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Pur-alpha regulates the developmental expression of RhoA and affects its downstream signaling ability in the mouse brain

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Abstract

Pur-alpha is an essential protein for postnatal brain development which localizes specifically to dendrites where it plays a role in the translation of neuronal RNA. Mice lacking Pur-alpha display decreased neurogenesis and impaired neuronal differentiation. Here we examined two Rho GTPases, Rac1 and RhoA, which play opposing roles in neurite outgrowth and are critical for dendritic maturation during mouse brain development in the presence and absence of Pur-alpha. Pur-alpha is developmentally regulated in the mouse brain with expression beginning shortly after birth and rapidly increasing to peak during the third week of postnatal development. RhoA levels analyzed by Western blotting rapidly fluctuated in the wild-type mouse brain, however, in the absence of Pur-alpha, a decrease in RhoA levels shortly after birth and a delay in the cycling of RhoA regulation was observed leading to reduced basal levels of RhoA after day 10 postnatal. Immunohistochemistry of brain tissues displayed reduced RhoA levels in the cortex and cerebellum and loss of perinuclear cytoplasmic labeling of RhoA within the cortex in the knockout mouse brain. While Rac1 levels remained relatively stable at all time points during development and were similar in both wild-type and Pur-alpha knockout mice, changes in subcellular localization of Rac1 were seen in the absence of Pur-alpha. These findings suggest that Pur-alpha can regulate RhoA at multiple levels including basal protein levels, subcellular compartmentalization, as well as turnover of active RhoA in order to promote dendritic maturation.

Keywords

Rho GTPase; Puralpha; Pur alpha; mouse; brain; development

INTRODUCTION

Pur-alpha is a multifunctional protein that is essential for postnatal development and increasingly recognized as a critical component in the translation of neuronal RNA (Gallia et al, 2001; Johnson et al, 2003; 2006). Pur-alpha is strongly conserved from bacteria through humans and has been most extensively characterized as a sequence-specific single-stranded DNA- and RNA-binding protein which directs both replication and gene transcription (see Gallia et al., 2000 and Johnson, 2003 for reviews). More specifically, in the nucleus, it associates with cellular DNA to activate or suppress transcription through binding to the

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CONFLICT OF INTEREST DISCLOSURE

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regulatory regions of a number of cellular genes including myelin basic protein, gata2, amyloid- β precursor protein, α -actin, TNF α , TGF β , and E2F1 as well as the Pur-alpha promoter itself (White et al, 2009). In addition, Pur-alpha regulates cell growth through directing cellular DNA replication as well as interacting with key cell cycle regulatory proteins including Rb, E2F-1, and several cyclins and cdks (Gallia et al, 1999, 2000). Pur-alpha is also known to promote repair of double stranded DNA breaks and loss of the PURA gene has been observed in adult myelogenous leukemia, further supporting its potential role as a cell cycle regulator and tumor suppressor protein (Johnson et al, 2003).

Insight on the role of Pur-alpha during development has been gained by observations in mice with homozygous deletion of the protein. Mice lacking Pur-alpha appear normal at birth but begin to exhibit failure to thrive at 7 to 10 days after birth when growth retardation becomes evident (Khalili et al., 2003). Animals progressively deteriorate, fail to gain body weight, and eventually expire by 23 days after birth. Heterozygous animals also display delays in weight gain though they eventually recover to the point where they are indistinguishable from wild type littermates. The severity of the phenotype seen in the knockout mice parallels the increase in Pur-alpha during development, which accelerates after 10 days postnatal to peak during the third week of postnatal development (Khalili et al., 2003). Most notable are defects in neuronal development throughout the cortex and in cerebellar Purkinje cells where the Pur-alpha knockouts fail to develop sufficient numbers of neurons and the neurons that are present lack proper dendritic structures, as seen by visualizing neurofilaments. In addition, hippocampal neurons fail to form synaptic connections in the absence of Pur-alpha, and exhibit a significant lack of Psd95 foci (Khalili et al., 2003).

Although Pur-alpha is a ubiquitous protein that is detected in organs and cells throughout the body, analysis of mouse brain tissues has shown intense immunolabeling of Pur-alpha in neurons, rather than other cells within the CNS, and in particular localized to the cytoplasmic compartment of neurons (Khalili et al, 2003). In fact, studies have demonstrated that Pur-alpha specifically localizes to the dendritic compartment of neurons, and further is localized at dendritic branch points where it has been found in complex with polyribosomes and hnRNP proteins suggesting it plays a role in local translation (Johnson et al, 2006). Partner proteins for Pur-alpha in polyribosomes include Staufen as well as FMRP and related fragile X mental retardation proteins, which have been associated with a number of disorders of the brain affecting neuronal development and synaptic plasticity (Johnson et al, 2006; Kanai et al, 2004).

Dendritic development and maintenance requires coordinated regulation of neurite retraction and outgrowth (Luo et al, 2002; Yoshihara et al, 2009). This is achieved, in part, by tight regulation of Rho GTPases that mediate cytoskeletal changes critical for neuronal morphogenesis and neuronal development (Govek et al., 2005). Rho GTPases are an evolutionarily conserved family of proteins (Boureux et al., 2007) that function as molecular switches cycling between inactive GDP- and active GTP-bound forms thereby linking G-protein coupled receptors to the intracellular downstream pathways. Several Rho guanine nucleotide exchange factors (RhoGEFs) have also been implicated in regulating neuronal processes with spatial and temporal activation within highly compartmentalized neuronal cells central to neurite development (Ethell and Pasquale, 2005). In addition, signal transduction via RhoGEFs affect a wide variety of neuronal functioning ranging from cell shape changes, cell motility, cell polarity, cellular development, neuronal morphogenesis, and neurotransmitter release (Schmidt and Hall, 2002). Proper control of the functions of Rho GTPases are important for development and disease processes ranging from oncogenesis, cardiovascular disorders and neurodegenerative diseases (Govek et al., 2005).

Two of the most widely studied RhoGTPases include the antagonistic RhoA and Rac1 proteins, which promote neurite retraction and outgrowth, respectively. Briefly, Rac1 promotes lamellipodia formation and membrane ruffling while RhoA causes growth cone collapse and neurite retraction, thus together with other Rho GTPases including Cdc42, these proteins are thought to direct dendrite formation and remodeling. Surprisingly little has been reported about the role of the Rac and Rho proteins in the developing brain and their impact on neurogenesis (Bolis et al, 2003; Kumanogoh et al, 2001). This is due, in part, to the fact that constitutive deletion of these proteins causes early embryonic lethality as they appear to be essential for early development (de Curtis, 2008). However, in vivo overexpression of constitutively active Rac1 induces smaller dendrites with increased density and synapses (Luo et al, 1996) while overexpression of constitutively active RhoA promotes spine retraction and reduces overall spine density (Govek et al, 2005).

Both RhoA and Rac1 have been strongly detected in the adult rat brain in regions throughout the brain including the cortex, hippocampus and cerebellum (Olenik et al, 1997). Rac1 is ubiquitously expressed in the brain, diffusely and homogeneously spread throughout the cells occasionally seen to label perinuclear cytoplasm of neurons (Bolis et al, 2003). Conditional knockout of Rac1 along with constitutive deletion of the neuron-specific Rac3 suggests complementary roles for these proteins during late brain development, in particular of the hippocampus (Corbetta et al, 2009; Gualdoni et al, 2007). In addition, subcellular localization of Rac1 and RhoA proteins has been shown to change during development, with one study reporting enrichment of RhoA in dendrites and compartmentalization of Rac1 to the axons in mature neurons (Santos Da Silva et al, 2004). The mechanisms responsible for axonal and dendritic localization of these proteins, or regulation in their basal expression levels are not yet known.

The temporal and spatial expression patterns of Rac1 and RhoA can provide important information regarding coordinated regulation of neurite outgrowth. Given our findings that Pur-alpha localizes specifically to the dendritic compartment of neurons, and its critical impact on dendrite formation in the developing mouse brain, we sought to examine the expression patterns and localization of Rac1 and RhoA in our Pur-alpha knockout mouse model.

MATERIALS AND METHODS

Cell culture and transfections

Cells utilized in this study include primary rat neurons prepared as described previously (Amini et al, 2009) and the human teratoma cell line, Ntera-2 or NT-2 cells obtained from ATCC. Cells were plated in DMEM containing 10% fetal bovine serum, and grown to 70–80% confluency. siRNA targeting bp 772–790 (5'-aca agt acg gcg tgt ttt at -3') of the human PURA gene (which is homologous with the mouse PURA gene) or scrambled non-targeting siRNAs were obtained as double stranded RNA oligonucleotides from Dharmacon. Cells were transfected with siRNAs using Oligofectamine according to the manufacturer's instructions (Invitrogen). For rat primary neurons, cells were harvested 36 h after transfection in lysis buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl, and 1% NP-40 containing protease inhibitors and lysates were utilized for Western blot analysis. For NT-2 cells, 24–48 h post transfection, cells were replated on collagen coated dishes, grown for another 48 h in serum containing media, then incubated with retinoic acid for an additional 48 h. Cells were then washed and incubated in serum free media for 2 h followed by treatment with 5 μ M L-a-lysophosphatidic acid (LPA) (Sigma, MO) for different time periods at 37° C. Cells were lysed by scrape loading in ice-cold lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, 1 mM EDTA and

2% glycerol plus protease inhibitors, after each LPA treatment and utilized for the Rho activation assay as described below.

Rho activation assay

Endogenous RhoA in the GTP bound active state was measured by association with the RhoA binding domain of Rhotekin (GST-RBD) using the RhoA activation assay kit (Upstate) as per the manufacturer's protocol. Lysates were incubated at 4°C for 45 min with GST-RBD bound glutathione-agarose beads. Beads were then washed three times with ice-cold lysis buffer and bound proteins were eluted by boiling in SDS sample buffer. In parallel, untreated lysates incubated with GDP or with non-hydrolysable GTP γ S at 22°C for 15 min were utilized as negative and positive controls, respectively. Samples were analyzed by Western blotting with anti-RhoA antibody as described below.

Mice with PURA gene deletion

Mice with targeted disruption of the PURA locus have been described previously (Khalili et al, 2003). Founders and F1 generation were confirmed by Southern and Western blot analysis (Khalili et al, 2003). Homozygous deletion of PURA is lethal during late development, therefore mice are maintained as heterozygous. Progeny are screened by PCR analysis using primer sets to distinguish, based on size of the amplicon, between the wild-type gene and the knockout which contains a neomycin cassette inserted into the PURA gene, as previously described (Khalili et al, 2003). Whole cell extracts were isolated from total brains of PURA+/+, PURA+/-, and PURA-/- age-matched littermates by homogenization in TNN buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, and 0.5% NP-40. The protein concentration was determined by the method of Bradford. Approval for the use of vertebrate animals was obtained by the Temple University Institutional Care and Use Committee and all vertebrate animal studies were carried out in compliance with all appropriate federal, state, and local laws and institutional regulation.

Western blot analysis

Equal amounts of whole cell protein extracts were fractionated by SDS $\pm$ PAGE minigel and proteins were transferred to PVDF membrane in transfer buffer containing 192 mM glycine, 25 mM Tris base, and 20% methanol. Membranes were incubated for 1 h in 1X TTBS solution containing 0.1% Tween-20, 100 mM Tris (pH 7.5), 0.9% NaCl, and 5.0% non-fat dry milk. After blocking, membranes were incubated with primary antibody for 2 h at room temperature followed by washing in 1X TTBS and incubation in secondary antibodies conjugated to alkaline phosphatase for 1 h at room temperature. Proteins were then visualized by autoradiography using the CDP-STAR chemiluminescence kit according to the manufacturer's instructions. Band intensity was determined on scanned images using Photoshop where images were inverted, and the histogram function was used to calculate band intensity and values for background subtraction.

Immunohistochemistry for Pur-alpha, Rac1 and RhoA

Formalin-fixed, paraffin-embedded sections of brain tissues from Pur-alpha knockout mice and their wild type age-matched littermates were sectioned at 4 μ M in thickness and stained with hematoxylin-eosin for routine histological analysis and characterization. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex system (Vectastain Elite ABC Peroxidase Kit, Vector Laboratories Inc, Burlingame, CA). After deparaffinization with xylene, rehydration, non-enzymatic antigen retrieval was performed in 0.01 M Citrate (pH 6.0) for 30 min at 95°C followed by cooling for 20 min. All sections were then quenched for endogenous peroxidase in methanol/3% H₂O₂ for 20 min. After blocking with serum/BSA, primary antibodies were added and incubated overnight in a

humidified chamber. Sections were then incubated with the appropriate biotinylated secondary antibody followed by detection with DAB (0.02% diaminobenzidine and 0.005% hydrogen peroxide), counterstaining with hematoxylin, and mounting with Permount. Sections were analyzed for relative expression level and subcellular localization. Original magnification of digitized images as indicated.

Antibodies

Antibodies utilized for immunoblotting and immunohistochemistry include monoclonal mouse anti-Pur-alpha antibody developed in the laboratory of Dr. Edward M. Johnson (clone 10B12), mouse monoclonal anti-Rho (clone 55, Upstate), mouse monoclonal anti-Rac1 (clone 23A8, Upstate). Mouse monoclonal anti-GRB2 antibody (clone 81, BD Transduction Laboratories) or anti-tubulin (clone B512, Abcam) were used as loading controls for western blotting.

RESULTS

Our previous results in the Pur-alpha knockout mouse model have revealed a critical role for Pur-alpha during development, particularly in the coordinated differentiation of neuronal cells throughout the brain. In particular, we observed a decrease in the total number of neurons and the level of neurofilaments as well as a reduction in the percentage of phosphorylated neurofilaments in the absence of Pur-alpha. Mice lacking Pur-alpha do not survive to maturity indicating that Pur-alpha is essential for normal development and heterozygous animals display an intermediate phenotype and subsequent delays during development, eventually becoming indistinguishable from their wild type littermates (Khalili et al, 2003). Given these findings and the well established reciprocal roles of Rho GTPases in promotion of neurite outgrowth and retraction, we examined two prominent members of this family, RhoA and Rac1, in the context of the Pur-alpha knockout mice. As shown previously (Khalili et al, 2003), Pur-alpha is a developmentally regulated protein whose levels are detected shortly after birth, increase to peak levels at 15–20 days postnatal, and decline slightly after 25 days to be maintained at relatively high levels throughout adulthood (Figure 1, Panels A and D). Pur-alpha levels are highest at the peak of myelination and neuronal differentiation in the mouse brain, which occurs during the third week after birth and significant levels of the protein are maintained in adults suggesting that Pur-alpha is required for normal brain homeostasis. In parallel, we examined brain tissue extracts from Pur-alpha heterozygous and knockout age-matched littermates at different developmental time points. In PURA+/- animals, decreased levels of Pur-alpha protein are detectable and also peak at day p15, though much lower levels are seen than in the PURA+/+ (Panels B and E). Of interest, the heterozygous animals (PURA+/-) produce less Pur-alpha protein than their control PURA+/+ littermates. Based on quantitation in Figure 2 and our previous studies, the Pur-alpha levels in heterozygous mouse brains are reduced to 25% of wild type levels at day 5 postnatal and 50% of wild type levels in adulthood (Khalili et al, 2003). Since changes in Pur-alpha levels of about 2-fold can affect cell proliferation (Stacey et al, 1999), this reduced level in the PURA+/- mice suggests haploinsufficiency of the Pur-alpha protein in certain tissues. As predicted, in mice with a targeted disruption of PURA, Pur-alpha protein is not detected (Panels C and F).

Surprisingly little is known about RhoA and Rac1 regulation during brain development. Analysis of extracts from wild type mouse brain tissues at different stages of development have uncovered distinct developmental patterns for RhoA in comparison with Rac1 levels. Western blotting of wild type mouse brain extracts analyzed in parallel with Pur-alpha demonstrated total protein levels of RhoA during brain development peaked at days 5, 10, and 20, while total RhoA levels decreased at 7, 15, and 26 days (Figure 1, Panel A), suggesting a rapid and tightly regulated fluctuation of RhoA occurs during waves of

corticogenesis. In mice heterozygous for Pur-alpha, a shift in the timing of RhoA cycling is observed in that a steady increase from days 2 to 7 is seen with a precipitous drop in RhoA levels from day 10 onward (Panel B). In the Pur-alpha knockout mice, steady state levels of RhoA were reduced between days 2 and 5 while remaining more constant from days 7 to 10 then drop to lower levels from day 10 onward. The lag in RhoA cycling seen in the heterozygotes where Pur-alpha levels are significantly reduced compared to wild type and the early drop in RhoA at day 5 followed by more moderate low level changes seen in the knockout mice suggest that regulation of the total fraction of RhoA in the brain is disrupted during brain development in the absence of Pur-alpha.

In contrast, we found that total Rac1 levels do not appear to be significantly altered in extracts from heterozygous or knockout mouse brains during development when compared to the wild type tissues. We observed that while Rac1 levels did appear to be downregulated at day 7 postnatal in extracts from wild type brains, total Rac1 levels remained fairly consistent during all other time points of brain development analyzed. Total levels of Rac1 protein did not alter substantially in brains from heterozygous or knockout Pur-alpha mice, suggesting that the basal levels of Rac1 and RhoA proteins are regulated by independent mechanisms.

To further examine the differences between RhoA and Rac1 levels in the presence and absence of Pur-alpha, we performed Western blot analysis of samples from PUR+/+, +/-, and -/- mouse brains to quantitate relative protein amounts at 5, 10, and 20 days postnatal. Similar to the results seen in Figure 1, we observed RhoA levels to peak at these time points, along with increasing levels of Pur-alpha (Figure 2, Panels A and B). However, heterozygous animals clearly had reduced levels of RhoA at later times during development and this pattern was even further reduced in the absence of Pur-alpha. As the total level of RhoA in most cells is generally thought to be maintained at steady state levels, these data suggest that such changes in the available pool of RhoA could have an impact on the active fraction of RhoA. We therefore examined the impact of Pur-alpha on the active fraction of RhoA in neuronal cells. The human teratoma cell line, NT-2, was differentiated with retinoic acid to produce cells with neuron-like process bearing morphology and treated with lysophosphatidic acid (LPA) to induce RhoA activation. Lysates were prepared from cells with and without transfection of siRNA targeting Pur-alpha and assayed for the active fraction of RhoA by Rho activation assay. As shown in Figure 2, Panel C, LPA treatment of NT2 cells induces rapid and transient RhoA activation which peaks within 5 minutes and quickly returns to baseline levels. Knockdown of Pur-alpha levels did not appear to have any noticeable impact on levels of the active RhoA fraction. As shown in Panel D, knockdown of Pur-alpha by siRNA in primary rat neuronal cultures lead to an increase in total RhoA protein levels as detected by Western blotting, demonstrating the negative regulatory effects of Pur-alpha on RhoA levels independent of any potential effect on RhoA GDP-GTP exchange. While the cell culture knockdown data could be seen to conflict with the developmental Western panels, we did note that at the highest levels of Pur-alpha, i.e. 20 and 26 day wild-type mice, some of the highest and lowest levels of RhoA, respectively, were observed. Taken together, these data suggest that Pur-alpha's ability to regulate RhoA in vivo is likely to be influenced by other pathways. The cell culture data suggest that under certain conditions in neurons, Pur-alpha can negatively regulate RhoA which fits with our observation that the Pur-alpha knockout mice exhibit neuronal loss.

To shed further light on the role Pur-alpha may play in RhoA regulation, we examined the patterns of Rac1 and RhoA expression during mouse brain development by immunohistochemistry for protein expression as well as subcellular localization. We selected one time point during early development (day 5 postnatal) and a second time point later in development (day 18 postnatal) when the downshift in RhoA expression was

observed by Western blotting in the Pur-alpha heterozygous and knockout mice. Brain sections of wild type (PURA+/+) and Pur-alpha knockout mice (PURA-/-) at 5 days and 18 days postnatal were examined for Pur-alpha, Rac1, and RhoA expression. As shown in Figure 3, modest levels of Pur-alpha were observed in the cytoplasm of cortical neurons and in Purkinje cells of the cerebellum at 5 days postnatal, a time point during development at which Pur-alpha is beginning to increase in brain (Panels Ai and ii). As expected, Pur-alpha was not detected in the cortex or the cerebellum of the knockout mice (Panels Aiii and iv). RhoA expression followed a similar pattern to that of Pur-alpha in the cortex and cerebellum and could be observed in neuronal cell bodies of the cortex as well as throughout the cerebellum (Bi and ii). Interestingly, in the absence of Pur-alpha, RhoA could no longer be detected in the cells of the external granular layer of the cortex (Panel Biii) while cytoplasmic labeling of neurons in the deeper layers of the cortex appeared more intensely labeled. Cells of the internal granule layer of the cerebellum in the absence of Pur-alpha also appeared more intensely labeled for RhoA than in the wild type cerebellum (Compare Panels Bii and iv).

In mouse brains at 18 days postnatal, at which time Pur-alpha begins to reach peak levels, Pur-alpha was observed to strongly label neurons throughout all layers of the cortex in a cytoplasmic pattern (Figure 4, Panel Ai). Likewise, the Purkinje cell layer of the cerebellum exhibited the same cytoplasmic localization (Aii). As expected, Pur-alpha was not detected in the cortex or the cerebellum of the knockout mice (Panels Aiii and iv). RhoA expression in wild type mice at 18 days postnatal clearly labeled cells of the molecular layer as well as neurons throughout the cortical layers with distinct perinuclear labeling of neurons of the external granular layer. In the absence of Pur-alpha, the molecular layer still exhibited expression of RhoA, however, the remaining layers of the cortex showed reduced expression and the distinct perinuclear labeling of neurons of the external granular layer was no longer observed (compare Panel Bi and iii). Cerebellar labeling of the molecular layer and white matter with RhoA was detected in wild type mice (Bii) but a reduced level of labeling in the molecular layer was seen in the knockout mouse brain at 18 days postnatal (Biv). These results demonstrate reduced overall expression of RhoA in neurons throughout the cortex and cerebellum in the absence of Pur-alpha in addition to loss of focal labeling of cortical neurons.

While Rac1 expression analyzed by Western blot did not show appreciable differences in the total amount of Rac1 detected in mouse brains at increasing ages of development, immunohistochemistry of mouse brains showed distinct changes in protein localization and labeling patterns. For instance, Rac1 levels were very high in the cortex with the majority of cortical neurons and cells of the internal granule layer of the cerebellum exhibiting Rac1 labeling at 5 days postnatal (Figure 4, Panels Ci and ii). The high level expression of Rac1 was particularly distinct in the external granular layer of the cortex of wild type mice (Ci). In the absence of Pur-alpha, however, labeling of neurons was reduced in all layers throughout the cortex, and in particular, the external granular and pyramidal layers were no longer prominently labeled with Rac1 (Compare Panels Ci and iii). Thus, while total amounts of Rac1 in mouse brain homogenates were not substantially altered, the expression pattern and the localization of Rac1 in the cortex was altered in the absence of Pur-alpha.

These data taken together demonstrate that the absence of Pur-alpha disrupts normal developmental patterns of RhoA and Rac1 in neurons throughout the cortex by affecting the overall levels of RhoA as well as the regional and subcellular localization of RhoA and Rac1 as determined by immunohistochemistry. Our findings also suggest that silencing of Pur-alpha leads to stabilization of RhoA, which would interfere with neurite outgrowth thereby resulting in the loss of neurons observed in mice lacking Pur-alpha.

DISCUSSION

There are many interesting similarities between Pur-alpha, Rac1, and RhoA. Like Pur-alpha, both Rac1 and RhoA are ubiquitous proteins that are expressed in most tissues throughout development and are critical to development as deletion of the proteins result in lethality. Unlike Pur-alpha, which increases during development to peak at three weeks postnatal, Rac1 levels remain relatively constant throughout brain development. In the absence of Pur-alpha, overall Rac1 levels were not substantially changed, however, the localization pattern in neurons was found to be altered. RhoA levels, on the other hand, were found to cycle during postnatal brain development and this pattern was substantially altered in the absence of Pur-alpha. In addition to the decrease in RhoA basal levels in the absence of Pur-alpha, the subcellular localization pattern of RhoA in neurons was altered as well. Pur-alpha exhibits spatiotemporal translocation from nuclei to cytoplasm during neuronal development, as shown by us and others, suggesting that translocation of Pur-alpha may impact the localization of Rac1 and RhoA in developing neurons (Khalili et al, 2003; Zeng et al, 2005). In addition, the change in RhoA but not Rac1 basal levels in the brain implicates Pur-alpha in regulation of RhoA protein levels and that maintenance of steady-state levels of Rac1 and RhoA occur by different mechanisms.

Pur-alpha is essential for development and maintenance of the nervous system as homozygous deletion of this protein results in premature lethality (Khalili et al., 2003). In the brain, levels of Pur-alpha appear shortly after birth and begin to dramatically increase after day 10 postnatal, peaking during the third week of mouse development and remaining at high levels in the adult brain. Transcriptional regulation of Pur-alpha is also developmentally regulated as Pur-alpha activity in the brain, as measured by binding activity to target promoter sequences in nuclear extracts prepared from mouse brain at different stages of development, follows a similar pattern as the total protein levels (Haas et al, 1993, 1995). The observed decrease in the basal levels of RhoA after day 10 postnatal suggests that Pur-alpha acts upstream to influence transcription or translation of RhoA.

Our previous findings in mice lacking Pur-alpha demonstrated a decrease in cellular replication in organs throughout the body as seen by reduced nuclear staining of MCM7 (Khalili et al, 2003). In the brain, a dramatic reduction in cellular replication was observed throughout the hippocampus and cerebellar external granular layer during development. Recent studies have demonstrated that RhoA signaling promotes neuronal migration as well as neural progenitor cell development (Richard et al, 2008), which suggests RhoA signal transduction as a potential pathway for inducing the deficit in neuronal progenitor cells observed in the absence of Pur-alpha. It should also be noted that Pur-alpha can act as a negative as well as a positive regulator of its well characterized downstream factors (see Gallia et al, 2001; Johnson et al, 2003; 2006). The data presented show a loss of coordinated RhoA expression during development in the Pur-alpha +/- and -/- mice which at times leads to inappropriate increases as well as decreases in RhoA levels. The cell culture model will provide us with a well controlled system to determine the potential molecular mechanism(s) by which Pur-alpha protein can impact RhoA.

We have previously shown that Pur-alpha negatively regulates the A β PP promoter, leading to upregulation of A β PP transcription and an increase in the level of A β PP protein in the absence of Pur-alpha (Darbinian et al, 2008). Similar observations were made in mouse brain tissue and fibroblast cells derived from Pur-alpha knockout mice in which increased A β PP was observed in neuronal cells in all regions of the brain analyzed (Darbinian et al, 2008). Interestingly, RhoA expression has also been seen to decrease in synapses and increase in dystrophic neurites of A β PP mice and appeared to co-localize with neurofibrillary tangles in brains of patients with Alzheimer's disease suggesting that altered

subcellular distribution of RhoA could be related to neurodegeneration (Huesa et al, 2010). These findings suggest cross talk between Pur-alpha and RhoA signaling which impacts A β PP levels and may point to a common pathway for neurodegenerative diseases.

In parallel with the in vivo expression patterns we have analyzed in the Pur-alpha knockout mice, these results suggest a regulatory effect for Pur-alpha on RhoA, which can affect the total pool of RhoA. It is also possible that Pur-alpha may have an impact on the activated fraction of the protein at certain points during development. Given the well established nuclear impact of Pur-alpha on transcriptional regulation and the neuronal localization of Pur-alpha to the cytoplasm of dendrites, Pur-alpha may target RhoA signaling by complementary mechanisms. Indeed, the impact of Pur-alpha on RhoA regulation, activation, and membrane association within dendrites would not be evident from the Western data shown above, though the subcellular alterations seen in the immunohistochemical findings do suggest such localized effects may result. In addition, the mechanism(s) by which Pur-alpha interferes with RhoA signaling may be indirect and remain to be elucidated.

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Figure 1. Dysregulation in pattern of RhoA during mouse brain development in the absence of Pur-alpha

Lysates were prepared from snap frozen whole mouse brain tissues harvested from wild type mice (A), mice heterozygous for targeted disruption of the PURA gene (B), or mice with homozygous deletion in PURA (C) at different stages of development including 2, 5, 7, 10, 15, 20, and 26 days after birth. Immunoblotting of whole cell extracts prepared from mouse brains show a gradual increase in Pur-alpha levels during development (Panel A). Reduced levels following a similar pattern of increase are seen in extracts from PURA^{+/-} mice, while Pur-alpha protein is undetectable at any time point in PURA^{-/-} mice. RhoA levels exhibited a cyclical pattern during brain development with decreased levels at 7, 15, and 26 days in wild type mice, but this pattern was perturbed in both the heterozygous as well as knockout Pur-alpha mice. Total Rac1 levels remained relatively unchanged at all time points in the tissues examined. Immunoblotting for an unrelated protein, GRB2, is shown as a control for equal loading. Quantitation of band intensity of Panels A, B, and C are shown in Panels D, E, and F, respectively. Band densitometry was normalized to GRB2 levels. Fold change for RhoA and Rac1 levels were calculated relative to levels of protein detected in brains at 2 days postnatal for each genotype, which were set as 1.0. Fold change for Pur-alpha was expressed as compared to the level detected at 2 days with the basal level of Pur-alpha set at 0.25 fold for heterozygous and 0.0 fold for Pur-alpha knockout based on quantitation of Figure 2, Panel A where a direct comparison of protein levels across the three genotypes of mice could be made.

Figure 2. Pur-alpha negatively regulates RhoA signaling protein during murine brain development

(A) Western blotting for Pur-alpha in wild type, heterozygous, and knockout Pur-alpha littermates were performed on whole cell extracts of 5, 15, and 20 days postnatal in parallel to allow direct comparison of relative proteins levels. (B) Quantitation of protein levels in Panel A by densitometry, normalized to tubulin as a loading control, and presented as fold change over levels detected in extracts from wild type mice at day 5 postnatal. Wild type mouse brains showed an increase in protein during development, while Pur-alpha levels in heterozygous mice demonstrated reduced levels due to haploinsufficiency and no detectable protein in Pur-alpha knockout mice. However, Rac1 levels at the same time points during development remained fairly consistent. (C) RhoA activation assay on human neuronal NT-2 cells treated with LPA to induce active RhoA in the presence and absence of siRNA targeting Pur-alpha shows a delay in turnover of active RhoA in the absence of Pur-alpha. Fold change over untreated (0 time point) is shown below. (D) Western blotting of rat primary neurons transfected with siRNA targeting Pur-alpha or non-targeting siRNA (nt) shows an increase in the total RhoA protein fraction upon decrease in Pur-alpha suggesting Pur-alpha down regulates total RhoA protein levels in neurons. GRB2 serves as loading control.

Figure 3. Aberrant expression pattern of RhoA and Rac1 in cortical neurons and cerebellum of mice lacking Pur-alpha at 5 days postnatal

The expression patterns of Pur-alpha, Rac1 and RhoA were observed in formalin fixed paraffin-embedded sections of brain cortex (Panels i and iii) and cerebellum (Panels ii and iv) from Pur-alpha knockout mice ($-/-$) and their wild type littermates ($+/+$) at 5 days postnatal. Pur-alpha expression at 5 days postnatal is relatively low and is localized to the cytoplasm of cortical neurons (Panel Ai) and to a lesser extent the Purkinje cell layer of the cerebellum (Aii). RhoA expression was relatively low in wild type mice at day 5 but increased expression was seen in some cells in the absence of Pur-alpha (Compare Bi and Biii). In contrast, Rac1 expression in cortical neurons was at high levels in the wild type mice at 5 days postnatal, but Rac1 levels were noticeably lower and with less perinuclear localization in the knockout mice (Compare Ci and Ciii). Hematoxylin counterstain, original magnification x200.

Figure 4. Aberrant expression pattern of RhoA and Rac1 in cortical neurons and cerebellum of mice lacking Pur-alpha at 18–19 days postnatal

The expression patterns of Pur-alpha, Rac1 and RhoA were observed in formalin fixed paraffin-embedded sections of brain cortex (Panels i and iii) and cerebellum (Panels ii and iv) from Pur-alpha knockout mice (–/–) and their wild type littermates (+/+) at 18–19 days postnatal. Pur-alpha expression at 19 days postnatal shows abundant perinuclear localization in the cytoplasm of cortical neurons (A-i) and the Purkinje cell layer of the cerebellum (A-ii). RhoA expression was relatively high and localized to the cytoplasm of cortical neurons in the presence of Pur-alpha at 18 days postnatal, but decreased expression was seen in the absence of Pur-alpha (Compare B-i and B-iii). While overall Rac1 expression in the cortex and cerebellar Purkinje cells was at high levels in wild type and knockout mice at 18 days postnatal (C-i and C-iii, respectively), the expression pattern of Rac1 showed less perinuclear localization in the knockout mice (C-ii and C-iv). Hematoxylin counterstain, original magnification x200.