Deletion of *Rictor* in neural progenitor cells reveals contributions of mTORC2 signaling to tuberous sclerosis complex

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Tuberous sclerosis complex (TSC) is a multisystem genetic disorder with severe neurologic manifestations, including epilepsy, autism, anxiety and attention deficit hyperactivity disorder. TSC is caused by the loss of either the *TSC1* or *TSC2* genes that normally regulate the mammalian target of rapamycin (mTOR) kinase. mTOR exists within two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Loss of either *TSC* gene leads to increased mTORC1 but decreased mTORC2 signaling. As the contribution of decreased mTORC2 signaling to neural development and homeostasis has not been well studied, we generated a conditional knockout (CKO) of *Rictor*, a key component of mTORC2. mTORC2 signaling is impaired in the brain, whereas mTORC1 signaling is unchanged. *Rictor* CKO mice have small brains and bodies, normal lifespan and are fertile. Cortical layering is normal, but neurons are smaller than those in control brains. Seizures were not observed, although excessive slow activity was seen on electroencephalography. *Rictor* CKO mice are hyperactive and have reduced anxiety-like behavior. Finally, there is decreased white matter and increased levels of monoamine neurotransmitters in the cerebral cortex. Loss of mTORC2 signaling in the cortex independent of mTORC1 can disrupt normal brain development and function and may contribute to some of the neurologic manifestations seen in TSC.

INTRODUCTION

Tuberous sclerosis complex (TSC) is a hamartomatous disorder caused by loss of function mutations in either the *TSC1* or *TSC2* genes that encode hamartin and tuberin, respectively. These proteins bind to one another and function at least in part to regulate the mammalian target of rapamycin (mTOR) serine/threonine kinase. mTOR itself is found within two functionally and molecularly distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is defined by the protein Raptor (regulatory associated protein of mTOR), mLST8, PRAS40 and DEPTOR and is exquisitely sensitive to rapamycin (1,2). mTORC1 regulates cell growth and protein translation through phosphorylation of multiple downstream effectors, including ribosomal protein S6 kinase and 4E-BP1 (3,4). mTORC1 regulates cell growth and protein translation through phosphorylation of multiple downstream effectors, including ribosomal protein S6 kinase and 4E-BP1 (3,4). mTORC2, in contrast includes mLST8, mSIN1 and *Rictor* (rapamycin insensitive component of TOR). mTORC2 is largely unaffected by rapamycin, although prolonged exposure may inhibit assembly and function of the complex (5). In contrast to mTORC1, much less is known about mTORC2, especially in the brain. Recent studies indicate that hamartin/tuberin may differentially regulate mTOR-containing complexes, normally inhibiting mTORC1 but activating mTORC2 (6–8). Loss of function mutations or deletions of the *TSC1* or *TSC2* genes, then, appear to result in increased mTORC1 but decreased mTORC2 signaling (8,9). Despite considerable progress in our understanding of these signaling pathways, there are still many questions regarding the specific contributions of dysregulated mTORC1 and mTORC2 signaling to the pathogenesis of TSC. While multiple cells can be affected, abnormalities of dorsal neural progenitor cells are likely responsible for much of the neuropathology and neurologic disease seen in patients with TSC (9).

mTORC2 phosphorylates the hydrophobic motif of several AGC kinases, including PKCα, Akt and SGK1 (10,11). Full
activation of Akt appears to require phosphorylation at Serine 473 by mTORC2 and PDK1 at Threonine 308 (12,13). Rictor is required for mTORC2 signaling and embryonic development as conventional Rictor knockout mice die around embryonic day 11 (E11) (14). To study the requirement of Rictor in specific tissues, a conditional floxed allele of mouse Rictor (RictorFlox) has been developed (15). Inactivation of Rictor throughout the brain was recently reported using Nestin-Cre mice (16). This caused widespread Rictor inactivation in both dorsal and ventral neural progenitor cells that give rise to almost all excitatory and inhibitory neurons. These mice were viable but demonstrated alterations in growth and behavior with decreased brain levels of dopamine (DA). No clear brain or cortical structural abnormalities were reported, although histopathologic analyses focused on the striatum (16).

To determine the role of Rictor and mTORC2 activity in the development of the cerebral cortex and to assess the relative contribution of mTORC2 signaling to the pathogenesis of TSC, we conditionally inactivated Rictor in dorsal neural progenitor cells using Emx1Cre knock-in mice (17). In contrast to Nestin-Cre, Emx1Cre during embryogenesis directs Cre expression to dorsal but not ventral neural progenitor cells. Dorsal progenitors generate almost all excitatory neurons, most astrocytes and a subset of oligodendrocytes in the dorsal cortex (18). RictorFloxFlox; Emx1Cre conditional knockout (CKO) mice (Rictor CKO) are viable and fertile, although they are smaller than control littermates. mTORC2 signaling was greatly decreased in the dorsal cortex, but mTORC1 signaling remained normal. Unlike Tsc1FloxFlox; Emx1Cre CKO mice (Tsc1 CKO) that we generated using the same Emx1Cre driver (9), brains from Rictor CKO mice have no gross abnormalities in structure or cortical layering. Cortical neuron size was decreased, and hypomyelination of the white matter was observed in the cerebral cortex. Behavioral testing in Rictor CKO mice revealed abnormal behaviors relevant to TSC, including significant hyperactivity and disordered sleep. These were associated with altered levels of several monoamine neurotransmitters in the prefrontal cortex. We find a clear role for Rictor during neurodevelopment and postnatal function. As other groups have identified mTORC1-independent and rapamycin-independent mechanisms in TSC (19–21), our results suggest an important role for mTORC2 and mTORC1 signaling during the pathogenesis of TSC.

RESULTS

Rictor CKO mice are viable and have impaired mTORC2 signaling

To assess the contribution of Rictor in dorsal neural progenitor cells of the cortex, floxed Rictor mice were bred with Emx1Cre animals to generate mice that are homozygous for the RictorFlox-floxed allele and heterozygous for Emx1Cre (Rictor CKO). Rictor CKO mice were born at expected Mendelian ratios, have a normal life span and both male and female Rictor CKO mice are fertile. To verify recombination and inactivation of the Rictor gene, protein extracts from dorsal cortex were analyzed by immunoblotting. Almost complete loss of Rictor protein was seen in older mice at P360 (Fig. 1A) as well as postnatal day 15 (P15, data not shown).

To assess the impact of Rictor deletion on mTORC2 signaling in the dorsal cerebral cortex, we examined levels of phospho-Akt (Ser473) and phospho-NDRG1 (Thr346) (8). Phosphorylation of both Akt and NDRG1 was greatly reduced following loss of Rictor, supporting a functional impairment of mTORC2 signaling (Fig. 1A and B). In contrast to changes in mTORC2 signaling, no alteration in phosphorylation of the mTORC1 downstream effector S6 (Ser235/236, Fig. 1) or (Ser240/244, Supplementary Material, Fig. S1) was seen. Mice examined at P9 and older ages also did not have changes in phospho-S6 levels (P15, Supplementary Material, Fig. S1). These findings indicate a specific loss of mTORC2 signaling, but unaltered mTORC1 signaling in the Rictor-deficient dorsal cortex.

Rictor deletion in the cerebral cortex impairs growth and brain development

Altered mTOR kinase activity in TSC has been closely linked to increased size of cells and organs. To address the impact of loss of mTORC2 signaling on total brain weight, we recorded brain and body weights in Rictor CKO mice when compared with littermate controls. At less than 10 weeks of age, both male (Fig. 1C) and female (Fig. 1D) Rictor CKO animals were significantly smaller than control littermates. Male and female Rictor CKO mice maintained smaller total weights than littermate controls, although the differences became no longer statistically significant in older male mice. Brain weights of Rictor CKO mice at P15 were also decreased when compared with littermates (Fig. 1E). However, when adjusted for body weight, the resulting brain-to-body weight ratios were not significantly different (Fig. 1F). These findings are in clear contrast to Tsc1 CKO mice that have increased brain weight and a greatly increased brain/body weight ratio in the setting of increased mTORC1 and decreased mTORC2 signaling in the dorsal cortex (9).

Neuronal size, layer formation and neuronal number

Tsc1 CKO mice have obvious alterations in neuronal layering that were not reversible by postnatal treatment with rapamycin, a potent mTORC1 inhibitor (9). While these findings may be attributed to the timing of rapamycin treatments (22), they may also be explained by the concomitant decreased mTORC2 signaling seen in Tsc1 CKO mice (9). To see if Rictor deletion in dorsal neural progenitor cells is sufficient to alter cortical layering, we analyzed the cortex of P15-day-old Rictor CKO mice for cortical layer thickness using markers restricted to either upper layer (Cux1) or lower layer (FoxP2) neurons. In contrast to the blatan changes seen in Tsc1 CKO mice, we did not observe any overt alterations in cortical thickness or the expression of upper layer neuronal marker Cux1 (Fig. 2A–C) or lower layer marker FoxP2 (data not shown). To determine the role of the Rictor gene on the control of neuronal size, we measured the cross-sectional area of Cux1 expressing upper layer neurons from P15 day cortex. These neurons had a moderate but statistically significant decrease in cross-sectional area when compared with Cux1-expressing neurons from littermate controls (Fig. 2D and E). Total numbers of upper
layer cortical neurons in \textit{Rictor} CKO cortex were not significantly different from littermate controls (data not shown). As mTORC1 signaling was not altered (Fig. 1), these findings support mTORC2 signaling as having a role in determining the size of cortical neurons but not their location within specific layers, although subtle defects may not be picked up by our immunofluorescence experiments.

Electroencephalography (EEG) slowing but absence of seizures in \textit{Rictor} CKO mice

Epilepsy is an extremely common finding in patients with TSC (23,24), and spontaneous and inducible seizures have been reported in several rodent models of TSC (9,25–27). We, then, examined whether loss of \textit{Rictor} in dorsal neural progenitor cells is sufficient to cause spontaneous seizures.
No obvious seizures were observed in any Rictor CKO mouse during routine handling. As seizures in mice may be subtle or brief, we also performed continuous video-electroencephalography (EEG) monitoring in adult Rictor CKO and littermate control mice. No spontaneous seizures or epileptiform discharges were seen for over 1000 h in aggregate data obtained from four pairs of Rictor CKO and littermate control mice (data not shown). However, a close
examination of background EEG frequencies using spectral analysis revealed increased power in the delta (0.5–4 Hz) and theta (4–8 Hz) frequency bands (Fig. 3). This excessive EEG slowing in Rictor CKO mice is non-specific, but suggests an underlying and widespread dysfunction of the cortical network (28,29).

**Oligodendrocytes and cortical myelination**

In addition to neuronal abnormalities, alterations in white matter have been proposed as an alternate mechanism for the developmental disorders frequently seen in patients with TSC (30–32). Neuroimaging of patients with TSC and several transgenic mouse models of TSC have demonstrated abnormalities in myelination (9,30,32,33). Such abnormalities may also potentially explain the diffuse EEG changes in Rictor CKO mice described above. To see if Rictor CKO mice had white matter alterations, P15 cortical sections were examined for expression of myelin basic protein (MBP), the major protein constituent of myelin. MBP immunofluorescence of the mesial dorsal cortex in control littermates demonstrated a fine branching myelin network (Fig. 4A). In contrast, Rictor CKO exhibited thicker MBP-positive processes with a fine branching myelin network (Fig. 4A). This provides additional evidence that mTORC2 signaling plays a role in myelination and likely impacts brain connectivity and function.

**Increased activity and decreased sleep in Rictor CKO mice**

Routine observation and handling of Rictor CKO mice suggested that they have increased activity when compared with control littermates. To better characterize these observations, we measured movements of adult Rictor CKO and control mice in an open field assay. We found significantly increased locomotor activity in Rictor CKO mice. Unlike age-matched control mice, these did not habituate over the 50 min test period (Fig. 5A). To determine if the increased activity was specific to a novel environment, we measured horizontal movement in a home cage assay over a 15 day period and found that the increased locomotor activity persisted throughout this prolonged observation (Fig. 5B).

As there is an increased incidence of sleep disorders associated with attention-deficit hyperactivity disorder (34–36) and in TSC (37–39), we used data from the prolonged EEG monitoring of Rictor CKO and control mice to identify any alterations in the proportion of time spent in sleeping. Strikingly, Rictor CKO mice had a >50% reduction in sleep time when compared with controls (Supplementary Material, Fig. S2). However, this decreased sleep did not persist after the first 24 h of each recording session suggesting that it is not due to a primary disruption in sleep homeostasis, but may be related to being placed in a novel environment.

**Decreased anxiety in Rictor CKO mice**

Patients with TSC have a high prevalence of anxiety disorders and hyperactivity (40,41). As Rictor CKO mice have increased activity, we hypothesized that the loss of Rictor in the dorsal cortex may also cause more complex behavioral abnormalities, including alterations in anxiety. We used the elevated zero maze as a measure of anxiety-related behavior in our mice (42–44). Surprisingly, Rictor CKO animals spent significantly more time exploring the open regions of the maze when compared with controls (Fig. 6A and B), suggesting a reduction in anxiety-related behavior. To confirm that the elevated zero maze results were not simply related to a non-specific increase in their activity and locomotion, we also measured running wheel activity, an assay of reward-motivated motor behavior. This has been used to model anxiety-related disorders in mice with decreased wheel running being associated with anti-anxiety drug treatment (45,46). Rictor CKO mice had less running wheel activity when compared with littermate controls, despite the overall increased locomotor activity (Fig. 6C). Finally, we used a marble-burying assay as an additional test of anxiety-related behavior that is also less dependent on locomotor activity (47,48). This behavioral test showed striking differences with Rictor CKO mice burying many fewer marbles than control littermates (Fig. 6D). Overall, these behavioral assays support a reduction in anxiety in Rictor CKO mice.

**Increased levels of multiple monoamine neurotransmitters**

Given these behaviors, we identified other previously reported mouse models that appeared to be broadly similar to Rictor CKO mice. These animal models were all noted to have altered levels of monoamine neurotransmitters (49–51). In addition, the previously reported pan-neuronal Rictor mouse model generated with Nestin-Cre had decreased levels of DA, but increased norepinephrine (NE) (16). To assess neurotransmitter levels, we took samples of prefrontal cortex from Rictor CKO and littermate control mice at 1 year of age to measure brain levels of DA, NE, serotonin (5-HT) and metabolites. In marked contrast to the decrease in prefrontal DA levels observed in the pan-neuronal Rictor Nestin-Cre mice (16), Rictor CKO mice had a significant increase in cortical DA, NE, 5-HT and the monoamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) (Fig. 7).
DISCUSSION

In this study, we demonstrate that the Rictor gene has a vital role in neurodevelopment. Our results provide highly important information about mTOR kinase function in TSC, specifically with regard to mTORC1 versus mTORC2 signaling. In contrast to mTORC1, mTORC2 signaling has not been well studied, despite having independent and likely essential functions in the brain and other organs. Although the exact mechanism by which TSC1 or TSC2 mutant cells have decreased mTORC2 signaling is unclear, it is possibly due to the loss of direct binding of the hamartin/tuberin complex to mTORC2. Alternatively, increased mTORC1 activates a negative feedback loop from p70S6K that inhibits IRS-1, thus preventing the PI3-Kinase-dependent activation of mTORC2 (52,53).

Our data did not support an independent role of Rictor/mTORC2 signaling in dorsal neural progenitor cells during the determination of neuronal position and cortical layering. The smaller neurons seen in the cerebral cortex of Rictor CKO mice, however, do support a role of mTORC2 in the control of neuronal size. This is in marked contrast to the very large cells such as those seen in TSC brain lesions (‘giant cells’) that are an almost invariable hallmark of increased mTORC1 signaling. The complex interaction of mTORC1 and mTORC2 with apparent opposing actions on cell size is of substantial interest as alterations in the size of neurons also likely affect function and connectivity. However, the increased neuronal cell size in TSC is likely predominately due to increased mTORC1 activity, and while this may be somewhat attenuated by concomitant decreased mTORC2 signaling, its overall contribution appears to be relatively minor. We favor an Akt-dependent mechanism for the decrease in neuronal size as our findings are very similar to those seen from the selective deletion of the Akt3(γ) isoform in mice (54).

The white matter abnormalities we find in Rictor CKO mice support a specific requirement for mTORC2 signaling during myelination, again likely working through an Akt-dependent mechanism. Enhanced PI3-Kinase signaling or overexpression of constitutively active form of Akt both activated mTORC1 with a subsequent increase in myelin formation (55). Furthermore, myelin can be stimulated by insulin, β1-integrin or neuregulin in an Akt-dependent manner (56,57). Similar to our data, a thin corpus callosum was seen following selective deletion of the Akt3(γ) isoform in mice (54). In both our Rictor CKO and previously published Akt deletion models (54,58), preserved S6 phosphorylation was seen, providing further support for an Akt-dependent but mTORC1-independent mechanism. These findings have clear translational significance for TSC, given reports of abnormal white matter in the cerebral cortex, sub-cortical regions and white matter tracts distal to the cortical tubers (30,32). Patients treated with everolimus, a mTORC1 inhibitor, have been reported to have some improvement in white matter abnormalities (59,60). Likewise, the white matter abnormalities we reported in Tsc1 CKO mice were also partially rescued by treatment with rapamycin (9). Although a partial rescue may be due to the relative timing and extent of mTORC1 inhibition therapy (22), additional mechanisms, including persistent abnormalities of mTORC2 signaling may also be involved. As our strategy targeted neural progenitor cells that specify neurons, astrocytes and oligodendrocytes (18), we cannot exclude that changes in myelination may be due to abnormal signaling in neurons, oligodendrocytes or the combination of the two. Ongoing studies targeting the Tsc1, Tsc2 or Rictor genes selectively in oligodendrocytes and not neurons will determine the specific role of mTORC1 and mTORC2 signaling to these cells and white matter development and function.

In contrast to multiple Tsc1 or Tsc2 CKO mouse models, we did not observe seizures in Rictor CKO mice either by observation or prolonged EEG monitoring. We did observe significant changes in the EEG frequencies with a relative increase in the power of the slower (delta and theta) frequency bands. This has previously been associated with disruptions in...
white matter connectivity (61–63). Although speculative, the widespread EEG abnormalities in Rictor CKO mice may be due to diffuse functional impairments of the cerebral cortex due to abnormal myelination. Again, more definitive data to connect these brain abnormalities will require oligodendrocyte-specific knockout of the Tsc1, Tsc2 and Rictor genes.

Figure 4. Myelination is abnormal in Rictor-deficient cortex with decreased expression of myelin-associated proteins. (A, B) Decreased expression of MBP in neuronal processes in Rictor CKO when compared with age-matched control mice. Low-power inset marked with asterisk denotes location from where images were obtained (Inset scale bar 1 mm). (C–F) Levels of the myelin components CNPase (D) and MBP (E) are significantly reduced in Rictor CKO (n = 3) cortex versus littermate controls (n = 3), whereas levels of the axonal marker PGP9.5 (F) are unchanged. Data expressed as mean ± SEM. Single and double asterisks denote statistical significance using Student’s t-test, P < 0.03 and P < 0.005, respectively. Corpus callosum thickness is reduced in the Rictor CKO when compared with littermate control. Low-power inset in (A) marked with pound sign denotes location from which images were obtained. The corpus callosum was readily visualized with MBP staining and its width measured in Rictor CKO (H) (n = 7) and littermate control mice (G) (n = 7) at P15. (I) Data were expressed as mean ± SEM. Asterisk denotes statistical significance using Student’s t-test, P < 0.01. Scale bars, 100 μm.
Deletion of Rictor was previously shown to reduce phosphorylation of Akt at Serine 473 and in many cell types, mTORC2 is thought to be the key kinase responsible for this phosphorylation (14,64). In comparison to other studies, some residual mTORC2 activity was seen in the brain of Rictor CKO mice. This may be attributable to tissue heterogeneity with the inclusion of non-targeted cells (16). In contrast to other models featuring the conditional deletion of Rictor in the brain and other organs (14–16,65,66), a reduction in Akt phosphorylation at the PDK1 consensus residue Threonine 308 was also seen in the brain of our Rictor CKO mice. Similar results, however, have been noted in a variety of cell lines (67) and support a model by which initial phosphorylation of Akt at Serine 473 promotes subsequent Threonine 308 phosphorylation (68,69). We generated Rictor-deficient neuronal cultures and Rictor-deficient mouse embryonic fibroblasts from Rictor<sup>Flox/Flox</sup> embryos. Using these two cell culture systems, we also found decreased levels of both Ser473 and Ser308 residues on Akt (Supplementary Material, Fig. S3A and B). We did not find any alterations in levels of activated PDK1 (Supplementary Material, Fig. S3C), suggesting that upstream signaling pathways are not altered in the cortex of Rictor CKO mice. Differential phosphorylation and signaling has been noted in other mouse tissues, including preserved phosphorylation of Akt at Serine 473 in muscle deficient for both Rictor and Raptor (70). We also cannot exclude a mechanism by which Akt is preferentially dephosphorylated at Threonine 308. Given the highly increased levels of DA in the cortex of Rictor CKO mice (Fig. 7), it is also possible that the activation of PP2A phosphatase by DA and G-protein-coupled receptors is a plausable cell non-autonomous mechanism for this observation (71). However, the decrease in Akt (Thr308) in two different Rictor-deficient cell culture systems argues for a cell-autonomous mechanism.

Given the very high prevalence of behavioral abnormalities in TSC, we investigated whether alterations in Rictor/mTORC2 activity were associated with abnormal behaviors in mice. Indeed, Rictor CKO mice had significant hyperactivity in both novel and familiar environments supporting a role for Rictor/mTORC2 in attention-deficit hyperactivity disorder that is common in patients with TSC (41). In contrast, we found significant reduction in anxiety-related behaviors using three different testing paradigms. This was unexpected, given that the clinical findings are increased anxieties in many patients with TSC; however, reduction in anxiety-like behavior has previously been described in several unrelated mouse models of hyperactivity (50,72,73). Anxiety in patients with TSC may also be related to the increased mTORC1 signaling that is not seen in Rictor CKO mice. A further possibility to explain the abnormal behaviors in these mice is the increased levels of multiple monoamines. We also found very significant differences in cortical monoamine levels when compared with Siuta et al. (16). This strongly suggests that different mechanisms are operative in our models, likely due to Rictor inactivation that is restricted to the dorsal cortex in the current CKO model versus the more widespread CKO of Rictor in dorsally and ventrally derived neurons. For example, Akt/GSK3β signaling cascade in the striatum is a well-documented regulator of DA-dependent behaviors in mice (71,72,74,75). Although we found a significant decrease in activated Akt in the dorsal cortex of Rictor CKO mice, this was not seen in the striatum (data not shown). It is possible that the loss of Rictor/mTORC2 activity in dorsal cortical neurons disrupts basal ganglia feedback circuits that would normally be responsible for restraining locomotor activity (76–78). Alternatively, the relatively small numbers of striatal medium spiny neurons that are expected to express Emx1 (79) and thus having an inactivated Rictor gene may be sufficient to cause behavioral changes through impaired Akt activation in the striatum (51,74,75).

In conclusion, Rictor CKO mice have disrupted development and brain function. Although this phenotype partially overlaps with that seen in Tsc1 CKO mice, comparisons of these two animal models provide genetic support that mTORC1 dysregulation likely causes much of the...
pathogenesis in TSC. However, there are clearly mTORC1-independent and rapamycin-independent abnormalities in patients with TSC, which may be due to persistent mTORC2 defects. Identifying such mechanisms will be highly important for the development of more efficacious therapies for patients with TSC.

MATERIALS AND METHODS

Rictor CKO mice

Mice expressing floxed Rictor alleles were a gift from Dr Mark Magnuson (Vanderbilt University, Nashville, TN) (15). Homozygous Rictor-floxed animals were maintained for over 2 years on a C57/BL6 genetic background and bred without difficulty. Emx1-Cre mice maintained on a C57/BL6 background were obtained from Jackson Laboratories (Strain #005628, Bar Harbor, ME). Rictor CKO mice are homozygous for the Rictor-floxed allele and heterozygous for Emx1Cre. Littermate RictorFlax/Flox, RictorFlax/Wt Emx1-Cre-negative and RictorFlax/Wt Emx1-Cre-positive (heterozygous conditional knockout) animals had no appreciable phenotypes and were used as controls. Genotyping of tail genomic DNA was performed using PCR as previously described (9). The Vanderbilt University institutional animal care and use committee approved all procedures involving mice.

Immunofluorescence

Brains of RictorEmx1Cre CKO and control littermates were processed using standard techniques (27,80). Briefly, animals were perfused with 4% paraformaldehyde, and brains were fixed overnight at 4°C in 4% paraformaldehyde and cryoprotected in 30% sucrose solution. Frozen sections were made at 10–20 μm and mounted on slides. For immunofluorescence experiments, brain sections were postfixed in 1% paraformaldehyde for three minutes and washed in PBS. Tissues were blocked in 5% goat serum with 0.1% Triton-X100 in PBS for 1 h. Primary antibodies were added to the blocking solution and slides were incubated overnight at 4°C. Primary antibodies and dilutions: NeuN 1:1000 (Millipore), Cux1 1:100 (Santa Cruz), FoxP2 1:10 000 (abcam), GFAP 1:1000 (Cell Signaling), phospho-S6 (Ser235-236) 1:200 (Cell Signaling) and MBP 1:200 (abcam). The next day, slides were washed in PBS and secondary antibodies (Alexa 488 and 568 fluorochromes, Invitrogen, CA, USA) were allowed to bind for 1 h. Slides were washed again in PBS and secondary antibodies (Alexa 488 and 568 fluorochromes, Invitrogen, CA, USA) were allowed to bind for 1 h. Slides were washed again in PBS and coverslipped with a 4′,6-diamidino-2-phenylindole-containing solution (Vectorshield Hard Set, Vector Labs). Negative controls for each experiment were performed by omission of the primary antibodies. Photomicrographs were obtained using a Zeiss epifluorescence microscope or a Zeiss LSM 510 confocal microscope. Low-power fluorescent images of whole sections were prepared as above and visualized with an IRDye 800
Statistical significance using Student’s t-test were calculated, and results were compared for statistical significance using Student’s t-test. Means and standard deviation were calculated, and results were compared for statistical significance using Student’s t-test. Means and standard deviation were calculated, and results were compared for statistical significance using Student’s t-test.

Cortical thickness
Brain sections were processed as above for immunofluorescence. Panoramic views were constructed using Adobe Photoshop CS5 for cropping. Any minor adjustments of brightness or contrast were done concurrently on photomicrographs of littermate control and Rictor CKO brain sections.

All experiments were performed three to seven times on brain sections from Rictor CKO mice and control littermates.

Cell size
Size of NeuN and Cux1 double-positive cells was quantified by importing images to ImageJ (NIH, version 1.38) and in a blinded fashion, >50 cells per animal from layers 2–4 of frontal cortex were measured using five independent control littermate and five Rictor CKO mice. Means and standard deviation were calculated, and results were compared for statistical significance using Student’s t-test.

Immunoblotting
Rictor CKO and control littermates were euthanized and brains were rapidly extracted, flash frozen in liquid nitrogen and stored at −80°C. The dorsal cortex was microdissected and homogenized in RIPA buffer consisting of 1X PBS with 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, along with protease and phosphatase inhibitor cocktails (Sigma) and clarified via centrifugation at 10,000×g for 10 min at 4°C. Protein concentrations were calculated using the Bradford method (Bio-Rad). Samples were diluted with NuPAGE LDS-loading buffer (Invitrogen) and separated by electrophoresis on 4–20% Bis-Tris gels, followed by the transfer to polyvinylidene difluoride membrane (Pall Corp., Pensacola, FL, USA). Membranes were blocked for 1 h at room temperature. Membranes were incubated with primary antibody at 4°C overnight in 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Primary antibodies: pS6 (Ser235/236), pS6 (Ser240/244), S6, pAkt (Ser473), pAkt (Thr308), Akt, Rictor, pNDRG1-Thr346, CNPase, pPKD1 (Ser241), p4E-BP1 (Thr37/46) and actin (all rabbit, from Cell Signaling, 1:1000 dilution). Additional antibodies: MBP 1:1000 (rat, abcam), PGP9.5 1:1000 (Serotec), pPKC alpha (Ser657, rabbit, Millipore) and actin 1:2000 (mouse, Sigma). Blots were washed with TBS-Tween and then probed with either horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) or with fluorescent-tagged secondary antibodies: Alexa 680 (rabbit, Invitrogen) and IRDye 800 fluorochromes (mouse and rat, Licor). For HRP-conjugated secondary antibodies, signal was developed using ECL western blotting substrate (Pierce, Rockford, IL, USA) and visualized on BioMax film (Kodak). Fluorescent-tagged secondary antibodies were visualized using an Odyssey fluorescence scanner. After visualization, digitized band densities were quantitated using ImageJ.

Neurochemical analyses
Prefrontal cortex samples were obtained from freshly dissected brains and consisted of 2 mm diameter punches from coronal brain slices. Samples were rapidly frozen in liquid nitrogen and stored at −80°C. Samples were analyzed for NE, DOPAC, DA, 5-HIAA, HVA and 5-HT by the Vanderbilt Neurochemistry Core Laboratory (Nashville, TN, USA) using HPLC with electrochemical detection (81).

Behavioral studies
All behavior experiments except for running wheel and home cage activity were conducted in the Vanderbilt Murine Neurobehavioral Core. Animals were acclimated to the core environment for at least 7 days prior to testing. Animals were housed on a 12 h light–dark cycle with free access to food and water. Rictor CKO and control mice were tested between P110 and P120. All testing was performed during the light cycle between 1200 and 1600 h. Prior to all tests, animals were acclimated for 20 min in an anteroom of the primary observation room.

Elevated zero maze
The maze apparatus was similar to previously published reports (82,83). The average light level over the open arms was 47 lux. Activity was recorded for 5 min. Data were acquired with ANY-Maze video tracking software (SDI, San Diego, CA, USA) and analyzed offline.

Open field
A commercial activity chamber measuring 10.75 × 10.75 inches (Med Associates, St. Albans, Vermont, USA) was used for measuring locomotor activity in a novel environment. Chambers were maintained inside ventilated, sound-attenuating

Figure 7. Monoamine levels are increased in Rictor CKO frontal cortex. Monoamine levels were significantly elevated in dorsal frontal cortex from Rictor CKO mice (n = 3) versus littermate controls (n = 6). Data were expressed as mean ± SEM. Asterisk denotes statistical significance using Student’s t-test, P < 0.05.
boxes. Animals were not otherwise handled for at least 3 h prior to the study. Activity was monitored for 50 min using Activity Monitor software (Med Associates, St. Albans, Vermont, USA).

Wheel running and home cage locomotion. Wheel running and horizontal activity in a home cage environment were measured in the Vanderbilt Mouse Metabolic Phenotyping Center using a commercial testing apparatus (Sable Systems International, Las Vegas, NV, USA). Rictor CKO and littermate controls were tested at 1 year of age. The mice were housed individually on a 12 h light–dark cycle with free access to food and water. Animals remained undisturbed for 15 days of testing, data were acquired using ExpeData software (Sable Systems International, Las Vegas, NV, USA).

Marble burying
Clean test cages with identical dimensions to the home cage were prepared with 2.5 inches of white bedding material. Fifteen black marbles were distributed evenly on top of the bedding in each cage away from edges and corners. One animal was placed in the center of each of the marble-filled cages. Mice were left in the marble-burying cages for 30 min. Marbles were considered buried if two-thirds or more of the marble was covered.

Video-EEG
Continuous video-EEG was performed on adult Rictor CKO and littermate control mice (n = 4 pairs). Prefabricated EEG headmounts (Pinnacle Technologies, Lawrence, KS, USA) were surgically implanted as described previously (27). EEG recordings were performed simultaneously on Rictor CKO and control pairs in adjacent recording chambers. Mice were allowed free access to food and water, and a 12 h light–dark cycle was maintained for the duration of each recording session. Up to three consecutive 24 h epochs were recorded for each mouse. Two channels of EEG data were recorded from the headmount, representative of rostral and caudal brain regions. Simultaneous video and electromyography were available to corroborate sleep and waking states. Visual analysis of EEG recordings as and EEG spectral power analysis (fast Fourier transform) was performed offline using commercial software (CareFusion Corporation, San Diego, CA, USA). The rostral EEG channel was selected for spectral analysis. The first 5 min of every hour of waking EEG was used for determination of absolute power values (µV²) on the delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (13–30 Hz) frequency bands. The values obtained for each 5 min epoch were averaged across the first and last 24 h of each 72 h recording session. To account for differences in the absolute power between individual mice, the mean power at each frequency band was normalized to the total power of all frequencies for each mouse.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


