Agrin regulates growth cone turning of *Xenopus* spinal motoneurons

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Summary

The pivotal role of agrin in inducing postsynaptic specializations at neuromuscular junctions has been well characterized. Increasing evidence suggests that agrin is also involved in neuronal development. In this study, we found that agrin inhibited neurite extension and, more importantly, a gradient of agrin induced repulsive growth-cone turning in cultured *Xenopus* spinal neurons. Incubation with a neutralizing antibody to agrin or expression of the extracellular domain of muscle-specific kinase, a component of the agrin receptor complex, abolished these effects of agrin. Agrin-induced repulsive growth-cone turning requires the activity of PI3-kinase and Ca²⁺ signaling. In addition, the expression of dominant-negative Rac1 inhibited neurite extension and blocked agrin-mediated growth-cone turning. Taken together, our findings suggest that agrin regulates neurite extension and provide evidence for an unanticipated role of agrin in growth-cone steering in developing neurons.

Key words: Axon guidance, Neurite outgrowth, PI3-kinase, Rac1, Calcium signaling

Introduction

Agrin was originally identified on the basis of its ability to induce the aggregation of acetylcholine receptors (AChRs) in muscle (Bezáková and Ruegg, 2003). In motoneurons, agrin is synthesized and released at the synaptic terminals to regulate the formation of postsynaptic AChR clusters at neuromuscular junctions (NMJs) through a receptor complex, which includes a receptor tyrosine kinase, muscle-specific kinase [MuSK (Glass et al., 1996; Kleiman and Reichardt, 1996)]. The absence of AChR clusters at NMJs of agrin knockout mice indicates the essential role of agrin in the maturation of NMJs (Gautam et al., 1996).

In addition to motoneurons, agrin is expressed by all neuronal populations in the central nervous system (CNS), and is implicated to have potential functions in the formation and maturation of central synapses (Cohen et al., 1997; Kroger and Schroder, 2002; O’Connor et al., 1994; Rupp et al., 1991). In addition, the prominent expression of agrin in neurons during axonal growth prior to synapse formation suggests that it may have a presynaptic function, e.g. in regulating axon extension. Consistent with this notion, aberrant arborization of motor axon terminals was observed in the agrin knockout mice (Gautam et al., 1996). Cell culture studies have also shown that neurite extension was inhibited by various isoforms of agrin (Bixby et al., 2002; Campagna et al., 1995; Mantych and Ferreira, 2001).

How agrin exerts its action on developing neurons is beginning to be explored. Treatment of primary neuronal cultures with agrin leads to the activation of cAMP-response element binding protein (Ji et al., 1998) and the expression of c-fos (Hilgenberg et al., 1999). To initiate its action on neurons, agrin is likely to first activate a cell-surface receptor. Evidence for agrin-binding sites on the surface of central neurons has recently been reported (Burgess et al., 2002; Hoover et al., 2003), although the identity of the receptor and the immediate cytoplasmic transduction events triggered by agrin binding to the neuronal surface remain unknown. At the NMJs, postsynaptic MuSK activation is known to mediate the effect of agrin in inducing AChR clusters. Unlike agrin, the expression of MuSK in mammals appears to be largely restricted to muscle, although MuSK transcripts could also be detected in neural tissues in lower vertebrates (Fu et al., 1999; Ip et al., 2000; Valenzuela et al., 1995). Thus, the possibility exists that the action of agrin on neurons may be mediated through a receptor tyrosine kinase that is homologous to MuSK, in a manner similar to that in the muscle cell.

In this study, we have examined the effects of agrin on neurite extension and steering using developing spinal neurons prepared from *Xenopus* embryos. We report that agrin inhibits neurite outgrowth in a dose-dependent manner and that a gradient of agrin results in Ca²⁺-dependent repulsive growth-cone turning. Furthermore, we show that the agrin-induced neuronal response depends upon the activity of Rac1. Taken together, our findings suggest that agrin regulates neurite extension and, more importantly, provide the first
demonstration of a novel role of agrin in the steering of growing axonal terminals.

Materials and methods

Preparation of cultures of Xenopus spinal neurons

Cultures of Xenopus spinal neurons were prepared from 1-day-old Xenopus laevis embryos as described (Ming et al., 1997). The culture medium was composed of 50% (v/v) Leibovitz medium (Invitrogen), 1% (v/v) fetal bovine serum (HyClone, Logan, UT, USA) and 49% (v/v) Ringer’s solution (115 mM NaCl, 2 mM CaCl$_2$, 2.5 mM KCl and 10 mM HEPES, pH 7.4). Cells were plated at a low density and maintained at room temperature (20-22°C) for 5-10 hours prior to treatment.

Neurite extension and growth cone turning assay for Xenopus spinal neurons

Fast-growing neurons were used for the neurite extension and growth-cone turning assay. Extending neurites were captured at different time intervals with a time-lapsed CCD (charge-coupled device) camera (TK-C1381; JVC, Yokohama, Japan) attached to a phase-contrast microscope (CK-40, Olympus, Tokyo, Japan) and analyzed using Scion Image programs. Only neurons with a neurite extension rate of more than 5 μm/hour prior to drug treatment were included for analysis. Neurite extension rate was normalized by comparing the extension rates of the neurons before and after the addition of drugs for the indicated periods. The growth-cone turning assay was carried out as described (Song et al., 1997). Briefly, microscopic gradients of drugs were produced with a micropipette placed 100 μm away from the center of the growth cone of an isolated neuron, at an angle of 45° with respect to the initial direction of neurite extension (indicated by the last 10 μm segment of the neurite). The turning angle was defined as the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the beginning and the end of the 1-hour period. Theoretical analysis and direct measurements of the gradient using fluorescent dyes have shown that, at a distance of 100 μm from the pipette tip, the concentration gradient across the growth cone (typical width 10 μm) is in the range of 5-10%, and the average concentration at the growth cone is about 10$^{-3}$-fold lower than that in the pipette. Microscopic images of neurites were captured and stored using Scion Image programs as previously described (Yuan et al., 2003). To determine the total length of neurite extension, the whole trajectory of the neurite at the end of the 1-hour period was measured with a digitizer. Only neurons with a neurite extension rate of more than 5 μm/hour over the 1-hour period were included in the analysis of turning angles.

All experiments were carried out at room temperature in modified Ringer’s solution (140 mM NaCl, 2.5 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$ and 10 mM HEPES, pH 7.4). Agrin was obtained from R&D Systems and prepared in 1×PBS containing 50 μg/ml of bovine serum albumin at pH 7.4. Data were reported as means ± s.e.m.; statistical significance was analyzed by Student’s t-test or one-way ANOVA.

cDNAs encoding MuSK mutant and Rac1 GTPase, and microinjection into Xenopus embryos

A MuSK cDNA fragment that lacked the tyrosine kinase domain was subcloned into pcDNA1 tagged with the Fc region of IgG to generate EC-MuSK (Yang et al., 1997). FITC-dextran and EC-MuSK cDNA were microinjected into one or two blastomeres of 2- or 4-cell-stage embryos with an Eppendorf transjector 5246 (Eppendorf, Hamburg, Germany). Injected embryos were incubated in 10% Ringer’s solution at room temperature (20-22°C) for 24 hours prior to culture preparation. The green fluorescence of FITC-dextran was used to identify the injected progeny cells as previously described (Ming et al., 1999). The cDNA construct encoding N17 dominant-negative Rac1 (DN-Rac1) fused with GFP was subcloned into pcDNA2 (a gift from D. Turner, University of Michigan, Ann Arbor, MI) at the Stul site. DN-Rac1 (generously provided by G. Bokoch, Scripps Research Institute, La Jolla, CA, USA) is a competitive inhibitor of Rac GTPase that binds irreversibly to guanine nucleotide-exchange factors, which are upstream regulators of GTPases. The plasmids were purified using Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany). The final concentration of cDNAs for microinjection was 0.2 μg/μl for DN-Rac1 and 0.5 μg/μl for GFP and EC-MuSK, and total amounts of 1.5 ng and 5 ng were injected, respectively.

Western blot analysis and GTPase activation assay

Expression of injected constructs was confirmed using western blot analysis. Five Xenopus embryos (stage 22-24) were collected and homogenized in 0.2 ml of lysis buffer (0.1% SDS, 1% Nonidet P-40, 1% glycerin, 50 mM HEPES, pH 7.4, 2 mM EDTA and 100 mM NaCl) by sonication. The homogenates were centrifuged at 13,000 g for 5 minutes. The supernatant was mixed with equal volume of 1,1,2-trichlorotrifluoroethane and centrifuged again to remove the yolk. The protein was incubated with antibodies against EGFP (polyclonal, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Rac1 (monoclonal, 1:1000; Upstate, Charlottesville, VA, USA) at 4°C overnight. Chemiluminant detection was performed using the Supersignal kit (Pierce, Rockford, IL, USA). GTPase activity was measured using a Rac/Rho activation assay kit (Upstate). Briefly, cultured cerebellar granule neurons from postnatal day 6 to 8 (P6-P8) were treated with agrin, the cells were washed with ice-cooled PBS, lysed at 4°C and incubated either with Pak1-PBD (Pak-binding domain of Rac1 and Cdc42) agarose, or Rhoetkin-binding Sepharose beads with constant rocking at 4°C. The proteins bound to the beads were washed three times with lysis buffer at 4°C, eluted in SDS sample buffer, and analyzed for bound Rac1 or Rho by western blotting using antibodies against Rac1 or Rho. GTPase activity was quantified by densitometry analysis of the blots.

Results

Extracellular gradient of agrin-induced repulsive growth-cone turning

In agrin-deficient mice, motor axons exhibit aberrant nerve terminal arborization and fail to induce AChR clusters (Gautam et al., 1996), suggesting that agrin might direct motor axons to synaptic sites on target cells. To examine whether agrin can indeed play a role in axon guidance, a microscopic gradient of agrin was applied to cultured Xenopus spinal neurons using a micropipette at 5 to 10 hours after plating, and growth-cone turning was examined as described previously (Yuan et al., 2003). Recombinant C-terminal rat agrin (Y$_4$Z$_8$) was applied to cultured Xenopus spinal neurons. Isolated neurons with fast-growing neurites were selected for neurite outgrowth assay at ~5-10 hours after plating. Only the neurons that showed a substantial amount of neurite extension (>5 μm/hour) prior to the addition of agrin were selected for the analysis. As shown in Fig. 1A,C, we observed a marked repulsive turning of the growth cones away from the source of agrin within 1 hour after the initial application of the agrin gradient, whereas a gradient of BSA had no effect on neurite extension (Fig. 1A). This repulsion could be completely abolished by the agrin-neutralizing antibody AGR-530, whereas the neutralizing antibody alone showed no obvious effect on growth-cone turning (Fig. 1B,C).

To further confirm the specificity of the inhibitory response to agrin, an alternative approach of expressing a fusion protein
Agrin induces growth cone turning

Agrin induces growth cone turning by a gradient of agrin. (A,B) Left: microscopic images of a neuron (cultured for 5-10 hours) at the beginning (0 min) and the end (60 min) of a 1-hour exposure to a drug gradient created by pulsatile application (arrows) with a micropipette. Right: superimposed traces depict the trajectory of neurite extension during the 1-hour turning assay. (A) Top, BSA (50 μg/ml in pipette); bottom, agrin (100 μg/ml in pipette). (B) Top, Ab, agrin-neutralizing antibody (200 μg/ml in pipette); bottom, agrin preincubated with the antibody (Agrin+Ab). Each value represents the average ± s.e.m.; *P<0.01 (one-way ANOVA). Scatter plots show the distribution of the turning angles for each growth cone of *Xenopus* neuronal growth cones induced by a gradient of BSA, agrin, agrin-neutralizing antibody (Ab) and agrin preincubated with the antibody (Agrin+Ab). Each value represents the average ± s.e.m.; *P<0.01 (Student’s t-test). Scatter plots show the distribution of neurite extension rate for the *Xenopus* neuronal growth cones examined. (D) Overexpression of a MuSK mutant inhibited agrin-induced growth-cone turning. Left: microscopic images of cultured *Xenopus* spinal neurons, prepared from embryos injected with either empty vector (Mock) or EC-MuSK, at the beginning (0 min) and the end (60 min) of a 1-hour exposure to an agrin gradient created by the pulsatile application (arrows) of agrin (100 μg/ml in the micropipette). Right: superimposed traces of neurites depicting the trajectory of neurite extension during the 1-hour turning assay. (E) Left: histogram showing the averaged turning angles of growth cones from neurons expressing different constructs in the presence of an agrin gradient applied with a micropipette (agrin, 100 μg/ml in the micropipette). Each value represents the average ± s.e.m.; *P<0.01 (Student’s t-test). Scatter plots show the distribution of neurite extension rate of each neuron.

Agrin inhibited neurite extension in *Xenopus* spinal neurons

Microscopic images were recorded using a time-lapse CCD camera, and the neurite extension rate was measured before and after the addition of agrin (20 ng/ml). We found that bath-applied agrin inhibited the neurite extension of *Xenopus* spinal neurons in a dose-dependent manner (Fig. 2A-C), whereas no significant inhibitory effect was observed in control neurons treated with bovine serum albumin (BSA, 50 μg/ml; Fig. 2A). To examine the specificity of agrin in inhibiting the extension of neurites, an agrin-neutralizing antibody (AGR-530) previously reported to block agrin-induced AChR clustering at the NMJ was used (Hilgenberg et al., 1999). Addition of this neutralizing antibody to the agrin solution used in the above neurite growth experiments completely abolished the inhibition effect of the agrin solution on neurite extension (Fig. 2D,E), whereas this neutralizing antibody alone did not affect neurite growth (data not shown). Interestingly, expression of EC-MuSK abolished the agrin-mediated inhibition of neurite outgrowth (Fig. 2D,E).

Dependence of agrin-induced growth-cone turning on Ca²⁺ and PI3-kinase

Both Ca²⁺ and phosphoinositide 3-kinases (PI3-kinase) play key roles in the signaling of axon guidance (Ming et al., 1999; Nishiyama, 2003). In *Xenopus* spinal neurons, growth-cone turning triggered by netrin 1, brain-derived neurotrophic factor (BDNF) and myelin-associated glycoprotein requires both (EC-MuSK) comprising the extracellular and transmembrane domains of MuSK fused to the Fc region of an immunoglobulin was utilized. A similar strategy of using truncated forms of receptor tyrosine kinases was shown to be effective in neutralizing the activity of their cognate ligands (Croll et al., 1998; McMahon et al., 1995). We expressed the fusion protein in *Xenopus* spinal neurons by injecting FITC-dextran together with a cDNA construct encoding EC-MuSK into the blastomeres of stage 22 *Xenopus* embryos. In cultures of dissociated spinal neurons, the green fluorescence provided a reliable marker for identifying progeny cells derived from injected blastomeres (data not shown) (Alder et al., 1995). Interestingly, overexpression of EC-MuSK in these neurons not only blocked agrin-induced repulsive growth-cone turning, but apparently converted repulsion into attraction (Fig. 1D,E).
Ca\textsuperscript{2+} signaling and PI3-kinase activity (Ming et al., 1997; Wong et al., 2002; Yuan et al., 2003). Consistent with a previous report (Zheng et al., 1996), we observed that neurons cultured in Ca\textsuperscript{2+}-free solution (CFS) exhibited a higher neurite extension rate (Fig. 3A,C). Unexpectedly, growth cones of neurons grown in CFS showed a marked attractive turning response towards the source of agrin (Fig. 3A). Moreover, Ca\textsuperscript{2+} release from internal stores also appeared to be necessary, as depletion of Ca\textsuperscript{2+} stores by pre-incubating the neurons with thapsigargin (TG) blocked the agrin-induced turning response (Fig. 3B,C). Taken together, these results indicate that agrin-induced repulsive growth cone turning depends on both extracellular Ca\textsuperscript{2+} and internal Ca\textsuperscript{2+} store. Treatments with PI3-kinase specific inhibitors, wortmannin or LY294002, also abolished agrin-induced repulsion (Fig. 4A,B). Taken together, these results indicate that, like the property of some axon guidance factors (the ‘group I’ (Ming et al., 2002; Ming et al., 1999)), agrin-induced repulsive growth-cone turning is dependent on both Ca\textsuperscript{2+} and PI3-kinase.

**Agrin downregulated Rac1 activity in cerebellar granule neurons**

Previous studies have shown that the small Rho family GTPase Rac1 is involved in the agrin-mediated signaling that triggers
Agrin induces growth cone turning (Weston et al., 2000). Because Rac1 is known to regulate neurite growth in a number of preparations (Daniels et al., 1998; Threadgill et al., 1997), we examined the potential role of Rac1 in agrin-induced neuronal responses. GTPase activity in cultured cerebellar granule neurons in response to agrin was examined by using a pulldown assay. We observed a marked decrease in Rac1 activity within 1 minute of agrin treatment, and the reduction persisted for up to 30 minutes, whereas Rho activity was not significantly affected (Fig. 5A). This finding suggests that decreased Rac1 activity may underlie the axon growth inhibition in response to agrin, although the modulation of neuronal Rac1 activity by agrin is different from that observed in muscle, where agrin was found to enhance Rac1 activity (Weston et al., 2000).

**Dependence of agrin-induced chemorepulsion on Rac1 activity**

We further examined whether Rac1 is also involved in the agrin-induced turning response by overexpressing a fusion construct of dominant-negative Rac1 (DN-Rac1) and GFP in *Xenopus* spinal neurons. The expression of DN-Rac1-GFP in *Xenopus* cells was confirmed by western blot analysis using antibodies against GFP and Rac1 (data not shown). Isolated neurons expressing DN-Rac1-GFP were identified by the expression of green fluorescence. Consistent with a previous report (Yuan et al., 2003), expression of DN-Rac1 caused a significant reduction in the neurite extension rate (Fig. 5B,C). When the agrin gradient was applied to neurons expressing DN-Rac1-GFP, we found that agrin-induced growth-cone turning was completely abolished, even when the observation period was extended to 2 hours (Fig. 5B,C), which suggests that Rac1 is indeed required for agrin-induced growth-cone turning.

**Discussion**

The key role of agrin in the induction of post-synaptic differentiation in muscle is well recognized. Although agrin is widely expressed in the nervous system, the functions and signaling mechanisms of agrin in neurons remain unclear. There is accumulating evidence that suggests a role for agrin in the regulation of synapse formation, neuronal differentiation, axon elongation and branching (Lai and Ip, 2003a; Lai and Ip, 2003b; Luo et al., 2003). Agrin knockout mice exhibit abnormal intramuscular nerve branching and nerve terminal differentiation (Gautam et al., 1996). Similarly, exuberant growth of motor axons and an absence of nerve terminal arborization are apparent in MuSK knockout mice (DeChiara et al., 1996). Most motor axons in agrin/MuSK knockout mice do not form secondary branches or arbors, but run long distances parallel to the myotube and eventually end without apparent specializations (DeChiara et al., 1996; Gautam et al., 1996). This phenotype suggests that agrin deficiency might result in axonal path-finding errors. Consistent with these studies, our results show that bath application of agrin inhibits the neurite extension of *Xenopus* spinal neurons. More importantly, we provide the first demonstration that the extracellular gradient of agrin triggers the turning of growth cones, which suggests an unexpected axon guidance function for agrin.
The calcium ion is a key intracellular messenger in regulating growth-cone extension (Gomez and Spitzer, 1999; Takei et al., 1998) and steering (Hong et al., 2000; Zheng, 2000). Similar to that observed for c-fos induction in neurons and AChR aggregation on cultured myotubes upon agrin treatment, there is a requirement for Ca^{2+} in neurite extension and growth-cone turning initiated by agrin. We also found that Ca^{2+} derived from both the extracellular space and intracellular stores is required for mediating the effect of agrin. Moreover, we observed that neurons incubated in CFS displayed a higher rate of neurite extension. This may be due to the removal of Ca^{2+} transients in growth cones, which has been reported to inhibit neurite extension (Lautermilch and Spitzer, 2000). In addition, a gradient of agrin-induced growth-cone attraction instead of repulsion in CFS. Depleting intracellular Ca^{2+} stores by the pre-incubation of neurons with thapsigargin blocked the agrin-induced turning response. This finding underscores the importance of intracellular Ca^{2+} in axon guidance signaling.

For a variety of surface receptors for mammalian growth factors, PI3-kinase is a crucial component of the initial cytoplasmic signaling pathways. The synthesis of the lipid product of PI3-kinase has been implicated in the rearrangement of the actin cytoskeleton, through the activation of the small GTP-binding protein Rac1 (Hawkins et al., 1995; Kundra et al., 1994; Nobes et al., 1995; Wennstrom et al., 1994). In the present study, we showed that pre-incubation of Xenopus spinal neurons with PI3-kinase inhibitors abolished agrin-induced growth-cone turning. Moreover, agrin inhibited IGF1-induced phosphorylation of Akt, a downstream target of PI3-kinase (data not shown). Taken together, our findings suggest that the interaction of agrin with its receptor expressed at the neuronal surface may regulate the cytoplasmic PI3-kinase, which in turn modulates the growth-cone extension.

Growth-cone turning involves remodeling of the growth cone and bending of the axon. This depends on reorganization of the actin filaments and microtubules, which are the primary cytoskeletal components of growth cones. Accumulating evidence shows that small GTPases of the Rho family, Rac1, Rho and Cdc42, regulate the organization of actin filament structures in growth cones in response to extracellular signals (Lundquist, 2003). Mutations of Rho GTPases result in axon guidance defects both in vitro and in vivo (Kaufmann et al., 1998; Luo et al., 1994; Yuan et al., 2003). In our study, we show that agrin can inhibit neuronal Rac1 activity. A gradient of inhibition of Rac1 activity across the growth cone may result in a gradient of actin filament polymerization, with reduced polymerization and a lower level of filopodial activity on the proximal side facing the pipette, leading to the repulsive turning of the growth cone. Expression of DN-Rac1 in Xenopus spinal neurons significantly inhibited neurite extension and abolished agrin-induced growth-cone turning by attenuating the action of agrin on Rac1-dependent actin filament polymerization. These findings, together with the report on the involvement of Rac1 in agrin-induced AChR aggregation in muscle (Weston et al., 2000), suggest that Rac1 is the downstream effector of agrin signaling in both muscle and neurons, and that agrin may inhibit axon extension and growth-cone repulsion through the inhibition of Rac1 activity.

Although there is ample evidence of an agrin-dependent signaling pathway in neurons (Hilgenberg et al., 1999; Ji et al., 1998; Karasewski and Ferreira, 2003), little is known about the identity of the neuronal agrin receptor. Recently, the domain of agrin that binds to its receptor in neurons was identified (Burgess et al., 2002; Hoover et al., 2003). Early studies on the expression profile of a component of agrin receptor complex, MuSK, in mammalian species indicate that it is largely restricted to skeletal muscle (Valenzuela et al., 1995). However, we have subsequently reported that MuSK transcripts could be detected in the developing neural tube and eye vesicles of Xenopus, and the postnatal cerebellum of chicken (Fu et al., 1999; Ip et al., 2000). To date, the agrin receptor in the CNS was unidentified. It is noteworthy that, in the present study, overexpression of EC-MuSK converts the repulsive agrin-induced growth-cone turning to an attractive property. Interestingly, a MuSK homolog was recently reported in zebrafish and suggested to be involved in axonal pathfinding (Zhang et al., 2004).
Agrin induces growth cone turning

Our findings on the ability of agrin to induce growth-cone turning imply that this molecule may function as an axon guidance molecule in development. Similar to axon guidance cues such as BDNF and netrin 1, the turning response induced by agrin also requires both Ca$^{2+}$ and PI-3 kinase. The action of many guidance cues on growth cones can be 'switched' between attraction and repulsion in a manner that depends on the level of cytosolic cyclic nucleotides (Jones and Werle, 2004), or the developmental stage (Hoch et al., 1993). It would be of interest to determine whether the switch from repulsion to attraction found for agrin in the present study is due to similar cytoplasmic mechanisms. As agrin is secreted by motoneuron nerve terminals during the synaptogenesis of NMJs, our findings suggest that these secreted agrin molecules might play a role in shaping the pattern of motor axonal terminal arbors through their action on the growth cones.

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