TrkB hyperactivity contributes to brain dysconnectivity, epileptogenesis, and anxiety in zebrafish model of Tuberous Sclerosis Complex

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Tuberous Sclerosis Complex (TSC) is a rare genetic disease that manifests with early symptoms, including cortical malformations, childhood epilepsy, and TSC-associated neuropsychiatric disorders (TANDs). Cortical malformations arise during embryonic development and have been linked to childhood epilepsy before, but the underlying mechanisms of this relationship remain insufficiently understood. Zebrafish have emerged as a convenient model to study elementary neurodevelopment; however, without in-depth functional analysis, the Tsc2-deficient zebrafish line cannot be used for studies of TANDs or new drug screening. In this study, we found that the lack of Tsc2 in zebrafish resulted in heterotopias and hyperactivation of the mTorC1 pathway in pallial regions, corresponding to the abnormal expression of genes involved in axon navigation. The mutants underwent epileptogenesis that resulted in nonmotor seizures and exhibited increased anxiety-like behavior. We further mapped discrete parameters of locomotion and have been linked to childhood epilepsy before, but the underling mechanisms of this relationship remain insufficiently understood. Zebrafish have emerged as a convenient model to study elementary neurodevelopment; however, without in-depth functional analysis, the Tsc2-deficient zebrafish line cannot be used for studies of TANDs or new drug screening. In this study, we found that the lack of Tsc2 in zebrafish resulted in heterotopias and hyperactivation of the mTorC1 pathway in pallial regions, which are homologous to the mammalian cortex. We observed commissural thinning that was responsible for brain dysconnectivity, recapitulating TSC pathology in human patients. The lack of Tsc2 also delayed axonal development and caused aberrant tract fasciculation, corresponding to the abnormal expression of genes involved in axon navigation. The mutants underwent epileptogenesis that resulted in nonmotor seizures and exhibited increased anxiety-like behavior. We further mapped discrete parameters of locomotor activity to epilepsy-like and anxiety-like behaviors, which were rescued by reducing tyrosine receptor kinase B (TrkB) signaling. Moreover, in contrast to treatment with vigabatrin and rapamycin, TrkB inhibition rescued brain dysconnectivity and anxiety-like behavior. These data reveal that commissural thinning results in the aberrant regulation of anxiety, providing a mechanistic link between brain anatomy and human TANDs. Our findings also implicate TrkB signaling in the complex pathology of TSC and reveal a therapeutic target.

Tuberic Sclerosis Complex (TSC) is an autosomal dominant disorder that is caused by loss-of-function mutations in the TSC1 or TSC2 genes (1), the products of which form a complex that negatively regulates mechanistic/mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a complex necessary for proper neuronal development (2). In TSC, epilepsy is the most prominent neurological symptom, which begins in the first year of life and often evolves into an intractable form (3). Epilepsy is accompanied by TSC-associated neuropsychiatric disorders (TANDs), including intellectual disability (ID) and anxiety (4). Epilepsy is thought to be caused by cortical malformations (5) that consist of heterotopias, tubers, and white matter (WM) dysconnectivity in TSC (4). Tuberous sclerosis patients are diagnosed in utero, suggesting that the pathology of TSC is acquired during embryonic development (6).

Cortical malformations result in childhood epilepsy and intractable or treatment-refractory seizures in various other diseases, underscoring the importance of proper cortex development (5). The existing mammalian models of TSC mimic human disease in many aspects, including cortical malformations and seizures (7–13). However, these models present difficulties in studying TSC pathology during embryonic development in utero when cortical malformations that lead to epileptogenesis arise (14). Thus, the present study comprehensively examined brain development and behavior in zebrafish, which provide an opportunity to study early neuronal development in vivo due to external development and body transparency. Although zebrafish do not contain a cortex per se, the homologous structures are present, the majority of which are localized to the pallium (15–17).

Previous studies investigated the utility of tsc2<sup>nu242</sup> mutant zebrafish as a model of TSC and reported abnormalities in pallial WM organization (including the disruption of WM with ectopic cell bodies), a decrease in locomotion, and abnormal brain activity in the optic tectum (18, 19). However, these studies did not elaborate potential disease mechanisms. Moreover, neurobehavioral changes that mimic symptoms of human TANDs were not investigated in tsc<sup>2nu242</sup>/<sup>nu242</sup> mutants. Therefore, in this study, we performed an in-depth analysis of changes in brain connectivity during development. We evaluated tsc<sup>2nu242</sup> fish behavior and mapped seizure activity and anxiety-like behavior to discrete parameters of locomotion. Finally, we tested the ability of drugs to rescue TSC-associated phenotypes and found that reducing tyrosine receptor kinase B (TrkB) signaling reversed brain dysconnectivity, epileptogenesis,

**Significance**

Tuberous Sclerosis Complex (TSC) is a hereditary disease that presents with early brain malformations, childhood epilepsy, and TSC-associated neuropsychiatric disorders (TANDs). Cortical malformations arise in utero and have been linked to childhood epilepsy before. Externally developing zebrafish seem convenient to study elementary neurodevelopment; however, without the in-depth functional analysis, the Tsc2-deficient zebrafish cannot be used for studies of TANDs. Here, we found that Tsc2-deficient zebrafish recapitulated symptoms seen in TSC patients on anatomical and behavioral levels, including aberrant brain morphology, thinning of brain connections, epileptogenesis, and increased anxiety-like behavior, which was rescued by reducing TrkB signaling, revealing a potential drug target. Moreover, we show that commissural thinning cause aberrant regulation of anxiety, providing a link between brain anatomy and emotion.

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and anxiety-like behavior. The latter phenotype was also reversed by inhibition of Rac1, which is potentially one of the downstream targets of TrkB.

Results

Lack of Tsc2 Leads to White Matter Disruption and Brain Dysconnectivity in Zebrafish. A disruption of WM organization is a common feature of TSC and was previously found in the telencephalon in tsc<sup>nu242/nu242</sup> mutants (19). Indeed, when we examined the telencephalon in tsc<sup>nu242/nu242</sup> fish, we confirmed the presence of ectopic cell bodies in tsc<sup>nu242/nu242</sup> by two-dimensional (2D) brain section imaging (Fig. 1A) and three-dimensional (3D) whole-brain imaging (SI Appendix, Fig. S1A). These cells presented higher levels of phosphorylated ribosomal protein S6 at serines 235 and 236 (P-Rps6; Fig. 1B) and represented the hyperactivation of mTorC1. The P-Rps6-positive cells in the pallium of tsc<sup>nu242/nu242</sup> mutants had higher intensity signals and larger cell bodies compared with pallial cells of tsc<sup>+/+</sup> fish (Fig. 1D and E and SI Appendix, Fig. S1 D and E). We also measured the thickness of the main brain commissures because human TSC patients exhibit WM disconnection that is associated with epileptic seizures and worse cognitive outcomes (12, 20–22). The tsc<sup>nu242/nu242</sup> anterior commissure (AC) that connects the brain hemispheres in the telencephalon was thinner than in tsc<sup>+/+</sup> fish (Fig. 2D and SI Appendix, Fig. S2A). The postoptic commissure was also thinner in tsc<sup>nu242/nu242</sup> mutants, whereas the size of the lateral tracts was similar to tsc<sup>+/+</sup> fish (SI Appendix, Fig. S2 B and C). The higher number of P-Rps6-positive cells negatively correlated with AC thickness, revealed by double immunostaining (Fig. 2 A and B).

To evaluate axon development that may underlie WM thinning, we crossed the tsc<sup>nu242</sup> line into transgenic Tg(ptf1a:GFP) background and examined them by live light-sheet imaging. ptf1a:GFP-positive neurons in the posterior tuberculum extend their axons toward the posterior commissure, where they cross the brain midline and innervate the other hemisphere (Fig. 2C). In tsc<sup>+/+</sup>, ptf1a:GFP-positive axons from one hemisphere crossed the brain boundary in one bundle. The same axons in tsc<sup>nu242/nu242</sup> presented disturbances in tract fasciculation (Fig. 2D). We were able to distinguish mild axonal phenotypes (e.g., one axon did not cross the midline in the bundle) and severe disturbances in axon bundling (e.g., axons crossed the midline stochastically; SI Appendix, Fig. S3A). Quantitative morphological analysis of ptf1a:GFP-positive axons of tsc<sup>nu242</sup> confirmed the impaired tract fasciculation in tsc<sup>nu242/nu242</sup> fish, reflected by a higher number of intersections at the midline compared with tsc<sup>+/+</sup> (Fig. 2E). Moreover, ptf1a:GFP-positive bundles in tsc<sup>nu242/nu242</sup> did not

Fig. 1. tsc<sup>nu242/nu242</sup> exhibit WM disruption and mTorC1 activation in the pallium. (A) Organization of gray matter and WM in coronal section through the AC. Circles represent cell bodies. Sample photographs of tsc<sup>nu242</sup> brain sections that were stained with DAPI are shown, together with the relative number of cell bodies in WM compartments in tsc<sup>nu242</sup> brains [H = 24.443, P = 4.92 × 10<sup>−6</sup>; 3.1 × 10<sup>−6</sup> for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup>, P = 0.017 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup> (Dunn’s test)]. (Scale bar, 40 μm.) (B) Representative images of coronal tsc<sup>nu242</sup> sections through the pallium that were immunostained with anti-P-Rps6 antibody (green). 5, subpallium. (Scale bars, 40 and 20 μm [magnification].) (C) The number of P-Rps6-positive neurons in the telencephalon in tsc<sup>nu242</sup> [H = 18.88, P = 7.95 × 10<sup>−5</sup>; P = 0.00071 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup>, P = 0.00117 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup> (Dunn’s test)]. (D) Mean phosphorylation levels of P-Rps6 per cell per fish in the telencephalon in tsc<sup>nu242</sup> [F = 6.772, P = 0.00335; P = 0.019 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup>, P = 0.0696 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup> (Dunn’s test)]. (E) Mean soma size of P-Rps6-positive neurons per fish in the telencephalon in tsc<sup>nu242</sup> [F = 21.48, P = 9.3 × 10<sup>−7</sup>; P = 4.1 × 10<sup>−7</sup> for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup>, P = 0.133 for tsc<sup>nu242/nu242</sup> vs. tsc<sup>+/+</sup> (Dunn’s test)]. *P < 0.05, **P < 0.005.

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**Fig. 2.** *tsc2<sup>vu242vu242</sup>* exhibit WM dysconnectivity resulting from aberrant axon elongation. (A) Representative horizontal optical sections through *tsc2<sup>vu242vu242</sup>* brains that were double-immunostained with anti-acetylated-Tubulin (ac-Tubulin; red) and anti-P-Rps6 (green) antibodies that show the AC (dashed lines) at 7.5 days post fertilization, together with the quantification of the width of the AC (F = 4.053, P = 0.0202; P = 0.011 for *tsc2<sup>vu242vu242</sup>* vs. *tsc2<sup>vu242vu242</sup>* with *tsc2<sup>vu242vu242</sup>* (Dunnett’s test)). (Scale bars, 30 μm.) (B) Correlation analysis of the number of P-Rps6–positive cells in the brain and the width of the AC, showing negative Spearman correlation and genotype clustering. (C) Schematic diagram of the localization of *ptfla:GFP*-positive neurons in the posterior tuberculum that extend their axons dorsally and cross the brain midline through the posterior commissure. (D) Representative confocal images of a dorsal view of *ptfla:GFP*-positive axons that show axonal tract fasciculation at the midline. The frequency of various *ptfla:GFP* axonal phenotypes with various severity was quantified. (Scale bar, 25 μm.) (E) Sholl analysis of *ptfla:GFP*-positive axon bundles at the midline of the *tsc2<sup>vu242</sup>* brains, including the number of intersections over the 3D distance from the soma [regression curves with confidence intervals; F = 23.84, P = 7.4 × 10<sup>−3</sup>; P = 2.3 × 10<sup>−5</sup>, *tsc2<sup>vu242vu242</sup>* vs. *tsc2<sup>vu242vu242</sup>* (Dunnett’s test); n = 16 *tsc2<sup>vu242vu242</sup>*; n = 13 *tsc2<sup>vu242vu242</sup>*, n = 15 *tsc2<sup>vu242vu242</sup>*, and the mean number of intersections with the data distribution. For *tsc2<sup>vu242vu242</sup>* the Sholl function converged on the value of 2 at a distance of 80 to 90 μm, representing two tracts that crossed the midline, one for each hemisphere. *tsc2<sup>vu242vu242</sup>* fish did not present this tendency, and the average number of axons at the midline was higher than in *tsc2<sup>vu242vu242</sup>*. (F) Frequency of parallelization of *ptfla:GFP* tracts from each hemisphere in the posterior commissure in the brain in *tsc2<sup>vu242vu242</sup>* fish (n = 27 *tsc2<sup>vu242vu242</sup>*; n = 31 *tsc2<sup>vu242vu242</sup>*, n = 18 *tsc2<sup>vu242vu242</sup>*). (G) Schematic diagram of the localization of *ath5:gap-RFP*-positive commissures in the brain in *tsc2<sup>vu242vu242</sup>* fish at 24 hpf, op, olfactory placodes; v, brain ventricle. (H) Representative confocal images of a frontal view of *ath5:gap-RFP*-positive neurons that show axon elongation (white arrows indicate axons) and the occurrence of various axonal phenotypes during olfactory medial tract development [genotype × phenotype: F = 8.677, P = 8.5 × 10<sup>−4</sup>; P = 0.077, *tsc2<sup>vu242vu242</sup>* vs. *tsc2<sup>vu242vu242</sup>* (Dunnett’s test); n = 18 *tsc2<sup>vu242vu242</sup>*; n = 60 *tsc2<sup>vu242vu242</sup>*, n = 25 *tsc2<sup>vu242vu242</sup>*]. (Scale bars, 100 μm.) *P < 0.05, **P < 0.005.
form parallel tracts, in contrast to tsc2–/–, which presented two parallel tracts (Fig. 2F).

To determine whether impairments in axonal development are a general phenotype of the mutants, we explored the axonal development of ath5:gap-RFP–positive olfactory neurons in Tg(ath5:gap-RFP):tsc2–/– zebrafish starting from 24 h post fertilization (hpf) when these neurons extended their axons to contact each other and crossed the hemisphere boundary through the AC, pioneering medial olfactory tracts (Fig. 2G). In living tsc2+/-;ath5:gap-RFP–positive neurons more often extended their axons improperly in various directions or did not extend them at all compared with tsc2+/+ (Fig. 2H and SI Appendix, Fig. S3B). This phenomenon persisted at 27 hpf, when ath5:gap-RFP–positive axons contacted each other at the midline, forming one tract in tsc2+/+ fish but not in tsc2+/-;ath5:gap-RFP or tsc2–/–;ath5:gap-RFP in which they were disturbed and exhibited deficient crossing of the midline (SI Appendix, Fig. S3C). The impairments in tract fasciculation were unrelated to disturbances in glial bridges, which were properly formed at the brain midline allowing axons to cross it (SI Appendix, Fig. S3D). These findings suggest deficiencies in sensing or responding to axon guidance cues. Higher messenger RNA (mRNA) levels of the dock3, dock4, and elmo2 genes, which are implicated in the Rac1-associated response to guidance cues, further support this hypothesis (SI Appendix, Fig. S4A). The increased expression of Dock3 and Elmo2 proteins was confirmed in the telencephalon of tsc2–/–, compared to controls (SI Appendix, Fig. S4 B–D). In tsc2+/-;ath5:gap-RFP, Elmo2-positive foci were found in the AC, while they were absent there in the tsc2–/–. Altogether, our results suggest that the lack of Tsc2 leads to commissural thinning and brain dysconnectivity, likely caused by disturbances in axon guidance signaling.

Increase in Epileptogenesis in tsc2+/-;ath5:gap-RFP Zebrafish Leads to Absence Seizures. Alterations of brain connectivity may result in epileptogenesis. Thus, we examined the basal activity in Tg(ath5:gap-RFP):tsc2–/–;ath5:gap-RFP fish and confirmed decreased locomotion in Tg(ath5:gap-RFP);tsc2–/–;ath5:gap-RFP fish that were disturbed and exhibited deficient crossing of the midline (SI Appendix, Fig. S2). The impairments in tract fasciculation were unrelated to disturbances in glial bridges, which were properly formed at the brain midline allowing axons to cross it (SI Appendix, Fig. S3). These findings suggest deficiencies in sensing or responding to axon guidance cues. Higher messenger RNA (mRNA) levels of the dock3, dock4, and elmo2 genes, which are implicated in the Rac1-associated response to guidance cues, further support this hypothesis (SI Appendix, Fig. S4A). The increased expression of Dock3 and Elmo2 proteins was confirmed in the telencephalon of tsc2–/–, compared to controls (SI Appendix, Fig. S4 B–D). In tsc2+/-;ath5:gap-RFP, Elmo2-positive foci were found in the AC, while they were absent there in the tsc2–/–. Altogether, our results suggest that the lack of Tsc2 leads to commissural thinning and brain dysconnectivity, likely caused by disturbances in axon guidance signaling.

Fish exhibiting an increase in anxiety-like behavior. The increase in velocity of tsc2+/-;ath5:gap-RFP may represent anxiety-like behavior, which has not been previously reported in Tsc2-deficient zebrafish. We observed disturbances in the axonal development of pfla1a:GFP–positive neurons in the posterior tuberculum in tsc2+/-;ath5:gap-RFP (Fig. 2D–F), which may result in alterations of connectivity of the fear/anxiety response axis (27). We evaluated whether tsc2+/-;ath5:gap-RFP exhibit anxiety-like behavior by subjecting tsc2–/– to a light-preference test. tsc2–/– fish spent more time in the light compartment, reflecting phototaxis. tsc2+/-;ath5:gap-RFP spent more time in the dark compartment compared with tsc2–/– (Fig. 4A), indicating that they were indifferent to light and implying decreased anxiety-like behavior. We also found that tsc2+/-;ath5:gap-RFP movements were more confined to the peripheral areas of the well compared with tsc2–/–, suggesting increased anxiety-like behavior. We then analyzed the activity of tsc2–/– in an open field to test anxiety-like behavior that is related to a novel environment and open areas. tsc2–/– spent less time exploring and spent more time near the edges compared with tsc2+/- (Fig. 4B), suggesting increased anxiety-like behavior.

Phototoxic zebrafish larvae exhibit anxiety-like behavior when subjected to a dark environment, resulting in hyperactivity. Switching the light on causes freezing because the sudden change in lighting conditions augments anxiety-like behavior even further, which is innately perceived by the fish as dangerous (27). tsc2+/-;ath5:gap-RFP mutants exhibited increased activity in the dark environment compared with tsc2–/–. These mutants also exhibited slightly more freezing during light, but this increase was not significantly different from tsc2–/–, which already presented very low activity (Fig. 4C). To validate the experimental setup, we subjected tsc2–/– fish to a light-preference test. tsc2–/– fish exhibited anxiety-like behavior in both the tsc2+/- and their control siblings, whereas RA increased anxiety-like behavior in tsc2+/- and tsc2–/–. However, RA did not significantly affect tsc2+/-, which had already exhibited increased anxiety-like behavior (Fig. 4D and SI Appendix, Fig. S7). These results were corroborated by changes in the amplitude of activity between light phases (Fig. 4E). Finally, cortisol levels in tsc2–/– were elevated compared with their control siblings (Fig. 4F).

The results from the light-preference test (Fig. 4A), sudden-light-changes test (Fig. 4C), and cortisol levels (Fig. 4F) suggest impairment in impulse control that contrasts with the anxiety hypothesis. To resolve this, we evaluated escape responses to repeated dark flashes and their habituation. The tsc2+/- exhibited lower or delayed responses to the first startle stimulus compared with tsc2–/–, but the startle habituation was similar to that of other fish (SI Appendix, Fig. S8). Although the total activity of tsc2+/- was lower, the fish indeed exhibited a startle response but to a lesser extent than siblings controls. These results do not confirm higher impulsivity and instead suggest that freezing behavior in response to the startle stimulus was attributable to higher preexisting levels of anxiety in tsc2–/–. Altogether, the results confirm increased anxiety-like behavior in tsc2+/- and recapitulate the human phenotype of TANDs (4).
Fig. 3.  

**tsc2^vu242^** exhibit decrease in activity, increase in epileptogenesis, and increase in response to ethosuximide treatment. (A) Activity analysis of tsc2^vu242^ fish that shows activity vs. time [F = 2.601, P = 0.0067; P = 2.38 x 10^-8 for tsc2^vu242^ vs. tsc2^+/-^, P = 7.42 x 10^-6 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)] and cumulative activity [F = 18.61, P = 4.76 x 10^-5; P = 4.16 x 10^-6 for tsc2^+/-^ vs. tsc2^+^, P = 1.22 x 10^-6 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)] in tsc2^+/-^ fish (n = 32) compared with tsc2^+/-^ fish (n = 85) and tsc2^+^ control siblings (n = 50) during 1 h of tracking. (B) Average velocity of high-velocity movements (>2 cm/s) of tsc2^+/-^, tsc2^+^, and tsc2^+^ controls [H = 31.73, P = 1.29 x 10^-10; P = 4.8 x 10^-10 for tsc2^+/-^ vs. tsc2^+^, P = 2.1 x 10^-7 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)]. (C) Representative images that show neuronal activity (without any stimulation) in the pallium in tsc2^+/-^ fish and the mean number of active cells, which increases in tsc2^+/-^ [F = 9.638, P = 0.0074; P = 0.009 for tsc2^+/-^ vs. tsc2^+^, P = 0.012 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)]. (Scale bar, 30 μm.) (D) Exemplary time-lapse photographs of STFBC of tsc2^+/-^ fish and quantification at 24 hpf [F = 8.59, P = 5 x 10^-5, P = 0.001 for tsc2^+/-^ vs. other genotypes (Dunnett’s test)] and 32 hpf [F = 7.535, P = 0.001; P = 0.012 for tsc2^+/-^ vs. tsc2^+^, P = 4 x 10^-4 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)]. (Scale bar, 100 μm.) (E) Representative tracks for each tsc2^+/-^ genotype after treatment with PTZ representing the entire 20 min and the first 5 s of tracking, showing the number of PTZ-induced seizures-like outbursts in the first 10 min of tracking for tsc2^+/-^, tsc2^+^, tsc2^+^, and tsc2^+^ fish [H = 34.667, P = 3 x 10^-6; P = 6.8 x 10^-6 for tsc2^+/-^ vs. tsc2^+^, P = 9.1 x 10^-8 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)]. Red tracks represent high-velocity movements (>2 cm/s). Green tracks indicate free swimming (movement within a range of 0.5 to 2 cm/s). Black tracks indicate free floating (<0.5 cm/s). (F) Time to first PTZ-induced outburst for tsc2^+/-^ compared with control siblings [H = 26.838, P = 1.49 x 10^-5; P = 3 x 10^-4 for tsc2^+/-^ vs. tsc2^+^, P = 2.1 x 10^-8 for tsc2^+/-^ vs. tsc2^+^ (Dunnett’s test)]. (G) Activity analysis of tsc2^+/-^ fish that were treated with ethosuximide, showing activity vs. time and cumulative activity of tsc2^+/-^ (n = 32) compared with tsc2^+^ (n = 56) and tsc2^+^ (n = 25) over 1 h of tracking. An increase in activity after acute ethosuximide treatment is considered to represent anxiety-like behavior, reflected by hyperactivity regardless of genotype [H = 40.312, P = 9.15 x 10^-9; P = 3.9 x 10^-6 for tsc2^+/-^ untreated vs. treated with ethosuximide, P > 0.05 for tsc2^+/-^ untreated vs. treated with ethosuximide, P > 0.05 for tsc2^+/-^ untreated vs. treated with ethosuximide for 24 h (Dunnett’s test)]. (H) Average velocity of high-velocity movements (>2 cm/s) of tsc2^+/-^, tsc2^+^, and tsc2^+^ fish after ethosuximide treatment [H = 9.5556, P = 0.02275, P = 0.08 for tsc2^+/-^ untreated vs. treated with ethosuximide, P > 0.05 for tsc2^+/-^ untreated vs. treated with ethosuximide for 24 h (Dunnett’s test)]. (I) Survival probability of tsc2^+/-^ fish after ethosuximide treatment vs. vehicle treatment. *P < 0.05, **P < 0.01, ***P < 0.005.
The thinner AC width in \textit{tsc2}^{vn242/vn242} mutants may reflect the WM thinning seen in TSC patients, which is associated with seizures (12, 20–22). In TSC patients, decreased density of the corpus callosum is correlated with epilepsy and such TANDs as autism and ID (22). Moreover, patients with TSC-associated refractory epilepsy have thinner and disorganized axon tracts (12). Similar fasciculation disturbances were seen in \textit{tsc2}^{vn242/vn242} \textit{ptfaa:GFP}-positive axons, which could explain these. These disturbances may result from impairments in axonal pathfinding and elongation. Consistently, previous studies found that lack of Tsc1 induces ectopic axons in vitro and WM defects in mice (31). The axon guidance pathways are regulated by external cues and their receptors that in turn activate a Dock–Elmo protein complex that subsequently activates Rac1, resulting in actin dynamics. Dock-3 and Dock-4 play pivotal roles in axon development. Dock-3 promotes axonal growth, forming the conventional Dock–Elmo complex, which is important for Rac1 activation in response to BDNF–TrkB signaling. Dock-4 promotes neurite differentiation to establish axon-dendrite polarity (32). Interestingly, in the present study, \textit{dock3}, \textit{dock4}, \textit{elmno2}, and \textit{rac1} mRNA levels were higher in \textit{tsc2}^{vn242/vn242} than in \textit{tsc2}^{+/+}. We further confirmed increased Dock4- and Elmo2-protein levels in \textit{tsc2}^{vn242/vn242} fish brains. Moreover, inhibition of Rac1 rescued \textit{tsc2}^{vn242/vn242} associated hyperactivity, but did not affect increased locomotion, suggesting that Rac1 involvement in anxiety-related behavior is separate from epilepsy.

Recently, zebrafish has become an accepted model for neuropsychiatric studies having applications in translational research. Zebrafish as highly complex vertebrates present a repertoire of complicated behaviors that can be used to study neurological and neuropsychiatric human disorders (33, 34). In the present study, \textit{tsc2}^{vn242/vn242} mutants exhibited lower activity but higher high-range velocity, suggesting that they swim in a burst-like manner. These high-amplitude movements can be caused by such TAND-related phenotypes as overactivity, impaired impulse control, or anxiety. Increased high-range velocity cannot represent hyperactivity because \textit{tsc2}^{vn242/vn242} total locomotion decreased. We hypothesized that the increased velocity of \textit{tsc2}^{vn242/vn242} resulted from anxiety-like behavior and the open field, sudden-light changes test and elevated cortisol levels confirmed this. However, the increased velocity could be potentially caused by impaired impulse control, which could explain the results of the light-preference test (higher risk taking and boldness) and would contradict the results of the open field. Impaired impulsivity would possibly result in elevated cortisol and hyperactive bursts, similar to the effects produced by anxiety. Therefore, we performed a startle analysis to repeated dark flashes which is relevant to anxiety, impulsivity, and ID reflected by escape responses to startling stimuli and habituation after repetition. \textit{tsc2}^{vn242/vn242} exhibited lower or delayed response to the first startle stimulus compared with \textit{tsc2}^{+/+} that exhibited no difference in habituation. This did not confirm higher impulsivity in \textit{tsc2}^{vn242/vn242} and further suggested anxiety as a cause for the observed phenotypes.

Because \textit{tsc2}^{vn242/vn242} fish remained at the edges of the well in the light–dark test, we propose that \textit{tsc2}^{vn242/vn242} suffer brain impairments and cannot react to dark conditions similarly to \textit{tsc2}^{+/+}. Startle response results do not necessarily contradict this hypothesis. Although we did not see differences in habituation of the \textit{tsc2}^{vn242/vn242} response compared with controls, the response to the startling stimulus was already impaired. Supporting the possibility of ID, we found perturbances in the fasciculation of \textit{ptfaa:GFP}-positive tracts from the posterior tuberculum in \textit{tsc2}^{vn242/vn242}, which may impair anxiety regulation. Another brain part, the habenula, is critical for processing emotions, social behavior, anxiety responses, and locomotion, and it is thought to act as a link between the forebrain and midbrain to produce emotional responses (35–37). The left habenula has

\textbf{Discussion}

TSC symptoms arise during brain development through mechanisms that are not fully understood. In the present study, \textit{tsc2}^{vn242/vn242} exhibited pallium malformations and thinner commissures, which were rescued by TrkB inhibition, together with behavioral abnormalities, suggesting that the observed neuroanatomical phenotypes were related to neuroanatomical changes.
been shown to regulate light preference behavior in zebrafish larvae (38). Other left–right asymmetries in brain morphology have also been linked to regulation of boldness and exploratory behavior (39). Therefore, it is likely that aberrant development of the brain hemisphere connections is responsible for the observed TAND-related phenotypes. Both the increased anxiety-like behavior in the open field and when subjected to sudden changes in light and the lack of light preference in \( tsc^{vu242/vu242} \) may thus result from disturbances in overall connectivity to the habenula.

Anxiety-like behavior can be induced by seizures (40), providing a link between epileptogenesis, seizures, and anxiety. Reductions of BDNF signaling in the hippocampus were shown to prevent spontaneous seizures and rescue anxiety-like behavior in rodent epilepsy models (26). Consistently, sustained Creb activity was shown to produce seizures in mice (41). In the present study, ANA-12 treatment rescued the activity impairment and reversed increased high-range velocity in \( tsc^{vu242/vu242} \). At the same time, ANA-12 also reduced TrkB and Creb activation and lowered the seizure threshold in \( tsc^{vu242/vu242} \), implicating TrkB signaling in epileptogenesis.

Reversal of AC thinning and anxiety-related hypervelocity by ANA-12 but not by VGN suggests that commissural connections substantially contribute to regulating anxiety and links brain anatomy with behavior. These results also implicate TrkB hyperactivity in both commissure development and anxiety. We also showed that Rac1 inhibition, similar to inhibition of TrkB, rescued anxiety-related velocity of \( tsc^{vu242/vu242} \). In line with our results, Rac1 overactivation was previously shown to inhibit the formation of long-term fear conditioning memory (42). Rac1 is required for commissural axon development specifically in the cortex and controls axon crossing through the AC and corpus callosum in mice (43, 44). \( tsc^{vu242/vu242} \) exhibited thinner AC and presented problems with axons crossing the brain midline. Therefore, it is probable that the disruption of commissural axons results in anxiety through Rac1. Moreover, Rac1 may act downstream of TrkB in axon development and synaptic crosstalk (30, 45, 46), raising an intriguing possibility that, also in TSC,
Fig. 5. ANA-12 rescues survival, brain dysconnectivity, impairment in locomotion, and anxiety-related hypervelocity in *tsc2vu242/vu242* mutants. (A) Quantification of activation of TrkB receptor judged by levels of P-TrkB and ratio of P-TrkB to TrkB by immunoochemistry (ELISA) revealed higher activation levels in *tsc2vu242/vu242* fish compared with control siblings and a decrease after ANA-12 treatment [*P*-TrkB: *H* = 4.355, *P* = 0.049; *P* = 0.05, *tsc2vu242/vu242* vs. *tsc2+/+* (Dunn’s test)]; *P* = 0.015, *tsc2vu242/vu242* untreated vs. treated with ANA-12 (Dunn’s test); P-TrkB/Tk8: *H* = 5.689, *P* = 0.05; *P* = 0.05, *tsc2vu242/vu242* vs. *tsc2+/+* (Dunn’s test); *P* = 0.049, *tsc2vu242/vu242* untreated vs. treated with ANA-12 (Dunn’s test)). (B) Quantification of levels of CREB phosphorylation by immunoochemistry (ELISA) revealed higher levels in *tsc2vu242/vu242* compared with controls and a decrease after ANA-12 treatment [genotype: *F* = 3.513, *P* = 4.66 × 10⁻⁵; treatment: *F* = 14.759, *P* = 8.33 × 10⁻⁵] genotype × treatment: *P* > 0.05; *P* = 0.012, *tsc2vu242/vu242* after ANA-12 vs. DMSO treatment (Tukey HSD test); *P* = 0.033, untreated *tsc2vu242/vu242* vs. *tsc2+/+* (Tukey HSD test)]. (C) Survival probability of *tsc2vu242/vu242* after various treatments. (D) Confocal images of *tsc2vu242* brains that were immunostained with anti-P-Rps6 antibody after ANA-12 treatment. (Scale bar, 30 μm.) (E) Cumulative activity of *tsc2vu242/vu242* after treatment with ANA-12 compared with *tsc2vu242/vu242* and *tsc2+/+* fish over 1 h of tracking, showing an increase in activity of mutant fish after treatment [*H* = 16.652, *P* = 2.42 × 10⁻⁵; *P* = 0.0172, *tsc2vu242/vu242* untreated vs. treated with ANA-12 for 24 h (Dunn’s test)]. (F) Cumulative activity of the *tsc2vu242/vu242* fish after the prevention of disease development by Rapa pretreatment compared with *tsc2vu242/vu242* and *tsc2+/+* fish over 1 h of tracking, showing an increase in activity of mutant fish after treatment [*H* = 32.18, *P* = 3.9 × 10⁻⁸; *P* = 2.0 × 10⁻⁸ for *tsc2vu242/vu242* untreated vs. rapamycin pretreatment (Dunn’s test)]. (H) Cumulative activity of *tsc2vu242/vu242* after the treatment with VGN or VGN-P to prevent disease development, respectively, compared with *tsc2vu242/vu242* and *tsc2+/+* fish over 1 h of tracking, showing improvements in activity after short VGN treatment but toxicity after longer treatment with VGN [*H* = 19.262, *P* = 6.566 × 10⁻⁵; *P* = 3.4 × 10⁻⁵ for *tsc2vu242/vu242* untreated vs. treated with VGN, *P* > 0.05 for *tsc2vu242/vu242* untreated vs. VGN-P (Dunn’s test)]. (I) Average velocity of high-velocity movements of *tsc2vu242/vu242* compared with *tsc2+/+* and *tsc2+/+* fish after treatment with ANA-12 [*H* = 14.686, *P* = 6.47 × 10⁻⁵; *P* = 0.015 for *tsc2vu242/vu242* untreated vs. treated with ANA-12 for 3 h, *P* = 0.003 for *tsc2vu242/vu242* untreated vs. treated with ANA-12 for 24 h (Dunn’s test)]. (J) Average velocity of high-velocity movements of *tsc2vu242/vu242* after the treatment with VGN or VGN-P to prevent disease development, respectively, compared with *tsc2+/+* and *tsc2+/+* fish [*H* = 3.286, *P* = 0.19]. *P* < 0.05, **P < 0.01, ***P < 0.005, ns: not significant.

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TrkB hyperactivity results in anxiety, causing Rac1 pathway dysregulation. Nevertheless, hyperactive Rac1/3 also caused excitatory–inhibitory imbalance and increased brain activity with impaired synchronization (47). This is coherent with our results as Rac1 can lead to higher brain activity and seizures. However, it is also possible that Rac1 affects anxiety by impairing synaptic crosstalk in other brain regions, independently from TrkB hyperactivity. In conclusion, the presented results implicate TrkB and Rac1 signaling in the pathology of TSC, expanding our understanding of the complex mechanisms that underlie this disease and revealing a potential therapeutic target.

Methods

Drug Treatments. PTZ, RA, VGN, ethoxussimide, and ANA-12 were purchased from Sigma-Aldrich. Rapamycin, dipyridamole, and W56 were obtained from MBL International, Cefarma S.A., and Toceis, respectively. Drugs were dissolved in E3, dimethylsulfoxide (DMSO), or glycerol for stock solutions, and were further diluted in E3. Drugs were administered directly into E3 with the same number of dechorionated fish. More information can be found in SI Appendix.

Image Acquisition and Analyses. Fluorescence images of crossections and whole mounts were acquired with a Zeiss LSM880 confocal microscope. For STFM imaging, the chorions were recorded using a Leica M60 microscope with a DCM290 camera every 10 ms for 3 min. Other in vivo imaging was performed with a Lighsheet Z.1 microscope (Zeiss). Image analyses were performed using Fiji (fiji.sc) (48) with a measurement tool, Skeletonize (2D/3D) (49), or 3D-Sholl analysis (50). Further details can be found in SI Appendix.

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Material and Data Availability. Manuscript and SI Appendix contain all data with the representative images only. The fish lines, materials, protocols, or imaging data are available upon reasonable request of the corresponding authors.

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