

Tanc2-dependent direct and regulated mTOR inhibition balances mTORC1/2 signaling in developing mouse and human neurons

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Abstract

mTOR signaling, involving mTORC1 and mTORC2 complexes, critically regulates neural development and is implicated in various brain disorders. mTORC1/2 components that stimulate mTOR kinase activity strongly affect neurodevelopment, but mTOR-inhibitory mTORC1/2 components do not, questioning the role of balanced mTOR regulation in neurodevelopment. We found a direct, regulated inhibition of mTOR by Tanc2, an adaptor/scaffolding protein with strong neurodevelopmental and psychiatric implications. While Tanc2-null mice show embryonic lethality, Tanc2-haploinsufficient mice survive but display mTORC1/2 hyperactivity accompanying synaptic and behavioral deficits reversed by mTOR-inhibiting rapamycin. Tanc2 directly interacts with and inhibits mTOR, which is suppressed by mTOR-activating serum or ketamine, a fast-acting antidepressant. Tanc2 and Deptor, known to inhibit mTORC1/2 but minimally affect neurodevelopment, distinctly inhibit mTOR in early- and late-stage neurons. Patient-derived Tanc2 mutations disable Tanc2 function, and human Tanc2 inhibits mTORC1/2. Therefore, Tanc2 represents a novel mTORC1/2 inhibitor with strong neurodevelopmental impacts, implicating mTOR inhibition in treating TANC2-related brain disorders and Tanc2 modulation in treating mTOR-related disorders.

Introduction

mTOR (mammalian target of rapamycin) signaling, a fundamental regulator of cellular growth and function¹⁻⁴, controls the development and function of the nervous system⁵⁻¹². mTOR signaling is also strongly associated with various brain disorders, including brain tumors, epilepsy, neurodegenerative disorders (e.g., Alzheimer's and Parkinson's diseases), neurocutaneous diseases (e.g., tuberous sclerosis complex and neurofibromatosis), intellectual disability, autism spectrum disorders (ASD), depression, and diseases of abuse (e.g., alcoholism)⁵⁻¹².

mTOR nucleates the formation of mTOR complex-1 (mTORC1) and -2 (mTORC2) by associating with both shared and distinct components. These include mLST8 and Deptor (for both mTORC1 and mTORC2), Raptor and PRAS40 (mTORC1 only), and Rictor, Protor, and Sin1 (mTORC2 only)¹⁻⁴. These mTOR-interacting proteins coordinate the activity, subcellular localization, and substrate interactions of mTOR¹⁻⁴. Loss of proteins that enable or stimulate mTORC1/2 function, namely mTOR, Raptor, Rictor and mLST, in mice leads to embryonic lethality¹. However, loss of mTORC inhibitors (Deptor and PRAS40) has no significant impact on embryonic development or postnatal growth or survival^{13,14}, leaving it unclear whether 'balanced' regulation of mTOR involving both mTOR activators and inhibitors is important for neural development.

Tanc2, a large (~ 200 kDa) multi-domain adaptor/scaffolding protein, is highly expressed in the brain^{15,16} and modestly expressed in other tissues (www.ebi.ac.uk/gxa/home)¹⁷. Tanc2 is present at both synaptic and non-synaptic sites in neurons. Synaptic Tanc2 directly interacts with PSD-95^{15,16}, an

abundant excitatory postsynaptic scaffolding protein^{18–20}, and also promotes synaptic capture of motor protein-transported vesicles²¹.

Functionally, the homozygous deletion of *Tanc2* in mice leads to embryonic lethality¹⁶. In humans, *TANC2* mutations are extensively associated with various neuropsychiatric disorders, including intellectual disability, ASD, developmental delays, and schizophrenia^{17,22–30}. Disruptive *TANC2* mutations were recently identified in 20 different patients with neurodevelopmental symptoms associated with psychiatric disorders¹⁷. These results suggest that *Tanc2* is a critical regulator of brain development and function, but the underlying mechanisms remain unclear.

Results

Abnormal behaviors and synaptic plasticity in *Tanc2*-mutant mice

To explore in vivo functions of *Tanc2*, we first characterized mice carrying a heterozygous deletion of the *Tanc2* gene (*Tanc2*^{+/-}), encoding the *Tanc2* protein (**Fig. 1a**). *Tanc2*^{+/-} mice, unlike homozygous *Tanc2*-mutant (*Tanc2*^{-/-}) mice¹⁶, do not exhibit embryonic lethality. However, *Tanc2*^{+/-} mice showed modestly decreased survival (~70% survival at postnatal day 7 [P7]), indicative of a dose-dependent impact of *Tanc2* deletion on mouse development and survival.

In behavioral tests, adult male *Tanc2*^{+/-} mice (2–5 months; male) showed impaired spatial learning and memory in the Morris water maze, but normal novel-object recognition (**Fig. 1b; Supplementary Fig. 1a**). These mice also displayed hyperactivity and anxiolytic-like behavior, but largely normal social and depression-like behavior, and as neonates, showed suppressed ultrasonic vocalizations upon mother separation (**Supplementary Fig. 1b–e and 2**). Female adult *Tanc2*^{+/-} mice showed behavioral abnormalities similar to those of males (**Supplementary Fig. 3**). These results indicate that *Tanc2*^{+/-} mice are more relevant to human disease conditions.

To better understand the impaired spatial learning and memory in *Tanc2*^{+/-} mice, we examined synaptic plasticity in the hippocampus. Long-term potentiation (LTP) induced by high-frequency stimulation (HFS) was suppressed at Schaffer collateral-CA1 pyramidal cell (SC-CA1) synapses of *Tanc2*^{+/-} mice (7–8 weeks) (**Fig. 1c**). In contrast, LTP at a younger age (4–5 weeks) was normal (**Supplementary Fig. 4a**), suggestive of age-dependent LTP impairment. Basal excitatory synaptic transmission and presynaptic release were unaltered at SC-CA1 synapses of *Tanc2*^{+/-} mice (3–4 weeks) (**Supplementary Fig. 4b,c**). Long-term depression (LTD) induced by low-frequency stimulation (LFS) was also suppressed at SC-CA1 synapses of *Tanc2*^{+/-} mice (3–4 weeks), whereas mGluR-dependent LTD was normal (**Fig. 1d; Supplementary Fig. 4d**).

The abovementioned decrease in LTD at 3–4 weeks, which contrasts with the normal LTP at a similar age (4–5 weeks), cannot be explained by the decrease in currents of NMDA receptors (NMDARs), which are known to regulate both LTP and LTD^{31,32}. We thus tested whether synaptic signaling downstream of

NMDAR activation, also known to control LTP/LTD^{31,32}, is altered by immunoblot analysis of neuronal signaling proteins.

mTOR hyperactivity in *Tanc2*-mutant mice

Intriguingly, mTOR activity, measured by mTOR phosphorylation (S2448) in immunoblot analyses, was markedly (~5-fold) increased in the whole brain of *Tanc2*^{+/-} pups (P14) without a change in total mTOR levels (**Fig. 1e**). This change was accompanied by hyper-phosphorylation of 4E-BP (T37/46), a downstream target of mTOR¹⁻⁴, but not S6 (S235/236), another mTOR target¹⁻⁴, likely owing to compensatory changes occurring in heterozygous mice (see the stronger changes induced by homozygous *Tanc2* deletion, below). In contrast, activities of PI3K (phosphoinositide 3-kinase), PTEN (phosphatase and tensin homolog) and TSC1/2 (tuberous sclerosis 1/2)—signaling proteins upstream of mTOR—were normal (**Supplementary Fig. 5a**), suggesting that they do not contribute to the mTOR hyperactivity.

Phosphorylation of Akt (S473), reflecting mTORC2 activity¹⁻⁴, was also strongly increased (**Fig. 1f**), suggesting that both mTORC1 and mTORC2 are hyperactive in the *Tanc2*^{+/-} brain (P14). Moreover, Ser-9 phosphorylation of GSK3b (glycogen synthase kinase 3b), a downstream target of Akt³³ that promotes LTD³², was increased (indicating reduced activity), in line with the suppressed LTD at *Tanc2*^{+/-} hippocampal synapses.

Interestingly, tests of *Tanc2*^{+/-} juveniles (P28) showed no significant changes in mTORC1 or mTORC2 activity, as indicated by immunoblot analyses of mTOR (S2448), S6 (S235/236), 4E-BP (T37/46), Akt (S473), and GSK3b (S9) (**Supplementary Fig. 5b**). This contrasts with results from *Tanc2*^{+/-} pups (P14) and suggests that the function of *Tanc2* is age-dependent, consistent with the strong decrease in *Tanc2* protein levels in the wild-type (WT) mouse brain after P14¹⁶.

Because the mTOR hyperactivity observed in *Tanc2*^{+/-} pups (P14) might represent indirect changes attributable to long-term deletion of *Tanc2*, we generated another *Tanc2*-mutant mouse line that carries a floxed *Tanc2* allele (*Tanc2*^{fl/fl}) for use in creating a conditional gene knockout (cKO) (**Supplementary Fig. 6**). Injection of AAV1-hSyn-Cre-EGFP into the hippocampus of *Tanc2*^{fl/fl} pups (P5–14) to produce local homozygous knockout of *Tanc2* induced hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473), GSK3b (S9) and mTOR (S2248) (**Fig. 1f**), indicative of mTORC1 and mTORC2 hyperactivity. These results collectively suggest that *Tanc2* deletion leads to mTORC1/2 hyperactivity at the pup (P7–14), but not juvenile (P21–28), stage.

Early rapamycin treatment normalizes LTP and behaviors in adult *Tanc2*^{+/-} mice

To gain mechanistic insight into how *Tanc2* deletion induces mTOR hyperactivity, we first tested whether *Tanc2* directly interacts with mTOR using protein-protein binding assays. Purified *Tanc2* protein directly interacted with purified mTOR protein (**Fig. 3a**). *Tanc2* also formed a complex with mTOR in the mouse

brain (**Fig. 3b,c**). This interaction was mediated by multiple regions of Tanc2 protein and the C-terminal region of mTOR containing FRB and kinase domains (**Fig. 3d–f**). Here, mTOR was found to additionally interact with Tanc1, a relative of Tanc2 that is strongly expressed in late stages of rat brain development (>P14) and regulates synapse development, but is not critical for mouse development^{15,16}.

The results described thus far suggest that Tanc2 directly interacts with mTOR, but do not speak to whether Tanc2 inhibits the kinase activity of mTOR. We tested this possibility by overexpressing Tanc2 in HEK293T cells, and found that this was sufficient to inhibit endogenous mTOR activity (**Supplementary Fig. 7**). Consistent with this, *in vitro* assays using purified proteins showed that Tanc2 directly inhibits mTOR kinase activity, as evidenced by decreased phosphorylation of the mTORC1 (mTOR + Raptor) target S6K in the presence of Tanc2 (**Fig. 3g**).

Serum and ketamine regulate the Tanc2–mTOR interaction

We next investigated whether Tanc2–mTOR interactions are regulated by extracellular influences, first testing serum, which is known to activate mTOR². Serum starvation promoted the colocalization and biochemical association of Tanc2 with mTOR in HEK293T cells within ~4 hours. This effect was reversed by serum replenishment for ~24 hours (**Fig. 4a,b**), suggesting that mTOR dissociates from Tanc2 upon serum stimulation. Moreover, the Tanc2–mTOR interaction induced by serum starvation was inhibited by rapamycin (**Fig. 4c,d**), suggesting that Tanc2 and rapamycin compete for binding to the mTOR FRB domain. Tanc1, which also associates with mTOR in the brain, interacted with mTOR in a serum- and rapamycin-dependent manner (**Supplementary Fig. 8**).

We next tested whether the Tanc1/2–mTOR interaction could be regulated in the brain of mice (P14) by ketamine-induced mTOR activation. Treatment with ketamine (10 mg/kg; *i.p.*), a fast-acting antidepressant known to stimulate mTOR signaling³⁴, rapidly (~30–60 minutes) increased mTOR activity and promoted synaptic localization of mTOR-associated proteins as well as PSD-95 (**Supplementary Fig. 9**), as previously reported³⁴. Importantly, ketamine treatment suppressed the Tanc1/2–mTOR interaction without affecting the Tanc1/2–PSD-95 interaction (**Fig. 4e**)^{15,16}, suggesting that Tanc1/2 bridges mTOR to PSD-95 at the synapse in a regulated manner.

Tanc2, Deptor, and Tanc1 distinctly inhibit mTORC1/2 in early- and late-stage neurons

Because Deptor, similar to Tanc2, also binds and inhibits mTORC1/2³⁵, we tested whether Tanc2 and Deptor show overlapping or distinct spatiotemporal expression patterns. Immunoblot analyses using cultured neurons and mouse brain extracts showed that Tanc2 protein was more strongly expressed in early stages (embryonic and early postnatal) and was less enriched at synapses (**Supplementary Fig. 10**). In contrast, Deptor and Tanc1 showed progressive increases in expression across postnatal stages and stronger synaptic enrichment in both cultured neurons and mouse brains, a pattern similar to that reported for rat Tanc1 and Tanc2^{15,16}.

These results suggest that *Tanc2* and *Deptor/Tanc1* may distinctly inhibit mTOR activity at different developmental stages. We thus sought to acutely knockdown *Tanc2* and *Deptor/Tanc1* in cultured mouse hippocampal neurons during early (days in vitro [DIV] 7–14) and late (DIV21–28) stages. Early-stage *Tanc2* knockdown induced hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473), and GSK3b (S9) (**Fig. 5a–c**), suggestive of mTORC1 and mTORC2 hyperactivity. Akt (T308) phosphorylation was unaltered, in line with the normal activities of mTOR-upstream proteins in *Tanc2*^{+/-} mice (**Supplementary Fig. 5**). In contrast, late-stage *Tanc2* knockdown had no effect on these phosphorylation events (**Fig. 5d–f**).

Late-stage, but not early-stage, knockdown of *Deptor* induced hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473) and GSK3b (S9) (**Fig. 5a–f**), suggestive of mTORC1 and mTORC2 hyperactivity. In addition, late-stage, but not early-stage, knockdown of *Tanc1* induced similar hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473), and GSK3b (S9) (**Fig. 5d–f**). *Tanc2* and *Deptor* double-knockdown did not produce additive effects at early or late stages, except with respect to early-stage (P7–14) mTOR phosphorylation (**Fig. 5a–f**). These results suggest that *Tanc2* and *Deptor/Tanc1* distinctly inhibit mTORC1/2 signaling at early and late stages of mouse brain development, respectively, in line with the embryonic lethality of *Tanc2*, but not *Deptor* or *Tanc1*, KO mice^{14,16}.

To determine whether neurons or glial cells are more important for *Tanc2*-dependent mTOR inhibition, we selectively knocked down *Tanc2* in neuron- or glia-enriched early-stage cultured hippocampal neurons (DIV7–14). Neuronal, but not glial, *Tanc2* knockdown induced mTOR hyperactivity, and, in line with this, *Tanc2* expression was much weaker in glial cells (**Supplementary Fig. 11**), suggesting that *Tanc2* is more important for mTOR inhibition in neurons than in glial cells at early stages.

Patient-derived *Tanc2* mutations suppress *Tanc2*-dependent mTOR inhibition

To determine whether there is a relationship between *Tanc2*-dependent mTOR inhibition and human brain disorders, we first tested whether specific *Tanc2* mutations associated with intellectual disability, schizophrenia, and ASD identified in humans (R760C, A794V, and H1689R)^{22,23,26} affected interactions with mTOR or inhibition of mTOR activity (**Fig. 6a**). Coimmunoprecipitation experiments showed that, of these mutants, only *Tanc2*-H1689R failed to biochemically associate with mTOR in HEK293T cells (**Fig. 6b,c**); however, all three *Tanc2* mutants failed to inhibit mTOR activity (**Fig. 6d–f**). Therefore, patient-derived *Tanc2* mutations disrupt the mTOR-binding and/or mTOR-inhibitory activity of *Tanc2*.

TANC2 in human neurons inhibits mTORC1 and mTORC2

Finally, we tested whether *Tanc2* inhibits mTOR activity in human neurons. To this end, we knocked down *TANC2* in human neural progenitor cells (NPCs) developing into mature neurons for 2 weeks using two independent *TANC2* knockdown constructs. Both *TANC2* knockdown constructs similarly increased phosphorylation of S6 (S235/236), 4E-BP (T37/46), and GSK3b (S9), although they exerted mixed effects on Akt (S473) phosphorylation (**Fig. 6g–i**). mTOR phosphorylation was unaltered, similar to the results

from mouse neurons (**Fig. 5**). These results collectively suggest that Tanc2 inhibits mTORC1/2 in both human and mouse neurons.

Discussion

The present study suggests that Tanc2 is a novel and regulated mTOR inhibitor that has strong neurodevelopmental impacts and therapeutic potential. The first important conclusion from our results is that Tanc2 binds to mTOR. In support of this, Tanc2 forms a complex with mTOR in heterologous cells and in the mouse brain. More directly, purified Tanc2 proteins form a complex with purified mTOR proteins. Tanc2 uses its multiple domains to associate with mTOR, whereas mTOR binds to Tanc2 through its C-terminal region, containing the FRB, kinase, and FATC domains. The latter is further supported by that rapamycin, known to bind to the FRB domain of mTOR, blocks the colocalization and biochemical association between Tanc2 and mTOR. This result suggests the possibility that rapamycin could be used to block the Tanc2–mTOR interaction and to promote mTOR activity in various contexts such as decreased mTOR activity in human disorders.

Tanc2 binds to mTOR in a regulated manner. The presence of serum, well known to activate mTOR, weakens the colocalization and biochemical association between Tanc2 and mTOR. In addition, ketamine, a fast-acting antidepressant known to promote excitatory synapse functions and mTOR activity³⁴, inhibits the Tanc2–mTOR interaction in the mouse brain. These results suggest that Tanc2 inhibits mTOR in a regulated manner to coordinate mTOR activity under various nutritional states and during brain development and neuronal or synaptic activities. Specific mechanisms that underlie the regulated Tanc2-mTOR interactions remain to be determined although they could be posttranslational modifications of mTOR or Tanc2 at binding interfaces or regulatory domains.

Perhaps the most important conclusion of the present study is that Tanc2 inhibits mTOR. This is supported by multiple lines of in vitro and vivo evidence. Most directly, purified Tanc2 inhibits the kinase activity of mTOR, as shown by the suppression of mTOR-dependent phosphorylation of an mTOR substrate (S6K). In addition, Tanc2 overexpressed in HEK293T cells inhibits mTOR, while mutant Tanc2 proteins carrying human mutations fail to bind and inhibit mTOR. Moreover, acute knockdown of Tanc2 increases mTOR activity in cultured mouse neurons at around developmental stages of strong Tanc2 expression. *Tanc2*^{+/-} mice show increased mTOR activity in both mTORC1 and mTORC2 complexes. In addition, cre-dependent acute knockout of Tanc2 in an independent Tanc2-mutant mouse line increases mTOR activity in mTORC1/2. In human neurons, Tanc2 knockdown increases mTOR activity in mTORC1/2 in neural progenitor cells developing into neurons. These results strongly suggest that Tanc2 binds to and inhibits mTOR in mouse and human neurons at early stages.

In addition to Tanc2, Tanc1 interacts with and inhibits mTOR in a rapamycin-dependent manner. Tanc1 expression sharply increases during postnatal stages of mouse brain development, whereas Tanc2 expression is relatively stronger at earlier stages. Deptor, a known mTOR inhibitor, also shows strong late-stage expression, similar to Tanc1. It is therefore possible that Tanc2, Tanc1, and Deptor distinctly inhibit

mTOR across different developmental stages. Indeed, our results indicate that Tanc2 and Tanc1/Deptor inhibit mTOR more strongly at around postnatal weeks 2 and 4, respectively. These results are in line with the differential impacts of homozygous Tanc2 and Tanc1/Deptor deletions in mice, where the deletion of Tanc2, but not Tanc1 or Deptor, leads to embryonic lethality^{14,16}.

Tanc2 and Tanc1 interact with the PSD-95 family of scaffolding proteins, known to mediate the molecular organization of multi-protein complexes at cell-to-cell junctions such as neuronal synapses in order to couple receptor activations with signaling pathways^{18,19}. Therefore, Tanc2 and Tanc1 may recruit mTORC1/2 complexes to PSD-95-based multiple protein complexes at excitatory postsynaptic sites. In line with this idea, Tanc2 has been suggested to recruit cargo dense core vesicles driven by the KIF1A motor protein to excitatory synapses²¹. Synaptically localized mTORC1/2 may be inhibited by local Tanc2 until mTOR activity is increased by the activation of synaptic receptors such as TrkB and mGluRs³⁶. The four known members of the PSD-95 family (PSD-95, PSD-93, SAP102, and SAP97) display differential spatiotemporal expression patterns; i.e. PSD-95 and PSD-93 are more abundant at later developmental stages whereas SAP102 expression is stronger at earlier stages. It is therefore possible that Tanc2 and Tanc1 may coordinate mTORC1/2 signaling at both synaptic and non-synaptic sites of PSD-95-enriched multi-protein complexes in developing neural and non-neural tissues.

The synaptic and behavioral phenotypes of *Tanc2*^{+/-} mice implicate Tanc2 in the regulation of synaptic plasticity and behaviors, including LTP, learning and memory, hyperactivity, and anxiety-like behavior, all of which are reversed by rapamycin-dependent mTOR inhibition. In humans, Tanc2 mutations have been extensively associated with various neurodevelopmental and neuropsychiatric disorders, including intellectual disability, schizophrenia, and ASD^{17,22-30}. These results, together with the embryonic lethality in *Tanc2*^{-/-} mice and strongly increased mTOR activity in *Tanc2*^{+/-} mice, suggest that Tanc2 regulates normal brain development and function by coordinating mTOR inhibition, and that rapamycin-dependent mTOR inhibition could be used to treat human patients with Tanc2 mutations and resulting mTOR hyperactivity. In addition, modulation of Tanc2 activity, i.e. anti-sense Tanc2 knockdown or virus-mediated Tanc2 overexpression, could be used to treat various mTOR-related brain disorders^{5-7,37-39}. These therapeutic potentials extend to non-neural tissues and non-brain mTOR-related disorders such as metabolic diseases^{1,2} because Tanc2 and Tanc1 are expressed in various non-neural tissues in mice and humans (www.ebi.ac.uk/gxa/home)^{16,17}.

In conclusion, our study reports that Tanc2 is a novel and regulated mTOR inhibitor with strong neurodevelopmental impacts, supporting the general notion that balanced mTOR regulation involving both mTOR activators and inhibitors is important for normal brain development and function. In addition, our results suggest that mTOR inhibition could be an effective strategy for treating human individuals with *TANC2* mutations suffering from neuropsychiatric disorders, including intellectual disability, ASD, developmental delays, and schizophrenia. In addition, Tanc2 modulations promoting or suppressing mTOR signaling could have therapeutic potential for the treatment of various mTOR-related peripheral and brain disorders.

Declarations

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Author contributions

S.K. and S.L. designed experiments and analyzed data; Y.L., H.K., and S.K. generated mice; S.K., Y.K., J.P., and H.J. conducted mouse behavioral experiments and analysis; S.K. and J.P. performed biochemical experiments and analysis; S.K., Y.K., W.S., J.D.R., K.K., and S.M.U. conducted slice electrophysiology experiments and analysis; S.L. performed cultured neuron experiments, protein-protein interaction experiments, and in vitro kinase assays; S.L. and K.K. performed virus injection experiments; D.W., S.L., C.Y. and M.L. performed imaging experiments and analysis; D.K. and S.L. performed human neuron experiments; S.K., J.H., W.D.H., and E.K. wrote the manuscript.

Declaration of interests

The authors declare that they have no competing financial interests.

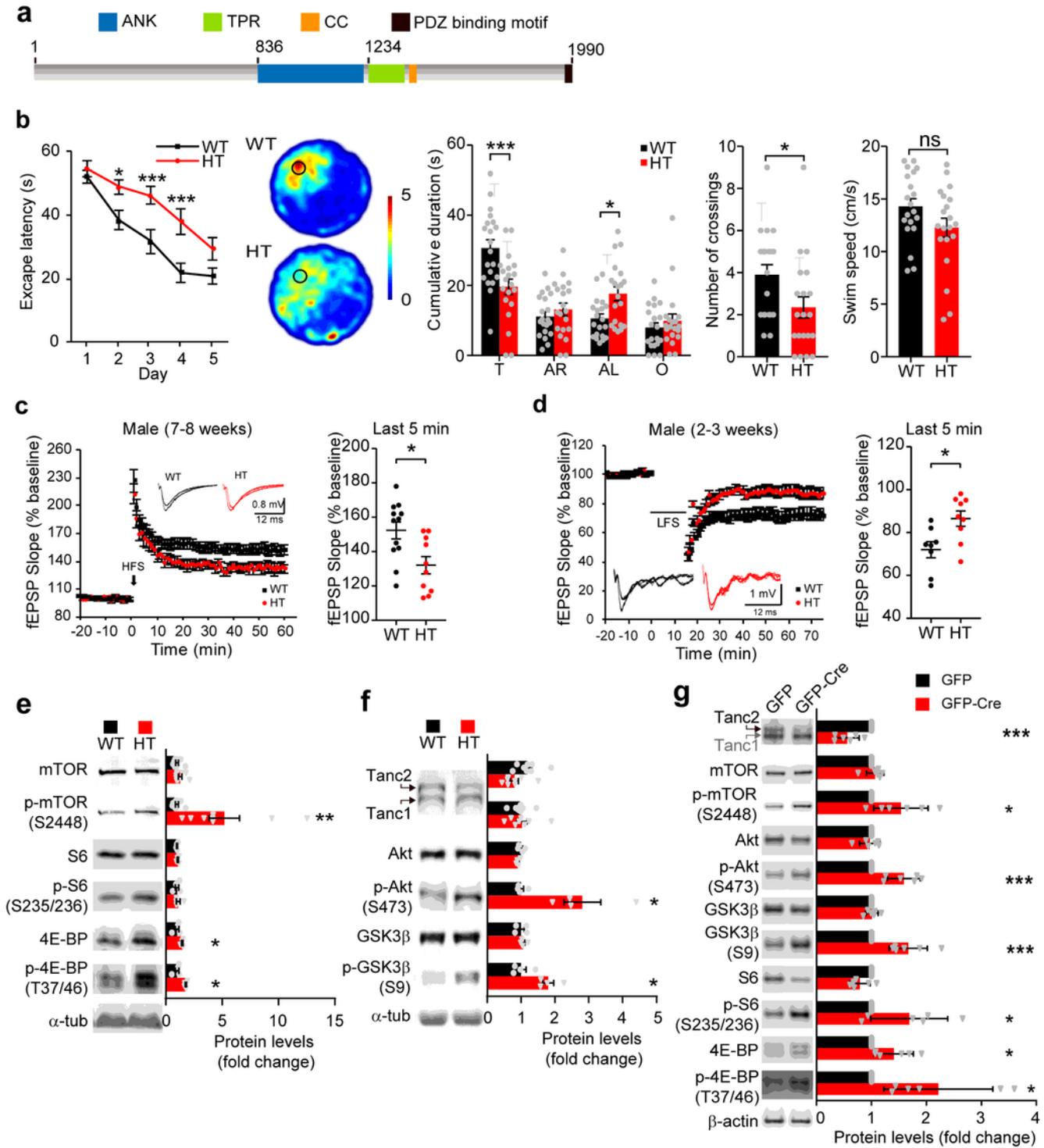
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Figures

Fig. 1**Figure 1**

Abnormal behaviors and synaptic plasticity and increased mTOR activity in Tanc2-mutant mice. (a) Domain structure of Tanc2. ANK, ankyrin repeats; TPR, tetratricopeptide repeats; CC, coiled-coil domain; PB, PDZ-binding motif. (b) Impaired spatial learning and memory in Tanc2^{+/-} mice (2–4 months; male) in the Morris water-maze test, as shown by the escape latency in the learning phase, the time spent in the target quadrant in the probe phase (T, target; AR, adjacent right; AL, adjacent light; O, opposite), and the

number of exact crossings over the former hidden platform in the probe phase. Note that swim speed was normal in *Tanc2*^{+/-} mice. (n = 13 mice [WT], and 11 [HT], *P < 0.05, ***P < 0.001, ns, not significant, two-way repeated measures/RM ANOVA and Student's t-test). (c) Suppressed HFS-LTP at SC-CA1 synapses of 7–8-week-old *Tanc2*^{+/-} mice (7–8 week-old male). (n = 12 slices, 5 mice [WT], and 10, 5 [HT], *P < 0.05, Student's t-test). (d) Suppressed LFS-LTD at SC-CA1 synapses of *Tanc2*^{+/-} mice (2–3 weeks; male). (n = 8, 6 [WT], and 9, 5 [HT], *P < 0.05, Student's t-test). (e) Increased mTOR activity in the brain of *Tanc2*^{+/-} (HT) mice (P14; male), as shown by Ser-2448 phosphorylation. Note that phosphorylation levels of 4E-BP1 (Thr-37/46), but not S6 (Ser-235/236), downstream of mTOR were also increased. For quantification, HT and WT signals were normalized to those of α -tubulin. See Supplementary Fig. 12 for uncropped images for this panel and all other immunoblot panels and Supplementary Table 1 for statistical details. (n = 4 mice [WT, HT], except for 8 for mTOR, p-mTOR, and *Tanc1/2*, *P < 0.05, **P < 0.01, Student's t-test). (f) Increased phosphorylation of Akt (S473) and GSK3 β (S9) upstream of mTOR. Note that Akt phosphorylation (S473) reflects activated mTOR in the mTORC2 complex. (n = 4 mice [WT, HT], *P < 0.05, Student's t-test). (g) Cre-dependent acute hippocampal *Tanc2* deletion leads to increased phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473), GSK3 β (S9) and mTOR (S2448), indicative of mTORC1 and mTORC2 hyperactivity. AAV1-Synapsin-GFP-Cre (or GFP alone as control) was injected into the hippocampus of *Tanc2*^{fl/fl} mice (P5–14). Note that S6 phosphorylation was increased, in contrast to the normal S6 phosphorylation observed in the *Tanc2*^{+/-} brain. (n = 3 independent experiments, *P < 0.05, ***P < 0.001, Student's t-test).

Fig. 2

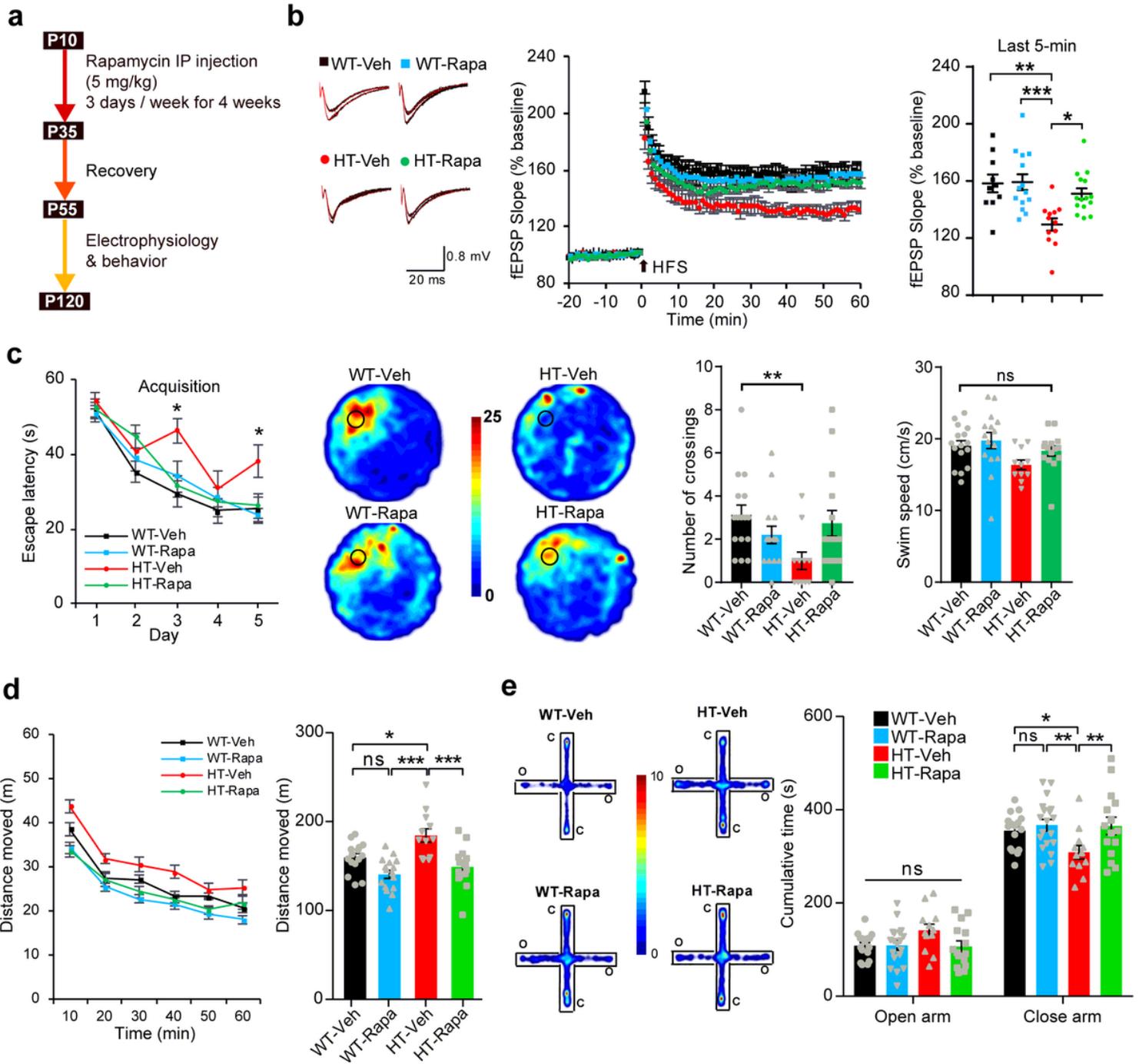


Figure 2

Early chronic rapamycin treatment normalizes LTP and abnormal behaviors in *Tanc2*^{+/-} mice. (a) Diagram showing early, chronic rapamycin treatment followed by electrophysiology and behavioral tests. (b) Early, chronic rapamycin treatment (P10–35) improves HFS-LTP at SC-CA1 synapses in adult *Tanc2*^{+/-} mice (7–8 weeks). (n = 10 slices, 5 mice [WT-Veh/vehicle], 14, 6 [WT-Rapa/rapamycin], 12, 5 [HT-Veh], and 15, 7 [HT-Rapa], *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA with Bonferroni test). (c) Early, chronic rapamycin treatment improves impaired spatial learning and memory in *Tanc2*^{+/-} mice

(7–8 weeks) in the Morris water-maze test, as indicated by escape latency and the number of crossings over the former platform location in the probe test. (n = 16 animals [WT-Veh], 15 [WT-Rapa], 11 [HT-Veh], and 15 [HT-Rapa], *P < 0.05, **P < 0.01, ns, not significant, two-way repeated-measures/RM ANOVA with Bonferroni test). (d) Early, chronic rapamycin treatment (P10–35) improves hyperactivity in Tanc2+/- mice (7–8 week; male) in the open-field test, as shown by distance moved over 60 minutes and total distance moved. (n = 15 mice [WT-Veh/vehicle], 16 [WT-Rapa/rapamycin], 12 [HT-Veh], and 14 [HT-Rapa], *P < 0.05, ***P < 0.001, ns, not significant, two-way ANOVA with Bonferroni test). (e) Early, chronic rapamycin treatment (P10–35) improves anxiolytic-like behaviors in Tanc2+/- mice (7–8 weeks; male) in the elevated plus-maze test, as shown by time spent in open/close arms. (n = 16 mice [WT-Veh], 19 [WT-Rapa], 12 [HT-Veh], and 15 [HT-Rapa], *P < 0.05, **P < 0.01, ns, not significant, two-way ANOVA with Bonferroni test).

Fig. 3

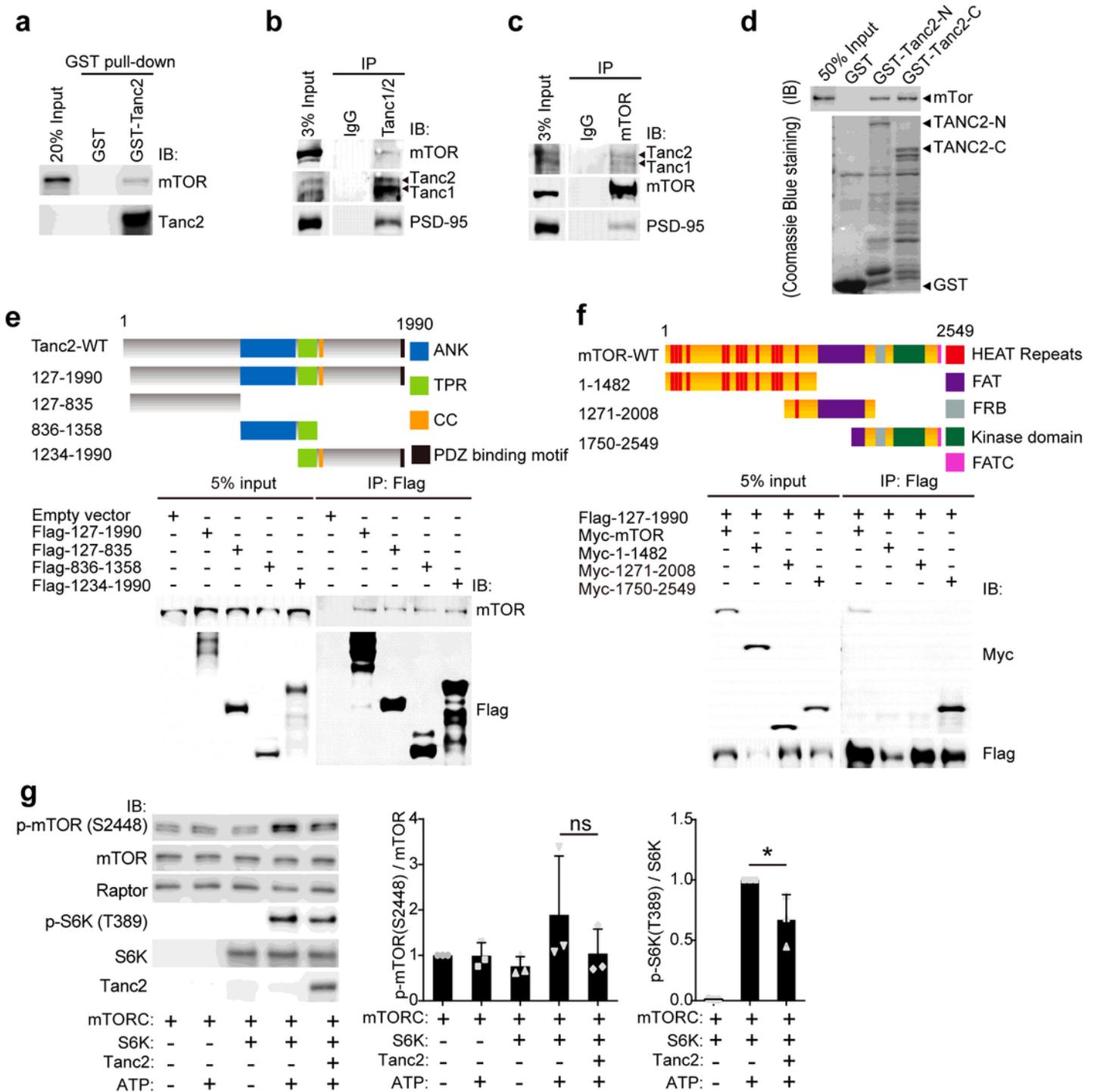


Figure 3

Tanc2 directly interacts with and inhibits mTOR (a) Purified Tanc2 protein directly interacts with purified mTOR protein. GST-Tanc2 (full-length) protein was used to pull down purified mTOR protein. Input, 20%. (b and c) Tanc1 and Tanc2 form a complex with mTOR in the mouse brain. Whole-brain lysates (P14; mouse) were immunoprecipitated (IP) with pan-Tanc or mTOR antibodies, followed by immunoblotting. Note that mTOR pull-down also coprecipitated PSD-95 through Tanc2. (d) Both N-terminal and C-terminal

regions of purified Tanc2 protein directly interact with purified mTOR. GST-tagged purified N- and C-terminal regions of human Tanc2 (aa 1-1358 and aa 1359-1990) were coupled to glutathione beads and incubated with purified mTOR proteins, followed by GST pull-down and immunoblot analysis. (e) Tanc2 forms a complex with mTOR in HEK293T cells through multiple regions of Tanc2. Lysates of HEK293T cells expressing deletion variants of Flag-Tanc2 (near-full-length, aa 127-1990; N-terminal region, aa 127-835; middle region, aa 836-1358; C-terminal region, aa 1234-1990) and mTOR (endogenous) were immunoprecipitated with Flag antibodies and immunoblotted with anti-Flag (for Tanc2) and mTOR antibodies. Note that all four deletion variants of Tanc2 interacted with mTOR, suggesting that multiple regions of Tanc2 are involved in mTOR binding. We used the near full-length Tanc2 because the full-length construct was unavailable at the time of the experiment; experiments repeated using the full-length Tanc2 construct yielded the same results. (f) The C-terminal region of mTOR containing FRB, kinase, and FATC domains is sufficient for complex formation with Tanc2. HEK293T cells expressing Myc-mTOR deletion variants and Flag-Tanc2 (aa 127-1990) were immunoprecipitated with Flag antibodies and immunoblotted with Myc and Flag antibodies. (g) Purified Tanc2 inhibits the kinase activity of purified mTORC1 (containing GFP-mTOR and mTOR-associated proteins such as Raptor), as shown by decreased phosphorylation of purified S6K (an mTOR substrate). In control experiments, EGFP-Tanc2 was replaced with purified EGFP protein (lanes 1–4; not probed). (n = 3 independent experiments, *P < 0.05, ns, not significant, one-way ANOVA with Tukey test).

Fig. 4.

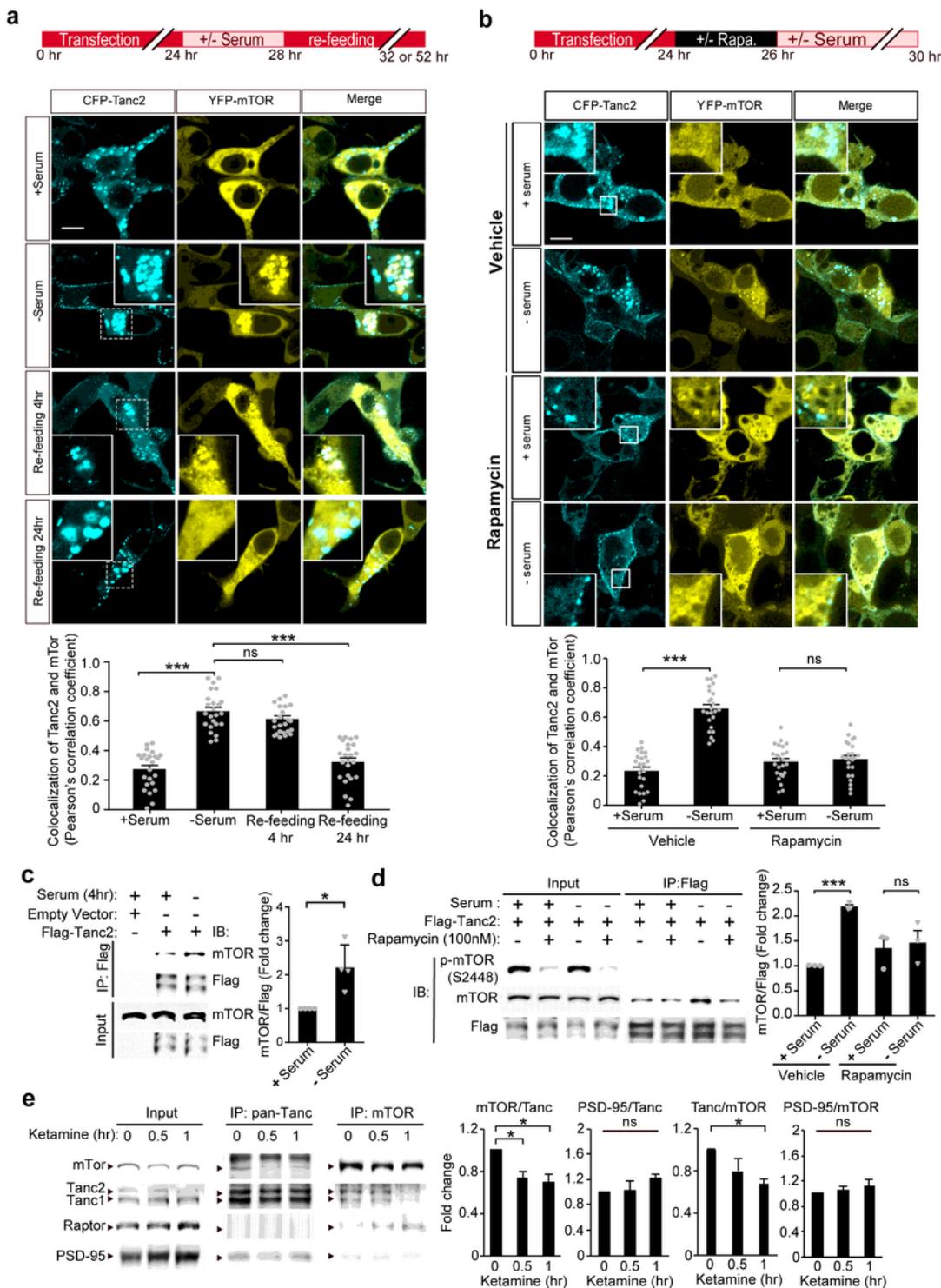


Figure 4

Serum and ketamine regulate the interaction of Tanc2 with mTOR. (a) Serum starvation induces colocalization of Tanc2 and mTOR in HEK293T cells, an effect that is reversed by serum re-feeding. HEK293T cells transfected with CFP-Tanc2 + YFP-mTOR were subjected to serum starvation (-serum) to inactivate mTOR, or to no serum starvation (control; +serum), for 4 hours followed by serum re-feeding for 24 hours while checking changes at 4-hr and 24-hr time points. The colocalization was quantified using

Pearson's correlation analysis of colocalized pixels (see Methods for details). (b) Rapamycin blocks serum starvation-induced Tanc2–mTOR colocalization in HEK293T cells. HEK293T cells expressing CFP-Tanc2 and YFP-mTOR were treated with rapamycin or vehicle for 2 hours before starting serum starvation. Colocalization was quantified by Pearson's correlation analysis of colocalized pixels (see Methods for details). (n = 24 cells from 3 independent experiments. ***P < 0.001, ns, not significant, Student's t-test). Scale bar, 10 μ m. (c) Increased biochemical association between Tanc2 and mTOR induced by serum starvation in HEK293T cells, as determined by coimmunoprecipitation (coIP). HEK293T cells expressing Flag-Tanc2 and mTOR (endogenous) in the presence and absence of serum starvation (4 hours) were immunoprecipitated with Flag antibody (for Tanc2) and immunoblotted as indicated. The lower Flag-Tanc2 band represents a degradation product. mTOR signals were normalized to Tanc2 signals for quantification. (n = 4 independent experiments, *P < 0.05, Student's test). (d) Rapamycin blocks the serum starvation-induced biochemical association of Tanc2 with mTOR in HEK293T cells, as determined by coimmunoprecipitation. HEK293T cells expressing Flag-Tanc2 and mTOR (endogenous) were treated with rapamycin or vehicle for 2 hours before starting serum starvation, followed by immunoprecipitation with anti-Flag antibodies (for Tanc2) and immunoblotting, as indicated. mTOR signals were normalized to Tanc2 (Flag) signals for quantification. (n = 3 independent experiments, ***P < 0.01, ns, not significant, Student's test). (e) Reduced biochemical association between Tanc1/2 and mTOR in the mouse brain (P13–14) upon ketamine treatment (10 mg/kg; i.p.), as shown by coIP experiments on whole-brain crude synaptosomes from ketamine-treated and -untreated mice using pan-Tanc or mTOR antibodies, followed by immunoblotting. Raptor was immunoblotted to show mTOR activation, and PSD-95 was immunoblotted for coIP with Tanc1/2 (positive control). (n = 4 independent experiments, *P < 0.05, ns, not significant, one-way ANOVA with Bonferroni test).

Fig. 5

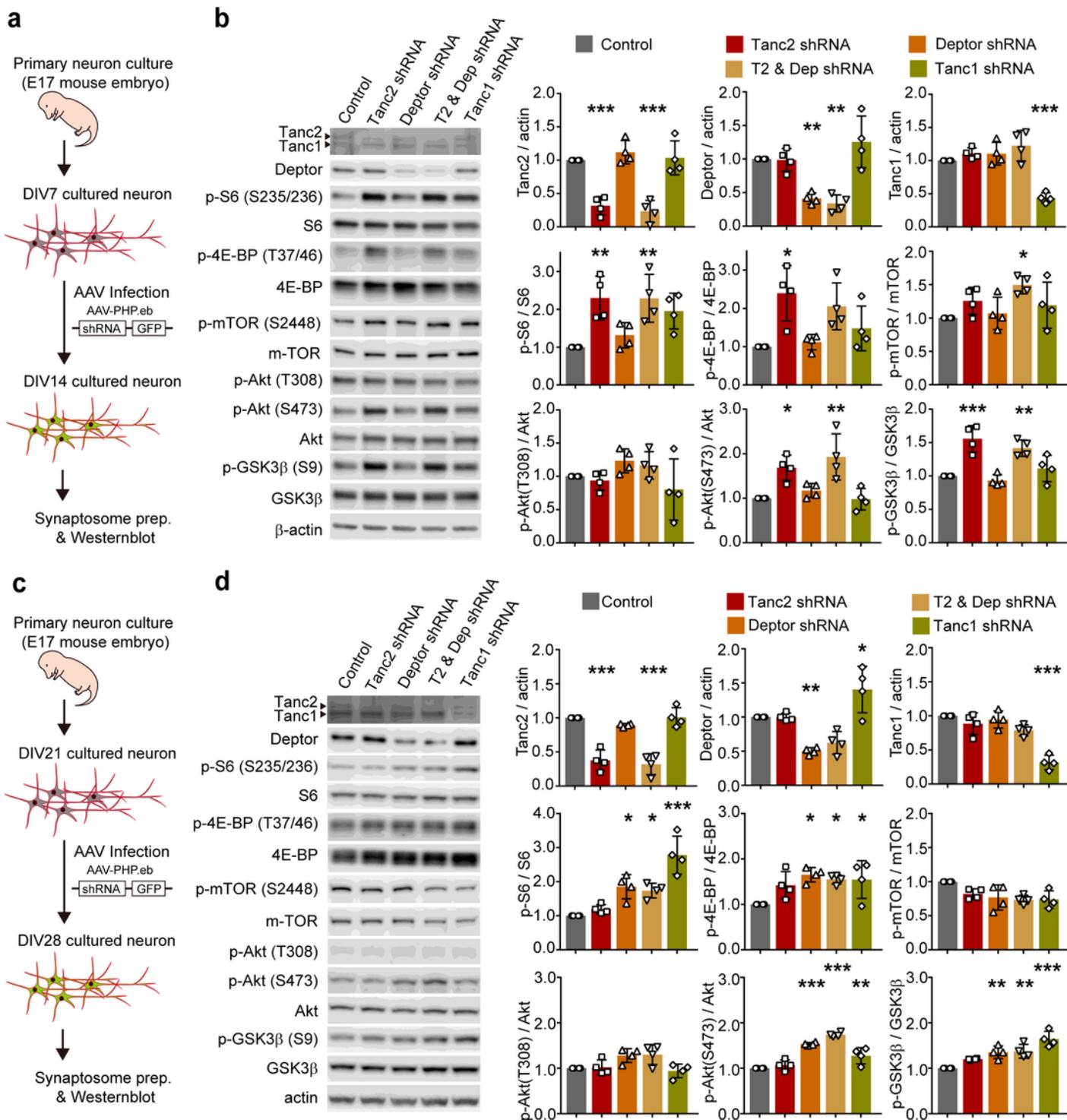


Figure 5

Acute knockdown of Tanc2, Deptor, and Tanc1 distinctly activate mTORC1/2 signaling in early- and late-stage neurons. (a) Schematic depiction of AAV1-shRNA-mediated acute knockdown of Tanc2, Deptor, or Tanc1 in cultured mouse hippocampal neurons (DIV7–14), and immunoblot analysis of crude synaptosomes for mTORC1/2 activity. (b) Early-stage (DIV7–14), acute knockdown of Tanc2, but not Deptor or Tanc1, leads to hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473) and

GSK3 β (S9), indicative of mTORC1 and mTORC2 hyperactivity. Note that knockdown of both Tanc2 (T2) and Depton did not exert synergistic effects except for p-mTOR, and that Akt phosphorylation at T308 (not S473) was unchanged, indicative of unaltered activities of proteins upstream of mTOR (n = 3 independent experiments; *P < 0.05, one-way ANOVA with Tukey test). (c) Schematic depiction of AAV1-shRNA-mediated knockdown of Tanc2, Depton, or Tanc1 in cultured mouse hippocampal neurons (DIV21–28) and immunoblot analysis of crude synaptosomes for mTORC1/2 activity. (d) Late-stage (DIV21–28) acute knockdown of Depton, or Tanc1, but not Tanc2, leads to hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), Akt (S473) and GSK3 β (S9), indicative of mTORC1 and mTORC2 hyperactivity (n = 3 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Tukey test).

Fig. 6.

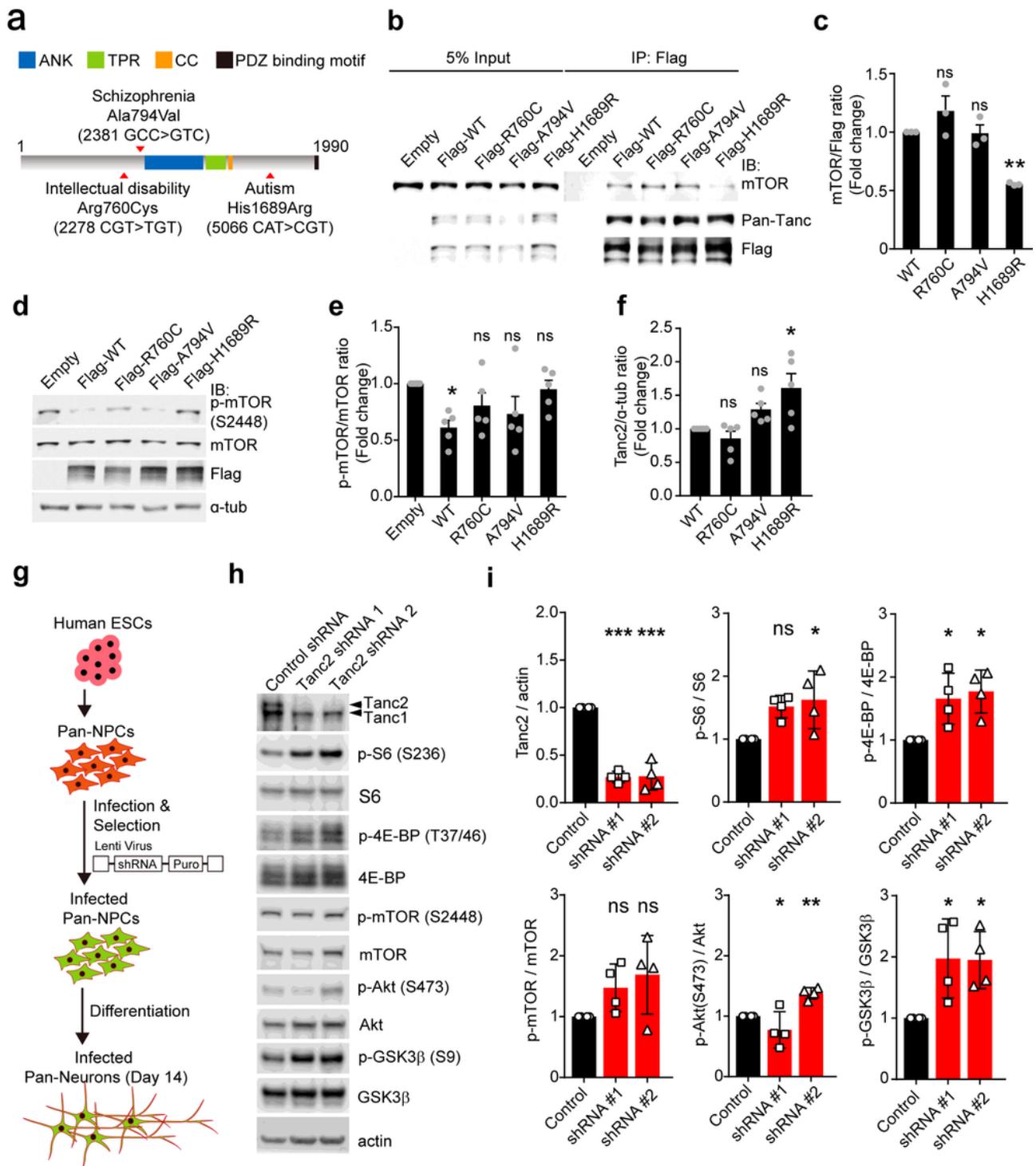


Figure 6

Patient-derived Tanc2 mutations suppress Tanc2-dependent mTOR inhibition, and acute knockdown of TANC2 in human neurons induces hyperactivity of mTORC1 and mTORC2. (a) A schematic showing the positions of TANC2 mutations identified in individuals with intellectual disability, schizophrenia, and ASD. (b and c) Flag-Tanc2-H1689R (human), but not the other Flag-Tanc2 mutants (Flag-Tanc2-R760C/A794V; human), fails to associate with mTOR (endogenous), whereas Flag-Tanc2-R760C and Flag-Tanc2-A794V

retain binding ability, as shown by coimmunoprecipitation experiments using HEK293T cell lysates. (n = 3 experiments, **P < 0.01, ns, not significant, one-way ANOVA with Bonferroni test). (d and e) All Tanc2 mutants (H1689R, R760C, and A794V) fail to suppress mTOR inhibition in HEK293T cells, as shown by immunoblot analyses of total and phosphorylated (Ser-2448) mTOR in cells expressing Flag-Tanc2-mutants. (n = 5 experiments, *P < 0.05, ns, not significant, one-way ANOVA with Bonferroni test). (f) Tanc2 mutations do not affect the total levels of Tanc2 proteins, except for an increase in the Tanc2-H1689R mutant, which is less likely to affect the interpretation of results from (B–E). Flag Tanc2 signals were normalized to α -tubulin signals. (n = 5 experiments, *P < 0.05, ns, not significant, one-way ANOVA with Bonferroni test). (g) Schematic of the experimental design to test if TANC2 knockdown increases mTORC1/2 activity in human neural progenitor cell (NPC)-derived neurons. Pan-NPCs infected with lentivirus particles for TANC2 knockdown were selected and subjected to neuronal maturation for two weeks before the analysis of mTORC1/2 signaling by immunoblot analysis. (h and i) Knockdown of TANC2 in human neurons leads to hyper-phosphorylation of S6 (S235/236), 4E-BP (T37/46), and GSK3 β (S9), indicative of increased mTORC1 and mTORC2 hyperactivity. Akt (S473) phosphorylation was increased and decreased by the #1 and #2 knockdown constructs, likely because of differential properties of the two constructs and compensatory cellular responses to adjust Akt activity. Human NPCs infected with two independent TANC2 knockdown lentivirus particles (#1 and #2) were subjected to the development into mature neurons and analyzed by immunoblot analyses. (n = 4 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Tukey test).

Supplementary Files

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