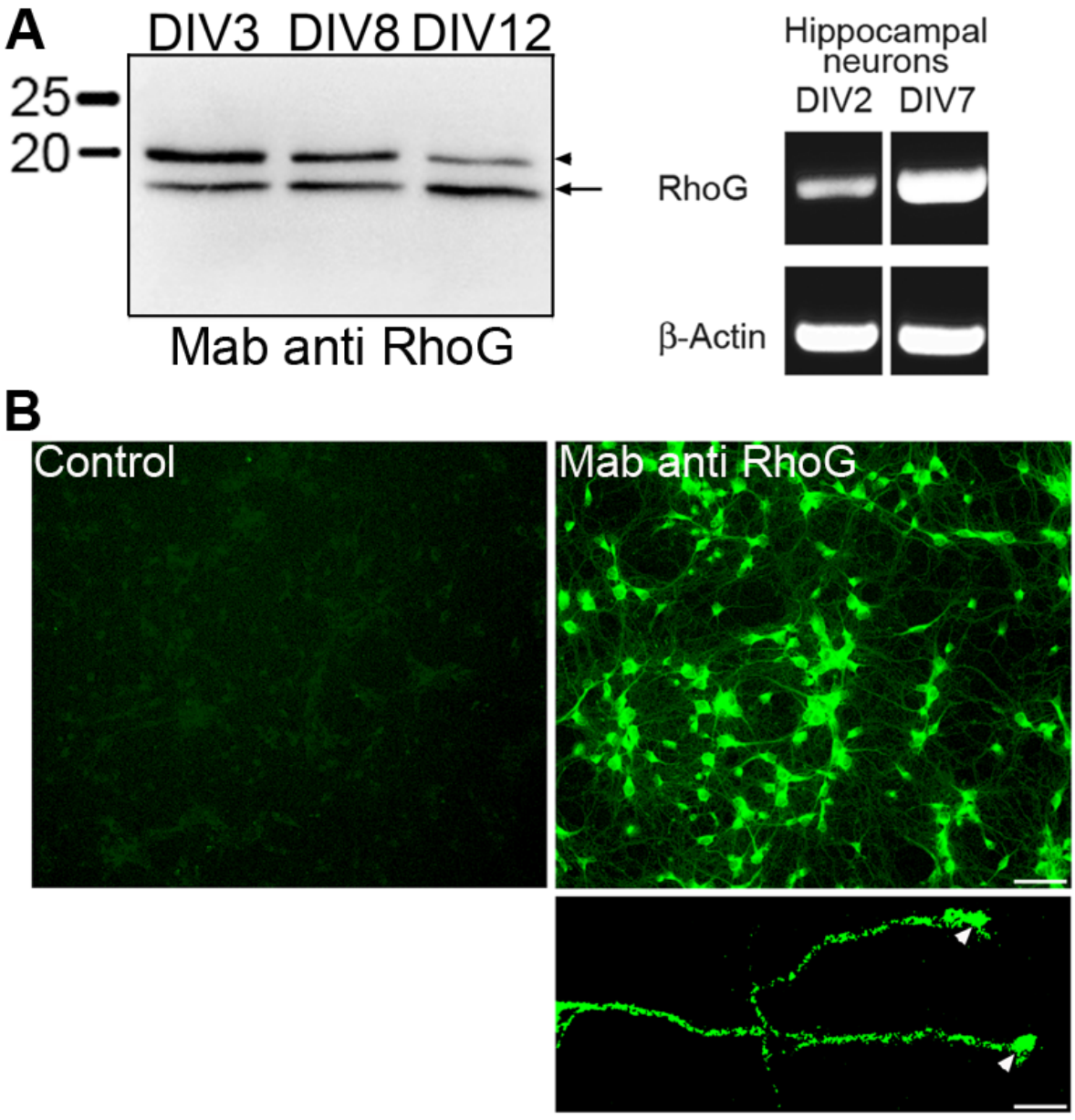
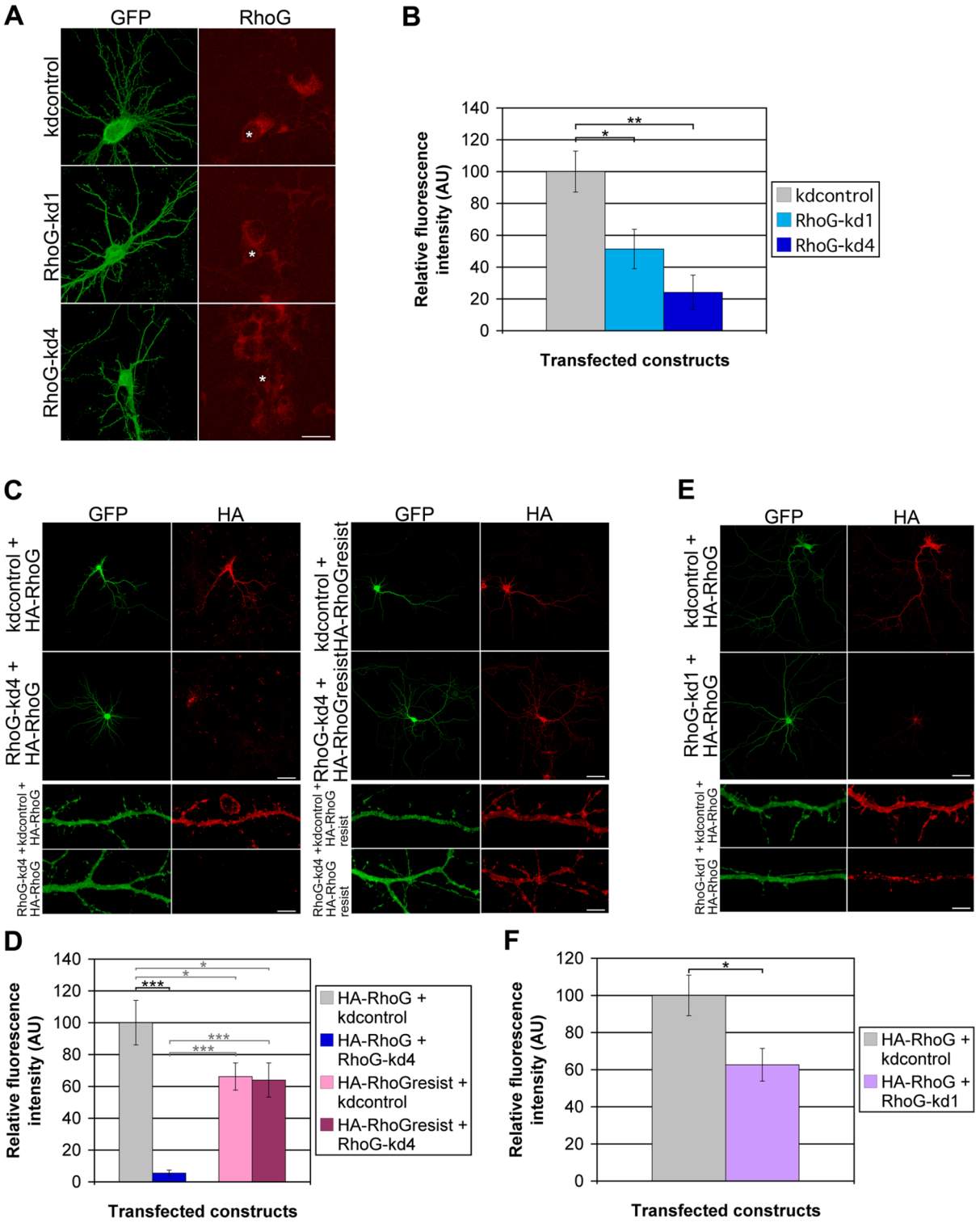
Supplementary data



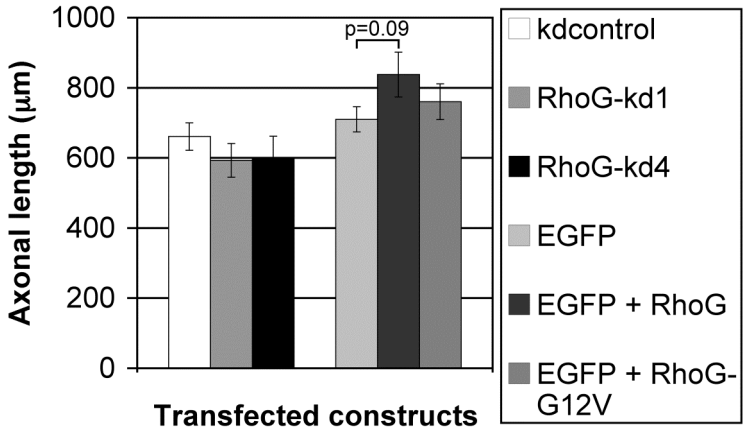
Supplementary Figure S1 RhoG is expressed in hippocampal neurons. (**A**) Detergent extracts of rat hippocampal neurons at different time points in culture were subjected to immunoblot analysis with a monoclonal antibody to RhoG (clone 1F3B3E5, Millipore #04-486, left-hand panel). The antibody specifically recognised two protein bands in the immunoblot. The lower band (arrow) corresponds to the RhoG protein which is derived from the published sequence (accession number XM_218977), as proved by immunoblot analysis

of the tagged recombinant protein (data not shown). The nature of the upper running band (arrowhead) is currently not clear. Only one PCR product corresponding to RhoG was obtained when amplifying from cDNA derived from rat hippocampal cells with primers that matched the 5´- end and the 3´- end of the published RhoG-encoding sequence (right-hand panel). (**B**) The monoclonal antibody to RhoG used for immunoblotting detected RhoG in primary hippocampal neurons in culture at DIV2. RhoG was located both in the cell bodies and in the processes of hippocampal neurons (upper right-hand image). As shown in higher magnification, RhoG was detected in axonal shafts and axonal growth cones (arrowheads in lower right-hand image). Scale bars, 80 μm and 15 μm.

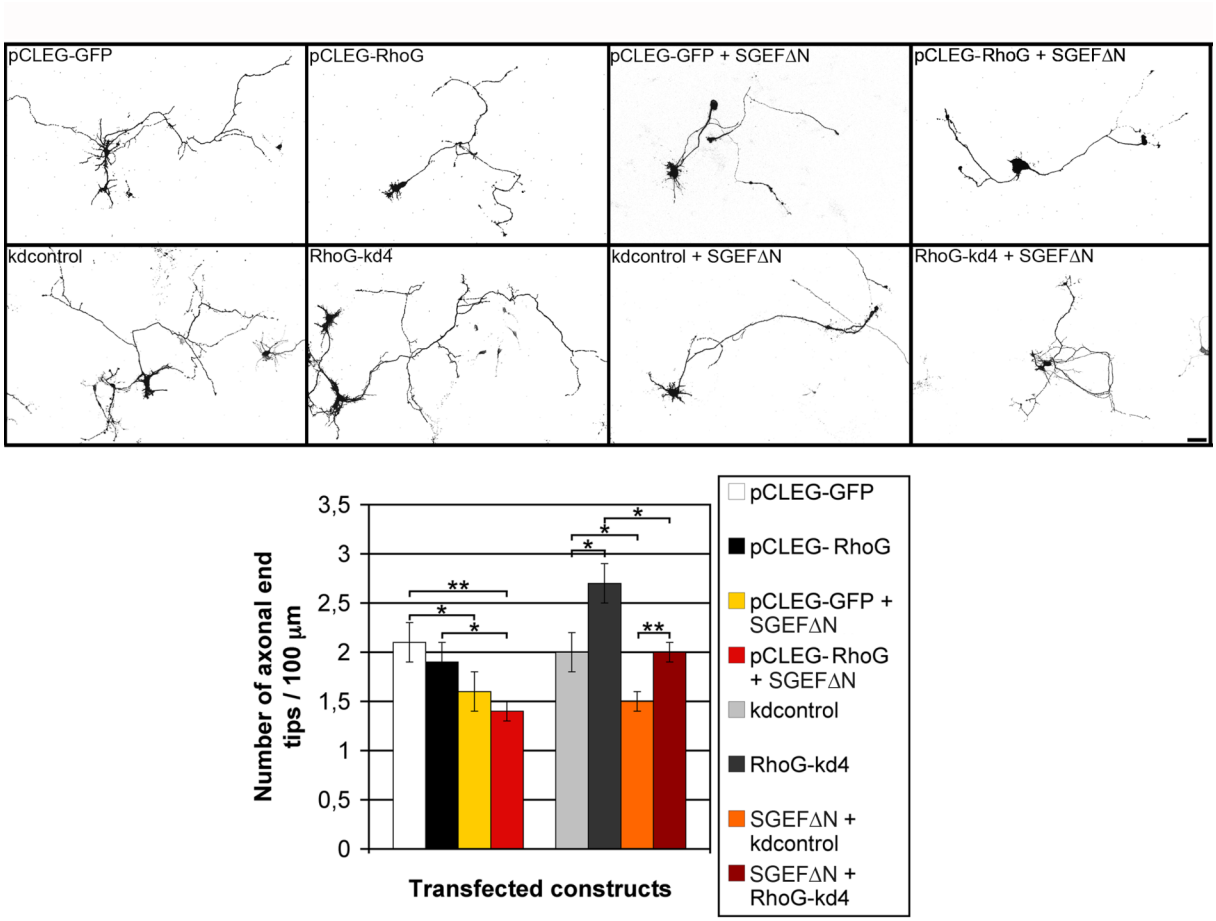


Supplementary Figure S2 The expression of RhoG is reduced by the knockdown constructs RhoG-kd1 and RhoG-kd4. (**A**) Primary hippocampal neurons in culture were transfected at DIV8 with the RhoG-specific knockdown constructs RhoG-kd1 or RhoG-kd4, and compared

to the corresponding control (kdcontrol) two days later after staining for endogenous RhoG (red) with the monoclonal antibody to RhoG (clone 1F3B3E5, Millipore #04-486). GFP staining was performed to show transfected neurons. Stars indicate examples of transfected neurons stained for RhoG. Scale bar, 20 μm. (**B**) The quantification of the RhoG-specific immunofluorescence signals (normalized) in neuronal cell bodies confirmed that both knockdown constructs significantly reduced the amount of endogenously expressed RhoG when compared to the control. Means (n = 20 neurons for kdcontrol and RhoG-kd4, n = 27 neurons for RhoG-kd1) ± s.e.m. (***P* < 0.005; **P* < 0.05). (**C, D**) Hippocampal neurons co-expressing either the RhoG-specific knockdown construct RhoG-kd4 together with the overexpression construct HA-RhoG, or the latter construct together with the knockdown control construct (kdcontrol) were stained for the HA tag of the overexpressed HA-RhoG two days after transfection at DIV10. The quantification of the immunofluorescence signals (normalized) in the primary dendrites showed that RhoG-kd4 very efficiently reduced the amount of overexpressed HA-tagged RhoG in neurons. Then RhoG-kd4 was co-expressed with a HA-RhoG overexpression construct (HA-RhoGresist(kd4)) harboring two silent point mutations in the binding site for the RhoG-kd4-derived siRNA to prevent binding of this siRNA. RhoG-kd4 was not able to reduce the amount of HA-RhoGresist(kd4) in hippocampal neurons compared to the knockdown control (kdcontrol). (**E, F**) Hippocampal neurons co-expressing either the RhoG-specific knockdown construct RhoG-kd1 together with the overexpression construct HA-RhoG, or the latter construct together with the knockdown control construct (kdcontrol) were stained for the HA tag of the overexpressed HA-RhoG two days after transfection at DIV10. The quantification of the immunofluorescence signals (normalized) in the primary dendrites showed that RhoG-kd1 significantly reduced the amount of overexpressed HA-tagged RhoG in neurons, although to a lesser extent than RhoG-kd4. Means (n = 20 neurons for HA-RhoG + kdcontrol and HA-RhoG + RhoG-kd4 co-expression, n = 24 neurons for all other experimental conditions) ± s.e.m. (****P* < 0.0005; **P* < 0.05). Scale bars, 50 μm and 5 μm.



Supplementary Figure S3 RhoG does not affect axonal length. The axonal morphology of hippocampal neurons, transfected with the indicated constructs at DIV2, was analysed after staining for GFP at DIV2+2. The axonal length (length of the longest axonal branch emanating from the neuronal cell body) was neither affected by the RhoG knockdown constructs nor by the RhoG-derived overexpression constructs when compared to the corresponding controls. Shown are the means ± s.e.m. (n = 40 neurons for kdcontrol, RhoG-kd1, RhoG-kd4, and RhoG; n = 80 neurons for EGFP and EGFP + RhoG-G12V).

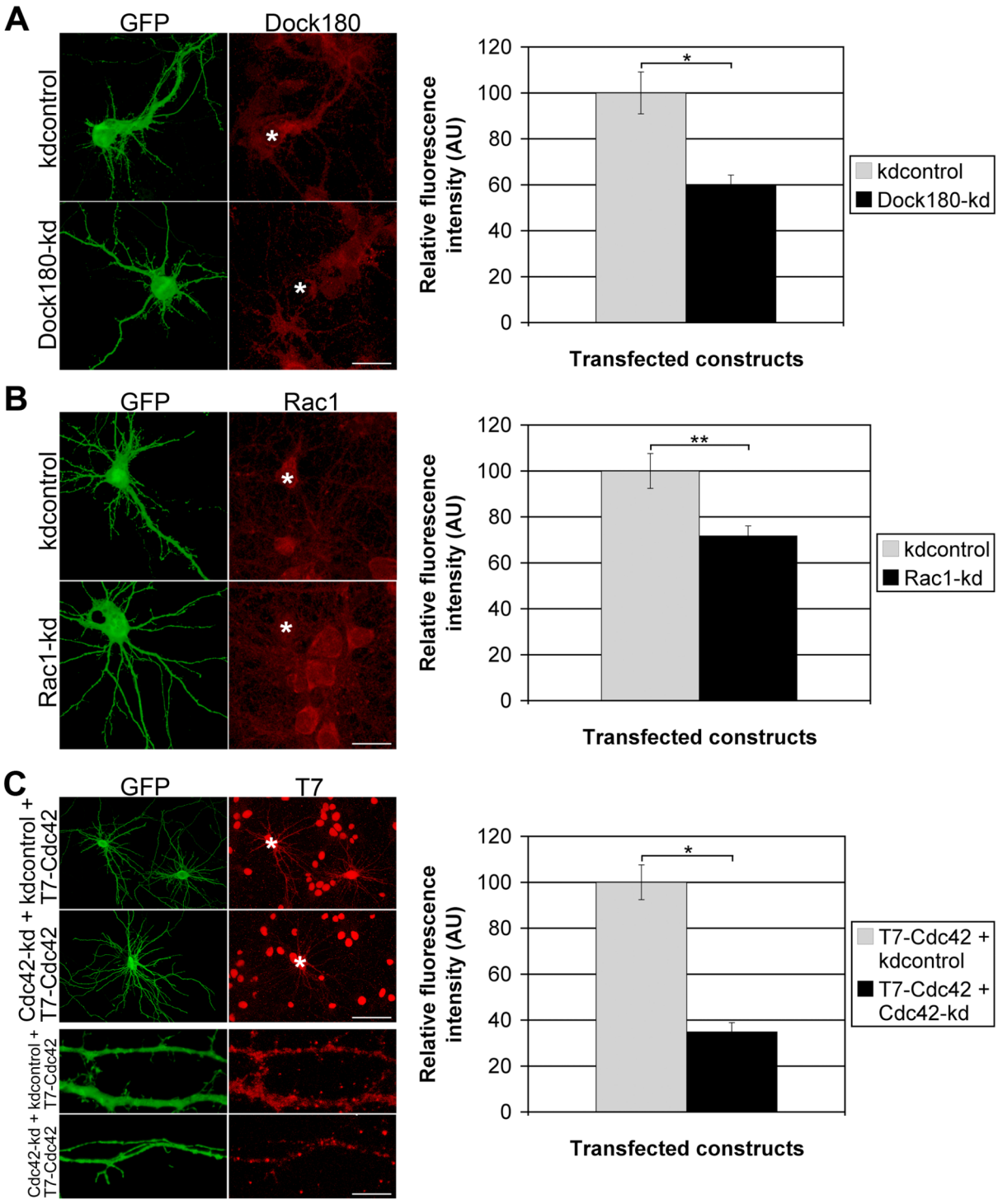


Supplementary Figure S4 A RhoG-activating SGEF construct reduces axonal branching. The construct SGEFΔN, which corresponds to the construct Myc-SGEFΔN (aa 364-871 of SGEF, GenBank accession NM015595; Ellerbroek *et al*, 2004), was co-transfected either with pCLEG-GFP or pCLEG-RhoG in hippocampal neurons at DIV2. The axonal morphology of the transfected neurons was analyzed after staining for GFP at DIV2+2.

The construct Myc-SGEFΔN comprises the DH/PH module of SGEF harboring the GEF activity for RhoG and a more C-terminal located SH3 domain conveying an intracellular targeting function (Ellerbroek *et al*, 2004).

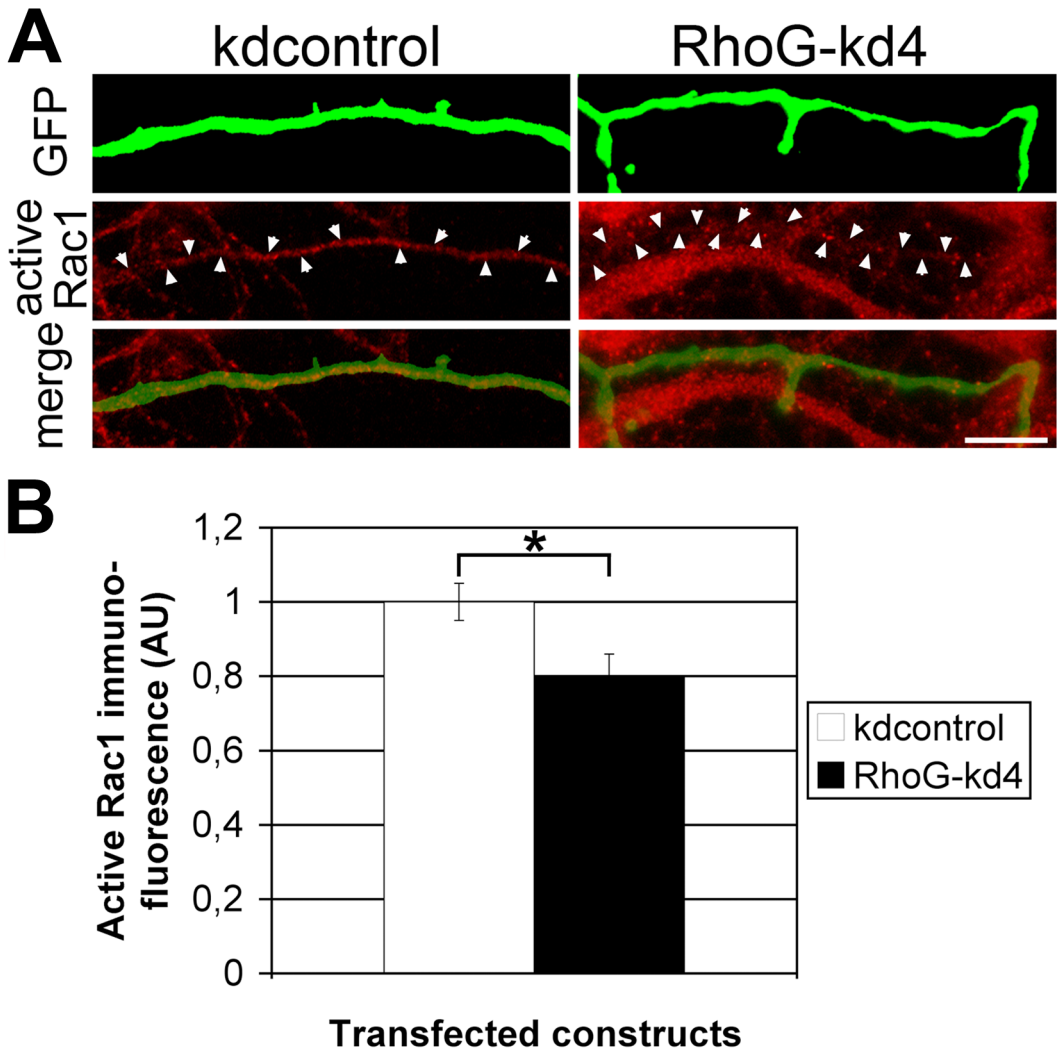
SGEFΔN reduced axonal branching when compared to the control (pCLEG-GFP), and SGEFΔN co-expressed with pCLEG-RhoG significantly reduced axonal branching when compared to pCLEG-RhoG expressed alone. The RhoG-specific knockdown construct RhoG-kd4 significantly rescued axonal branching when co-expressed with SGEFΔN, indicating that SGEFΔN reduced axonal branching at least partially via RhoG. Means (n = 80

neurons for SGEFΔN + kdcontrol and SGEFΔN + RhoG-kd4 co-expression, n = 40 neurons for all other experimental conditions) ± s.e.m. (**P < 0.005; *P < 0.05). Scale bar, 20 μm.



Supplementary Figure S5 The expression of Dock180, Rac1, and Cdc42 in hippocampal neurons is reduced by the corresponding knockdown constructs. Primary hippocampal neurons in culture were transfected at DIV8 with the indicated constructs and stained two days after transfection either with an antibody to Dock180 (goat polyclonal antibody to

Dock180, sc-6167, Santa Cruz Biotechnology), Rac1 (mouse monoclonal antibody to Rac1, clone 23A8, Millipore), or the T7-tag of co-transfected T7-Cdc42 (mouse monoclonal antibody to the T7 tag, #69522-3, Novagen), respectively. Stars indicate examples of transfected neurons that were analyzed. Scale bars for (**A**, **B**), 20 μm. Scale bars for (**C**), 50 μm and 5 μm. The quantification of the immunofluorescence signals (normalized) in neuronal cell bodies (**A**, **B**) and primary dendrites (**C**) confirmed that the knockdown constructs Dock180-kd, Rac1-kd, and Cdc42-kd significantly reduced the amount of the corresponding proteins Dock180, Rac1, and T7-Cdc42, respectively, when compared to the controls. Means [n = 20 neurons (**A**), n = 22 neurons (**B**), and n = 24 neurons (**C**)] ± s.e.m. (**$P < 0.005$; *$P < 0.05$).

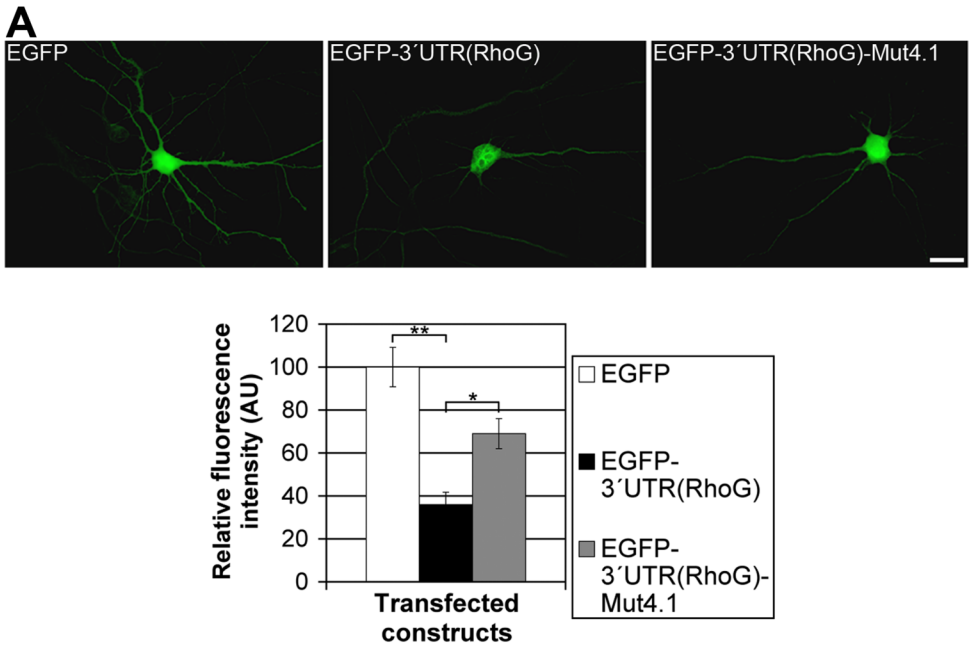


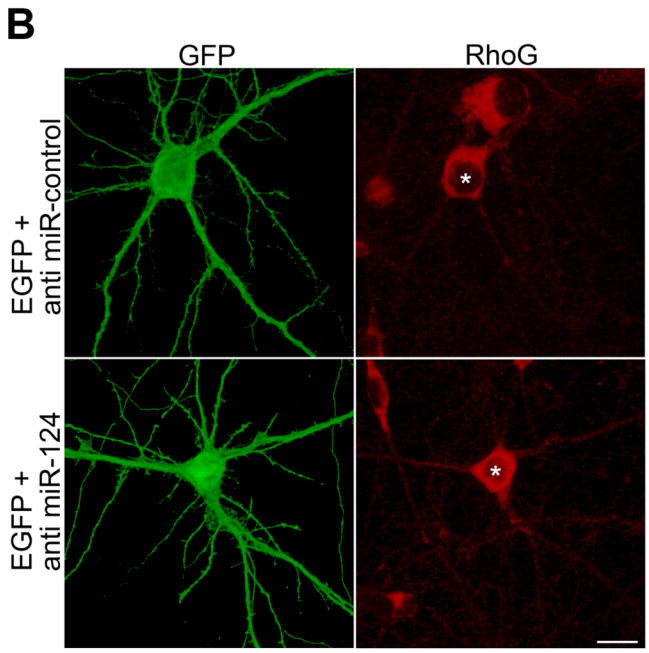
Supplementary Figure S6 Knockdown of RhoG expression reduces the amount of active Rac1 in axons of hippocampal neurons. (**A**) Hippocampal neurons were transfected at DIV2 with the indicated constructs and stained at DIV2+2 for GFP (green) or active Rac (red), respectively. Shown are axonal sections. For active Rac staining (arrowheads), the monoclonal antibody anti active Rac1, which specifically recognises Rac-GTP (NewEast, #26903), was used. Scale bar, 15 μm. (**B**) The shRNA construct RhoG-kd4 caused a reduction in the axonal immunofluorescence of anti active Rac1 staining. Means (n = 12 neurons) ± s.e.m. (*$P < 0.05$).

Rattus norvegicus RhoG, mRNA

ctcccccgccgcggggagggcaccaggtcactacttccagtggggtccagaagagggaggag
gcactgcctccacggtagtaactttacaacacaatgcagagcatcaagtgtgtggtggtggg
tgatggggccgtaggcaagacgtgccttctcatctgctatacaactaatgccttccccaagg
aatacatccccactgtgttcgacaattacagcgcccagagtgcggttgatgggcgcaccgtg
aacctaaacctgtgggacactgccggccaggaggagtacgaccgcctccgcacccctttccta
cccccagaccaacgtcttcgtcatctgtttctccattgccagtccaccctcctatgagaacg
tgaggcacaaatggcacccagaggtgtgtcaccactgccctgatgtgcccatcctcctggtg
ggcaccaagaaggacctcagagcccagcctgataccctacggcgcctcaaggagcagggtca
agcgcccatcacaccacagcagggccaggcgctggccaagcagatccacgctgtgcgctacc
tcgagtgctcagcgctgcagcaagacggtgtcaaggaggtgtttgccgaggctgtccgggcg
gtgctcaacccaacaccgataaagcgtgggcggtcctgcatcctcttgtgacctggcactc
agcttggaggctatcccttgtccccctaccaattgtgccttggcgccttgtctgccccagc
tgtgccttaaggactaattctggcacccttgcctgggaactccctgaatgccttttattcc
ttaaggaggtacacagggagaggggctttgggccccaccccactctgcttgggaataccaag
tattccatgagttcacctcaaacgggttgacctttctaagaggctaacccagtgctcccctc
catttcctgctactgaccagttcatccagctttccacaaagttgttgctgcctgatgtgatg
cctcctcaagataggggctctaacctctctctgccttagctcttggagtaagctctccaaga
tgttctctccctctcccaagggaggagccacagaatcctcagaagaaaaatgtgccctaacc
tgccccatgtgcccaggacttatgcgcctgctgactgactcaggccccatgctcctgggggc
ctttcctacccccatcagcatcaataaaacctcctgtctccagtgaaaaaaaaaaaaaaaaa
aaaaaaa)

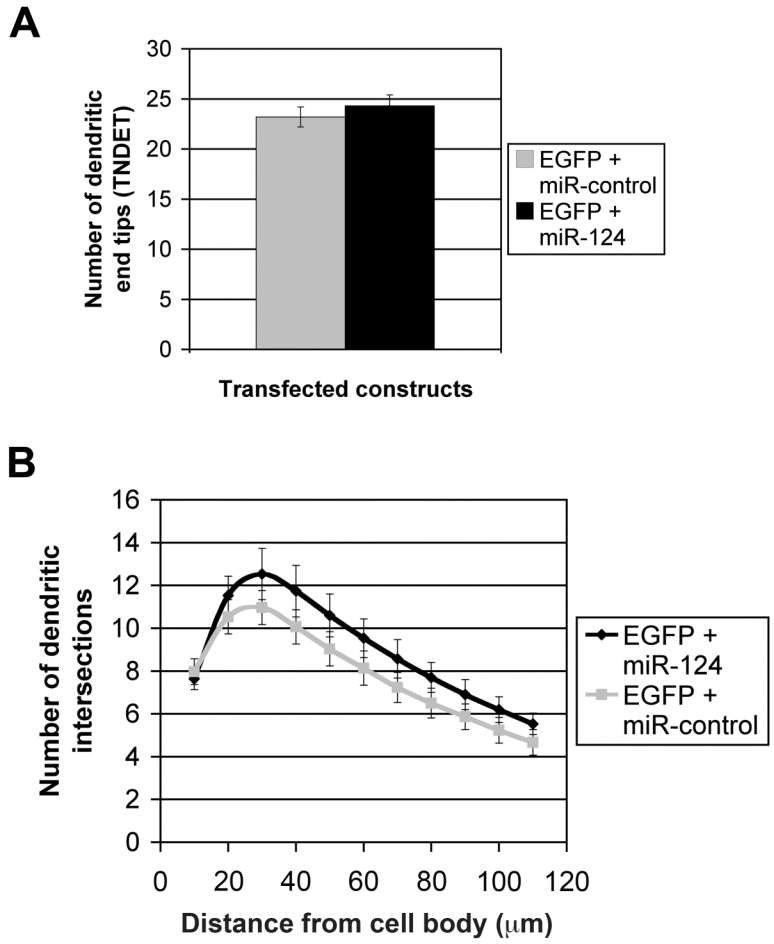
Supplementary Figure S7 The 3´UTR of the RhoG gene comprises two binding sites for the microRNA miR-124, which are highly conserved between the species rat, mouse, and human. The “seeds” of the two miR-124-binding sites (positions 45 – 51 and 74 – 80 of the 3´UTR), which are conserved between the species specified above, are highlighted in red. A third miR-124 binding site (position 352 – 358 of the 3´UTR), highlighted in green, is not conserved. The first codon of the open reading frame (atg) and the stop codon (tga) are highlighted in yellow. The 412 bp fragment of the 3´UTR that was used to clone the sensor construct EGFP-3´UTR(RhoG) is shown in parenthesis (blue). The full-length 3´UTR of RhoG containing the endogenous polyadenylation signal which was used to clone the sensor construct EGFP-3´-UTRfull(RhoG) is also shown in parenthesis (orange).





Supplementary Figure S8 miR-124 regulates RhoG expression by binding to the miR-124-binding sites in the 3´UTR of the RhoG mRNA. (**A**) The EGFP expression of the sensor construct EGFP-3´UTR(RhoG) was downregulated by endogenous miR-124. Point mutations in the miR-124 binding sites reduced the degree of regulation. Means (n = 100 neurons) ±

s.e.m. (**P < 0.005; *P < 0.05). Scale bar, 20 μm. (**B**) Anti miR-124 increases RhoG expression in hippocampal neurons. Primary hippocampal neurons were transfected with the indicated constructs at DIV8 and stained for endogenous RhoG at DIV8+2. GFP staining was performed to show transfected neurons. Stars indicate examples of transfected neurons that were representative for the respective experimental condition. Scale bar, 12 μm.



Supplementary Figure S9 miR-124 does not increase dendritic branching in DIV14+3 hippocampal neurons. DIV14 primary hippocampal neurons were transfected with the indicated constructs and stained three days later for GFP. The complexity of the dendritic trees of transfected neurons were analyzed by determining the number of dendritic end tips (**A**) and by performing Sholl analysis (**B**). Means [n = 60 neurons (**A**), and n = 30 neurons (**B**)] ± s.e.m. miR-124 did not increase TNDET compared to miR-control, and miR-124 only slightly but not significantly led to a shift of the Sholl graph (indicating more dendritic branching) compared to miR-control.