miR-124-regulated RhoG reduces neuronal process complexity via ELMO/Dock180/Rac1 and Cdc42 signalling

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Introduction

MicroRNAs, a class of small noncoding RNAs that posttranscriptionally regulate gene expression, are likely to have key roles in neuronal development and plasticity (Schratt et al., 2006; Fineberg et al., 2009; Gao, 2010). In particular, the nervous system-specific microRNA miR-124 has been pointed out to promote neuronal differentiation at the transcriptional as well as the posttranscriptional level. miR-124 contributes to the regulation of chromatin assembly and accessibility by facilitating a switch of BAF53 subunits in ATP-dependent chromatin-remodelling complexes (Yoo et al., 2009). This switching of subunits relieves the repression of BAF53b, which is critical for activity-dependent dendritic outgrowth.

Additionally, at the level of transcription, miR-124 inhibits the NRSF/REST complex, a global repressor of nervous system-specific transcription, by targeting the expression of the SCP1 phosphatase, which in turn is implicated in the function of the NRSF/REST complex (Visvanathan et al., 2007). Posttranscriptionally, miR-124 stimulates neuronal differentiation by downregulating the expression of the RNA-binding protein PTBP1, inducing a switch towards nervous system-specific alternative splicing (Makeyev et al., 2007). Consequently, miR-124 induces neurite outgrowth in the CNS catecholaminergic CAD cell line. Furthermore, miR-124 reduces the expression of several transcription factors including Lhx2 and CREB1 either to prevent apoptosis in the developing retina and regulate proper axonal development of dentate gyrus granule neurons (Sanuki et al., 2011), or to constrain serotonin-induced synaptic facilitation (Rajaehupathy et al., 2009), respectively. In addition, miR-124 promotes adult neurogenesis in the subventricular zone stem cell niche by repressing the expression of the transcription factor Sox9 (Cheng et al., 2009).

By modulating chromatin-remodelling complexes, global transcriptional and alternative splicing regulators as well as transcription factors, miR-124 indirectly affects the expression of several target genes involved in neuronal differentiation (Gao, 2010). So far, little is known about miR-124 target interactions that directly control signalling pathways to define morphological characteristics of differentiated neurons such as axonal shape or dendritic tree complexity. We were interested in identifying novel target interactions of miR-124 that directly connect gene expression to a discernable phenotype related to neuronal process differentiation.Using the algorithms PicTar (Krek et al., 2005), miRanda (John et al., 2004), TargetScan (Lewis et al., 2005), and MiRtarget2 (Wang and El Naqa, 2008) for microRNA target prediction, we found the small GTPase RhoG as a strong candidate for miR-124-regulated gene expression. RhoG has been demonstrated to be critical for proper regulation of the actin cytoskeleton in several organisms and cell types: this GTPase mediates phagocytosis of apoptotic cells (deBakker et al., 2004), ICAM1-dependent endothelial cup assembly necessary for transendothelial migration of leucocytes (van Buul et al., 2007), and clathrin-independent T-cell receptor internalization and troygocytosis (Martinez-Martin et al., 2011). Furthermore, RhoG is involved in the invasion of pathogenic bacteria (Patel and Galán, 2006; Roppenser et al., 2009), and the migration of cancer cells (Hiramoto-Yamaki et al., 2010). In neural development, RhoG was demonstrated to promote progenitor cell proliferation in the cerebral cortex (Fujimoto...
et al., 2009). However, most of the previous research on the role of RhoG in neuronal differentiation has been based on experimental investigations using the rat pheochromocytoma PC12 cell line (Katoh et al., 2000; Estrach et al., 2002; Katoh and Negishi, 2003). In this cell line, RhoG stimulates neurite outgrowth depending on an active ELMO/Dock180 signalling module (Katoh and Negishi, 2003).

Here, we set out to explore the functions of RhoG in neuronal process differentiation in primary hippocampal neurons in culture and in the native brain in vivo. Our results demonstrate that RhoG inhibits axonal branching via the ELMO/Dock180/Rac1 signalling pathway. In addition, RhoG reduces dendritic tree complexity through the small GTPase Cdc42. We validate the predicted regulation of RhoG expression by the microRNA miR-124 in hippocampal neurons, and connect this regulation to the stimulation of neuronal process complexity.

Results

RhoG reduces dendritic tree complexity

Up to now it was found that RhoG stimulates neurite outgrowth in the rat pheochromocytoma PC12 cell line (Katoh et al., 2000; Estrach et al., 2002; Katoh and Negishi, 2003). This GTPase is expressed in brain tissue (Ishikawa et al., 2002) and in hippocampal neurons in culture (Supplementary Figure S1). To elucidate the function of RhoG in neuronal process elaboration in primary neurons, we performed loss-of-function and gain-of-function experiments in hippocampal neurons in cell culture and in the native mouse brain in vivo.

For loss-of-function experiments, we used the independent shRNA constructs RhoG-kd1 and RhoG-kd4 (Meller et al., 2008) to reduce the amount of RhoG endogenously expressed in hippocampal neurons. To assess the efficiency and specificity of these RhoG knockdown constructs, we performed three different experiments: (1) HA-tagged RhoG was co-expressed with the knockdown constructs RhoG-kd1 and RhoG-kd4, respectively, in HEK293 cells. Cell extracts were subjected to immunoblot analysis with an antibody directed to the HA tag. The quantification of the immunopositive bands confirmed that RhoG-kd4 very efficiently reduced the amount of overexpressed RhoG compared with the control (kdcontrol). Similarly, RhoG-kd1 significantly decreased RhoG expression, although to a lesser extent (Figure 1B). (2) Hippocampal neurons were transfected with either RhoG-kd1, RhoG-kd4, or the corresponding control construct. Two days after transfection, the neurons were stained with a monoclonal antibody directed to RhoG (Supplementary Figure S1). The quantification of the RhoG-specific immunopositive signals in neuronal cell bodies confirmed that both knockdown constructs, RhoG-kd1 and RhoG-kd4, significantly reduced the amount of endogenously expressed neuronal RhoG compared with the control (kdcontrol). Similarly, RhoG-kd1 significantly decreased RhoG expression, although to a lesser extent (Figure 1B). (3) HA-tagged RhoG was co-expressed with the knockdown constructs RhoG-kd1 and RhoG-kd4, respectively, in hippocampal neurons. These neurons were stained with an antibody to the HA tag (Supplementary Figure S2C and E) and the immunopositive signals in the primary dendrites were quantified. RhoG-kd4 almost entirely prevented the expression of HA-tagged RhoG in hippocampal neurons (Supplementary Figure S2D). RhoG-kd1 significantly reduced the amount of overexpressed RhoG in neurons, but less efficiently than RhoG-kd4 (Supplementary Figure S2F).

Finally, we co-expressed RhoG-kd4 together with the RhoG overexpression construct RhoGresist(kd4) harbouring two silent point mutations in the binding site for the RhoG-kd4-derived siRNA (see Materials and methods). When co-expressing RhoG-kd4 together with RhoGresist(kd4) in hippocampal neurons, RhoG-kd4 was not able to reduce the expression of RhoGresist(kd4) (Supplementary Figure S2C and D).

We then employed the validated shRNA constructs RhoG-kd1 and RhoG-kd4 to investigate the function of RhoG in neuronal process elaboration. We found that knockdown of endogenous RhoG expression by RNA interference using the shRNA constructs RhoG-kd1 and RhoG-kd4 promoted dendritic tree complexity by increasing the total number of dendritic end tips (TNDET) of days in vitro 7 (DIV7) cultured primary hippocampal neurons (Figure 1A and C). Consistent with this finding, overexpression of either RhoG or the dominant-positive mutant RhoG-G12V reduced TNDET (Figure 1A and C). Next, a rescue experiment was performed using RhoGresist(kd4) to prove that the RhoG-kd4-mediated dendritic phenotype was caused specifically by a reduction of endogenously expressed RhoG. In fact, RhoGresist(kd4) decreased TNDET similar to RhoG even in the presence of co-expressed RhoG-kd4, which led to a depletion of endogenously expressed RhoG (Figure 1A and C). In the presence of the weaker knockdown construct RhoG-kd1 (Supplementary Figure S2D and F), RhoG function could be rescued simply by overexpression of RhoG (Figure 1A and C).

The relevance of RhoG for dendritic tree differentiation was further investigated by conducting Sholl analysis, which quantifies the number of times dendrites from a neuron cross concentric circles of increasing diameters around the neuronal cell body (Brandt et al., 2007). The Sholl analysis confirmed that overexpression of RhoG decreased dendritic tree complexity by reducing dendritic branching, while knockdown of endogenous RhoG increased dendritic branching (Figure 1D). Additionally, the results of the rescue experiments were corroborated. The Sholl analysis further indicated that the inhibitory effect of RhoG on dendritic tree complexity became manifest predominantly in the proximal to middle part of the dendritic tree.

To demonstrate the in vivo relevance of these results, we employed the in-utero electroporation (IUE) technique (Brandt et al., 2007) to elicit in mouse brains acute manipulations of the amount of functional RhoG in cornu ammonis 1 (CA1) pyramidal neurons (Figure 1E and F). Knockdown of endogenous RhoG expression by the respective shRNA constructs resulted in an increase in the number of apical dendritic end tips of CA1 pyramidal neurons due to enhanced dendritic branching. In line with this outcome, the number of apical dendritic end tips of CA1 neurons overexpressing RhoG was significantly reduced (Figure 1G and H). Together, we demonstrate that RhoG reduces dendritic tree complexity in primary neurons in culture and in the native mouse brain in vivo.

RhoG inhibits axonal branching

The expression of RhoG not only during dendritogenesis but additionally during axonogenesis (Supplementary Figure S1) suggests that this protein may also be involved in regulating axonal morphology. We verified this assumption by
employing the same constructs as described above either to reduce endogenous RhoG expression or to overexpress wild-type RhoG as well as dominant-positive RhoG in DIV2 hippocampal neurons. In contrast to the weaker knockdown construct RhoG-kd1, the stronger knockdown construct RhoG-kd4 (Figure 1B; Supplementary Figure S2) significantly
increased the number of axonal end tips/100 μm without having an effect on axonal length (Figure 2A and B; Supplementary Figure S3). Furthermore, the dominant-positive construct RhoG-G12V, but not wild-type RhoG led to a reduction in the number of axonal end tips/100 μm. As it was seen for the RhoG knockdown constructs, neither RhoG nor RhoG-G12V affected the axonal length compared with the EGFP control (Figure 2A and B; Supplementary Figure S3). The fact that only dominant-positive RhoG-G12V but not RhoG elicit a functional effect was also observed in another context. Namely it was found that RhoG-G12V, but not RhoG stimulates neural progenitor cell proliferation (Fujimoto et al., 2009). One possible explanation for this difference could be that RhoG-activating guanine nucleotide exchange factors (GEFs) are limiting during axonogenesis. To support this hypothesis experimentally, we expressed the deletion construct SGEFΔN, which is derived from the RhoG-activating GEF SGEF (SH3-containing GEF) in hippocampal neurons. This construct comprises the DH/PH module of SGEF harbouring the GEF activity for RhoG and a more C-terminal located SH3 domain conveying an intracellular targeting function (Ellerbroek et al., 2004). We observed that SGEFΔN significantly reduced axonal branching (Supplementary Figure S4). To ascertain whether SGEFΔN reduces axonal branching via activation of RhoG, SGEFΔN was co-expressed together with RhoG-kd4 in hippocampal neurons, and axonal branching was analysed. Knockdown of endogenous RhoG expression by RhoG-kd4 was able to rescue axonal branching under the condition SGEFΔN + RhoG-kd4 when compared with the corresponding control condition SGEFΔN + kdcontrol (Supplementary Figure S4). This finding suggests that a substantial amount of neuronal RhoG is inactive (particularly during RhoG overexpression) but could be activated by a GEF (e.g., SGEFΔN) to reduce axonal branching. Collectively, these results reveal that RhoG inhibits axonal branching.

**RhoG inhibits axonal branching via ELMO/Dock180/Rac1 signalling**

A functional ELMO/Dock180 signalling module leading to Rac1 activation has been presented to be essential for RhoG stimulating neurite outgrowth in PC12 cells (Katoh and Negishi, 2003). The functional significance of the ELMO/Dock180 module was also ascertained for phagocytosis as well as engulfment of apoptotic cells (Gumienny et al., 2001; deBakker et al., 2004; Elliott et al., 2010; Lu et al., 2011), and the invasion of pathogenic bacteria (Handa et al., 2007; Roppenser et al., 2009). Based on these published data, the question arose whether this signalling pathway is also fundamental for RhoG-inhibited axonal branching. Overexpression of the dominant-negative RhoG-F37A and RhoG-G12VF37A mutants, unable to bind to ELMO (Katoh and Negishi, 2003), in DIV2 hippocampal neurons resulted in a considerable increase in axonal branching (Figure 3A and B). These results suggest that the ELMO/Dock180/Rac1 signalling pathway is important for RhoG-mediated inhibition of axonal branching. We further corroborated these data by examining the effects of the dominant-negative ELMO deletion construct ELMO-D625, which cannot bind to Dock180 (deBakker et al., 2004; Meller et al., 2008), and the dominant-negative Rac1 construct Rac1-T17N on axonal branching. We found that both constructs significantly increased the number of axonal end tips/100 μm (Figure 3A and B). Moreover, the knockdown of either Dock180 or Rac1 expression by the shRNA constructs Dock180-kd and Rac1-kd (Meller et al., 2008; Supplementary Figure S5A and B), respectively, resulted in increased axonal branching (Figure 3A and B). In addition, RhoG knockdown led to a reduction in the amount of active, GTP-bound Rac1 in axons (Supplementary Figure S6). To further demonstrate the importance of the ELMO/Dock180/Rac1 signalling module for RhoG-mediated inhibition of axonal branching, overexpression of RhoG-G12V was combined with knockdown of Rac1. We found that dominant-positive RhoG did not inhibit axonal branching if Rac1 expression was reduced by RNA interference with Rac1-kd (Figure 3A and B). Therefore, these findings demonstrate that RhoG inhibits axonal branching via the ELMO/Dock180/Rac1 signalling pathway.

**RhoG inhibits dendritic branching dependent on the small GTPase Cdc42**

Surprisingly, however, RhoG-F37A, which is unable to bind to ELMO and to stimulate Rac1 activation via Dock180, did not increase but rather decreased the total number of

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**Figure 2** RhoG inhibits axonal branching. (A) The axonal morphology of hippocampal neurons, transfected with the indicated constructs at DIV2, was analysed after staining for GFP at DIV2 +2 (arrowheads). Scale bar, 15 μm. (B) RhoG-kd4 increased, while RhoG-G12V decreased the number of axonal end tips. Mean values (n = 80 neurons for EGFP and EGFP + RhoG-G12V, n = 40 neurons for all other conditions) ± s.e.m. (**P < 0.005; *P < 0.05).
Figure 3 RhoG inhibits axonal branching via ELMO/Dock180/Rac1 signalling. (A) The axonal morphology of hippocampal neurons, transfected with the indicated constructs at DIV2, was analysed after staining for GFP at DIV2+2 (arrowheads). Scale bar, 20 µm. (B) RhoG-F37A and RhoG-G12VF37A, RhoG mutants that cannot bind to ELMO, strongly increased the number of axonal end tips. The same effect was determined for ELMO-D625, Rac1-T17N, Dock180-kd, and Rac1-kd. Under the condition of Rac1 knockdown, RhoG-G12V did not decrease the number of axonal end tips. Mean values (n = 80 neurons for Dock180-kd and kdcntrol, n = 40 neurons for all other conditions) ± s.e.m. (***P<0.0005; **P<0.005; *P<0.05).
dendritic end tips in DIV7 hippocampal neurons (Figure 4A and B). In addition, knockdown of Rac1 also decreased TNDET, and overexpression of RhoG combined with knockdown of Rac1 synergistically affects TNDET (Figure 4A and B). These results imply that RhoG does not reduce dendritic tree complexity via the ELMO/Dock180/Rac1 sig-

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**Figure 4** RhoG inhibits dendritic branching dependent on Cdc42. (A) The dendritic phenotype of hippocampal neurons, transfected with the indicated constructs at DIV7, was analysed after staining for GFP at DIV7 + 3. Note that RhoG-F37A led to a considerable reduction in dendritic tree complexity, but still increased axonal branching (arrow). Scale bar, 15 μm. (B) RhoG-F37A decreased TNDET to a similar degree as RhoG did. Knockdown of Cdc42 increased, whereas knockdown of Rac1 decreased TNDET. Combining Rac1 knockdown with RhoG overexpression synergistically decreased TNDET. Under the condition of Cdc42 knockdown performed by expression of Cdc42-kd, RhoG no longer decreased TNDET. Under these conditions, TNDET equals that of Cdc42-kd expression alone. Similarly, knockdown of Cdc42 led to a rescue of TNDET when co-expressed with RhoG-F37A. Mean values (n = 40 neurons) ± s.e.m. (***P < 0.0005; *P < 0.05). (C) Neurons transfected as described above were analysed by Sholl analysis for all experimental conditions. For each point of the Sholl graph, the mean values (n = 24 neurons) ± s.e.m. are shown (***P < 0.0005; **P < 0.005; *P < 0.05). Light blue stars, Cdc42-kd + RhoG compared with kdcontrol + RhoG; dark blue stars, Cdc42-kd + RhoG-F37A compared with kdcontrol + RhoG-F37A; green stars, Rac1-kd + RhoG compared with kdcontrol + RhoG; orange stars, kdcontrol + RhoG-F37A compared with kdcontrol + RhoG; grey stars, Cdc42-kd + RhoG compared with Cdc42-kd + RhoG-F37A.
nalling pathway. Instead, we found that the small GTPase Cdc42 is imperative for RhoG-mediated inhibition of dendritic branching. Knockdown of Cdc42 with the shRNA construct Cdc42-kd (Leemhuis et al., 2010; Supplementary Figure S8C) led to an increase in TNDET. Additionally, the decrease in TNDET mediated by RhoG was completely reversed in the presence of Cdc42-kd. Actually, even though Cdc42 knockdown was performed concurrent to RhoG overexpression, we could not determine any reduction of TNDET when compared with the respective Cdc42 knockdown alone (Figure 4A and B). A similar outcome could be observed when combining overexpression of RhoG-F37A with knockdown of Cdc42 (Figure 4A and B). Sholl analysis of all experimental conditions confirmed the results presented above (Figure 4C). Only a subtle difference could be noticed when comparing the effects of RhoG overexpression with RhoG-F37A overexpression in the distal part of the Sholl graph. RhoG-F37A decreased the number of dendritic intersections in the distal part of the dendritic tree to a higher extent than RhoG. Taken together, these data reveal that RhoG reduces dendritic branching via Cdc42 signalling.

The microRNA miR-124 regulates the expression of RhoG

According to the algorithms PicTar (Krek et al., 2005), miRanda (John et al., 2004), TargetScan (Lewis et al., 2005), and MiRtarget2 (Wang and El Naqa, 2008) for microRNA target prediction, the 3′UTR of the RhoG gene comprises two miR-124-binding sites (Figure 5A; Supplementary Figure S7). These sites are highly conserved in mice, rats, and humans, while the 3′UTRs derived from the genes coding for Mtl and Mig-2 (the functional equivalents of RhoG in Drosophila and Caenorhabditis elegans, respectively) do not contain miR-
124-binding sites. To experimentally verify the miR-124-regulated gene expression of RhoG, we used a sensor construct comprising large parts of the 3′ UTR of the RhoG target gene with the two miR-124-binding sites downstream of an EGFP expression cassette (Figure 5B). This sensor construct, EGFP–3′UTR(RhoG), was transfected into HEK293 cells together with vector-based miR-124 and the expression of EGFP was analysed by immunoblotting. It was conspicuously reduced in comparison to the expression of the EGFP control construct, which contains the EGFP expression cassette but lacks the 3′UTR of the RhoG target gene (Figure 5C). In contrast, the construct RhoG–3′UTR(RhoG)–Mut4.1 harbouring two point mutations in the ‘seeds’ of both miR-124-binding sites (see Materials and methods) was expressed as high as the unregulated EGFP control construct (Figure 5C).

To examine whether endogenously expressed miR-124 (Krichevsky et al., 2003; Deo et al., 2006; Sanuki et al., 2011) was able to reduce the sensor construct expression in primary hippocampal neurons, we measured the fluorescence intensity of EGFP 4 h after transfection of these neurons (Figure 5D; Supplementary Figure S8A). A clear reduction of EGFP expression was observed when using the sensor construct EGFP–3′UTR(RhoG) (Supplementary Figure S8A). A rescue of EGFP expression was observed in neurons expressing EGFP–3′UTR(RhoG)–Mut4.1. This indicated an expression regulation depending on the miR-124-binding sites in the 3′UTR of the sensor. To further substantiate this finding, we analysed a sensor construct comprising the full-length RhoG 3′UTR including the endogenous polyadenylation signal [EGFP–3′UTRfull(RhoG)]. Nearly identical results were observed (Figure 5E and F). If miR-124 regulated RhoG expression, an increased expression of endogenous RhoG would be expected when inhibiting miR-124. Therefore, anti-miR-124, which should capture the endogenously expressed miR-124, was co-transfected together with EGFP into hippocampal neurons. Then a quantification of the RhoG-specific immunopositive signals in neuronal cell bodies of transfected neurons was performed (Supplementary Figure S8B). We detected a significant increase in RhoG expression when inhibiting endogenous miR-124 by the transfected anti-miR-124. Therefore, anti-miR-124, which should capture the endogenously expressed miR-124, was co-transfected together with EGFP into hippocampal neurons. Then a quantification of the RhoG-specific immunopositive signals in neuronal cell bodies of transfected neurons was performed (Supplementary Figure S8B). We detected a significant increase in RhoG expression when inhibiting endogenous miR-124 by the transfected anti-miR-124 (Figure 5G). Finally, we wanted to verify that miR-124 regulates RhoG expression by an interaction with the miR-124-binding sites in the 3′UTR of the RhoG-encoding message. To this end, anti-miR-124 was co-transfected with the sensor construct EGFP–3′UTR(RhoG) or the corresponding EGFP control construct, respectively, in hippocampal neurons.

Figure 6 miR-124 increases dendritic complexity by inhibiting RhoG expression. (A) The dendritic phenotype of hippocampal neurons, transfected with the indicated constructs at DIV7, was analysed after staining for GFP at DIV7 + 3. Scale bar, 15 μm. (B) Anti-miR-124 decreased TNDET. RhoG-kd4, co-expressed with anti-miR-124, rescued TNDET. miR-124 increased TNDET. Co-expression of RhoG with miR-124 abolished the increase of TNDET induced by miR-124. Mean values (n = 40 neurons for all experiments with anti-miR, n = 80 neurons for all experiments with miR precursors) ± s.e.m. (***P < 0.0005; *P < 0.05). Blue stars, anti-miR-124 + kdcntrol compared with anti-miR-control + kdcntrol; light blue stars, anti-miR-control + RhoG-kd4 compared with anti-miR-control + kdcntrol; dark blue stars, anti-miR-124 + RhoG-kd4 compared with anti-miR-124 + kdcntrol; orange stars, miR-124 + pCLEG–GFP compared with miR-control + pCLEG–GFP; yellow stars, miR-control + pCLEG–RhoG compared with miR-124 + pCLEG–GFP; brown stars, miR-124 + pCLEG–RhoG compared with miR-124 + pCLEG–GFP.
miR-124 increases dendritic and axonal complexity by inhibiting RhoG expression

We showed that RhoG expression is endogenously regulated by the microRNA miR-124 (Figure 5). Therefore, the question arose whether miR-124 could have an impact on dendrite elaboration by inhibiting RhoG expression. To tackle this question, we first determined the effects of either enhanced miR-124 expression or capture of endogenous miR-124 by anti-miR-124 on dendrite morphology. We found that miR-124 increased, while anti-miR-124 decreased TNDET (Figure 6A and B). Obviously, RhoG is not the only target for expression regulation by miR-124 (Gao, 2010; Sanuki et al., 2011). To substantiate that miR-124 increases dendritic tree complexity through inhibition of RhoG expression, we co-expressed miR-124 together with RhoG lacking the miR-124-binding sites in the 3’UTR. This overexpressed RhoG, which is not susceptible for miR-124, abolished the increase of TNDET induced by miR-124. In addition, TNDET could be rescued when reducing endogenous RhoG expression with the shRNA construct RhoG-kd4 in the presence of anti-miR-124 (Figure 6A and B). All these results were confirmed by performing Sholl analysis (Figure 6C).

Overexpression of wild-type RhoG was sufficient to reduce dendritic tree complexity but not to inhibit axonal branching (Figures 1C, D, H and 2B). Thus, we assumed that increasing endogenous RhoG expression by means of anti-miR-124 would not reduce axonal branching. In fact, we found that miR-124, leading to a cellular knockdown of RhoG, but not anti-miR-124, resulted in an upregulation of RhoG expression, affecting axonal branching by increasing the number of axonal end tips/100 µm (Figure 7A and B). This effect of miR-124 on axonal branching was likely to be mediated specifically by RhoG, because RhoG-G12V was shown to abrogate the increase in axonal branching, which was induced by miR-124 (Figure 7A and B). Taken together, these findings indicate that miR-124 regulates axonal and dendritic tree complexity by inhibition of RhoG expression (Figure 7C).

Discussion

We unravelled RhoG as a novel target for miR-124-dependent expression regulation, and assigned a precise developmental function to this regulation: miR-124 stimulates axonal and dendritic tree complexity by inhibiting RhoG expression (Figure 7C). RhoG, in fact, was uncovered to reduce axonal and dendritic tree complexity in primary neuronal cell culture and in the mouse brain in vivo. These results are surprising as it was shown that RhoG overexpression promotes neurite outgrowth both in the PC12 cell line (Katoh et al., 2000; Estrach et al., 2002; Katoh and Negishi, 2003) and in rat superior cervical ganglion (SCG) neurons in culture (May et al., 2002). The apparent discrepancies in the outcome of RhoG-regulated process differentiation in PC12 cells and SCG neurons in comparison to hippocampal neurons could be attributable to the fact that most of the previous studies were based on RhoG overexpression, or did employ dominant-negative as well as constitutively active mutants of RhoG, which can exhibit nonspecific or nonphysiological effects resulting from perturbation of several Rho GTPases (Wang and Zheng, 2007). In our present study, we combine overexpression of wild-type RhoG, constitutively active, or dominant-negative RhoG mutants with different knockdown
approaches employing RNA interference, and get concordant results. More likely, however, the observed difference in the functional outcome of RhoG-driven neuronal process morphogenesis in PC12 cells and SCG neurons compared with hippocampal neurons may stem from differences in the cellular systems analysed. It is tempting to speculate that RhoG could function in a lineage-specific manner. In fact, SCG neurons belong to the sympathetic nervous system, and PC12 cells are derived from a tumour of the sympatheticadrenaline lineage (Anderson, 1993; Unsicker, 1993), thus differing from hippocampal neurons in origin.

Although RhoG reduces neuronal process complexity in both dendrites and axons, we found subtle differences. Whereas the dominant-positive RhoG mutant RhoG-G12V reduced dendritic and axonal branching, wild-type RhoG only inhibited dendritic but not axonal branching. Furthermore, while both RhoG knockdown constructs increased dendritic tree complexity, only the stronger knockdown construct RhoG-kd4 stimulated axonal branching. In line with these results is our finding that anti-miR-124, leading to an increase in RhoG expression, reduced dendritic but not axonal complexity. All of these observations may be explained by a limited availability of RhoG-activating GEFs during axon, but not during dendrite differentiation. Several GEFs being able to activate RhoG have been described as Vav family members (Schuebel et al., 1998; Samson et al., 2010), Trio (Bellanger et al., 2000; Blangy et al., 2000; Estrach et al., 2002), Kalirin (May et al., 2002), and Ephexin4 (Hiramoto-Yamaki et al., 2010). Hence, it is also conceivable that different GEFs activate RhoG for reducing either axonal or dendritic tree complexity, respectively.

Different aspects support our hypothesis that overexpressed RhoG may be functionally inactive due to limited RhoG-stimulating endogenous GEF activity under certain experimental conditions: (1) Several previous studies on RhoG function in different experimental settings only used dominant-positive RhoG-G12V or RhoG-Q61L constructs but not wild-type RhoG to demonstrate RhoG-specific biological effects (Katoh and Negishi, 2003; van Buul et al., 2007; Yamaki et al., 2007; Meller et al., 2008). (2) A recent study showed that dominant-positive RhoG-G12V but not wild-type RhoG promotes the proliferation of neural progenitor cells whereas reduction of endogenous RhoG expression via RNA interference decreased this proliferation (Fujimoto et al., 2009). (3) In this study, we demonstrate that overexpression of the SGEFAN construct, which comprises the DH/PH module harbouring the GEF activity for RhoG, reduced axonal branching in hippocampal neurons in a RhoG-dependent manner (Supplementary Figure S4). The SGEFAN is derived from SGEF, a GEF that is active on RhoG but not on Rac1 (Ellerbroek et al., 2004).

The striking upregulation of miR-124 during the time of axonal and dendritic development suggests that this microRNA and its target genes may play specific roles during these processes. Actually, several recent studies reported on a relevance of miR-124 in neuronal process formation. In the CAD cell line, derived from CNS catecholaminergic neurons, miR-124 induces the outgrowth of long neurites, which branch and form complex networks (Makeyev et al., 2007). In addition, in mouse P19 embryonal carcinoma cells differentiating to neurons, miR-124 promotes neurite outgrowth (Yu et al., 2008). Interestingly, this outgrowth can be blocked by co-expression of constitutive-active Cdc42, but only to a lesser extent by constitutive-active Rac1, suggesting that inhibition of Cdc42 is necessary for miR-124 function. In a well-characterized model system to study neuronal process differentiation (Dotti et al., 1988), we specified the function of miR-124 and established this microRNA to contribute to dendritic tree complexity by repressing the expression of RhoG, which reduces dendritic tree complexity through Cdc42. On the first view contrary to our results, Edbauer et al. (2010) did not observe any effects of miR-124 expression on dendritic growth or arborization in hippocampal neurons in culture. However, they used DIV14 + 3 hippocampal neurons, while our experiments were performed with DIV7 + 3 neurons. When using DIV14 + 3 neurons in our experimental setting, in agreement with Edbauer et al. (2010), we neither found a significant increase in TNFε elicited by miR-124 nor a significant shift of the Sholl graph caused by miR-124 (Supplementary Figure S9). However, miR-124 still led to a small (although not significant) shift of the Sholl curve to more dendritic tree complexity at DIV14 + 3 (Supplementary Figure S9B). Dendritic growth in hippocampal neurons in culture starts around DIV4, and the dendritic tree of most neurons is well elaborated around DIV14. It is therefore likely that miR-124 has no longer a significant impact on dendritic growth and arborization during the time when dendritogenesis is largely completed but spinogenesis proceeds. In line with our data are also the results obtained by Yoo et al. (2009), who showed that miR-124 stimulates activity-dependent dendritic growth by switching BAF53a to BAF53b expression. Together, a model arises, in which miR-124 promotes the elaboration of dendritic trees.

Finally, we delineated different signalling pathways involved: RhoG inhibits axonal branching via ELMO/Dock180/Rac1, while reducing dendritic tree complexity dependent on Cdc42 (Figure 7C). Previously, it has been shown that RhoG is able to drive two different signalling pathways: the ELMO/Dock180-stimulated activation of Rac1 (Katoh and Negishi, 2003) and the phosphatidylinositol 3-kinase (PI3K)-mediated phosphorylation of Akt (Murga et al., 2002; Harada et al., 2011). The latter pathway was published to be important for RhoG-dependent regulation of cell survival and anoikis as well as RhoG-promoted neural progenitor cell proliferation in the cerebral cortex (Fujimoto et al., 2009; Harada et al., 2011). The former pathway was identified as critical for phagocytosis and engulfment of apoptotic cells (Gumienny et al., 2001; de Bakker et al., 2004; Elliott et al., 2010; Lu et al., 2011), the invasion of pathogenic bacteria (Handa et al., 2007, Roppenser et al., 2009), cell migration (Katoh et al., 2006), and neurite outgrowth of PC12 cells (Katoh and Negishi, 2003). However, the relevance of RhoG-induced, and Rac1-dependent effects on the actin cytoskeleton has been discussed controversially (Meller et al., 2008). Furthermore, it has been suggested that RhoG may independently activate Rac1 and Cdc42 (Gauthier-Rouvière et al., 1998).

Our results support the hypothesis that RhoG can independently activate Rac1 and Cdc42. The first indication was the finding of the divergent effects of the mutant RhoG-F37A on axonal versus dendritic branching. RhoG-F37A contains a mutation in the effector region of RhoG that prevents RhoG-F37A from binding to ELMO (Katoh and Negishi, 2003). However, based on studies of a F37A mutant derived
from Rac1, which is structurally similar to RhoG, it is likely that RhoGF37A still can bind GEFs (Lamarche et al., 1996; Schwartz et al., 1998). This way, Rho-F37A would function in a dominant-negative manner for the RhoG/ELMO/Dock180/Rac1 signalling pathway by inhibiting the activation of endogenous RhoG through these GEFs. Consequently, Rho-G-F37A increases axonal branching. On the other hand, it was shown biochemically and functionally that the F37A mutant of Rac1 can still activate several signalling molecules (Lamarche et al., 1996; Schwartz et al., 1998). Based on these results, it is likely that RhoG-F37A can still activate other signalling molecules independent from ELMO1. Our data support the view that Rho-G-F37A decreased dendritic branching by means of two different effects: (1) Rho-G-F37A, like RhoG, stimulates the activity of Cdc42, which is inhibitory for dendritic branching, probably through a so far unknown signalling intermediate; (2) RhoG-F37A cannot increase Rac1 activity, which is stimulatory for dendritic branching (Figure 4B and C). This interpretation could also give an explanation for the subtle difference, which could be observed when comparing the effects of RhoG and Rho-G-F37A on the distal part of the dendritic tree (Figure 4C). Together, the results of our present study strengthen the view that RhoG is capable of activating either of these two key molecules (Rac1 and Cdc42) dependent on the cellular and developmental context.

A relevance of Rac1 signalling for the regulation of axonal branching in the Drosophila giant fibre system was formerly proposed (Allen et al., 2000). Another study highlighted the dependence of axon growth, guidance, and branching regulation of Drosophila mushroom body neurons on differential activation of Rac1, Rac2, and Mtl (the functional equivalent of RhoG in Drosophila) signalling (Ng et al., 2002). The same study showed that even Mtl single mutants represent an axon branching phenotype. Similarly, redundant functions of Cdc-10, Rac2, and Mlg-2 (the functional equivalent of RhoG in C. elegans) in axon branching and pathfinding of several neuron classes in C. elegans were demonstrated (Lundquist et al., 2001; Struckhof and Lundquist, 2003).

Finally, a potential significance of Cdc42 for inhibition of dendritic branching in Drosophila neurons was suggested (Gao et al., 1999). Furthermore, Cdc42 knockout as well as dominant-negative Cdc42 led to increased branching of the endfoot of radial glial cells, which function as neural stem cells (Yokota et al., 2010). Thus, our results strengthen an unappreciated facet of Rac1 and Cdc42 functions in neuronal process development, and point to the view that RhoG is a cellular regulator of Rac1 and Cdc42 activity, in turn regulated by the microRNA mir-124.

Materials and methods

DNA constructs

Most of the 3’UTR sequence of the RhoG mRNA (shown in Supplementary Figure S7), which comprised the miR-124-binding sites, was amplified from rat brain cDNA with the following primer pair: 5’-CACGAATTCCTGGACTCTGCTCGACTGGCTATCTTCTCA-3’ and 3’-CACGGGATCCGCTCTCTCTCTCTGGGAGGGAGAGAGATCAT–5’. The amplified sequence was inserted into the 3’UTR of the EGFP gene carried on a modified pEGFP-C1 vector (Clontech) with a stop codon inserted at the 5’end of the MCS to give the sensor construct EGF–3’UTR (RhoG). The construct EGF–3’UTR (RhoG–Mut4.1) carries the following mutations in the “seeds” of the conserved mir-124-binding sites: ‘GTGCCCT’ of site 1 is mutated to ‘GTGCCCC’ and ‘GTGCCCTA’ of site 2 is mutated to ‘GTGCCCTG’. The sensor construct EGF–3’UTR(RhoG) comprising the full-length 3’UTR sequence of the RhoG message including the endogenous polyadenylation site was derived from an PCR amplification product from rat brain cDNA with the following primer pair: 5’-CACGAAATTCGGCCTCAAGTCGCAGCTGGGGTTAATGCTTCGACCC-3’ and 5’-CACAGATTCATCAAGCTGAGCAGCAGCTGGGGTATCTTTCCGTCAGTCG-3’, and was cloned into the modified pEGFP-C1 vector as described above. The construct EGF–3’UTR(RhoG–Mut4.1) was cloned analogous to EGF–3’UTR (RhoG)–Mut4.1.

The RhoG construct was cloned into the vectors pCMV–HA or pCMV–myc (Clontech). The primer pair used for amplification from rat brain cDNA was as follow: 5’-CACGAATTCCTGGACTCTGCTCGACTGGCTATCTTCTCA-3’ and 3’-CACGAAATTCGGCCTCAAGTCGCAGCTGGGGTTAATGCTTCGACCC-3’. The construct RhoGresist(kd4) was derived from the construct RhoG by introducing two point mutations into the binding site for RhoG–kd4 (ACTTTCGGCATATGCTTC) using the QuickChange Site-directed Mutagenesis kit (Stratagene). For the IUE experiments, RhoG was subcloned into the pCLE vector (Brandt et al., 2007). This vector, derived from the pCLE vector and used for gain-of-function experiments, contains a CMV/MLV promoter, an EF1α enhancer, and an internal ribosomal entry site (IRES) for the translation of GFP as a reporter (Chen et al., 2005).

Mutations of RhoG were introduced using the QuickChange Site-directed Mutagenesis kit (Stratagene) and the following primer pairs were used to generate the constructs RhoG-G12V and RhoG-F37A: 5’-CACGAATTCCTGGACTCTGCTCGACTGGCTATCTTCTCA-3’ and 3’-CACGAAATTCGGCCTCAAGTCGCAGCTGGGGTTAATGCTTCGACCC-3’ and 5’-GCCGTCGTAATGGCAGGATGCAAGATTCGCGTAGATCCGGGAGGAGACCGA-3’.

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Cell culture and transfection
Primary hippocampal neurons were prepared from embryonic days 18–19 Wistar rat pups as described (Brandt et al., 2007). The cells were cultured on poly-L-lysine-coated glass coverslips in neurobasal A medium supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine, and the antibiotics penicillin and streptomycin at a density of 150 000 cells/well.

Hippocampal neurons were transfected with Effectene (Qiagen) according to the manufacturer’s instructions. HEK293 cells were transfected with FuGene6 according to the manufacturer’s instructions. Anti-miR-124, anti-miR-control, miR-124 precursor, and miR-control precursor were purchased (Ambion). The final concentration in the cell culture experiments was 40 nM for anti-miR-124 and anti-miR-control, and 30 nM for miR-124 precursor and miR-control precursor.

Immunohistochemistry and immunocytochemistry
P10 mice were deeply anaesthetised and sacrificed by transcardial perfusion with 4% paraformaldehyde. Vibratom sections (50 μm thick) were prepared and, after permeabilization in PBS supplemented with 5% fetal calf serum and 0.2% Triton X-100, incubated with a polyclonal antibody to GFP (ab6556, Abcam). Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) was used. Hippocampal cells in culture were indirectly stained after permeabilization with a polyclonal antibody to GFP (ab6556, Abcam), and co-stained with a monoclonal antibody to endogenous HA or mcherry (clone 9E10, Roche) tag. Stainings for endogenously expressed RhoG, Dock180, and Rac1 were performed with a monoclonal anti-RhoG antibody (clone 1F3B3E5, Millipore #04-486), a polyclonal anti-Dock180 antibody (sc-6167, Santa Cruz Biotechnology), and a monoclonal anti-Rac1 antibody (clone 23A8, Millipore). Active, GFP–Rac1 was detected with a monoclonal anti-active Rac antibody (NewEast, #26903). T7-Cdc42 was visualized by an indirect immunostaining with a monoclonal antibody to the T7 tag (#69522-3, Novagen). The secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 568-conjugated goat anti-mouse (Molecular Probes), and Cy3-conjugated goat anti-mouse (Dianova) were used according to the manufacturer’s instructions. Cells were imaged with a fluorescence microscope (Olympus, BX50) equipped with a Cool SNAP ES digital camera (Roper Scientific). For fluorescence imaging, the filters used were U-MWIG, U-MNIBA, and U-MWU2 (Olympus) were used. For higher magnification pictures, an oil immersion objective (PLAN APO × 60, 1.4 NA) was used.

In-utero electroporations
The IUE experiments were carried out with C57BL/6 mice as described in accordance with a protocol approved by the local animal welfare committee (Brandt et al., 2007). The morning of a detectable vaginal plug and the first neonatal day were considered to be embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively. Plasmids were prepared using EndoFree Plasmid Kit (Qiagen, Hilden, Germany). Pregnant mice were anaesthetised at embryonic day 0.5 and postnatal day 0 (P0), respectively. Plasmids were electroporated into the uterus wall through the uterine wall with a CUY21 EDIT (Nepagene) or a ECM830 (BTX) square wave electroporator and a pair of platinum electrodes (CUY650P5). Electric pulses were delivered to embryos through the uterine wall into the lateral ventricle of the embryo using a pulled glass capillary (World Precision Instruments).

Cell transfection was performed after 3 days later for analysis. The quantification of fluorescence intensities in neurite outgrowth and synaptogenesis in the giant fiber system. Mol Cell Neurosci 16: 754–765


References

Conflict of interest
The authors declare that they have no conflict of interest.
Rhö reduces neuronal process complexity

K Franke et al


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