The neuronal Arf GAP centaurin α1 modulates dendritic differentiation

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Summary

Centaurin α1 is an Arf GTPase-activating protein (GAP) that is highly expressed in the nervous system. In the current study, we show that endogenous centaurin α1 protein is localized in the synaptosome fraction, with peak expression in early postnatal development. In cultured dissociated hippocampal neurons, centaurin α1 localizes to dendrites, dendritic spines and the postsynaptic region. siRNA-mediated knockdown of centaurin α1 levels or overexpression of a GAP-inactive mutant of centaurin α1 leads to inhibition of dendritic branching, dendritic filopodia and spine-like protrusions in dissociated hippocampal neurons. Overexpression of wild-type centaurin α1 in cultured hippocampal neurons in early development enhances dendritic branching, and increases dendritic filopodia and lamellipodia. Both filopodia and lamellipodia have been implicated in dendritic branching and spine formation. Following synaptogenesis in cultured neurons, wild-type centaurin α1 expression increases dendritic filopodia and spine-like protrusions. Expression of a GAP-inactive mutant diminishes spine density in CA1 pyramidal neurons within cultured organotypic hippocampal slice cultures. These data support the conclusion that centaurin α1 functions through GAP-dependent Arf regulation of dendritic branching and spines that underlie normal dendritic differentiation and development.

Keywords

Arf; GAP; PI 3-kinase; Dendrite; Neuronal; Development

Introduction

Phosphoinositide (PI) 3-kinases are essential for neuronal survival, differentiation and plasticity (see Kutsol et al., 2001; Rodgers and Theibert, 2002; Engelman et al., 2006). Centaurin α1 (also named p42IP4 and PIP3BP) has been proposed to be a neuronal PI 3-kinase target that functions in the regulation of Arf GTPases (reviewed by Jackson et al., 2000; Rodgers and

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/15/2683/DC1
Theibert, 2002; Hawkins et al., 2006). Centaurin α1 is highly enriched in the nervous system and expressed in a variety of neurons, with peak mRNA expression in early postnatal development (Hammonds-Odie et al., 1996; Stricker et al., 1997; Tanaka et al., 1997; Aggensteiner and Reiser, 2003). Centaurin α1 has two pleckstrin homology (PH) domains that mediate PtdIns(3,4,5)P3 binding (Tanaka et al., 1997). In addition, centaurin α1 possesses a highly conserved domain present in GTPase-activating proteins (GAPs) for Arfs (Jackson et al., 2000). Arfs are a family of GTPases that function in vesicular trafficking and cytoskeletal organization (Randazzo et al., 2000; Nie and Randazzo, 2006; D’Souza-Schorey and Chavrier, 2006). Centaurin α1 does not display in vitro Arf GAP activity, but binds Arfs in vitro, colocalizes with Arfs in vivo, and overexpression induces a decrease in Arf6 GTP levels, indicative of Arf GAP activity (Thacker et al., 2004; Venkateswarlu et al., 2004).

Of the six Arf genes identified, Arf1 and Arf6 are the best characterized. Arf1 is involved in vesicular trafficking in the Golgi, trans-Golgi network (TGN) and endosomal compartments whereas Arf6 regulates trafficking in the plasma membrane/recycling endosomal compartment and cytoskeletal organization at the cell periphery (Donaldson and Jackson, 2000; Nie and Randazzo, 2006; D’Souza-Schorey and Chavrier, 2006). Arfs facilitate coat protein assembly onto budding membranes, and activate PtdIns(4)P 5-kinase and phospholipase D (PLD). Arf6 has also been shown to directly affect the Rho-GTPase family member, Rac1 (D’Souza-Schorey and Chavrier, 2006; Cotton et al., 2007). Arfs are regulated by GTP-exchange factors (GEFs), which facilitate GTP binding, and by Arf GAPs which stimulate GTP hydrolysis (Donaldson and Jackson, 2000; Nie and Randazzo, 2006). Arf GAPs have also been proposed to function with Arfs in coat assembly and disassembly (Nie and Randazzo, 2006).

In addition to its characterized roles in trafficking, Arf6 has been implicated in neuronal differentiation. Expression of inactive mutants of the Arf GEF ARNO or EFA6A, or the constitutively GDP-bound Arf6 mutant enhances dendritic branching, axonal outgrowth and spine density (Hernandez-Deviez et al., 2002; Hernandez-Deviez et al., 2004; Sakagami et al., 2004; Miyazaki et al., 2005). Conversely, expression of an inactive mutant of the Arf GAP p95-APP1 or the constitutively GTP-bound Arf6 mutant inhibits neurite extension, blocks dendritic filopodia and affects spines (Albertinazzi et al., 2003; Zhang et al., 2003; Gauthier-Campbell et al., 2003; Miyazaki et al., 2005; Choi et al., 2006). These studies have led to the proposal that Arf6-GDP is a stimulator of axonal and dendritic outgrowth and branching, whereas Arf6-GTP is an inhibitor of these structures.

To date, the requirement for an endogenous Arf GAP in neuronal differentiation has not been established. In the current study, we have characterized the developmental expression of endogenous centaurin α1 and tested its requirement for differentiation in cultured neurons and neuronal slices. Centaurin α1 is expressed in the brain during prenatal and early postnatal development. It localizes to developing dendrites and following differentiation is localized in dendritic spines and synaptic regions. Knocking down centaurin α1 levels by siRNA or expression of a GAP-inactive mutant centaurin α1 leads to a dramatic decrease in dendritic branching and dendritic spines. Overexpression of centaurin α1 shows the opposite phenotype with increased dendritic arborization and spine-like protrusions. The results reported herein support the function for centaurin α1 in Arf-dependent regulation of dendritic differentiation.

**Results**

**Developmental expression and localization of endogenous centaurin α1 in rodent brain**

In the present study, we investigated the developmental expression and localization of endogenous centaurin α1 in rodent brain and cultured neurons. Centaurin α1 protein expression is detectable as early as E16 and increases during prenatal and postnatal brain development (Fig. 1A). Peak expression is observed between 2 and 4 weeks postnatally in brain and...
expression persists in the adult at approximately half the peak developmental levels. This protein expression is in concordance with published mRNA and protein expression data (Aggensteiner and Reiser, 2003) and corresponds to a period of robust neuronal differentiation and synaptogenesis in the hippocampus (Melloni and DeGennaro, 1994). We detected two closely migrating protein bands, irrespective of the specific antibody or lysis protocol used, which showed variable resolution. Whether this doublet results from post-translational processing, alternative splicing, or proteolysis is currently under investigation.

In fractionated P23 rat brain, endogenous centaurin α1 is present in the supernatant (S2) and crude microsomal and synaptosomal fraction (P2) in approximately equal levels, consistent with previous results showing both cytosolic and membrane association for centaurin α1. Further fractionation of P2 was performed to yield the purified synaptosome fraction, an enriched synaptic fraction that contains both presynaptic proteins, including syntaxin and synapsin, and post-synaptic proteins such as PSD95 (Fig. 1B) (Gordon-Weeks, 1987). Centaurin α1 is also present in purified synaptosomes, with an enrichment over P2 comparable with the other synaptic proteins examined (Fig. 1B). The centaurin α1 protein associated with synaptosomes showed a peak developmental expression at P28, similar to the expression of total centaurin α1 protein (Fig. 1C). Centaurin α1 expressed in the adult is also associated with the synaptosome fraction. This result is in agreement with immunohistochemistry results that show centaurin-α1/p42IP4 localizes to synaptic regions in neuronal sections (Kreutz et al., 1997; Sedehizade et al., 2002) and is consistent with the identification of centaurin α1 in the post-synaptic density fraction by proteomic analysis (Yoshimura et al., 2004).

Expression of endogenous centaurin α1 in dissociated cultured hippocampal neurons

Centaurin α1 is expressed in the cell soma and processes of hippocampal neurons in the CA1, CA3 and dentate gyrus regions (Fig. 1D and data not shown). Cultured hippocampal neurons provide an excellent model system to study neuronal differentiation and synaptogenesis (Goslin and Banker, 1989; Papa et al., 1995), therefore we examined the expression and localization of centaurin α1 in primary hippocampal cultures. Neurons were dissociated from E18 rat brain and cultured from 3 to 21 days in vitro (DIV). Centaurin α1 protein expression, measured by immunoblot analysis, could be detected at 3 DIV and expression increased in culture with maximal expression at the latest time examined, 21 DIV (Fig. 2A). Thus, the expression of centaurin α1 in cultured neurons paralleled its in vivo expression. Furthermore, the expression of centaurin α1 in developing cultured hippocampal neurons also mirrored that of the dendritic spine marker spinophilin, the presynaptic marker synaptotagmin and the postsynaptic marker PSD95 (Fig. 2A).

At 3 DIV, neurons extend short immature processes. In these neurons, centaurin α1 localized in a punctate pattern in the cell soma, as well in the numerous short immature processes (Fig. 2B and supplementary material Fig. S1). Punctate staining in undifferentiated neurons is consistent with association of centaurin α1 with membrane compartments. By 7 DIV, after processes have begun to differentiate into the lone axon and multiple dendrites, centaurin α1 was localized in puncta throughout the cell soma, and in the numerous dendritic-like processes, but not in the axon. Dendrites typically branch repeatedly to form a complex tree-like structure (arbor) that received inputs from other neurons at the synapse. After synapse formation by 14 and 21 DIV, centaurin α1 was localized throughout the processes, and often localized in large puncta, suggestive of a spine or synaptic location (see below).

Localization of endogenous centaurin α1 in dissociated cultured hippocampal neurons

The localization of centaurin α1 was compared with well-characterized neuronal marker proteins, the dendritic marker MAP2, the axonal marker tau, two presynaptic markers: synaptotagmin and SV2, the spine marker spinophilin, and the excitatory postsynaptic marker
PSD95 (Huber and Matus, 1984). Levels of colocalization between centaurin α1 and these marker proteins in dendritic segments were quantified (Sailer et al., 2004; Swanwick et al., 2004; Zhang et al., 2006). At 7 and 14 DIV, centaurin α1 localized to both the cell soma and to MAP2 positive dendrites (Fig. 3). Interestingly, although centaurin α1 localized within the MAP-2 positive dendrites, only an average 21% of centaurin α1 staining colocalized with MAP2. Centaurin α1 was usually found at the lateral regions of the dendrite and to a lesser extent in the central core region where microtubules, stained with beta-tubulin and MAP2 are located (Fig. 3 and supplementary material Fig. S2) (Kaec et al., 2001). The centaurin α1 that did colocalize with MAP2 was often localized near dendritic branch points (Fig. 3). By contrast, centaurin α1 did not appear to localize to axonal processes marked by tau (Fig. 3).

Approximately 15% colocalization with tau was observed (supplementary material Fig. S2) (Binder et al., 1985). We estimate an approximate 5-7% nonspecific or background colocalization between proteins known to reside in different compartments. The 15% level of colocalization is above background but probably represents the overlap of closely apposed staining between dendrites and axons (see below).

Centaurin α1 staining within MAP2-positive dendrites was punctate and found closely juxtaposed to, but not usually colocalizing with, the presynaptic markers synaptotagmin and the synaptic vesicle-2 protein (SV2) (Fig. 3 and supplementary material Fig. S2) (Fletcher et al., 1991). Close apposition between centaurin α1 (found in dendrites) with the presynaptic markers was suggestive of a postsynaptic localization for centaurin α1. We observed approximately 18% colocalization between centaurin α1 and either of the presynaptic markers in dendritic segments. A similar extent of colocalization (15%) has been reported between presynaptic and postsynaptic markers (Zhang et al., 2006). When centaurin α1 was compared with the postsynaptic marker PSD95 or the spine marker spinophilin, many puncta showed exact colocalization (Fig. 3). We measured an overall 28% colocalization with PSD95 and 30% colocalization with spinophilin (Fig. 3 and supplementary material Fig. S2) (Hunt et al., 1996; Allen et al., 1997), and consistent with the observation that centaurin α1 localizes to more than one compartment, throughout the dendrite, dendritic spines and the postsynaptic region.

Knockdown of centaurin α1 by siRNA in dissociated cultured hippocampal neurons

The developmental expression and dendritic localization highlighted the possibility that centaurin α1 might function in dendritic differentiation in neurons. To address this, endogenous centaurin α1 levels were knocked down in hippocampal neurons using double-stranded siRNA transfection by Amaxa nucleofection, which yields >90% transfection efficiency of siRNA (Gresch et al., 2004) (Fig. 4). Following centaurin-α1-specific siRNA treatment, there was a significant decrease in centaurin α1 protein levels of 60% at 3 DIV and a more moderate decrease of 40-45% at 7 DIV as assessed by immunoblot analysis (Fig. 4A). Centaurin α1 siRNA transfection resulted in a reduction of centaurin α1 staining by immunofluorescence, with a substantial depletion of centaurin α1 from dendritic processes but a relative sparing of some perinuclear centaurin α1 staining, suggesting that different pools of centaurin α1 may have different turnover rates (Fig. 4C).

At 3 DIV, GFP-control-transfected neurons contained several dendrites, which generally displayed a few branches and several filopodia (Fig. 4 and supplementary material Fig. S3). We measured axonal and dendritic length and the number of terminal tips – a well-established measure of branching (Uylings and van Pelt, 2002; Kumar et al., 2005). Decreasing centaurin α1 levels at 3 DIV inhibited dendritic branching and length but did not affect the axon (Fig. 4C). Knockdown of centaurin α1 produced an average 70% decrease in the number of terminal tips and a 73% inhibition of dendritic length at 3 DIV (Fig. 4D). At 7 DIV, the terminal tips
were reduced by 46%, and the dendritic length was reduced by 34%, suggesting that these processes were recovering as the centaurin α1 levels recovered. As with 3DIV, no statistically significant effect on axonal length was observed at 7DIV. By 14 DIV, the levels of centaurin α1 protein had returned to control (Fig. 4A), and the dendritic morphology appeared normal, indicating that the effects of centaurin α1 knockdown were reversible (supplementary material Fig. S3).

Knockdown of centaurin α1 at 3 and 7 DIV also inhibited lamellipodia and filopodia (Fig. 4). Lamellipodia are thin, broad, veil-like extensions that contain a dynamic array of branched actin filaments. Filopodia are finger-like projections containing tight bundles of actin. Both structures are crucial to the growth and differentiation of neuronal processes, and are shown to be involved in dendritic branching and spine formation (reviewed by Luo, 2002; Chen and Ghosh, 2005).

To demonstrate the specificity of the siRNA effects, we tested whether expression of an siRNA-resistant cDNA plasmid could rescue the siRNA-dependent inhibition of dendritic outgrowth and branching. The human centaurin α1 CDNA is divergent from the rat cDNA in several nucleotides and therefore is more resistant to the rat siRNAs. Expression of the human centaurin α1 plasmid in siRNA-treated neurons produced an overexpression of human centaurin α1 of approximately three- to fivefold at 3 and 7 DIV (Fig. 4B). This expression fully restored dendritic length and branching in the siRNA-treated neurons at both 3 and 7 DIV (Fig. 4D,E). In fact, the rescued neurons showed a statistically significant 34% and 91% increase in number of terminal tips at 3 and 7 DIV, respectively, and a 64% and 102% increase in dendritic length at 3 and 7 DIV, respectively, compared with control scrambled siRNA-treated neurons (Fig. 4E). There was not a statistically significant change in axon length. These data support the conclusion that centaurin α1 is required for early dendritic differentiation in cultured neurons.

We next assessed the effects of overexpressing wild-type and mutant rat centaurin α1 on neuronal morphology. Amaxa nucleofection yields approximately 50% transfection efficiency of mammalian expression plasmids in neurons (Gresch et al., 2004). Immunoblot analysis was used in parallel cultures to determine the levels of overexpression of centaurin α1 at 3, 7 and 14 DIV following transfection (supplementary material Fig. S4A). Centaurin α1 levels were normalized to the actin levels. Given the 50% transfection efficiency, we expect a nine- to tenfold increase of centaurin α1 levels at 3DIV, and a fourfold increase at 7 and 14 DIV in the transfected neurons (supplementary material Fig. S3 and Fig. 4).

**Overexpression of wild-type centaurin α1 in developing dissociated hippocampal neurons**

The staining pattern of expressed centaurin α1, assessed with an anti-Flag antibody, was similar to the staining of the endogenous protein with localization in the cell body and dendrites in a punctate pattern. However, unlike the endogenous protein, expressed centaurin α1 also localized to the axon. Overexpression of centaurin α1 at 3 DIV led to an increase in filopodia and branches arising from the cell soma and the dendrites (Fig. 5A and supplementary material Fig. S4). At 3 DIV, expression of centaurin α1 led to a statistically significant 22% increase in terminal tips (Fig. 5C). A statistically significant 15% increase in total dendritic length was also observed. By 7 DIV, centaurin α1 overexpression led to a 54% increase in the number of terminal tips and a 17% increase in the dendritic length. Expression of centaurin α1 also increased the size and number of lamellipodia and filopodia at 3 and 7 DIV (supplementary material Fig. S4). At 7 and 14 DIV, the expressed Flag-centaurin α1 colocalized with spine and synaptic markers to a similar extent as the endogenous protein (data not shown). Although localized to the axon, no effects on the axonal processes were observed at 3 or 7 DIV. By 14 DIV, centaurin α1 levels remained elevated, and there was also a clear increase in dendritic branching and filopodia (Fig. 5B); however, as the neuronal processes were so long and complex at this stage, we were unable to quantify these effects. Thus, expressed centaurin α1
localizes to the same compartments as endogenous centaurin α1 and enhances dendritic branching and filopodia in developing cultured neurons. Interestingly, rat centaurin α1 was not as effective as human centaurin α1 in enhancing dendritic outgrowth and branching in rat hippocampal neurons, which could be a result of differences in the proteins or their expression levels.

**Overexpression of a GAP-inactive mutant centaurin α1**

Centaurin α1 is an Arf GAP (Venkateswarlu et al., 2004). Mutation of the conserved arginine in the GAP domain has been shown to eliminate GAP activity in all Arf GAPs examined, including centaurin α1. Furthermore, expression of GAP mutants can result in inhibition of endogenous Arf GAPs (Randazzo and Hirsch, 2004; Venkateswarlu et al., 2004). Similarly to the expressed and endogenous wild-type protein, the GAP mutant localized to dendrites in a punctate pattern (Fig. 6) and colocalized with markers of spines and synapses (data not shown).

Overexpression of the GAP mutant R49K centaurin α1 in hippocampal neurons led to a significant decrease in dendritic branching and dendritic length at 3, 7 and 14 DIV (Fig. 6A and supplementary material Fig. S5). Expression of R49Kcentaurin α1 at 3 DIV produced a 35% decrease in the number of terminal tips and a 30% decrease in dendritic length (Fig. 6B). At 7 DIV, a 30% decrease in terminal tips and a 22% decrease in dendritic length was observed (Fig. 6B). These effects were statistically significant (P<0.05). No significant effects on the axon length were observed at 3 DIV or 7 DIV. Likewise, the GAP inactive mutant did not appear to inhibit the size of lamellipodia. At 14 DIV, there was a discernible inhibition of dendritic branching, but as described above, the processes were so long and complex that the effects could not be quantified. Although not as robust, the inhibitory effects of the GAP-inactive mutant were similar to those observed with siRNA, and consistent with the GAP-inactive mutant acting as a dominant-negative inhibitor. These results suggest that the GAP activity of centaurin α1 is required for its regulation of dendritic branching.

**Overexpression of wild-type and GAP-inactive centaurin α1**

At 14 DIV the centaurin α1 expression remained elevated about fourfold (supplementary material Fig. S4A). Thus, we could address whether centaurin α1 functions during later stages of dendritic differentiation, during dendritic filopodia and spine formation. Dendritic spines are regions where excitatory synapses form when filopodia are contacted by the axon (Tada and Sheng, 2006). At 14 DIV, neurons display mature spines, immature spines and dendritic filopodia. We classified all three structures as dendritic protrusions for analysis (Kumar et al., 2005). Neurons overexpressing wild-type centaurin α1 for 14 DIV showed a dramatic (73%) increase in the number of dendritic protrusions (Fig. 7). In addition, there was an increase in the number of long and branched filopodia in the centaurin α1 expressing neurons. By contrast, expression of the GAP-inactive mutant R49K centaurin α1 produced a statistically significant reduction in dendritic protrusions of 15% (Fig. 7). By this stage of development, few lamellipodia were observed in either control or transfected neurons. These data suggest that centaurin α1 may function at later stages of dendritic differentiation in spine development or spine maintenance.

**Centaurin α1 localizes to dendritic spines and regulates dendritic spine density in CA1 neurons**

To address this further, we overexpressed centaurin α1 or the GAP-inactive mutant in organotypic hippocampal slice cultures, which provide another valuable experimental preparation for investigating dendritic development (Gahwiler et al., 1997). An advantage of slice cultures is the preservation of neuronal integrity and circuitry, allowing neurons to undergo development in culture that parallels in vivo development (Lo et al., 1994; Alonso et al., 2004). Slices were cotransfected with eYFP and either wild-type centaurin α1 or R49K
centaurin α1, or with dsRed as a control. CA1 pyramidal cells were identified by their characteristic morphology and location within the hippocampus (Fig. 8). In agreement with its endogenous localization in dissociated hippocampal cultures, expressed centaurin α1 was detected in dendritic regions and in dendritic spines (Fig. 8). Over 98% of cells expressing eYFP also expressed the cotransfected plasmid (data not shown), consistent with results showing that over 90% of neurons in slices transfected with two plasmids express both proteins (Klimaschewski et al., 2002; Wirth et al., 2003).

Expression of wild-type centaurin α1 in P9 slices for 36 hours did not affect dendritic spine density in CA1 pyramidal neurons compared with the dsRed control (8.5±0.5 spines/10 μm with dsRed vs 8.0±0.5 spines/10 μm with centaurin α1) (Fig. 8). Thus endogenous centaurin α1 levels may facilitate maximal differentiation in the P9-11 slice cultures. However, expression of the GAP-inactive mutant R49K centaurin α1 significantly reduced spine density by 39% (5.2±0.6 spines/10 μm; P<0.00016 compared with dsRed control) (Fig. 8). Inhibition of dendritic spines by R49K centaurin α1 expression in organotypic slices was more robust than its effects in dissociated neurons and provides further evidence that centaurin α1 functions through its GAP activity to regulate spine formation or stability.

Discussion

The results reported herein demonstrate that centaurin α1 is a developmentally expressed Arf GAP that is required for dendritic differentiation in developing neurons. In the brain, centaurin α1 protein expression peaks during the first few postnatal weeks and is enriched in the purified synaptosome fraction. In developing hippocampal neurons, centaurin α1 localizes to dendrites, dendritic spines and synapses. Knockdown of centaurin α1 levels by siRNA in cultured dissociated hippocampal neurons significantly reduces dendritic branching and length, but the axon is unaffected. This inhibition is specific because it could be rescued by expression of siRNA-resistant human centaurin α1. Likewise, expression of a dominant-negative GAP mutant centaurin α1 in cultured neurons and organotypic hippocampal slices inhibits dendritic branching and spine density, respectively. By contrast, overexpression of wild-type centaurin α1 increases dendritic branching and dendritic protrusions. As a modulator of dendritic arborization and spine density, centaurin α1 may directly impact the number and dynamics of synapses in the developing brain.

Centaurin α1 protein was detectable in brain at E16, a stage of rapid proliferation, differentiation and migration of neuronal cells. Centaurin α1 protein levels peaked between 2 and 4 weeks postnatally, in accordance with data reported for centaurin α1 mRNA (Aggensteiner and Reiser, 2003). Centaurin α1 expression increased in developing cultured neurons between 3 and 21 DIV. This temporal expression is similar to that reported for the spine marker spinophilin, the early presynaptic markers synapsin I and bassoon, and postsynaptic marker PSD-95 (Melloni and DeGennaro, 1994; Allen et al., 1997; Zhai et al., 2001; Bresler et al., 2001). This corresponds to a broad period of neuronal differentiation, including dendritic arborization, spine formation and synaptogenesis in the developing rat cortex and hippocampus (Gaarskjaer, 1981; Melloni and DeGennaro, 1994).

The results from siRNA knockdown and GAP mutant expression, and its localization in dendrites and spines during development support a role for centaurin α1 in both dendritic branching and spine regulation. There is now a strong precedent for proteins serving a dual function in both dendritic branching and spine dynamics. For example, members of the Rho-GTPase family, Rac1, Cdc42 and Rnd1/2 have been shown to function in both dendritic branching and spine development (see Govek et al., 2005; Watabe-Uchida et al., 2006; Ishikawa et al., 2006). PSD-95 is a protein involved in dendritic spine maturation that plays an additional role in regulating dendrite outgrowth and branching (Charych et al., 2006). The
origin recognition core (ORC) complex protein is required for dendritic branching and initiating dendritic spine formation (Huang et al., 2005). In addition, EFA6, an Arf GEF, and Arf6 have been reported to regulate the development of dendrites and dendritic spines (Sakagami et al., 2004; Miyazaki et al., 2005; Choi et al., 2006). Many of these proteins, including centaurin α1, are also expressed in the adult brain and are localized to synapses. As regulators of dendritic spine dynamics, these proteins and centaurin α1 could also function in synaptic remodeling in the adult brain.

One way that centaurin α1 could serve a dual role in development in branching and spine dynamics and later in synaptic remodeling, is through the regulation of lamellipodia and filopodia (see Luo, 2002; Jaffe and Hall, 2005; Dijkhuizen and Ghosh, 2005; Lippman and Dunaevsky, 2005; Newey et al., 2005). siRNA knockdown of centaurin α1 in cultured neurons reduced lamellipodia and filopodia, whereas overexpression of wild-type centaurin α1 enhanced these structures. Lamellipodia are found on the cell body, growth cones, and spines, and are thought to be the precursors of primary dendrites and possibly additional branch formation. Filopodia are formed on primary dendrites and when stabilized, become the source for further branch addition. Later in development, filopodia are thought to serve as the precursors of immature spines, which, after contact by an axon, can become mature spines where excitatory synapses are formed. Studies are underway to determine whether centaurin α1 is required for the initiation/formation, growth or stability of filopodia and lamellipodia.

Arf GAP activity is required for centaurin α1 regulation of dendritic branching, dendritic filopodia and spines. Our results correspond well with studies in which increasing Arf6-GDP levels enhances dendritic branching and spine density, whereas increasing Arf6-GTP levels inhibits these processes (Hernandez-Deviez et al., 2002; Miyazaki et al., 2005). Our results are also consistent with centaurin α1 functioning with Arf6 to regulate the formation or stabilization of filopodia. Regulation of dendritic filopodia, thought to be the precursors of spines, may directly impact spine density. Interestingly, overexpression of the Arf6 GEF EFA6A also increases mature spine density at the expense of filopodia, suggesting that Arf6 functions in both the production or stabilization of emerging filopodia and also in the conversion of filopodia to mature spines (Choi et al., 2006). How Arf6 regulates filopodia and spines has not yet been elucidated, but may involve control of lipid-modifying enzymes, vesicular trafficking and/or regulation of the Rho GTPase family (Radhakrishna et al., 1999; Sany and Casanova, 2001; Hernandez-Deviez et al., 2002; Krauss et al., 2003; Miyazaki et al., 2005; Cotton et al., 2007).

The regulation of centaurin α1 localization and activity in neurons are important issues for future studies. The observation that Arf GAPs, such as centaurin α1 and Arf-GEFs, such as EFA6 and ARNO are potentially regulated by PtdIns(3,4,5)P3, and found in dendritic and synaptic regions, lends support to the hypothesis that PI 3-kinase coordinately regulates GEFs and GAPs that control the Arf pathways required for dendritic development. In addition, centaurin α1 has multiple identified binding partners in addition to PtdIns(3,4,5)P3, including protein kinases and cytoskeletal proteins, which could provide input to centaurin α1 regulation from a variety of signaling pathways (Dubois et al., 2001; Dubois et al., 2003; Zemlickova et al., 2003; Thacker et al., 2004; Venkateswarlu et al., 2005; Striker et al., 2006). In turn, centaurin α1 may also provide cross-talk to other signaling pathways such as the Ras-MAP kinase pathway, which was shown to be diminished by reducing centaurin α1 expression (Hayashi et al., 2005).
Materials and Methods

Plasmids

Rat Flag-centaurin α1 and the GAP mutant R49K constructs were previously described (Thacker et al., 2004). Human Flag-centaurin α1 constructs were kindly provided by Kanamalapudi Venkateswarlu (Venkateswarlu et al., 2005) and Muahamed Hawadle (Neural Development Unit, UCL Institute of Child Health, UK). eGFP vectors were obtained from Clontech. The siRNAs were selected using the Invitrogen BLOCK-iT RNAi Designer. The siRNA sequences 348, 5′-GAAUUCUUGCCUCUCAUCTT-3′ and 804, 5′-AAAGCCUCUUCUGUCUUTT-3′ were from Integrated DNA Technologies.

Antibodies

A polyclonal antibody against centaurin α1 was previously described (Hammonds-Odie et al., 1996). Goat polyclonal anti-centaurin α1 was from Abcam. Anti-Flag M5 monoclonal antibody and anti-Flag polyclonal antibody and mouse anti-syntaxin were from Sigma. Goat anti-synapsin-1 was from Santa-Cruz Biotechnology. Mouse anti-synapsin-1 monoclonal antibody and mouse anti-synaptophysin monoclonal antibody were from Chemicon International. Goat anti-Neurabin II (A-20) polyclonal antibody, antibodies mouse anti-MAP-2 and rabbit anti-MAP-2 were from Zymed Laboratories. Mouse anti-PSD-95, clone K28/43 was from Upstate Biotechnology, Lake Placid, NY. Oregon Red-X phalloidin, Alexa Fluor 488 anti-rabbit, Cascade Blue anti-rabbit and anti-mouse secondary antibodies were from Molecular Probes. Texas-Red anti-mouse and anti-rabbit secondary antibodies were from Vector Laboratories. Mouse anti-β-tubulin, anti-SV2 and anti-actin were from Developmental Studies Hybridoma Bank, University of Iowa, IA. Mouse anti-tau was a gift from Gail Johnson, University of Alabama at Birmingham, AL.

Subcellular fractionation

Synaptosomes were prepared from 2- to 3-week-old rats as described (Blackstone et al., 1992). Briefly, forebrains were homogenized with a glass homogenizer, centrifuged at 1000 g for 15 minutes at 4°C, and the resulting post-nuclear supernatant (S1) was centrifuged at 10,000 g for 15 minutes to yield the crude synaptosomal and microsomal pellet (P2). The washed crude synaptosomal/microsomal P2 pellet was resuspended in 0.8 M sucrose, layered onto 1.2 M sucrose, and centrifuged at 230,000 g for 15 minutes. The gradient interface was collected, and layered onto 0.8 M sucrose and centrifuged at 230,000 g for 15 minutes. The pellet contained pure synaptosomes (Syn). Synaptosomes were prepared from different developmental ages as previously described (Patterson and Skene, 1999) by a modification of the Ficoll flotation method (Gordon-Weeks, 1987).

Dissociated neuronal culture

Hippocampal neurons were cultured from embryonic day 18 (E18) rats as described (Price and Brewer, 2001). Briefly, embryonic brains were removed and hippocampi were digested with papain (Worthington) at 37°C. Neurons were resuspended in Neurobasal medium containing B-27 supplement, 10 IU/ml penicillin-streptomycin and L-glutamine (Gibco-Invitrogen), referred to as NBMplus. Dissociated neurons were plated on poly-DL-lysine (Sigma) coated glass coverslips and cultured for 3-21 days in NBMplus medium at 37°C in 5% CO₂.

Dissociated neuron transfections

For studies in early development (3-7 DIV) after dissociation, neurons were transfected via Nucleofection (Amaxa Biosystems). Dissociated neurons (3×10⁶ cells) were centrifuged and resuspended in 100 μl rat neuron Nucleofector solution (Amaxa Biosystems). 3 μg plasmid DNA or 3 μg of each siRNA was added to the neuronal suspension in a 2 mm electroporation chamber.
cuvette and electroporated with program O-03. NBMplus medium was added and neurons were plated at a density of 8×10⁴ cells per well in a 12-well plate at 37°C in 5% CO₂. For studies in 14 DIV cultures, hippocampal neurons were transfected at 1 DIV using a modified calcium phosphate protocol (Kohrmann et al., 1999). 3-5 μg of DNA was added in 250 mM CaCl₂, 2× BBS (250 mM NaCl, 1.5 mM Na₂PO₄, 50 mM BES (Sigma), pH 7.1) was added to the Ca-DNA mixture. The transfection solution was added immediately to the coverslips and incubated at 2.5% CO₂ at 36.5°C. After 30-45 minutes, coverslips were washed twice with HBS (135 mM NaCl, 4 mM KCl, 1 mM Na₂HPO₄, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 20 mM HEPES, pH 7.35).

Immunocytochemistry

Neurons were fixed in 4% formaldehyde (Tousimis Research) in phosphate buffer (23 mM NaH₂O₄, 80 mM Na₂HPO₄) for 20 minutes, permeabilized with 0.25 % Triton X-100 in 4% formaldehyde in PBS for 10 minutes, washed three times with 1× PBS, then blocked in 10% BSA in 1× PBS for 1 hour at 37°C. Coverslips were incubated overnight with primary antibodies, washed three times in 1× PBS and incubated with secondary antibody for 2 hours at 25°C. Coverslips were washed three times with 1× PBS, once with ddH₂O, then dried and mounted with Vectashield (Vector Laboratories, CA).

Neuronal imaging

Images were acquired with an Olympus IX 70 inverted microscope with epifluorescence optics using the 40× and 100× objective with a 1.5× tube lens attached giving a resolving power of 150×. Images were captured using Retiga 1300 cooled CCD, firewire, high resolution, monochromatic camera (QImaging; Burnaby, British Columbia, Canada), and images were deconvolved using IPLab microtome extension and merged with IPLab Spectrum software from (Scantalytics). The percentage colocalization in dendritic segments from cultured neurons was measured using the IPLab extension, Fluorescence CV Images were processed using Adobe Photoshop.

Evaluation of neuronal morphology

Images were obtained using the 40× objective and analyzed and quantified using image Pro Plus 4.1 software (Media Cybernetics). The number of dendritic terminal tips, which is a measure of the dendritic complexity, not including the longest extension (axon) and its branches, was determined as described (Uylings and van Pelt, 2002; Hernandez-Deviez et al., 2002; Gerecke et al., 2004; Kumar et al., 2005). The length of each dendritic extension was measured by tracing along its length and the lengths were subsequently summed for the total dendritic length. A range of 25-50 neurons was measured for each condition. Results are expressed as means of treatments normalized to means of control. Values were compared by Student's t-test (two-tailed) with P<0.05 considered statistically significant.

Dissociated neuron protrusion analysis

Images were acquired with an 100× objective with a 1.5× tube lens attached giving a resolving power of 150×. Spines and filopodia were identified as small protrusions <7 μm in length at right angles from dendrite and had no further branches according to (Papa et al., 1995; Kumar et al., 2005). The total number of protrusions on the dendritic length was measured using Imaged software (National Institute of Health, MD). Protrusion density was calculated by counting the number of spines per unit length of parent dendrite, and normalized to 10 μm of dendrite. The total dendritic length for protrusion density analysis was as follows: GFP control was 910.2 μm, Flag-centaurin α1 777.1 μm and Flag-centaurin α1-R49K 822.1 μm. Values were compared by Student's t-test (two-tailed) with P<0.05 considered statistically significant.
Organotypic slice cultures

Transverse hippocampal slices (500 μm) were prepared from postnatal day 7-9 rats with a custom-designed wire slicer (California Fine Wire Company, Grover Beach, CA) and maintained as previously described (Tyler and Pozzo-Miller, 2001; Tyler and Pozzo-Miller, 2003). All tissue culture reagents were purchased from Life Technologies.

Biolistic transfections and Immunocytochemistry

After 4 DIV, culture medium was completely changed to culture medium containing antibiotic and antymycotic. Cultured slices were transfected 4 hours later using the Helios Gene Gun (Bio-Rad) as described (Alonso et al., 2004). Gold particles (25 mg of 1.6 mM) (Bio-Rad) were coated with 26.7 μg pDsRed2-N1 (dsRed) (Clontech), as a control plasmid and 26.7 μg enhanced yellow fluorescent protein (eYFP) (Clontech) to visualize dendrites and spines, or 26.7 μg eYFP and either 53.3 μg Flag-centaurin α1 or Flag-R49K centaurin α1. A time of 36 hours was used for transfection because this provides reliable slice viability as previously characterized (Tyler and Pozzo-Miller, 2001; Tyler and Pozzo-Miller, 2003). Cultured slices were fixed in 4% paraformaldehyde in 100 mM phosphate buffer (PB) for 40 minutes at RT, then rinsed in PBS. Slices were incubated overnight at 4°C in blocking buffer (1× PBS, 0.25% Triton X-100, 5% normal goat serum), followed by overnight incubation at 4°C in blocking buffer with anti-Flag polyclonal antibody (1:1000). After washing in PBS, slices were incubated for 3 hours at 25°C in blocking buffer with Alexa Fluor 546 anti-rabbit secondary (1:2500). Slices were rinsed in PBS and mounted with Vectashield (Vector, CA).

Quantitative spine density analysis by laser-scanning confocal microscopy

Fluorescent images of CA1 pyramidal neurons were acquired with a laser-scanning confocal microscope (LSCM; Olympus Fluoview 300, NY), using an oil-immersion 60× (1.2 NA) or 100× (1.65 NA) objective lens (Olympus). eYFP was excited with the Ar laser, dsRed was excited with the Kr laser and detected with standard FITC filters. Optical z-sections were acquired at 0.5 μm steps through the apical dendritic tree of the neurons. Measurements were performed on secondary and tertiary dendrites. Dendritic projections with lengths between 1 and 3 μm were identified as spines and counted off-line using ImageJ software (National Institutes of Health, Bethesda, MD) as described previously (Tyler and Pozzo-Miller, 2003; Alonso et al., 2004). Spine counts were obtained from a total apical dendritic length of 891 μm in control (eYFP) slices (five cells from four slices), 1855 μm in centaurin-α1-transfected slices (nine cells from six slices) and 1214 μm in R49 centaurin-α1-transfected slices (seven cells from five slices). Data were normalized per 10 μm of dendritic length.

Immunoblot analysis

Samples were separated by SDS-PAGE, and transferred to PVDF membranes (Bio-Rad). Membranes were probed with primary antibodies, followed by horseradish-peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies (Santa Cruz Biotechnology), and secondary antibodies were detected using Supersignal West Dura Extended Duration substrate (Pierce Chemical).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant MH50102 to A. Theibert. We thank Elizabeth Sztul for critical discussion and reading of the manuscript, Kanamarlapudi Venkateswarlu and Muahamed Hawadle for human centaurin α1 expression...
plasmids, Albert Tousson and the UAB Imaging Facility for assistance with fluorescent imaging, Kim Gereke for analysis of dendritic morphology and Yuying Wu for assistance with the embryonic hippocampal neuron dissociation.

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Fig. 1.
Developmental expression and localization of endogenous centaurin α1 in the rodent brain.
(A) Immunoblot analysis of centaurin α1 protein from lysates (25 μg total lysate per lane) from
whole brain at different developmental stages from E10 to P42 (=adult). (B) Immunoblot
analysis of centaurin α1 and synaptic markers in fractionated P23 whole rat brain. H, whole
brain homogenate; P2, crude microsomes and synaptosomes; S2, supernatant; Syn, purified
synaptosomes; 20 μg of each fraction was loaded per lane. (C) Immunoblot analysis of
centaurin α1 in the synaptosome fraction prepared from the developing brain. 10 μg of each
fraction was loaded per lane. (D) Immunohistochemistry with anti-centaurin α1 antibody in
hippocampus from P28 rat. DG, dentate gyrus.
Fig. 2.
Expression of endogenous centaurin α1 in primary cultured dissociated hippocampal neurons. (A) Immunoblot showing developmental expression of centaurin α1 and the specific synaptic proteins PSD95, spinophilin (Spino) and synaptotagmin (Syn) in primary cultured neurons at 3, 7, 14 and 21 DIV. 20 μg of total lysate was loaded per lane. (B) Indirect immunofluorescence shows endogenous centaurin α1 localization (red) and levels at 3, 7, 14 and 21 DIV. Bars, 10 μm.
Fig. 3.
Localization of endogenous centaurin α1 in primary cultured dissociated hippocampal neurons. Indirect immunofluorescence was used to visualize endogenous centaurin α1 localization (red) compared with microtubule associated protein 2 (MAP-2), tau, synaptophysin (syn), PSD-95 and spinophilin (Spin) in 14 DIV neurons. Arrows indicate areas of juxtaposition; arrowheads indicate areas of colocalization. Bars, 10 μm.
Fig. 4.
Effects of knockdown of centaurin α1 protein by siRNA in dissociated cultured hippocampal neurons. Hippocampal neurons were transfected after dissociation (0 DIV) and cultured for 3, 7 and 14 DIV. (A,B) Immunoblot analysis of endogenous centaurin α1 in hippocampal cultures. Total cell lysates (12.5 μg lysate per lane) were probed with anti-centaurin α1 and an anti-actin antibodies, which were used to assess actin levels for calculation of the knockdown percentage. (A) Lane 1, scrambled (Scr) siRNA 3 DIV; lane 2, rat centaurin α1 (rCena1) siRNA 3 DIV; lane 3, Scr siRNA 7 DIV; lane 4, rCena1 siRNA 7 DIV; lane 5, Scr siRNA 14 DIV; lane 6, rCena1 siRNA 14 DIV. (B) Lane 1, Scr siRNA; lane 2, rCena1 siRNA; lane 3, rCena1 siRNA plus human Flag-centaurin α1. (C) Indirect immunofluorescence showing endogenous centaurin α1 (red) compared with β-tubulin (green) to visualize neuronal morphology at 3 and 7 DIV. Double-stranded scrambled RNA control is shown in left-hand panels and double-stranded siRNA to rat centaurin α1 in the right-hand panels. Bars, 20 μm. (D) Rescue by expression of human centaurin α1. Neurons were transfected with rat centaurin α1 siRNA, GFP
and human centaurin α1. Indirect immunofluorescence showing endogenous plus heterologous centaurin α1-(red), β-tubulin (blue) to visualize neuronal morphology and GFP (green) to label neurons co-expressing human Flag centaurin α1 at 3 and 7 DIV. Bars, 20 μm. (E) Quantification of the effects of siRNA knockdown of centaurin α1 at 3 and 7 DIV and rescue with human centaurin α1 compared with scrambled siRNA control. The data were quantified from three independent experiments (n=30 at 3 DIV, n=25 at 7 DIV cells for each condition). Data represent mean ± s.e. *P<0.05.
Fig. 5.
Effects of overexpression of wild-type centaurin α1 in developing dissociated hippocampal neurons. Hippocampal neurons were transfected by Nucleofection after dissociation (0 DIV) and cultured for 3, 7 and 14 DIV. (A) Fluorescence (GFP, green) and indirect immunofluorescence (β-tubulin; red, 3 DIV; blue, 7 and 14 DIV; Flag-tagged centaurin α1, red) were used to visualize transfected neurons and neuronal morphology. GFP control (left panels). GFP plus Flag-centaurin α1 (right panels). Insets show higher magnification. Bars, 20 μm. (B) Quantification of the effects of expression of FLAG-centaurin α1 at 3 and 7 DIV compared with GFP control. The data was quantified from three independent experiments.
(n=50 cells from 3 DIV and n=30 cells from 7 DIV, respectively, for each condition). Data represent mean ± s.e. *P<0.05.
Fig. 6.
Effects of overexpression of a GAP-inactive mutant centaurin α1 on dendritic differentiation. Hippocampal neurons were transfected after dissociation (0 DIV) and cultured for 3-14 DIV. (A) Fluorescence (GFP, green) and indirect immunofluorescence (β-tubulin, red) were used to visualize transfected neurons and neuronal morphology. Expression of GFP control (left panels) compared with GFP plus Flag-R49K centaurin α1 (right panel). Boxed areas show a higher magnification. Bars, 20 um. (B) Quantification of the effects of overexpression of Flag-R49Kcentaurin α1 for 3 and 7 DIV compared with GFP control. The data was quantified from three independent experiments (n=50 cells at 3 DIV and 30 cells at 7 DIV). Data represent mean ± s.e. *P<0.05.
Fig. 7.
Effects of overexpression of wild-type and GAP-inactive centaurin α1 on dendritic protrusions in dissociated cultured hippocampal neurons. Dissociated hippocampal neurons were transfected at 1 DIV and cultured for 13 DIV. (A) Fluorescence (GFP, green) and indirect immunofluorescence (spinophilin or neurabin, red) were used to visualize transfected neurons and morphological effects. Expression of GFP control (left panels) compared with GFP plus Flag-centaurin α1 (middle panel) and GFP plus Flag-R49K centaurin α1 (right panel). Bars, 10 μm. (B) Quantification of the number of dendritic protrusions per 10 μm dendrite length in neurons expressing GFP, GFP plus Flag-centaurin α1 and GFP plus Flag-R49K centaurin α1. Data represent mean ± s.e. *P<0.05.
Fig. 8.
Centaurin α1 localizes to dendritic spines and regulates dendritic spine density in CA1 neurons in organotypic hippocampal slice cultures. (A) Representative dendritic segments from CA1 pyramidal cells in organotypic hippocampal slice cultures transfected with eYFP and Flag-centaurin α1. Centaurin α1 was visible in the dendritic shaft and spines (arrows). Bar, 25 μm. (B) Representative dendritic segments of CA1 pyramidal neurons from organotypic hippocampal slice cultures transfected with eYFP and either dsRed, Flag-centaurin α1 or Flag-R49K centaurin α1. Bars, 2 μm. (C) Quantification of spine density in each condition. Data represent mean ± s.e. *P<0.00016.